NOVEL DICHLOROACETATE PRODRUGS FOR IMPROVED TARGETING OF
BREAST CANCER CHEMOTHERAPEUTICS

A DISSERTATION IN
Pharmaceutical Sciences
and
Molecular Biology & Biochemistry

Presented to the Faculty of the University
of Missouri-Kansas City in fulfillment of
the requirements for the degree

DOCTOR OF PHILOSOPHY

by

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2019
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University of Missouri-Kansas City, 2019

ABSTRACT

It is well documented that most cancer cells utilize aerobic glycolysis or Warburg metabolism instead of mitochondrial oxidative phosphorylation for cell energy production. Dichloroacetate is a synthetic small molecule with proven anticancer effect. As a pyruvate mimic, dichloroacetate is a glycolytic inhibitor that inactivates pyruvate dehydrogenase kinase causing the activation of pyruvate dehydrogenase. This activity enables pyruvate conversion to acetyl-CoA leading to mitochondrial remodeling via the reversal of the Warburg effect and the release of pro-apoptotic mediators into the cytoplasm. The primary aim of this dissertation is to synthesize dichloroacetate prodrugs that improve DCA targeting to different breast cancer subtypes, triple negative and ER+ breast cancers to have enhanced cancer cell death.

The general hypothesis and aims of this work are introduced in Chapter 1. A literature review is provided on cancer etiology, breast cancer epidemiology, and the influence of DCA on cancer, specifically breast cancer metabolism in Chapter 2.

Chapters 3 and 4 detail the physicochemical analysis of phenoxyethyl dichloroacetamide (PE-DCA) and functionalized glucose-dichloroacetamide (G-DCA),...
respectively. Physicochemical analysis include: LC/MS, FT-IR, SEM, P-XRD, and $^{13}$C and $^1$H NMR spectroscopy. Chapter 3 focuses specifically on the physicochemical analysis of PE-DCA. PE-DCA was synthesized with a yield of 87.11%, and molecular weight was quantified using LC/MS and corresponded to the expected molecular weight of 247 g/mol. The chemical structure and formula of PE-DCA were confirmed using FTIR and NMR spectroscopy. XRD and SEM analysis indicated that the morphological structure is different suggesting that the structural integrity of native DCA in comparison to that of the native DCA, which could influence cellular uptake of the prodrug. The physicochemical properties of G-DCA are presented in Chapter 4. G-DCA was synthesized in a two-step process with yields >97% for each step. Using LC/MS, the molecular weight was confirmed to be 502.09 g/mol. G-DCA’s chemical structure and formula were further confirmed using FTIR and NMR spectroscopy. XRD and SEM analysis illustrate that G-DCA has a contrasting morphology and composition than that of native DCA. The differences in the physicochemical properties of the prodrugs can give insight on their metabolic profile.

The cell viability and toxicity activity of MDA-MB 231, MCF-7, and MCF-10A (normal breast epithelial cells) when treated with PE-DCA and G-DCA for 24 h, respectively, are described in Chapters 5 and 6. As compared to native DCA, with CC$_{50}$ values of 25 mM and 25-30 mM in MDA-MB 231 and MCF-7 cell lines, respectively, CC$_{50}$ values for PE-DCA (MDA-MB 231=14.72 µM; MCF-7=47.60 µM) and G-DCA (MDA-MB 231=16.22 µM; MCF-7=97.54 µM) were substantially lower, further proving that the prodrugs enhance DCA’s targeting to cancer cells to reverse the Warburg effect.

The metabolic profiles of the two cancerous cell lines after treatment with G-DCA, PE-DCA, and DCA in different media environments (pyruvate, low glucose and high
glucose) are elucidated in Chapter 7. Intracellular ROS and autophagosome production were quantified in the various conditions to explore the relationship between ROS production and the induction of autophagy when exposed to different media environments. Results for both cancer cell lines indicate an increase intracellular ROS production in high glucose media which further exploits the similarities in the metabolic profiles of breast cancer subtypes. Moreover, related to autophagosome production, upon drug treatment in varying concentrations and media conditions, the relationship between ROS and autophagy induced apoptosis was explored.

The intracellular DCA uptake in MCF-10A, MCF-7, and MDA-MB 231 cell lines after treatment with DCA, PE-DCA, and G-DCA are quantified in Chapter 8. Results showed that the DCA prodrugs are more cell targeted to cancer cells than normal breast epithelial tissues further proving that because of DCA conjugation, DCA’s target specificity is more refined.

This dissertation gives a comprehensive analysis of the metabolic and physicochemical characteristics of the novel DCA prodrugs and their influence on breast cancer proliferation and cell death.
The faculty members listed below, appointed by the Dean of the School of Graduate Studies have examined a dissertation titled “Novel Dichloroacetate Prodrugs for Improved Targeting of Breast Cancer Chemotherapeutics.” presented by Danielle N. Thomas, Candidate for the Doctor of Philosophy Degree and certify that, in their opinion, it is worthy of acceptance.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Dr. Russell Melchert. During my tenure at the UMKC School of Pharmacy, he has pushed me to be a more adept researcher. His advice and encouragement in my pursuit for a career in academia has been more than helpful during my doctoral education. I have gained invaluable insight on the kind of leader, researcher, and advisor I will be as I pursue a career in academia.

My committee members, Drs. Thomas Johnston, Kathleen Kilway, Shin Moteki, and Xiaolin Yao have imparted upon me priceless feedback that has guided my dissertation research. I am more than appreciative of their availability to meet as a committee and personally to give me much needed feedback and advice to strengthen my research. All my committee members have been my professors throughout my graduate career, and I have gained a breadth of knowledge from them through our scholastic interactions as well. As I pursue my post-doctoral career, I hope to motivate my future colleagues and students in the same manner they have done for me.

I would also like to extend my gratitude to the Chair of the Division of Pharmacology & Pharmaceutical Sciences, Dr. Gerald Wyckoff. He has been my mentor as a master’s student in Cell & Molecular Biology, the Preparing Future Faculty, and my doctoral education. His steadfast dedication to the success of the students within the division is inspiring as I endeavor in my career.

I am thankful for the many professors on the UMKC campus: Dr. Dhananjay Pal for his assistance in cell culture assays. Dr. Rachael Allen for her support, mentorship, and kindness during my entire graduate tenure. Dr. Karen Bame for her exceptional research advice in scholarly support. Dr. Keith Busek for his assistance in the synthesis reactions of
my DCA prodrugs. Drs. James Murowchick, Zhonghua Peng, and Donggao Zhao for their exceptional services in the physicochemical analysis of the DCA prodrugs. The faculty, students, and staff of the Faculty Senate, School of Biology, Spencer Chemistry, School of Pharmacy, Student-Athlete Support, Services Office, and the UMKC community at large who have contributed to my personal and scholarly growth during my time at UMKC.

I am grateful for my colleagues in Dr. Youan’s laboratory past and present: Dr. Fohona Coulibaly for his encouragement to takeover my DCA prodrug dissertation and work and his assistance, advice, and support in my research endeavors. Dr. Albert Ngo for his support, encouragement, and assistance. Omowumi Akinjole and Abdullah Alsahai for their friendship, motivation, and leadership. Furthermore, an extreme appreciation goes to Mark Rayhart for his dedication in improving the synthesis of the DCA prodrugs. Also, many thanks to Navid Ayon for his assistance on DCA intracellular uptake studies and Vrinda Gote for her aide in cell culture protocols. Moreover, I appreciate the support and friendship of my fellow classmates of the UMKC School of Pharmacy Division of Pharmacology and Pharmaceutical Sciences.

I would like to give special thanks to the administrative staff, past and present at the School of Pharmacy, especially that of Tamica Lige, Sharon Self, Gwen Huke, Jeannie Westmoreland, Joyce Johnson, Shana Eisentrager, Jane Poe and Nancy Bahner. I must express my sincere gratitude to the School of Graduate Studies, Missouri Black Caucus, South Central Gifted Scholar’s Fund, UMKC Women’s Council Graduate Assistance Fund, Pharmacy Foundation, BioNexus, and National Institute of Health for financial support. I must express my sincere gratitude to the School of Graduate Studies, Missouri Black Caucus,
South Central Gifted Scholar’s Fund, UMKC Women’s Council Graduate Assistance Fund, Pharmacy Foundation, BioNexus, and National Institute of Health for financial support.

Lastly, I am beyond grateful for my family and friends. Thank you to my husband, Michael Carter and children, Michael, Morgan, and Malachi for their support as I pursue my education far from home (Los Angeles). Their sacrifices, motivation, encouragement, patience, and love do not go unnoticed. Thank you to my friends and extended family, especially that of Dr. Nora Ekeanya-Smith, Leandra McLaurin, April Nunn, for keeping me grounded and encouraged during my doctoral education. I am completely indebted to my mother, Connie Jacobs-Thomas and all her sacrifices, support, and love she has bestowed upon on me. I would not be where I am without her guidance. Her grace and strength are an example of the women I want to become.
For Connie
CHAPTER 1
INTRODUCTION

1.1 Statement of Problem

Cancer is a major epidemiological and public health concern worldwide. As of 2018, for women in the United States, breast cancer is among the leading causes of death with lung and colon cancers being the first and third, respectively. Relatedly, in 2018, over 200,000 new cases of breast cancer accounted for 30% of new cancer diagnosis amongst women.\(^1\) Cancer is defined as abnormal cell growth caused by the accumulation of malformed or uncorrected DNA damage. This overgrowth has the potential to invade other parts of the body leading to metastasis due to errors in the cell cycle. These errors are influenced by genetic, environmental, and molecular implications.\(^2\) Cancer cells can evade cell death or apoptosis via several pathways, such as a mutation in the cell-cycle tumor suppressor protein 53 (TP53), that when functional, can stimulate the apoptotic intrinsic pathway causing the activation, repression, and transactivation of genes that lead to cancer cell death.\(^3\)

Cancer cells are extremely adaptable due to genomic instability driven by factors that include, but are not limited to: metastasis potential due to the deformation of normal cell integrity and overexpression of growth factors that can lead to not only malignant cell proliferation, but sustained angiogenesis and evasion of apoptosis.\(^2\) This genomic instability can lead to multidrug resistance (MDR) of chemotherapeutic drugs. Although significant progress has been made in understanding and ability to control the progression of various cancers, still, the similarity in various molecular pathways and cancer cell adaptability, has resulted in a poor performance of most anticancer drugs. In fact, it is extremely rare to find an essential target that is unique to cancer cells despite the level of malignancy or anatomical
Classical chemotherapeutic options have limitations, such as inefficient tumor targeting which can lead to many side effects in non-target tissues, drug hydrophobicity and poor water insolubility that can affect drug administration, and drug resistance after repeated treatments. Since the introduction of earlier chemotherapeutic treatments, the notable breakthroughs in cancer therapy have mainly been the introduction of new cytotoxic agents with novel, but often non-selective or specific mechanisms of action. Because of these limitations, safer and more cell-targeted treatment options are critical.

The exceptional reliance of most cancer cells on aerobic glycolysis for ATP production (known as Warburg effect) has been proven to be a striking feature of most tumors. The Warburg effect, first postulated by Otto Warburg in the 1920s, describes the mitochondrial remodeling in cancer cell genesis since oxidative phosphorylation occurs in the mitochondria in normal cells. In normal cells, a majority of ATP, about 95%, is produced via oxidative phosphorylation with the remaining 5% due to aerobic glycolysis. Moreover, cancer cells rely on aerobic glycolysis to make 50-70% of their ATP. In order to accommodate for the increased rate of aerobic glycolysis, cancer cells upregulate enzymes involved in metabolism via glucose transporter proteins. The Warburg effect can be used to describe cancer cell resistance to apoptosis. Glycolytic enzymes, such as lactate dehydrogenase (LDH) increase hypoxic conditions in the cell, increase intracellular NADPH levels, decrease in ROS causing anti-apoptosis, chemoresistance, and metastasis intitiation.

Furthermore, because of the high dependency of cancer cells on aerobic glycolysis, there is a critical need for amplified uptake of glucose across the plasma membrane via glucose transporter proteins upregulation. As a result, glycolysis inhibitors, such as
dichloroacetate (DCA) can be utilized as potential drugs to enhance targeting in cancer chemotherapeutics. DCA, a pyruvate mimic, stimulates oxidative phosphorylation by targeting pyruvate dehydrogenase kinase (PDK), the inhibitor of pyruvate dehydrogenase (PDH) causing pyruvate to be oxidized in the mitochondria and the ultimate reversal of the Warburg effect.\textsuperscript{8,11} Through the reversal of the Warburg effect, pro-apoptotic mediators, such as Cytochrome-C are released in the cytoplasm due to a decrease in mitochondrial membrane potential (MMP).\textsuperscript{4} These molecular mechanisms of DCA allows for the selective induction of cancer cell death and lower lactate levels intra- and extracellularly, signifying increased oxidation in the mitochondria and the ultimate reversal of the Warburg effect.\textsuperscript{12}

Although DCA has been used in humans for over 30 years to treat symptoms associated with lactic acidosis by decreasing intra- and extracellular lactate levels, preclinical studies of DCA suggest it is an efficient potential cancer chemotherapeutic agent.\textsuperscript{4} However, because of its high dose requirement (25-50 mg/kg/day), rapid clearance (4.82L/h), short half-life (1h), and the strong neurotoxicity associated with its use, a targeted delivery approach of this anticancer agent is critical to maximize its benefits.\textsuperscript{13}

To enhance cellular uptake and anti-cancer action of DCA, engineering novel DCA analogues, such as PE-DCA and G-DCA can potentially overcome some of the DCA limitations to be.\textsuperscript{13} For example, glucose transporter, GLUT 1, has been known to be overexpressed in breast cancer tumors favoring tumor growth. In a study of glucose transporter protein expression in 70 breast cancer cases, 46.7% of the patients exhibited overexpression of GLUT 1.\textsuperscript{14} This suggests that glucose is a proven ligand for DCA for improved cell targeting. Moreover, phenoxyethyl derivatives are synthesized as non-nucleoside reverse transcriptase inhibitors (NNRTIs) that can be cytotoxic to in-vitro cancer
cells. Although these NNRTI derivatives are often used in anti-HIV treatment, phenoxyethyl derivatives can inhibit the endogenous reverse transcriptase in cancer cells and alter the oxidative stress in mitochondria leading to apoptosis.

1.2 Hypothesis

It is hypothesized that the DCA conjugates phenoxyethyl dichloroacetamide (PE-DCA) and a functionalized glucose dichloroacetamide (G-DCA) will be more cancer cell specific leading to enhanced apoptosis as compared to native DCA due to the reversal of the Warburg effect.

1.3 Objectives

- To synthesize both PE-DCA and G-DCA using novel synthetic pathways
- Perform physicochemical characterization of both prodrugs via Fourier-transform infrared spectroscopy (FTIR), Proton and Carbon Nuclear Magnetic Resonance ($^1$H and $^{13}$C NMR) Spectroscopy, LC-Mass spectrometry (LC/MS), Powder X-Ray Diffraction (P-XRD), and Scanning Electron Microscopy (SEM)
- Perform in-vitro cytotoxicity and cell viability testing via Lactate Dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays of both prodrugs compared to native DCA on triple negative breast cancer (MDA-MB 231), ER+ breast cancer (MCF-7), and normal breast epithelial (MCF-10A) cell lines
- Measure intracellular drug uptake of the two prodrugs on the above cell lines using LC/MS at various concentrations
• Measure reactive oxygen species (ROS) of MCF-7 and MDA-MB 231 cell lines when treated with the two prodrugs to determine the reversal of Warburg effect

• Measure autophagy of the MCF-7 and MDA-MB 231 cell lines when treated with the two prodrugs as an indication of cell death
CHAPTER 2
LITERATURE REVIEW

2.1 Cancer and the Cell Cycle

There are over 200 types of cancer that affect most of the body, including organ tissues, blood vessels, bone, etc. Cancer is defined as abnormal cell growth that can both invade and damage the normal tissues of the body in a localized or metastasized manner. In the United States, 1 in 3 and 1 in 2 women and men, respectively will develop cancer at some point in their lifetime. This abnormal cell growth is related to mutations in regulating the cell cycle, which describes the process by which eukaryotic and prokaryotic cells prepare for cell division and proliferation (Figure 2.1).

Figure 2.1: Eukaryotic cell cycle illustrating various checkpoints, by El-Aouar Filho et al., is licensed under CC BY 3.0
In normal functioning cells, the cell cycle is divided into four distinct phases: G1, S, G2, and M and are regulated by various cyclin and cyclin-dependent kinase (CDK) interactions at specific checkpoints (Table 2.1). G1, G2, and M checkpoints. These checkpoints regulate the advance of cells through the phases of the cell cycle (Table 2.2)

Table 2.1: Description of cell cycle activity in normal mammalian cells

<table>
<thead>
<tr>
<th>Cell State</th>
<th>Phases</th>
<th>Abbreviation</th>
<th>Activity</th>
<th>Cyclin-CDK Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>Gap 0</td>
<td>G0</td>
<td>The cell is no longer in the cell cycle nor dividing</td>
<td>Cyclin C/CDK3</td>
</tr>
<tr>
<td>Interphase</td>
<td>Gap 1</td>
<td>G1</td>
<td>Cell increases in size in preparation for the S phase; Regulated by the G1 checkpoint</td>
<td>Cyclin D/CDK4,6, Cyclin E/CDK2</td>
</tr>
<tr>
<td>DNA Synthesis</td>
<td>S</td>
<td>DNA replication; Regulated by the S checkpoint</td>
<td>Cyclin A/CDK2</td>
<td></td>
</tr>
<tr>
<td>Gap 2</td>
<td>G2</td>
<td></td>
<td>Accelerated cell growth and protein synthesis</td>
<td>Cyclin A/CDK1</td>
</tr>
<tr>
<td>Cell Division</td>
<td>Mitosis</td>
<td>M</td>
<td>Cell division; Regulated by the Mitosis checkpoint</td>
<td>Cyclin B/CDK1</td>
</tr>
</tbody>
</table>
Table 2.2: Eukaryotic cell checkpoints and function

<table>
<thead>
<tr>
<th>Checkpoint</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Committed step; Cells pass this checkpoint if they are at an adequate size, have appropriate nutrients, growth factors, etc. If so, cells continue to S phase. Cells that do not meet these requirements go to G0</td>
</tr>
<tr>
<td>G2</td>
<td>If cells are the appropriate size with adequate protein reserves and chromosomal replication, cells continue to the M phase. If DNA damage errors are detected, G2 will pause to fix said errors. If irreparable, cell will undergo apoptosis or cell death</td>
</tr>
<tr>
<td>M</td>
<td>If chromosomes are attached on the metaphase plate, cell division will continue, leading to cell proliferation. If not, cells will undergo apoptosis</td>
</tr>
</tbody>
</table>

The G1 checkpoint is the restriction point, in which cells irreversibly commit to the cell cycle. If cell growth is not adequate, the cell will go to the resting (G0) phase until conditions improve. Checkpoint regulation is dictated through the binding of cyclins to CDKs that activate CDK phosphorylation of specific target proteins and signal the cell to continue to the next phase of cycle (Figure 2.2). Upon cyclin degradation, the CDK becomes deactivated, signaling the cell’s exit from a phase.\(^{18}\) Negative regulatory molecules, such as Retinoblastoma (Rb) monitor cell growth by inhibiting cell cycle progression until the cell is ready to advance.\(^ {19}\) For example, in the progression of G1 to S phase and in response to elevated cell growth, Cyclin E binds to CDK2 to phosphorylate Rb proteins in order to cause inactivation and trigger the release of transcription factor, E2F, which then binds to DNA to turn on gene expression and allow the cell to progress to the S phase (Figure 2.3).\(^ {19}\)

Similar to Rb proteins, negative regulatory inhibitors, such as tumor suppressor proteins, like the master gene regulator, p53, can further control the cell cycle at the G1/S checkpoint in response to genomic instability.\(^ {20}\) In normal eukaryotic cells, in response to DNA damage caused by a variety of factors, such as UV radiation, accumulation of reactive
Figure 2.2: The Formation of the Cyclin-CDK complex to initiate cell cycle progression\textsuperscript{21}

Figure 2.3: Retinoblastoma (Rb) protein phosphorylation to activate cell cycle progression\textsuperscript{21}
reactive oxygen species (ROS), etc., p53 can trigger repair mechanisms at the site of DNA damage so cells can continue to divide. As illustrated in Figure 2.4, the repair mechanism, such as nuclear excision repair, is unsuccessful, cells with the mutated genes, undergo cell death or apoptosis. Contrasting, when p53 is abnormal or mutated, as illustrated in over 50% of breast cancer cases, the cell cycle continues despite the presence of DNA damage, leading to the over-proliferation of cells with faulty or nonfunctional p53 that can eventually become cancerous. The genesis of cancerous tumors is a response to dysfunctional cell cycle regulation.

![Figure 2.4: Normal and abnormal tumor suppressor p53 function](image)

**2.2 Genetic Influences on Cancer Etiology**

The process of oncogenesis is characterized by changes in molecular and epigenetic factors, as well as abnormal cell division, as aforementioned, that cause dysfunctional
altering of tissue proliferation in normal cells leading to cancer cell growth. Cancer cells divide uncontro\%lably due to a variety of mutations in genetic mechanisms, such as the cell cycle. Proto-oncogenes are normal functioning genes that promote cell proliferation and division. When mutated, proto-oncogenes become oncogenes which have the potential to cause cancer. Mutations in oncogenes are often missense and affect a single allele leading to heterozygosity.\textsuperscript{25} The first oncogene characterized was \textit{c-myc} for chromosome translocation activation in Burkitt’s lymphoma, as well as breast cancer. With this translocation, the proto-oncogene is translocated from chromosome 8 to the chromosome 14 leading to abnormal expression and the manifestation of cancer.\textsuperscript{26} The transition of proto-oncogenes to oncogenes involves gain of function mutations via point mutations, gene amplification, and chromosomal translocation that will eventually lead to cancer induction (Figure 2.5).\textsuperscript{27}

![Diagram](image)

**Figure 2.5:** Pathway of transition of proto-oncogenes to oncogenes is licensed under CC BY 3.0 \textsuperscript{28}
Mutations in tumor suppressor genes, such as p53, are oncogenic and are defined by loss of function that lead to inactivation of regulatory proteins and activation of oncogenes. Tumor suppressor genes encode and regulate proteins that are associated with cell proliferation. The five main class of proteins encoded by tumor suppressor genes are: DNA repair enzymes, apoptotic promoters, cell cycle checkpoint control, hormone receptors, and intracellular proteins for cell cycle regulation. Mutations in tumor suppressor genes have recessive control on oncogenes which contrasts the dominant control of mutations in proto-oncogenes. Both tumor suppressor genes and proto-oncogenes play a primary role in cancer proliferation.

There are seven types of proteins (Figure 2.6) that modulate cell growth and when there are mutations in these proteins due to gain and loss of function mutations derived from proto-oncogenes and tumor suppressor genes, cancer can arise. For example, mutations in Class Ia proteins can activate growth factor receptors that can eventually induce cancers. Class VII cell cycle control proteins lead to increased mutations in other classes due to their regulatory function. Over-expression of receptor tyrosine kinases (Classes II and III proteins), such as HER-2/neu are the cause of 15-30% of breast cancers. Furthermore, amplification of HER-2/neu decreases effectiveness and activity of estrogen receptors and leads to continuous cell proliferation of cancerous tumors. These kinases add phosphate groups to specific target proteins causing the receptor to be constitutively activated leading to cancer cell proliferation. Mutations in these protein classes contribute to the defining characteristics of cancerous cells.

The hallmarks of cancer are defined by mutations derived from proto-oncogenes and tumor suppressor genes describe cancer cell proliferation and metastasis. The primary
hallmarks include selective growth, vascularization, immune modulation, and altered stress response to evade apoptosis and promote survival (Figure 2.8). Cancer cells can sustain growth signaling because they do not require stimulation via

Figure 2.6: Diagram of the seven protein classes for cell growth regulation

growth signals for proliferation contrasting that of normal cells that are regulated via homeostatic control.\textsuperscript{31} Cancer cells’ independence on growth factors directly correlates to their ability to produce their own growth factor ligands that can respond to receptor stimulation. Moreover, cancer cells can stimulate normal cells via signal transduction in order to sequester and supply the growth factors needed for cancer cell proliferation.\textsuperscript{34} Cancer cells’ dysfunctional proliferation by evading growth suppressors define the biological shift of pre-cancerous cells to that of cancerous cells.\textsuperscript{35} Tissue structure in normal cells are more organized and uniform. Hyperplasia is reversible and results from uncontrolled cell division in which cells lose their structural integrity. If this growth remains unregulated, dysplasia occurs relating to a severe lack of tissue uniformity. Severe dysplasia, defined as cancer, is localized with cells not yet metastasized or invaded to other parts of the body (Figure 2.8).
Cancer cells are able to evade apoptosis by altering mechanisms used to sense DNA damage and eventually preventing the downstream signaling of apoptosis. Likewise, sustained angiogenesis allows for cancer cells to have a constant supply of oxygen and other nutrients that promote cell viability. Vascularization in cancer cells occurs by exploiting physiological processes, such as wound repair in order to activate angiogenesis. For instance, the VEGF gene regulates vessel growth in postnatal and adult development. VEGF signaling is controlled by tyrosine kinases, that when overexpressed can lead to oncogene formation. The upregulation of neovasculature pathways can cause instances of hypoxia in cancer cells, related to another important hallmark of cancer cells. Dysregulated metabolism can be explained via the Warburg effect. Postulated by Nobel Prize winner, Otto Warburg in 1924, the hypothesis describes the production of ATP in cancerous cells via aerobic glycolysis leading to a decrease in both mitochondrial respiration and remodeling (decrease in lactate fermentation). This mitochondrial
deactivation decreases the mitochondrial membrane potential due to a loss of growth factors and energetic supply leading to hyperpolarization of various voltage gated channels preventing cytochrome c release to trigger secretion of various apoptotic factors. Because of mutations derived from tumor suppressor genes and proto-oncogenes are associated with oncogenesis, cancer cells are able to adapt to oxygen deficient conditions through shifting to glucose fermentation and lactate production in the cytosol. Although aerobic glycolysis is less efficient in energy production than that of mitochondrial oxidative phosphorylation, as exhibited in normal cells, it does lead to enhanced biomass and metabolites needed for cancer cell proliferation and survival. Understanding the molecular framework of cancer pathology can allow for efficient therapeutic options.

### 2.3 Breast Cancer Molecular Epidemiology

According to the Centers for Disease Control and Prevention (CDC), breast cancer is the most common cancer amongst all women despite race and ethnicity. In the United States, each year, there are over 200,000 and 2,000 new cases of breast cancer diagnoses in women and men, respectively. Breast cancer is defined as the abnormal proliferation of malignant cells in the breast tissue.

A majority of breast cancers initiate in the lobules or the ducts, which are the hollow vessels that drain milk to be excreted from the nipples. Figure 2.9 illustrates the presence of a malignant tumor within the lobules (milk producing glands) of the breast tissue. Often breast cancer can spread to other parts of the body, especially when malignant tumors reach the lymph nodes causing metastasis. There are at least ten different breast cancer types which
are classified by the location of the cancer within the breast tissue, as well as the cancer invasiveness (Table 2.3). Within those types, there are several molecular subtypes for breast cancer that are defined by the genes the cancer expresses (Table 2.4).
<table>
<thead>
<tr>
<th>Type</th>
<th>Abbreviation</th>
<th>Subtypes</th>
<th>Location</th>
<th>Treatment</th>
<th>Survival Rate/Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal carcinoma in situ</td>
<td>DCIS</td>
<td>-</td>
<td>Cancer starts within the milk duct and does not spread into surrounding breast tissue</td>
<td>Lumpectomy, radiation therapy, and hormone therapy</td>
<td>Risk increases with age; 25% of breast cancers are DCIS; Less than 1% of patients die or have risk of metastasis if have pure DCIS</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>IDC</td>
<td>Medullary ductal carcinoma</td>
<td>Manifests in milk ducts and metastasizes to other tissues</td>
<td>Lumpectomy, mastectomy, chemotherapy, radiation therapy, hormone therapy</td>
<td>Accounts for 3-5% of all breast cancers; Common in women with BRCA1 mutation</td>
</tr>
<tr>
<td>Mucinous (Colloid) ductal carcinoma</td>
<td></td>
<td></td>
<td>Cancer initially manifests in the milk ducts and spread to mucus lining in other parts of the body</td>
<td>Lumpectomy, mastectomy, chemotherapy, hormone therapy, radiation therapy</td>
<td>1-7% of all breast cancers; Tends to effect elderly women between the ages of 56-above</td>
</tr>
<tr>
<td>Papillary ductal carcinoma</td>
<td></td>
<td></td>
<td>Cancer initially manifests in milk ducts and move to the papules; less likely to be in lymph</td>
<td>Lumpectomy, mastectomy, chemotherapy, hormone therapy, radiation therapy</td>
<td>Less than 3% of breast cancers</td>
</tr>
<tr>
<td>Tubular ductal carcinoma</td>
<td></td>
<td></td>
<td>Manifests in milk ducts then spread to the</td>
<td>Lumpectomy, mastectomy, chemotherapy, hormone therapy</td>
<td>Can account for 8-27% of breast cancers</td>
</tr>
<tr>
<td>Type</td>
<td>Abbreviation</td>
<td>Subtypes</td>
<td>Location</td>
<td>Treatment</td>
<td>Survival Rate/Prevalence</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
<td>----------</td>
<td>----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Lobular carcinoma in situ</td>
<td>LCIS</td>
<td>-</td>
<td>tubules; often slow growing</td>
<td>therapy, radiation therapy&lt;sup&gt;53&lt;/sup&gt;</td>
<td>30-40% for women with LCIS and 12.5% for women on average&lt;sup&gt;57&lt;/sup&gt;</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>ILC</td>
<td>-</td>
<td>Cancer spreads to the lobules&lt;sup&gt;57&lt;/sup&gt;</td>
<td>Mastectomy, hormone therapy&lt;sup&gt;57&lt;/sup&gt;</td>
<td>Accounts for about 10% of breast cancers&lt;sup&gt;58&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inflammatory</td>
<td></td>
<td>-</td>
<td>Swelling and redness at the site of the lump&lt;sup&gt;59&lt;/sup&gt;</td>
<td>Lumpectomy, mastectomy, chemotherapy, hormone therapy, radiation therapy&lt;sup&gt;58&lt;/sup&gt;</td>
<td>Less than 1% of breast cancers&lt;sup&gt;59&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male breast cancer</td>
<td></td>
<td>-</td>
<td>Cancer forms in breast glands that can develop as a side effect of various medications&lt;sup&gt;60&lt;/sup&gt;</td>
<td>Lymph node surgery, chemotherapy, hormone therapy, radiation therapy&lt;sup&gt;60&lt;/sup&gt;</td>
<td>Less than 1% of breast cancers&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 2.4: Breast Cancer Molecular Subtypes

<table>
<thead>
<tr>
<th>Name</th>
<th>Receptor Profile</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>Hormone-receptor positive (estrogen and/or progesterone); HER-2 negative</td>
<td>30-70% of all breast cancers</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Hormone-receptor positive (estrogen and/or progesterone); HER-2 negative or positive</td>
<td>10-20% of all breast cancers</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>Hormone receptor (estrogen and progesterone) and HER-2 negative</td>
<td>15-20% of all breast cancers</td>
</tr>
<tr>
<td>HER-2 enriched</td>
<td>Hormone receptor negative (estrogen and progesterone) and HER-2 positive</td>
<td>5-15% of all breast cancers</td>
</tr>
</tbody>
</table>

Breast cancer can be classified by two main groups: carcinomas and sarcomas.

Carcinomas represent most breast cancers and are defined as cancers that arise from the epithelial tissues of the breast, such as the lobules and ducts. Contrastingly, representing less than 1% of breast cancers, sarcomas arise from supporting cellular structures, such as blood vessels. The most common types of breast cancer are invasive ductal carcinoma in which cancer can grow outside the milk ducts and spread to other parts of the body and invasive lobular carcinoma in which cancer initially manifests in the lobules of the breast and can metastasize to other parts of the body.

Risk of acquiring any type of breast cancer can relate more to genetic and hormonal, rather than that of environmental factors. Although, ionizing radiation as well as electromagnetic fields are exogenous factors that cause the manifestation of breast cancer amongst women, especially those patients that have been exposed to radiation via medical
procedures and atomic bomb survivors. Histologically, breast cancers can be classified based on the presence or lack of molecular markers, such as estrogen, progesterone, and human epidermal growth factor (HER-2). Over 700,000 breast cancer cases are hormone receptor positive. Estrogen is a female hormone made primarily in the ovaries that is responsible for sexual development and reproductive functions. Between the stages of menstruation, offset by puberty to that of menopause, estrogen stimulates the growth of normal breast epithelial tissue. Unlike that of the uterus, which sheds its lining due to hormonal fluctuations in estrogen and progesterone during menstruation, breasts can have a buildup of estrogen leading to enhanced risk of developing hormone receptor positive (estrogen and/or progesterone) based cancers. Because of this taking estrogen based oral contraceptives and menopausal hormone replacement therapy, can increase the risk of breast cancer. Risk factors, including age of first menstruation, age of first full term pregnancy, etc. also influence breast cancer etiology. Age and gender play a significant role in breast cancer prevalence with women over the age of 50 comprising 80% of breast cancer cases. This age disparity can be characterized mostly by reproductive hormones that can aid in breast cancer oncogenesis. Estrogen receptor overexpression in normal breast epithelial can increase the risk of developing breast cancer in women leading to decreased apoptosis, increased DNA damage, and amplified proliferation of breast cancer cells.

The HER-2 protein is a type of epidermal growth factor, tyrosine kinase, and an oncogene that contributes to over 20% of invasive breast cancer cases when overexpressed. Both estrogen and HER-2 proteins signal transduction pathways are related to the malignant proliferation of breast cancer cells in 85% of breast cancer cases. HER-2 amplified breast cancers often metastasize to viscera and the CNS, as well as have high proliferation rates and
low estrogen and progesterone receptor levels.\textsuperscript{72} Many HER-2 positive cancers also have estrogen receptor positive expression (although in a smaller ratio as compared to HER-2) that are related to p53 dysregulation and increased aneuploidy within the cancer genome.\textsuperscript{17} HER-2 overexpression can be lost in some tumors after endocrine therapy causing these tumors to become HER-2 negative and evading cell death. Similarly, estrogen receptors can downregulate HER-2 protein and vice-versa further explaining the aggressive nature of triple negative breast cancer (TNBC).\textsuperscript{71}

As mentioned, genetics plays a crucial role in the etiology of breast cancer. Hereditary breast cancers are related to mutations in the \textit{BRCA1} and \textit{BRCA2} tumor suppressor genes and account for 5-10\% of breast cancer cases.\textsuperscript{73} The \textit{BRCA1} gene is composed of 22 exons that encode for a protein of 1863 amino acids.\textsuperscript{74} Most of the mutations associated with this gene involve missense related to insertion and deletions of base pairs. The \textit{BRCA2} gene is larger than that of \textit{BRCA1}, with 10.3kb of open reading frame and is associated with over 1800 germline mutations related to deletions, insertions, and loss of function mutations in breast cancers.\textsuperscript{73} Women with the \textit{BRCA1} mutation have a 50-65\% chance of developing breast cancer by the age of 70.\textsuperscript{75} In contrast, mutations in the \textit{BRCA2} gene account for 45\% of breast cancer development by the age of 70.\textsuperscript{76} Moreover, \textit{BRCA1} is associated more with TNBC and are more aggressive as compared to \textit{BRCA2} associated breast cancers that are primarily hormone receptor positive (estrogen or progesterone receptors) breast cancers that manifest in post-menopausal women.\textsuperscript{73} Understanding the pathogenesis of breast cancer susceptibility genes can give more insight into the etiology of cancer in order to improve therapeutic options.
2.4 Breast Cancer Chemotherapeutics

Chemotherapy is a systematic therapeutic treatment to either limit cancer cell viability by slowing cancer cell growth or triggering apoptosis of cancer cells. Current chemotherapeutic options are plagued with issues of poor water solubility, resistance, cytotoxicity on normal cells, poor targeting, etc. Most chemotherapeutic medications are given intravenously (IV), intramuscularly (IM), subcutaneous (SC), and orally via a pill or tablet.

Breast cancer chemotherapeutic treatments can be given after surgery (adjuvant) to kill any remaining cancer cells; before surgery (neoadjuvant) to shrink tumor size before surgery for localized breast cancer; and for metastasized advanced breast cancer. There are several different categories of drugs used for adjuvant, neoadjuvant, and advanced staged breast cancers that can be used in a combinatorial manner (Table 2.5). The anthracycline drug, doxorubicin cures about 20-35% of patients when used in adjuvant therapy and low level metastases access. In order to enhance anthracycline drug delivery, minimize dilapidating side effects of cardiotoxicity, and increase therapeutic efficiency, liposomal formulations are used. Both pegylated and non-pegylated liposomal encapsulated doxorubicin are on the market in Europe, Canada, and the United States with maximum tolerated dosages being 50 mg/m² every 4 weeks and 75 mg/m² every 3 weeks, respectively. Taxanes are commonly used anti-cancer agents despite their poor solubility. Currently, there are two FDA approved drugs for prevention of breast cancer: Raloxifene Hydrochloride and Tamoxifen Citrate. These drugs are aromatase inhibitors that block the estrogen
overexpression in women leading to lower risk of breast cancer. Depending on baseline risk of the patient, the drugs, when used concomitantly, and lower risk of invasive and DCIS breast cancers by 40%. The drugs also have implications in preventing osteoporosis.\textsuperscript{82}

Table 2.5: Types of breast cancer chemotherapeutics

<table>
<thead>
<tr>
<th>Chemotherapy</th>
<th>Mechanism of Action</th>
<th>Drug Example</th>
<th>Dose/Route</th>
</tr>
</thead>
</table>
| Anthracyclines\textsuperscript{83} | Cytotoxic drugs from antibodies that inhibit DNA and RNA synthesis\textsuperscript{84} | Doxorubicin, Epirubicin\textsuperscript{84} | **Doxorubicin:** 60–75 mg/m\textsuperscript{2} via IV for 21-day intervals as monotherapy and 40–60 mg/m\textsuperscript{2} via IV for 21–28-day intervals in combination therapy; Max recommended cumulative dose: 450–500 mg/m\textsuperscript{2} \textsuperscript{84}  
**Epirubicin:** 100–120 mg/m\textsuperscript{2} via IV for 3–4-week intervals; Max cumulative dose: 900 mg/m\textsuperscript{2} \textsuperscript{84} |
| Taxanes\textsuperscript{85} | Identified in yew plants and cause microtubule dysfunction inhibiting cell division\textsuperscript{85} | Paclitaxel, Docetaxel\textsuperscript{85} | **Paclitaxel:** 175 mg/m\textsuperscript{2} via IV over 3h for 3 weeks 4x for monotherapy and 175 mg/m\textsuperscript{2} via IV over 3h for 3 weeks after 6 months relapse for aggressive cancers\textsuperscript{86}  
**Docetaxel:** 60–100 mg/m\textsuperscript{2} via IV over 3 weeks for monotherapy and 75 mg/m\textsuperscript{2} via IV for 21-day intervals in |
<table>
<thead>
<tr>
<th>Chemotherapy</th>
<th>Mechanism of Action</th>
<th>Drug Example</th>
<th>Dose/Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-fluorouracil (5-FU)</td>
<td>Acts as an anti-metabolite that acts as a pseudo-nutrient to kill cancer cells</td>
<td>Adrucil</td>
<td>500-600 mg/m² via IV on days 1 and 8 of a 28-day schedule</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Acts as an alkylation agent to damage genome of cancer</td>
<td>Cytoxan</td>
<td>600 mg/m² IV with other anti-cancer drugs</td>
</tr>
</tbody>
</table>

Table 2.6: Targeted breast cancer treatment options

<table>
<thead>
<tr>
<th>Therapy Type</th>
<th>Definition</th>
<th>Drug Function</th>
<th>Drug Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone</td>
<td>Inhibits or stops the growth of hormone sensitive tumors (ER+ or PR+)</td>
<td><strong>Block ovarian function</strong>- drugs act as gonadotropin-releasing hormone (GnRH) agonists that interfere with pituitary gland signals that stimulate estrogen production in ovaries.</td>
<td>Zoladex® and Lupron®</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Block estrogen production</strong>- drugs act as aromatase inhibitors that impede estrogen production. Used in both pre and post-menopausal women to regulate estrogen production</td>
<td>Arimidex®, Femara®, Aromasin®, (permanently inactivates aromatase)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Block estrogen effects</strong>- drugs impede cell</td>
<td>SERMs: Nolvadex®, (tamoxifen)</td>
</tr>
<tr>
<td>Therapy Type</td>
<td>Definition</td>
<td>Drug Function</td>
<td>Drug Examples</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>proliferation. Selective estrogen receptor modulators (SERMs) bind to estrogen receptors to block estrogen binding. Other anti-estrogen drugs bind to the receptor to initiate cell death</td>
<td>Other anti-estrogen drugs: Faslodex®</td>
</tr>
<tr>
<td>PARP inhibitors</td>
<td>Blocks DNA repair signaling</td>
<td>Block the effects of growth factors in HER-2 negative breast cancers</td>
<td>Olabarib®</td>
</tr>
<tr>
<td>mTOR inhibitors</td>
<td>Blocks mTOR to prevent angiogenesis</td>
<td>Block the effects of growth factors in HER-2 negative breast cancers</td>
<td>Everolimus®</td>
</tr>
<tr>
<td>CDK inhibitors</td>
<td>Blocks activity of cyclin dependent kinases</td>
<td>Block the effects of growth factors in HER-2 negative breast cancers</td>
<td>Palbociclib®</td>
</tr>
<tr>
<td>Tyrosine kinase</td>
<td>Blocks signals for tumor cell growth</td>
<td>Block the effects of growth factors in HER-2 positive breast cancers</td>
<td>Lapatinib®</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal antibodies</td>
<td>Uses laboratory grade antibodies to target cancer cell proliferation and survival metabolites</td>
<td>Block the effects of growth factors in HER-2 positive breast cancers</td>
<td>Trastuzumab®</td>
</tr>
<tr>
<td>Immunotherapies</td>
<td>Triggers the immune system to kill cancer cells</td>
<td><strong>Immune checkpoint inhibitors</strong>-drugs that restore immune response to breast cancer cells. PD-L1 is a protein overexpressed in tumor cells and when blocked can slow down TNBC growth.</td>
<td>Tecentriq®[^93^]</td>
</tr>
</tbody>
</table>
Because cancer cells can develop resistance to standard therapies, novel therapy approaches seek to mitigate these concerns. ER+ breast cancers, endocrine therapy drugs are used combinatorialy for enhanced ER+ breast cancer cell death. CDK inhibitor drugs, like Palbociclib, is a CDK4/6 inhibitor. These CDKs act on the cell cycle and activate cyclin D1. Even in hormonal resistance, ER+ breast cancer cell depends on the CDK4/6-cyclin D1 interaction for proliferation. CDK inhibitor drugs can downregulate the phosphorylation of Rb to mediate G1-S phase cell cycle arrest. Moreover, with respect to TNBCs, the most aggressive breast cancer cell type, targeted therapies are limited because TNBC molecular targets can overlap with that of normal breast epithelial cells. Many TNBC targeted therapies work to down regulate the expression of cell survival mechanisms in cancer, such as VEGF for angiogenesis, growth factors, and DNA repair mechanisms. Targeting molecular modalities in breast cancer can lead to more effective tailored breast cancer treatments in patients.

2.5 Dichloroacetate Metabolism and the Warburg Effect

Dichloroacetate (DCA) is the small synthetic salt derived from the halogenated organic acid, dichloroacetic acid. Dichloroacetic acid is found naturally in the red microalgae Asparagopsis taxiformis, like other halogenated organic acids, such as dibromoacetic acid. Also, trace amounts of dichloroacetic acid are found in drinking water and can be used as a disinfecting by-product for swimming pools. Like that of trichloroacetic acid (TCA), dichloroacetic acid can be used for cosmetic purposes, such as chemical peels. Moreover, in pregnant patients, dichloroacetic acid is administered in concentrations up to 85% in alcohol for the treatment of genital warts. Similarly, the acid can be utilized as a cauterizing agent
for other skin maladies, such as callous removal, in-grown nails, and cyst removal in the reproductive system.  

The salt compound of dichloroacetic acid is produced via water chlorination and is often ionically bound to a sodium cation (Figure 2.11). Sodium dichloroacetate, what is often referred to as DCA, has potential antineoplastic activity via the activation of apoptotic pathways in cancer cells through a decrease in extracellular lactate production, increase in reactive oxygen species (ROS) intracellularly, and the inhibition of glycolysis. DCA is often used in the treatment of lactic acidosis by reducing plasma lactic acid concentration via the activation of pyruvate dehydrogenase (PDH). This activation stimulates the Krebs cycle by oxidizing pyruvate to acetyl-CoA, rather than fermentation, which decreases lactate accumulation in the cell.

Figure 2.10: (A) Dichloroacetic Acid and (B) Sodium Dichloroacetate salt

Dichloroacetic acid and its salt derivative have a variety of benefits, including lipotropic on diabetic rats. DCA derivatives, such as diisopropylammonium dichloroacetate (DIPA), exert a prolonged hypoglycemic effect in diabetic rats without altering the blood glucose levels in normal rats. When used concurrently on diabetic rats, DIPA and DCA decreased fatty acid oxidation in muscle cells, reduced intracellular citrate levels leading to the reactivation of phosphofructokinase (PFK). Chlorinated acetic acids, like DCA, were used to synthesize dialkylamino acetates, like pangamic acid or vitamin
Vitamin B, as with thiamine, in high dosages (75x the recommended daily allowance) can reduce cancer cell proliferation. This occurs in a similar mechanism to that of DCA by inhibiting PDK activation causing reduced PDH phosphorylation, induction of apoptosis, reduction in glucose consumption and lactate production in human neuroblastoma (SK-N-BE) and pancreatic (Panc-1) cancer cell lines as compared to DCA.104

Due to DCA’s small molecular weight (150 g/mol), it is able to diffuse through most plasma and mitochondrial membranes.105 Relatedly, DCA is rapidly absorbed leading to its high oral bioavailability. Unfortunately, due to DCA’s ease in traversing cellular membranes through pyruvate transport pathways, it can easily cross the blood brain barrier (BBB) causing strong neurotoxicity.106 Other limitations such as high dose requirement (25-50 mg/kg/day), rapid clearance (4.82L/h), short half-life (1 h) and the strong neurotoxicity, have limited its clinical use, further illustrating the need for a targeted delivery approach of this anticancer agent is critical to maximize its benefits.13

DCA is dehalogented to glyoxylate, which is inactive toward the pyruvate dehydrogenase complex (PDC) due to the enzyme glutathione transferase zeta1 (GSTZ1) in the cytosol and mitochondrial.88 Chronic administration of DCA can lead to inhibition of DCA metabolism. DCA is metabolized to glyoxylate in the liver by the enzyme GSTZ1 and long term exposure of DCA leads to the inhibition of GSTZ1 causing decreased protein expression and deactivation of the phenylalanine/tyrosine catabolic pathway. GSTZ1 is overexpressed in cancer, especially breast cancer, which may lead to resistance to the antitumorgenic effects of chemotherapeutic metabolic regulator drugs, such as DCA.107 Therefore, there is a need to enhance DCA’s targeting efficiency as a protective measure.
As a glycolytic inhibitor, DCA can be utilized to enhance targeting in cancer chemotherapeutics. DCA, a pyruvate mimic, stimulates oxidative phosphorylation by targeting PDK, the inhibitor of PDH causing pyruvate to be oxidized in the mitochondria and the ultimate reversal of the Warburg effect.\textsuperscript{8,11} As aforementioned, cancer cells, even in aerobic environments, favor glycolytic metabolism instead of mitochondrial oxidative phosphorylation. In normal cells, oxidative phosphorylation produces about 36 ATP for every glucose molecule as compared to the two ATP produced via glycolysis. Cancer cells often overexpress glucose transporters on the mitochondrial surface in order to enhance glucose uptake and increase ATP production for cell survival.\textsuperscript{105}

Cancer cells reliance on aerobic glycolysis and lactate fermentation to produce energy is less efficient in energy production, but allows the genesis of metabolites for cancer cells to grow. In cancer cells, PDK regulates pyruvate flux into the cytoplasm and, through the inhibition of PDH, PDK ensures aerobic or cytoplasmic glycolysis and limits mitochondrial oxidative phosphorylation for energy production. PDH is activated when the carboxylate group of DCA is bound to the Arg-154 residue of PDK forming a salt bridge. The chlorine molecules of DCA are located within the hydrophobic pocket.\textsuperscript{108} PDK inhibition happens rapidly \textit{in vivo} within 15-30 minutes, but diminishes within 12-24 hours after oral administration.\textsuperscript{88} DCA, as a pyruvate mimic, can reverse the Warburg effect and activate PDH to switch cancer cells’ metabolic profile to oxidative phosphorylation from cytoplasmic glucose oxidation and eventual apoptosis.

In a study of reversing apoptosis resistance in pulmonary arterial smooth muscle cells when treated with DCA and platelet-derived growth factor (PDGF), results show that when used concomitantly, DCA and PGDF activate the phosphatidylinositol 3-kinase
(P13K)/protein kinase B (Akt) apoptotic pathway as compared to DCA and PDGF alone. Moreover, mitochondrial hyperpolarization was reversed in patients suffering from glioblastoma multiforme when treated with DCA. DCA also enhanced p53 function, inhibited angiogenesis, hypoxia-inducible factor-1a (HIF1a), which is a pro-apoptotic factor. Furthermore, an *in vitro* study illustrated that DCA can trigger apoptosis at concentrations higher than 25 mM at 48 h, as well as promote ROS production in a variety of cancer lines, including lung, leukemia, and breast. In a study analyzing DCA metabolic function on cisplatin-sensitive human ovarian cancer (A2780) and the cisplatin-resistant clone (A2780/DD0P) cell lines, DCA treatment resulted in decreased expression of mitochondrial uncoupling protein 2 (UCP2). This decrease in expression is related to increases oxidative stress by ROS causing a metabolic switch from glycolysis to oxidative phosphorylation. Because DCA acts as a glycolytic inhibitor and cancer cells often have a resistance to apoptosis because of enhanced mitochondrial dysfunction, DCA is an effective candidate for cancer therapy. Since DCA can trigger apoptotic pathways through the reversal of the Warburg effect, DCA could act as an attractive chemotherapeutic when used prior or concurrently with other anti-cancer drugs to increase the effectiveness of DCA and perhaps decrease the toxicity limitations of other chemotherapeutics.
CHAPTER 3
SYNTHESIS AND PHYSICOCHEMICAL ANALYSIS OF PHENOXYETHYL DICHLOROACETAMIDE (PE-DCA) PRODRUG

3.1 Rationale

Due to the conjugation of 2-phenoxyethylamine and DCA, the physicochemical properties of phenoxyethyl dichloroacetamide (PE-DCA; Compound 3) have changed compared to native DCA suggesting improved targeting efficiency and cytotoxic effects on breast cancer cells. Understanding the physicochemical properties of PE-DCA, like other anticancer therapeutic agents, is critical in the optimization of drug delivery and formulation. These changes can address the limitations of not only DCA, such as high dose requirements, but also common limitations of cancer chemotherapeutics, such as poor solubility. The enhancement of physicochemical and biological properties of PE-DCA can influence the pharmacokinetic and clinical behavior of PE-DCA on breast cancer.

3.2 Materials

2-Phenoxyethylamine, dichloroacetic anhydride (DCA-A), triethylamine (TEA), and dichloromethane were purchased from Sigma Aldrich (St. Louis, MO) and used without any further purification. Deionized water for all the experiments was obtained through a Millipore Milli Q water purification system (Millipore Corp., Danvers, MA). All other chemicals were of analytical grades and used as obtained from the suppliers.

3.3 Methods

3.3.1 Synthesis of Phenoxyethyl Dichloroacetamide (PE-DCA; Compound 3)

As depicted in the synthetic scheme in Figure 3.1, PE-DCA was synthesized by reacting 2-phenoxyethylamine (1; 0.55g, 4.00 mmol) with dichloroacetic anhydride (2; 0.46
mL, 3.00 mmol) and a catalytic amount of triethylamine (TEA, 0.42 mL, 3.00 mmol). The components were mixed initially at 0 °C for 1 h in dichloromethane (DCM), then allowed to warm at room temperature for 12 h. The reaction contents were poured on to water and extracted with saturated sodium bicarbonate (10 mL), hydrochloric acid (0.1 M, 10 mL), and then saturated sodium chloride (three times; 10 mL). The organic layer was dried with magnesium sulfate and filtered. The solvent was removed under reduced pressure. The residue was removed under column chromatography (silica gel; eluent:10% methanol in DCM) yielding 2,2-dichloro-N-(2-phenoxyethyl)acetamide (3; PE-DCA; 0.364 g; > 87% yield).

![Figure 3.1: Synthetic scheme of PE-DCA.](image)

**3.3.2 Proton Nuclear Magnetic Resonance (\textsuperscript{1}H NMR spectrum of #3)**

PE-DCA was characterized via 400 MHz \textsuperscript{1}H NMR spectrometer (Varian Inova, Palo Alto, CA). Approximately, PE-DCA (13 mg) was dissolved in deuterochloroform (700 µL; CDCl\textsubscript{3}) and added to an NMR tube (Sigma Aldrich, St. Louis, MO) for evaluation. NMR data was processed using VNMRJ. Spectra peaks were further confirmed using ChemDraw Professional v16.

**3.3.3 Carbon Nuclear Magnetic Resonance (\textsuperscript{13}C NMR spectrum of #3)**

PE-DCA was further characterized via 400 MHz spectrometer (operating at 100 MHz for \textsuperscript{13}C; Varian Inova, Palo Alto, CA). PE-DCA (13 mg) was dissolved in deuterochloroform
(700 µL; CDCl₃) and added to an NMR tube (Sigma Aldrich, St. Louis, MO) for evaluation. NMR data was processed using VNMRJ software. Spectra peaks were further confirmed using ChemDraw Professional v16.

3.3.4 Fourier-transform infrared spectroscopy (FTIR)

FTIR analysis of the final PE-DCA product was conducted using Agilent Cary 630 FTIR (Agilent Technologies, Santa Clara, CA). The FTIR apparatus uses Microlab software Version 4.0. About 2 mg of PE-DCA product was used for the analysis with values within 600-4000 cm⁻¹ range.

3.3.5 Liquid Chromatography-Mass Spectroscopy (LC/MS)

To elucidate the structure of the final PE-DCA product, LC/MS analysis was performed with electrospray ionization (ESI) in a positive ion mode on QTrap® API-3200 mass spectrometer equipped with Shimadzu quaternary pump, vacuum degasser, DAD detector and autosampler (Shimadzu Scientific Instruments, Columbia, MD). Data acquisition and processing were performed by Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA, USA).

3.3.6 Powder X-Ray Diffraction (XRD)

XRD analysis was performed to assess crystallinity of PE-DCA compared to native DCA. This experiment was executed at room temperature on a MiniFlex automated XRD instrument (Rigaku, Woodland, TX). The instrument utilizes a Cobalt tube, Fe-filtered Cu K-alpha radiations at 30 kV and 15 mA. The pattern was run in step scan mode with 0.05° step size and 3 second count time per step (1°2Θ/minute). Jade 8+ (Material Data, Inc, 56 Livermore, CA) was employed to process the diffraction patterns. No specific sample preparation was needed for this analysis.
3.3.7 Scanning Electron Microscopy

Surface morphology of PE-DCA compared to native DCA was evaluated with scanning electron microscopy (SEM) CM12 (FEI, Hillsboro, OR) at 80 kV accelerating voltage. A small amount solid sample was placed on a copper grid covered with carbon film and air dried. Digital images are acquired with an ORIUSTM SC 1000 11 Megapixel CCD camera (Gatan, Pleasanton, CA).

3.3.8 Melting Point Range Detection

The melting point of PE-DCA was determined using a Thomas Hoover Uni-Melt Capillary Melting Apparatus was used. The temperature was adjusted slowly with the sample (placed in capillary tube) on the capillary stage. The melting point was determined from the start of melting until its completion and measured in triplicate. The reported resulted from the average. Chemglass Melting Point Tubes-Closed One End capillary tubes were used (Thomas Scientific, Swedesboro, NJ).

3.4 Results and Discussion

After synthesis and purification, the observed product was composed of fine white crystals. Melting point range was determined to be 89.4 - 92.7 °C. FTIR spectroscopy, NMR (1H and 13C) spectroscopy, and LC-mass spectrometry (LC-MS) were used as characterization tools to verify the PE-DCA chemical structure, molecular weight, and purity.

PE-DCA was synthesized with a yield of 87.11%. LC/MS was performed on positive mode (M+1). Analysis indicated a value of 248 m/z (Figure 3.2) suggesting an exact mass of 247 g, which is consistent with the expected value as delineated in ChemDraw Professional (Table 3.1). Other peaks on the LC-MS spectra represent secondary product peaks based on the relative abundance of chlorine isotopes (Figure 3.2). Because LC-MS analysis was done
under positive mode, the PE-DCA final product contains the $^{35}\text{Cl}$ isotope with an 100% relative abundance as confirmed through analysis (Table 3.1). Moreover, analysis of the full PE-DCA spectra show not only the chlorine isotopic peaks associated with the final PE-DCA product, but also that of contaminates, at 153.9818 and 265.0499 m/z (Figure 3.3). The neutral chemical formula for PE-DCA contaminate at 153.9818 m/z was determined to be $\text{C}_4\text{H}_5\text{Cl}_2\text{NO}$ with a proposed chemical structure deduced (Figure 3.4A and B). The structure represents the dichloroacetamide portion of PE-DCA (2,2-dichloro-N-ethylacetamide) which has a 15% relative abundance as compared to PE-DCA (~70%, Figure 3.3). The other contaminate peak at 265.0499 m/z has an additional NH$_3$ group as compared to the final PE-DCA product. Further analysis can ascertain the chemical structure, but the neutral chemical formula was determined to be $\text{C}_{10}\text{H}_{14}\text{Cl}_2\text{N}_2\text{O}_2$ (Figure 3.5). This contaminate as a relative abundance, according to LC-MS of 20% (Figure 3.3).

Figure 3.2: LC/MS EMS spectrum showing PE-DCA molecular weight of 247 g/mol
Table 3.1: Chemical analysis of PE-DCA

<table>
<thead>
<tr>
<th>Chemical Formula</th>
<th>C_{10}H_{11}Cl_{2}NO_{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exact Mass</td>
<td>247.02 g</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>248.10 g/mol</td>
</tr>
<tr>
<td>m/z</td>
<td></td>
</tr>
<tr>
<td>35Cl: 247.02 (100.0%)</td>
<td></td>
</tr>
<tr>
<td>37Cl: 249.01 (63.9%)</td>
<td></td>
</tr>
<tr>
<td>36Cl: 248.02 (10.8%)</td>
<td></td>
</tr>
<tr>
<td>39Cl: 251.01 (10.2%)</td>
<td></td>
</tr>
<tr>
<td>38Cl: 250.02 (6.9%)</td>
<td></td>
</tr>
<tr>
<td>40Cl: 252.01 (1.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.3: Full LC-MS spectrum of PE-DCA
In addition to being present in the MS spectrum of PE-DCA, this molecule is a product in the MS/MS of both PE-DCA and "PE-DCA + plus NH_3" (m/z 265).
Figure 3.4: A: LC-MS spectra for PE-DCA contaminate at 153.98179 m/z and B: Zoomed spectra
Furthermore, FTIR comparison spectra for 2-phenoxyethylamine (PE), dichloroacetic anhydride (DCA-A), and PE-DCA were obtained to further prove the presence of the final pure PE-DCA product. As illustrated in Figure 3.6, defining spectra of PE-DCA are as follows: N-H bend peaks for secondary amides at 1650 and 3330 cm\(^{-1}\), respectively; C-H bending bands of the aromatic group are located at 650-900 cm\(^{-1}\); and C-C stretch bands of the aromatic ring at about 1620 cm\(^{-1}\).
The $^1$H and $^{13}$C NMR spectra was performed to confirm the presence of the final PE-DCA product. The proton NMR data is depicted in Figure 3.7 and characterized PE-DCA peaks: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.34 – 7.23 (m, 2H), 6.98 (tt, $J = 7.4$, 1.1 Hz, 2H), 6.93 – 6.86 (m, 2H), 5.93 (s, 1H), 4.08 (dd, $J = 5.5$, 4.7 Hz, 2H), 3.73 (td, $J = 5.7$, 4.7 Hz, 2H) ppm. The amine group (NH) is hidden under the triplet at 7.02 ppm due to broadening of the baseline. The resonance at 1.5 ppm represents an impurity, such as a methyl group. There are small amounts of impurities in the PE-DCA final product derived from secondary products of the conjugation reaction, and those present can be associated with the contaminates presented in the LC-MS spectra (Figures 3.4 and 3.5).
Figure 3.7: $^1$H NMR spectrum of PE-DCA.

The carbon NMR peaks for the PE-DCA final product are given in Figure 3.8: $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 39.86, 65.95, 66.27, 114.54, 121.48, 129.62, 158.20, 164.27 ppm. All eight carbon resonances are observed (Figures 3.8), and there are no additional carbon peaks in the spectra signifying that the PE-DCA product is rather pure.
Figure 3.8: $^{13}$C NMR spectrum for PE-DCA

Powder X-Ray Diffraction (P-XRD) analysis of DCA confirm its crystalline structure (Figure 3.9A). PE-DCA was also determined to be crystalline in structure using parallel, monochromatic radiation (Figure 3.9B). The sharp peaks of DCA are not present in the PE-DCA compound suggesting that through the chemical reaction of 2-phenoxyethylamine and dichloroacetic anhydride, the conjugation has led to a new compound (Figure 3.9C). This relates to the variation in crystalline structure of native DCA as compared to PE-DCA. As illustrated in the Scanning Electron Microscopy (SEM) data, native DCA is composed of stacked rod-like crystal morphology as visualized with 10,000x and 2000x microscopy (Figure 3.10A-C).
Figure 3.9: P-XRD data for: (A) Native DCA, (B) PE-DCA, and (C) Stacked Native DCA and PE-DCA
The final PE-DCA crystalline structure has a completely different structural morphology than that than native-DCA (Figure 3.10A-D) which further confirms XRD data that the S_N2 conjugation of 2-phenoxyethylamaine and dichloroacetic anhydride forms a completely novel formulation, PE-DCA. Based on SEM analysis, PE-DCA is composed of groups of spheres (Figure 3.11A) and the remnants of the native-DCA crystalline morphology of fragmented rods and flattened plaques (Figures 3.11B-D). The structural integrity of native-DCA crystals have been compromised in the conjugation to form PE-DCA which can have an influence on the intracellular uptake of this formulation on breast cancer cells.
3.5 Conclusion

Assessing the physicochemical properties of PE-DCA can allow for a better understanding of the therapeutic effects of the formulation on breast cancer cells. Understanding the physical and chemical properties can aid in elucidating the mechanism of actions of the drug via bioanalytical assays. Because the molecular weight of PE-DCA (248 g/mol) differs from that of DCA (150 g/mol), it may not be easily able to diffuse through plasma and cellular membranes, suggesting enhanced selectivity to cancer cell types.

It has been illustrated that, PE-DCA, like other aryloxyethylamine derivatives, can be used as herbicides, especially when chloro-substituted rather than bromo. The structure of
PE-DCA is similar to that of other phenoxyethanamine derivatives, such as the anti-cancer drug, Tesmilifene (DPPE), suggesting that PE-DCA, like that of DPPE, can have enhanced cytotoxic and antineoplastic activity on metastatic breast cancer cells.\textsuperscript{114} Tesmilifene is a small anti-cancer drug that reached phase III clinical trials for aggressive metastatic breast cancers in the early 2000s (Figure 3.12). When co-administered with doxorubicin, a chemotherapy medication used to treat various cancers, including breast cancer by blocking the growth enzyme, topoisomerase 2 to arrest cancer cell proliferation, breast cancer patients had a 50\% higher survival rate than those patients treated with doxorubicin alone.\textsuperscript{115} Tesmilifene has a binding affinity for the antiestrogen receptor binding site (AEBS) in the cellular microsomes of cytochrome P450 enzymes and P-glycoprotein leading to efflux inhibition and decreased cancer cell proliferation.\textsuperscript{116-117} AEBS is highly expressed in triple negative breast cancers compared to estrogen receptor positive breast cancers due to a lack of estrogen receptors, which suggests that the PE-DCA prodrug may have therapeutic capabilities for the aggressive TNBC.\textsuperscript{118}

![Figure 3.12: Tesmilifene (DPPE) anti-cancer drug (Mol. Wt. = 283.42 g/mol)](image)

Furthermore, phenoxyethyl derivatives are synthesized as non-nucleoside reverse transcriptase inhibitors (NNRTIs) that can be cytotoxic to in-vitro cancer cells.\textsuperscript{15} Although these NNRTI derivatives are often used in anti-HIV treatment, phenoxyethyl derivatives can
inhibit the endogenous reverse transcriptase in cancer cells and alter the oxidative stress in mitochondria leading to apoptosis.\textsuperscript{16}

Related to the functional groups of the PE-DCA final product, the chloroacetamide group can act as an effective scaffold in inhibiting Fibroblast Growth Factor Receptors (FGFR).\textsuperscript{117} Deregulation of FGFR signaling via point mutations and chromosome translocation can aid in breast cancer cell proliferation, as well as in lung and prostate cancers.\textsuperscript{119} In cancer cells, when FGFRs bind to their specific ligand, abnormal upregulation of cancer proliferation pathways, such as Ras-dependent mitogen-activated protein kinase (MAPK) are expressed, as well as a loss of cell integrity and polarity.\textsuperscript{120} The FGFR1 isoform is expressed in 10\% of breast cancers, especially that of estrogen receptor positive, leading to diminished survival rate.\textsuperscript{121} In order to inhibit FGFR function in enhancing cancer cell proliferation, chloroacetamide can be used to make irreversible FGFR inhibitors for those cancerous tumors that have FGFR mutations. Chloroacetamide drug conjugates were used for the treatment of squamous non-small-cell lung cancer. The chloroacetamide group was able to covalently bind to the cysteine residue of the FGFR1 P-loop, which led to the irreversible inhibition of FGFR phosphorylation, decreasing cancer cell progression.\textsuperscript{122} Similarly, the conjugation of 2-phenoxyethylamine with that of DCA allows for enhanced targeting efficiency in breast cancer cells due the implication of anti-proliferative capabilities of PE-DCA’s functional groups. Further analysis of the physicochemical characteristics of PE-DCA can clarify its mechanism of action.
CHAPTER 4
PHYSICOCHEMICAL ANALYSIS OF GLUCOSE DICHLOROACETAMIDE (G-DCA) PRODRUG

4.1 Rationale

Due to the conjugation of a functionalized glucose and DCA via a two-step substitution and addition reaction, the physicochemical properties of glucose dichloroacetamide (G-DCA) have changed compared to native DCA suggesting improved targeting and cytotoxic effects on breast cancer cells. The understanding the physicochemical properties of G-DCA, like other anticancer therapeutic agents, can improve drug delivery and formulation. This conjugation can address the limitations of not only DCA, such as high dose requirement, but common limitations of cancer chemotherapeutics, such as resistance. Enhancements in physicochemical and biological properties of G-DCA as compared to native DCA can influence the pharmacokinetic and clinical behavior of G-DCA on breast cancer.

4.2 Materials

Ethanolamine, dichloroacetic acid anhydride (DCA-A), triethylamine (TEA), and dichloromethane (DCM) were purchased from Sigma Aldrich (St. Louis, MO). 2,3,4,6-Tetra-O-acetyl-beta-D-glucopyranosyl 2,2,2-trichloroacetimidate was purchased from TCI (Portland, OR). Deionized water for all the experiments was obtained through a Millipore Milli Q water purification system (Millipore Corp., Danvers, MA). All other chemicals were of analytical grades and used as obtained from the suppliers.
4.3 Methods

4.3.1 Synthesis of Functionalized Glucose-Dichloroacetamide (G-DCA; Compound 8)

(2R,3R,4S,5R,6R)-2-(Acetoxyethyl)-6-(2,2-dichloroacetamido)ethoxy)tetrahydro-2H-pyran-3,4,5-triy triacetate (G-DCA) will be referenced as glucose dichloroacetamide. As illustrated in Figure 4.1, the first step of the G-DCA synthesis is performed in the following manner. Dichloroacetic anhydride (2; 0.61 mL, 3.82 mmol) was added dropwise to ethanolamine (Compound 4; 1.15 g, 3.82 mmol) in DCM (10 mL) in a round bottom flask. TEA was then added dropwise (0.50 mL, 3.82 mmol), and the mixture was allowed to react overnight. The reaction was worked up by washing it with saturated sodium bicarbonate solution followed by 1M HCl and brine. After vacuum distillation of the solvent, the resulting yellow crystals (1,1-dichloro-3-((hydroxymethyl)amino)propan-2-one) were recrystallized using a minimal amount of hot methanol (>97% yield). In the second step, 2,2-dichloro-N-(2-hydroxyethyl)acetamide (5; 50 mg; 0.29 mmol) was then reacted with 2,3,4,6-tetra-O-acetyl-beta-D-glucopyranosyl 2,2,2-trichloroacetimidate (6; 148 mg, 0.343 mmol, TCI) in a round bottom flask. The reaction was performed under argon gas to remove any moisture. Afterwards, the reaction was cooled down in an acetone/dry ice water bath at -20 °C. Boron trifluoride ethyl etherate (37 µL; 0.30 mmol) was added dropwise over a 2 hour time period to the reaction and which the reaction was warmed to room temperature for an hour. The reaction was then quenched with TEA (50 µL, 0.36 mmol). The solvent was removed under reduced pressure leaving a yellow adhesive paste. The residue was removed under column chromatography (silica gel; eluent: 10% methanol in DCM) to give the final product of ((2R,3R,4S,5R,6R)-2-(acetoxyethyl)-6-(2,2-dichloroacetamido)ethoxy)tetrahydro-2H-pyran-3,4,5-triy triacetate) (7; 0.5861 g, yield
An additional step of hydroxide group deprotection of the functionalized G-DCA via sodium methoxide and dry methanol was conducted and in the future, that product can be utilized to determine if a deprotected G-DCA has enhanced cytotoxicity on breast cancer cells as compared to the functionalized G-DCA product. Moreover, a deprotection method, like that of Wu et al. using MeOH/THF (2 mL; 2:1, v/v). For future reactions, Potassium carbonate can be employed.\textsuperscript{123} Having a functionalized glucose with protecting groups has implications of increased reactivity as well as protection from degradation during intracellular uptake of the drug.\textsuperscript{124}

Figure 4.1: Synthesis of the two-step reaction to form functionalized G-DCA

4.3.2 Proton Nuclear Magnetic Resonance (\textsuperscript{1}H NMR spectrum of 7)

G-DCA was characterized via 400 MHz spectrometer (Varian Inova, Palo Alto, CA). Approximately, G-DCA (13 mg) was dissolved in deuterochloroform (CDCl\textsubscript{3}, 700 \textmu L) and added to an NMR tube (Sigma Aldrich, St. Louis, MO) for evaluation. NMR data was
processed using VNMRJ. Spectra peaks were further confirmed using ChemDraw Professional v16.

4.3.3 *Carbon Nuclear Magnetic Resonance* (13C NMR spectrum of 7)

G-DCA was further characterized via 13C NMR spectroscopy (Varian Inova, Palo Alto, CA). G-DCA (13 mg) was dissolved in deuterochloroform (CDCl₃, 700 μL) and added to an NMR tube (Sigma Aldrich, St. Louis, MO) for evaluation and added to an NMR tube (Sigma Aldrich, St. Louis, MO) for evaluation. NMR data was processed using VNMRJ software. Spectra peaks were further confirmed using ChemDraw Professional v16.

4.3.4 *Fourier-transform infrared spectroscopy (FTIR)*

FTIR analysis of the final G-DCA product is conducted using Agilent Cary 630 FTIR (Agilent Technologies, Santa Clara, CA). The FTIR apparatus uses Microlab software Version 4.0. About 2mg of PE-DCA product is used for the analysis with values within 600-4000 cm⁻¹ range.

4.3.5 *Liquid Chromatography-Mass Spectroscopy (LC/MS)*

To elucidate the structure of the final G-DCA product, LC/MS analysis was performed with electrospray ionization (ESI) in a positive ion mode on QTrap® API-3200 mass spectrometer equipped with Shimadzu quaternary pump, vacuum degasser, DAD detector and autosampler (Shimadzu Scientific Instruments, Columbia, MD). Data acquisition and processing were performed by Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA, USA).

4.3.6 *Powder X-Ray Diffraction (XRD)*

XRD analysis was performed to assess crystallinity of G-DCA compared to native DCA. This experiment was executed at room temperature on a MiniFlex automated XRD
instrument (Rigaku, Woodland, TX). The instrument utilizes a Cobalt tube, Fe-filtered Cu K-alpha radiations at 30 kV and 15 mA. The pattern was run in step scan mode with 0.05° step size and 3 second count time per step (1°2θ/minute). Jade 8+ (Material Data, Inc, 56 Livermore, CA) was employed to process the diffraction patterns. No specific sample preparation was needed for this analysis.

4.3.7 Scanning Electron Microscopy

Surface morphology of G-DCA compared to native DCA was evaluated with scanning electron microscopy (SEM) CM12 (FEI, Hillsboro, OR) at 80 kV accelerating voltage. A small amount solid sample was placed on a copper grid covered with carbon film and air dried. Digital images are acquired with an ORIUSTM SC 1000 11 Megapixel CCD camera (Gatan, Pleasanton, CA).

4.4 Results and Discussion

FTIR spectroscopy, NMR (\(^1\)H and \(^{13}\)C) spectroscopy, and LC-mass spectrometry (LC-MS) were used as characterization tools to elucidate the G-DCA chemical structure, molecular weight, and purity. The product of step 1, 2,2-dichloro-N-(2-hydroxyethyl)acetamide, was synthesized with a yield greater than 97%.

LC-MS analysis confirmed the molecular weight to be 172.01 g/mol (Figure 4.2). Characteristic chlorine isotope peaks at 170.2, 172.1, and 174.1m/z represent \(^{35}\)Cl, \(^{37}\)Cl, and \(^{39}\)Cl isotopes, respectively. Furthermore, FTIR spectra for 2,2-dichloro-N-(2-hydroxyethyl)acetamide was obtained to further prove the presence of the product before continuing to Step 2. As illustrated in Figure 4.3, defining peaks of the spectra of 2,2-dichloro-N-(2-hydroxyethyl)acetamide are as follows: N-H bend peaks for secondary amides
at 1550 cm\(^{-1}\) and 3330 cm\(^{-1}\), respectively; strong carbonyl stretch peak at 1650 cm\(^{-1}\); O-H broad peak at 3200-3600 cm\(^{-1}\); and C-Cl stretch 800 cm\(^{-1}\).

Figure 4.2: LC/MS spectrum for the 2,2-dichloro-N-(2-hydroxyethyl)acetamide product showing relative abundance of chlorine isotopes
Figure 4.3: FTIR spectrum for 2,2-dichloro-N-(2-hydroxyethyl)acetamide

\(^1\)H and \(^{13}\)C NMR spectra were performed to confirm the presence of the 2,2-dichloro-N-(2-hydroxyethyl)acetamide. A proton NMR emulation (Figure 4.4) for the first step of the G-DCA synthesis: \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.01 (s, 1H), 5.93 (s, 2H), 3.81 – 3.74 (m, 5H), 3.53 – 3.44 (m, 5H), 1.40 (s, 1H), and 1.22 (s, 1H) ppm. The hydrogen of the OH peak is expected to be around 4.20 ppm but is not present because of an impurity peaks included trace water. The resonances below 3 ppm represent impurity peaks from the initial reaction, such as alcohols and hydrocarbon groups. Additional purification methods can be employed in the future at this step. Moreover, characteristic carbon spectra for the 2,2-dichloro-N-(2-hydroxyethyl)acetamide product: \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 164.78, 101.51, 66.31, 61.16, 42.60, 30.28, and 29.67 ppm (Figure 4.5). There are four characteristic resonances for the four carbons of the 2,2-dichloro-N-(2-hydroxyethyl)acetamide product. The smaller
peaks represent impurities from the TEA solvent and associated with side products from the conjugation reaction.

Figure 4.4: $^1$H NMR spectrum for 2,2-dichloro-N-(2-hydroxyethyl)acetamide
Figure 4.5: $^{13}$C NMR spectrum for 2,2-dichloro-N-(2-hydroxyethyl)acetamide

The final step in the G-DCA synthesis results in the formation of a functionalized glucose product, (2R,3R,4S,5R,6R)-2-(acetoxymethyl)-6-(2-(2,2-dichloroacetamido)ethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate. This step utilizes the product from step 1, 2,2-dichloro-N-(2-hydroxyethyl)acetamide, and a protected glucose compound, 2,3,4,6-tetra-O-acetyl-beta-D-glucopyranosyl 2,2,2-trichloroacetimidate. The final product was synthesized with over a 97% yield.

LC-MS Spectrum for the final product was obtained to confirm the presence of G-DCA. The expected molecular weight of G-DCA product was confirmed to be 502.09 g/mol (Figure 4.6). Characteristic chlorine isotopic ($^{35}$Cl and $^{39}$Cl) peaks are present at 499 and 502.9 m/z. Moreover, peaks at 460 and 416 m/z illustrate the loss of acetyl groups (acetyl
groups=43 amu) due to the electropositive carbon of the acetyl group leading to salt formation. **Figure 4.7** shows the isotopic emulation for the G-DCA product as well characteristic chlorine isotopic peaks further the presence of the G-DCA final product.

![Figure 4.6: LC-MS spectrum for G-DCA final product](image)

**Expected mass: 502.09 g/mol**

**Ac= 43 amu**
The neutral chemical formula for the G-DCA contaminate (Figure 4.8) at 153.9818 m/z was determined to be C₄H₅Cl₂NO and is also present in the PE-DCA spectra suggesting that this contaminate may be a byproduct of DCA conjugation reactions (Figure 3.4A and B). The structure represents the dichloroacetamide portion of G-DCA (2,2-dichloro-N-ethylacetamide). Another identified contaminate product present in the G-DCA product is at 102.12794 m/z with a neutral formula of C₆H₅N (Figure 4.9). This contaminate represents diisopropylamine (DIPA). DIPA, a nuclear transcriptional repressor, has implications of anti-cancer activity.¹²⁵ In the synthesis of G-DCA, DIPA is a byproduct. There are studies of
using diisopropylamine dichloroacetate (DADA), an inhibitor of the pyruvate dehydrogenase kinase 4 isoform, as a therapeutic agent for influenza, various cancers, and other metabolic disorders.\(^\text{126}\)

Figure 4.8: LC-MS spectrum of G-DCA contaminate
Figure 4.9: LC-MS spectrum of a secondary G-DCA contaminate
The characteristic proton NMR resonances for the G-DCA and DIPA products are shown in Figure 4.10: $^1$H NMR (400MHz, CDCl$_3$) \( \delta \) 6.96 (s, 1H), 6.68 (s, 2H), 6.20 (s, 2H), 5.94 (d, \( J = 3.9 \) Hz, 1H), 5.19 (t, \( J = 9.5 \) Hz, 1H), 5.12 – 5.02 (m, 1H), 4.98 (dd, \( J = 9.7, 8.0 \) Hz, 1H), 4.52 (d, \( J = 8.0 \) Hz, 1H), 4.27 – 4.10 (m, 3H), 3.88 (ddd, \( J = 9.9, 5.9, 3.6 \) Hz, 1H), 3.82 – 3.65 (m, 2H), 3.65 – 3.52 (m, 2H), 3.49 – 3.41 (m, 1H), 3.18 (qd, \( J = 7.3, 4.8 \) Hz, 11H), 2.22 – 1.95 (m, 20H), 1.95 – 1.77 (m, 3H), and 1.36 (t, \( J = 7.3 \) Hz, 16H) ppm. The resonances between 6-7 ppm represents the loss of acetyl groups. There are product contaminates with one to two acetyl groups missing as represented in the LC-MS spectrum at 485 m/z (one acetyl group removed) and 416 m/z (two acetyl groups removed) m/z (Figure 4.6).

The characteristic carbon NMR peaks for both the G-DCA and DIPA products are depicted in Figure 4.11: $^{13}$C NMR (101 MHz, CDCl$_3$) \( \delta \) 171.20, 170.71, 170.19, 169.42, 164.24, 163.45, 100.64, 91.86, 90.34, 90.12, 72.60, 71.95, 71.18, 69.84, 68.16, 68.01, 66.27, 62.40, 61.91, 61.31, 58.27, 46.88, 40.07, 39.68, 20.64 (d, \( J = 17.5 \) Hz), and 8.70 ppm. The peak at 20.64 ppm represents a deprotected carbon of the G-DCA final product (contaminant). There are other contaminates present in the final G-DCA product which can further be removed through additional purification steps. Carbohydrates, such as glucose are hydroscopic and prone to degradation due to instability of the OH bonds which can further explain additional contaminate peaks.
Figure 4.10: $^1$H NMR spectrum of final G-DCA product
Figure 4.11: $^{13}$C NMR spectrum of final G-DCA product

P-XRD analysis of DCA confirm the crystalline structure of DCA and PE-DCA (Figure 3.9). G-DCA was also determined to be an amorphous solid using parallel, monochromatic radiation (Figures 4.12A-C). The sharp peaks of DCA are not present in the G-DCA compound suggesting that through the chemical synthesis, the conjugation to form a functionalized glucose has led to a new compound which can be associated with the variation in morphological structure of native DCA and PE-DCA compared to G-DCA (Figures
4.12B-C). As illustrated in the SEM data, native DCA is composed of stacked rod-like crystal morphology as visualized with 10,000x and 2000x microscopy (Figure 3.10). The structural integrity of native-DCA crystals have been compromised in the conjugation to form G-DCA which can have an influence on the intracellular uptake of this formulation on breast cancer cells (Figure 4.13).

Figure 4.12: P-XRD data for G-DCA final product: A) DCA and G-DCA, B) PE-DCA and G-DCA, C) DCA, PE-DCA, and G-DCA
4.5 Conclusion

Assessing the physicochemical properties of G-DCA can allow for a better understanding of the therapeutic effects of the formulation on breast cancer cells. Understanding the physical and chemical properties can aid in elucidating the mechanism of actions of the drug via bioanalytical assays. Because the molecular weight of G-DCA (502.09 g/mol) differs from that of DCA (150 g/mol), it may effect its ease to passively diffuse through plasma and cellular membranes, suggesting enhanced selectivity to specific cell types.

Glucose conjugated chemotherapeutics show specific recognition to GLUTs since these transporters are over-expressed on tumorigenic cells. It is proven that glucose conjugated chitosan particles have specific interactions with cancer cells and are promising chemotherapeutic delivery agents. In contrast, there are also anti-cancer drugs that inhibit
GLUT and glycolysis activity in order to induce glucose deprivation apoptotic pathways.

The role of glucose can be exploited in drug therapy depending on cancer type to cause cell death and anti-proliferation safely. The G-DCA prodrug can have a dual effect on cancer cells-DCA acts as a glycolytic inhibitor causing the reversal of the Warburg effect and enhanced cellular uptake via GLUTs because of the conjugation of a functionalized glucose. Like other glucose conjugated drugs, G-DCA may not inhibit GLUT activity, but it can use these transporters to uptake elevated levels of G-DCA into the cell to have enhanced cancer killing by reversing the Warburg effect.
CHAPTER 5

BIOLOGICAL ANALYSIS OF BREAST CANCER CELL RESPONSES TO PHENOXYETHYL DICHLOROACETAMIDE (PE-DCA) PRODRUG

5.1 Rationale

DCA is a glycolytic inhibitor that acts as a pyruvate mimic to inhibit PDK causing the reversal the Warburg effect by switching cellular metabolism to oxidative phosphorylation from glycolysis. Because of the defective mitochondrial integrity in cancer cells due to their hypoxic environment, DCA is more selectively cytotoxic toward cancer cells.\textsuperscript{128} It has been illustrated that, PE-DCA, like other aryloxyethylamine derivatives can be used as herbicides, especially when chloro- substituted rather than bromo.\textsuperscript{113} The structure of PE-DCA is similar to that of other phenoxyethanamine derivatives, such as the anti-cancer drug, Tesmilifene (DPPE), suggesting that PE-DCA, like that of DPPE, can have enhanced cytotoxic and antineoplastic activity on metastatic breast cancer cells.\textsuperscript{114} Therefore, enhancing mitochondrial targeting of anticancer agents via 2-phenoxyethylamine conjugation with DCA, can be an effective strategy in improving DCA’s cytotoxicity on breast cancer cells. The conjugation of 2-phenoxyethylamine with that of DCA allows for enhanced targeting efficiency to breast cancer cells to the implication of anti-proliferative capabilities of PE-DCA’s functional groups. Further analysis of the bioanalytical characteristics of PE-DCA can clarify its mechanism of action on cancer cells.

5.2 Materials

\textsuperscript{\textdagger} 2-Phenoxyethylamine, dichloroacetic acid anhydride (DCA-A), triethylamine (TEA), and dichloromethane were purchased from Sigma Aldrich (St. Louis, MO). Normal breast epithelial (MCF-10A), estrogen receptor positive breast cancer (MCF-7), and triple negative
breast cancer (MDA-MB 231) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The CellTiter 96® AQueous One Solution Proliferation assay kit with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] reagent was purchased from Promega (Madison, WI). The LDH Cytotoxicity Detection Kit was purchased from Takara (Shiga, Japan). Deionized water for all the experiments was obtained through a Millipore Milli Q water purification system (Millipore Corp., Danvers, MA). All other chemicals were of analytical grades and used as obtained from the suppliers.

5.3 Methods

5.3.1 Cell Culture

Complete growth media for estrogen receptor positive breast cancer cells (MCF-7): Dulbecco’s Modified Eagle Medium (DMEM, Gibco) as base medium; 10% final volume of fetal bovine serum (FBS, ATCC); 1% final volume of 200mM L-Glutamine, 10mM non-essential amino acid (NEAA), and penicillin/streptomycin antibiotic, respectively (Gibco); and 70% final volume of 10µg/mL of bovine pancreas insulin (Sigma Aldrich). Complete growth media for TNBCs (MDA-MB 231): L-15 base media (ATCC) and 10% final volume of FBS. Complete growth media for normal breast epithelial cells (MCF-10A): Dulbecco’s Modified Eagle Medium F-12 (DMEM-F12) was used as base medium (Gibco); 5% final volume of horse serum (Invitrogen); 20ng/mL of 100µg/mL stock of epidermal growth factor (EGF, Invitrogen); 0.5mg/mL of 1mg/mL stock of hydrocortisone (Sigma Aldrich); 100ng/mL of 1mg/mL stock of bovine pancreas insulin (Sigma Aldrich); and 1% of final volume of penicillin/streptomycin antibiotic (Gibco). All three cell lines were cultured and maintained at 37°C in 5% CO₂ humidified atmosphere. Culture media was changed every 1-2 days until cells reached confluence.
5.3.2 Cell Viability Assay (MTS Assay)

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega) was used to analyze cell viability. To perform the cell viability (MTS) assay, cells were seeded to 96-well plates in 100µL of serum free medium at a density of 5x10³ cells/well for each of the three cell lines (MCF-7, MDA-MB 231, MCF-10A) for 24h. The cells were treated with 100 µL PE-DCA prodrug, PE-DCA and G-DCA (1:1 prodrug mixture), native 2-phenoxyethylamine, and native DCA (Sigma Aldrich) samples in the concentration range of 50-10,000µM. Treated cells were incubated for 24h at 37ºC with 5% CO₂ and 95% relative humidity. The amount of formazan MTS assay product formed was determined by adding 20µL of MTS reagent to the culture wells. The plate was then further incubated for 4 h at 37ºC in a humidified, 5% CO₂ atmosphere. The absorbance was measured at 490 nm using a BioRad microplate reader (BioRad Hercules, CA, USA). Cells treated with medium and 1% Triton-X-100 were used as negative and positive controls, respectively. The percent (%) cell viability was determined using the following equation:

Cell Viability (%) = \frac{Absorbance of sample}{Absorbance of negative control} \times 100 \quad \text{(Equation 5.1)}

The absorbance represents the amount of formazan product formed in viable cells. All experiments were conducted in triplicates and cytotoxicity results were rated according to ISO-standard 10993-5 as non-cytotoxic (cell viability higher than 75%), slightly cytotoxic (cell viability ranging from 50% to 75%), moderately cytotoxic (cell viability ranging from 25% to 50%), and severely cytotoxic (cell viability lower than 25%).

5.3.3 Cell Cytotoxicity Assay via Lactate Dehydrogenase (LDH Assay)

Lactate dehydrogenase (LDH) is released from cells when membrane integrity is compromised. LDH levels can be measured extracellularly from the culture supernatant. In
the LDH colorimetric assay, LDH is released into the cell culture media when cellular membrane integrity is compromised. LDH reduces NAD+ to NADH and H+ via the oxidation of lactate to pyruvate. The tetrazolium salt is protonated leading to the red color of the formazan salt. MCF-10A, MCF-7 and MDA-MB 231 cells were seeded into a 96-well plate at a density of $1 \times 10^3$ cells/well in 100 µL in DMEM media (Gibco) containing 10% FBS (ATCC). The cells were treated with 100 µL PE-DCA prodrug, PE-DCA and G-DCA (1:1 prodrug mixture), native 2-phenoxyethlamine, and native DCA (Sigma Aldrich) samples in the concentration range of 50-10,000µM. Treated cells were incubated for 24h at 37ºC with 5% CO$_2$ and 95% relative humidity. Extracellular LDH release was measured from the culture supernatant quantified via the LDH assay kit (Takara Bio Inc). Cells treated with medium and 1% Triton-X-100 were used as negative and positive controls, respectively. Absorbance was read at 490nm using the BioRad microplate reader (BioRad Hercules, CA, USA). The percent extracellular LDH release was expressed via the following formula:

$$LDH\ release\ (% ) = \frac{Absorbance\ of\ sample - Absorbance\ of\ negative\ control}{Absorbance\ of\ positive\ control} \times 100$$

(Equation 5.2)

5.3.4 Statistical Analysis

All experiments were performed at least in triplicate (n=3) and the results were expressed as mean ± standard error (SE) to compare mean values. ANOVA and pos-hoc Tukey tests were employed to compare mean values within each treatment group. A p value of $\leq 0.0001$ is considered as statistically significant.

5.4 Results and Discussion

MTT and LDH assays were performed to quantify cell viability and toxicity on breast cancer and normal breast epithelial cell lines after prodrug treatment. Cytotoxicity results
were rated according to ISO-standard 10993-5 as non-cytotoxic (cell viability higher than 75%), slightly cytotoxic (cell viability ranging from 50% to 75%), moderately cytotoxic (cell viability ranging from 25% to 50%), and severely cytotoxic (cell viability lower than 25%). In previous chapters, physicochemical characteristics of the PE-DCA prodrug were determined and based on the overall chemical synthesis in conjugating 2-phenoxyethylamine and DCA, PE-DCA may have enhanced targeting efficiency to breast cancer cells due the implications of anti-proliferative capabilities of PE-DCA’s functional groups.

MTT cell viability assay on normal breast epithelial cells (MCF-10A) indicate that after 24hr treatment, the conjugated prodrugs, prodrug physical mixture, as well as DCA are non-cytotoxic on normal breast epithelial cells (Figure 5.1). It is noted that DCA is mildly cytotoxic on normal cells, which is further illustrated in the results below. Because of the implication of enhanced targeting efficiency of the novel DCA prodrugs to cancer cells as compared to native DCA, the prodrug physical mixture was tested as well. The prodrug physical mixture is a 1:1 ratio of PE-DCA and G-DCA. As shown, in Figure 5.1, the prodrug mixture is non-cytotoxic to normal breast epithelial at the denoted concentration range. The fact that the novel prodrugs, as well as the prodrug mixture are non-cytotoxic on normal cells emphasizes the potential safety and efficacy of the prodrugs as anti-cancer therapeutics.

LDH assay results for MCF-10A cells do not exactly correlate with that of the MTT assay, since DCA conjugated prodrugs and DCA act as metabolic enhancers (Figure 5.2). Because cell supernatant is used for the LDH assay, there is a lower number of available cells for this assay as compared to the MTT assay which can explain the discrepancy between percent cytotoxicity and cell viability. Furthermore, MTT conversion into formazan is quite accelerated, which may be seen as an overestimation in cell viability. Moreover,
the enzyme LDH can catalyze the conversion of pyruvate to lactate via the fermentation pathway especially in cancer cells as signified by the Warburg effect.\textsuperscript{132} Despite this discrepancy, based on ISO standards, the prodrugs, prodrug physical mixture, and DCA are classified as either non-cytotoxic or slightly cytotoxic on normal breast epithelial tissues, further emphasizing its potential as an anti-cancer therapeutic.

Figure 5.1: MTT cell viability of normal breast epithelial (MCF-10A) cell line treated with PE-DCA, G-DCA, Native DCA, and prodrug physical mixture for 24 h. Data represents mean $\pm$ SE. A $p$ value of $\leq 0.001$ is considered as statistically significant.
Cell viability and toxicity assays were performed on ER+ (MCF-7) and TNBC (MDA-MB 231) cell lines. Phenoxethylamine derivatives act as CNS depressants and are used in tranquilizers and anti-psychotic medications. On TNBC cells, cell viability is significantly lower in cells treated with PE-DCA as compared with 2-phenoxethylamine (PE) alone (Figure 5.3). This further indicates the importance of the conjugation of PE to DCA in enhancing DCA anti-cancer proliferation. LDH assay results, further confirm the increased cellular cytotoxicity of PE-DCA treated TNBC cells as compared to PE alone (Figure 5.4). Based on ISO standards, at the concentration range, PE-DCA is moderately cytotoxic of TNBC cells.
Figure 5.3: MTT cell viability of TNBC (MDA-MB 231) cell line treated with PE-DCA and PE alone for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 5.4: LDH cell toxicity of TNBC (MDA-MB 231) cell line treated with PE-DCA and PE alone for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Compared to native DCA, cell viability of TNBC cells is significantly lower than that of native DCA further illustrating the enhanced targeting efficiency of the conjugated prodrug (Figure 5.5). As illustrated in Figure 5.5, DCA barely has an effect on TNBC cells perhaps due to the fact that the CC$_{50}$ for DCA is between 20-30 mM and the experimental treatment group concentrations are substantially lower than that to elicit a response.$^{111}$

![Graph showing MTT cell viability of TNBC (MDA-MB 231) cell line treated with PE-DCA and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.](image)

Figure 5.5: MTT cell viability of TNBC (MDA-MB 231) cell line treated with PE-DCA and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

As aforementioned, discrepancies in LDH analysis for DCA could relate to DCA being a metabolic enhancer (Figure 5.6). DCA, is a small molecule that acts as a pyruvate mimic that via the inhibition of PDK, PDH activity is enhanced causing an increase in mitochondrial pyruvate flux, promotion of mitochondrial oxidative phosphorylation, decreased lactate production (by inhibiting LDH), and an overall reversal of the Warburg effect. Despite the difference in MTT and LDH assays, PE-DCA is more cytotoxic on TNBC cells as compared to native DCA (Figure 5.6).
Figure 5.6: LDH cell toxicity of TNBC (MDA-MB 231) cell line treated with PE-DCA and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant. Moreover, in comparing the two novel prodrugs PE-DCA and G-DCA, both prodrugs are moderately cytotoxic to TNBC cells as confirmed with both MTT and LDH assays. (Figures 5.7 and 5.8). Similarly, MTT and LDH data for the prodrug physical mixture and PE-DCA, respectively show that the prodrug physical mixture is more cytotoxic to TNBC cells than that of native DCA (Figures 5.9 and 5.10). Data also suggests that the prodrug physical mixture does not have an additive effect on TNBC cells even though it is composed of G-DCA and PE-DCA prodrugs which have cytotoxic effects on TNBC cells separately. Further metabolic studies of the novel prodrugs can confirm if the prodrug physical mixture has a dampening effect on TNBC cells as well as other cancers when used together. Because TNBC is the most aggressive form of cancer, initial MTT and LDH viability and cytotoxicity data illustrates PE-DCA’s promising potential as an anti-cancer chemotherapeutic PE-DCA prodrug for TNBC.
Figure 5.7: MTT cell viability of TNBC (MDA-MB 231) cell line treated with PE-DCA and G-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 5.8: LDH cell toxicity of TNBC (MDA-MB 231) cell line treated with PE-DCA and G-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 5.9: MTT cell viability of TNBC (MDA-MB 231) cell line treated with PE-DCA, Prodrug Physical Mixture, and DCA for 24 h. Data represents mean ± SE. A p value of ≤0.05 is considered as statistically significant.

Figure 5.10: LDH cell toxicity of TNBC (MDA-MB 231) cell line treated with PE-DCA, Prodrug Physical Mixture, and Native DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Similar analysis was conducted on ER+ (MCF-7) breast cancer cells. Compared to PE alone, PE-DCA is more cytotoxic on MCF-7 cells (Figures 5.11 and 5.12) but is moderately cytotoxic as compared to analysis in MDA-MB 231 TNBC cells (Figures 5.3 and 5.4). This further illustrates the potential of PE-DCA being a better agent for more aggressive cancers.

Figure 5.11: MTT cell viability of ER+ (MCF-7) cell line treated with PE-DCA and PE alone for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 5.12: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with PE-DCA and PE alone for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant

Compared to native DCA and G-DCA, PE-DCA is slightly cytotoxic to ER+ breast cancer cells (Figures 5.13 and 5.14). Moreover, in comparison to the prodrug physical mixture, PE-DCA is more effective in cancer killing, but there are discrepancies between the MTT and LDH data for the prodrug physical mixture (Figures 5.15 and 5.16). Cell viability is higher for the MTT assay as compared to the LDH assay for the prodrug physical mixture. This can be attributed to DCA being a metabolic enhancer and its mechanism of action as a pyruvate mimic causing the reversal of cancer cell metabolism from lactate fermentation to that of mitochondrial oxidative phosphorylation (LDH is inhibited).
Figure 5.13: MTT cell viability of ER+ (MCF-7) cell line treated with PE-DCA, G-DCA, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 5.14: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with PE-DCA, G-DCA, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 5.15: MTT cell viability of ER+ (MCF-7) cell line treated with PE-DCA, Prodrug Physical Mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 5.16: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with PE-DCA, Prodrug Physical Mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
CC$_{50}$ values for PE-DCA on TNBC and ER+ breast cancer cell lines were calculated based on MTT data for the two cell lines (Figures 5.17 and 5.18). As previously mentioned, the CC$_{50}$ for native DCA is 20-30 mM for breast cancer. By adding the 2-Phenoxyethylamine to DCA in a novel prodrug synthesis, CC$_{50}$ values are significantly lower in both cancerous cell lines further proving that PE-DCA is a promising therapeutic agent and that DCA’s targeting is enhanced through the conjugation. CC$_{50}$ values range from 25-30 mM in aggressive breast cancers, such as TNBC, and is ~25 mM in ER+ breast cancers.$^{130}$ Table 5.1 compares the CC$_{50}$ values for PE-DCA in MCF-7 and MDA-MB 231 cell lines as compared to literature showing a 2038 and 525 fold increases in MDA-MB 231 and MCF-7 cell lines, respectively.

![Graph](image)

Figure 5.17: CC$_{50}$ of PE-DCA on TNBC cells (MDA-MB 231)
Figure 5.18: CC$_{50}$ of PE-DCA on ER+ breast cancer cells (MCF-7)

Table 5.1: CC$_{50}$ value comparison of PE-DCA and Native DCA on MDA-MB 231 and MCF-7 cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Native DCA</th>
<th>PE-DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB 231</td>
<td>25-30mM</td>
<td>14.72µM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>25mM</td>
<td>47.60µM</td>
</tr>
</tbody>
</table>

5.5 Conclusion

In normal cells, in aerobic environments, cells yield about 36 ATP for every molecule of glucose consumed in glycolysis and mitochondrial oxidative phosphorylation. In hypoxic conditions, such as that of the cancer tumor microenvironment, pyruvate is converted to lactate via LDH activation. DCA, is a small molecule that acts as a pyruvate mimic that, via the inhibition of PDK, PDH activity is enhanced causing mitochondrial pyruvate flux increase, promotion of mitochondrial oxidative phosphorylation, decreased lactate production, and an overall reversal of the Warburg effect. This activity suggests that DCA may have an indirect effect on LDH inhibition in causing cancer cells to “switch” from lactate fermentation to that oxidative phosphorylation after DCA treatment and that DCA can
affect cell metabolism by repolarizing the mitochondrial membrane as a glycolytic inhibitor. Elevated LDH levels are a hallmark of malignant tumors allowing for LDH, especially the isoform LDH-A, to be a plasma biomarker for various cancers. Furthermore, enhanced levels of mitochondrial pyruvate via DCA or LDH inhibition, can promote glucose oxidation rather than aerobic glycolysis denoting the reversal of the Warburg effect. Inhibiting LDH, increases mitochondrial pyruvate, similar to that of DCA which is a pyruvate mimic.

PE-DCA shows increased cytotoxicity in TNBC cells as compared to ER+ breast cancer cells which can be attributed to the metabolic profile differences of the two cancers. Estrogens influence metabolic processes, such as glucose and lipid uptake via the expression of antagonistic effectors, AMP-activated kinase (AMPK) and peroxisome proliferator activated receptor (AKT). When AKT expression is increased as a response to estrogen levels, AMPK is activated acting as a sensor to reprogram metabolism. TNBC cells have both dysfunctional mitochondrial activity as well as an enhanced glycolytic metabolism. DCA is a glycolytic inhibitor and therefore, with the addition of 2-phenoxyethylamine, PE-DCA can have increased anti-proliferation activity as compared to native DCA. TNBC cells have increased glucose uptake leading to elevated cell proliferation rates so enhancing DCA’s glycolytic inhibitory function via 2-phenoxyethylamine conjugation, will limit cell proliferation. This is in part due to lower rates of mitochondrial oxidative phosphorylation for energy production. TNBC cells rely on glycolysis compared to other molecular subtypes of breast cancer even under normoxic conditions due to compromised mitochondrial respiration. TNBC cells’ metabolic profile confirms that TNBC cells are more sensitive to glycolytic inhibitors as compared to ER+ breast cancers.
The implication that PE-DCA is more TNBC cell targeted relates to the chemical structure of PE-DCA being similar to that of other phenoxyethanamine derivatives, such as the anti-cancer drug, Tesmilifene (DPPE), suggesting that PE-DCA, like that of DPPE, can have enhanced cytotoxic and antineoplastic activity on metastatic breast cancer cells.\textsuperscript{114} Tesmilifene has a binding affinity for the antiestrogen receptor binding site (AEBS) in the cellular microsomes of cytochrome P450 enzymes and P-glycoprotein leading to efflux inhibition and decreased cancer cell proliferation.\textsuperscript{116-117} AEBS is highly expressed in TNBC as compared to ER+ breast cancers due to a lack of estrogen receptors.\textsuperscript{118} Further metabolic analysis, such as intracellular ROS and autophagy induction will be reviewed in upcoming chapters to further understand the mechanism of action of PE-DCA on different breast cancer subtypes.

Moreover, PE-DCA shows enhanced therapeutic activity to MCF-7 cells as compared to native DCA. MCF-7 cell lines are able to modulate between aerobic glycolysis and mitochondrial oxidative phosphorylation metabolic pathways depending on glucose concentration in the tumor microenvironment.\textsuperscript{138} In high glucose availability, the 17B-estradiol (E2) regulates glycolysis by activating AKT in order to inhibit the Krebs cycle. In depressed glucose concentrations, E2 inhibits glycolysis by activating the Krebs cycle causing upregulation of PDH, stimulating cell proliferation. DCA is a pyruvate mimic that inhibits PDK activity, leading to PDH activation. This inhibition, in MCF-7 cells, in partnership with a low glucose microenvironment, can decrease MCF-7 proliferation by reversing the Warburg effect and triggering pro-apoptotic pathways.\textsuperscript{138}
CHAPTER 6
BIOLOGICAL ANALYSIS OF BREAST CANCER CELL RESPONSES TO GLUCOSE DICHLOROACETAMIDE (G-DCA) PRODRUG

6.1 Rationale

It has been documented that glucoconjugation can improve targeting and selectivity of anticancer therapeutics. Linking chemotherapeutics to glucose or other sugars can be an effective strategy in targeting the Warburg effect, the cancer hallmark that defines how cancer cells grow through aerobic glycolysis.

There are various examples of the implication of sugar conjugation on enhancing chemotherapeutic targeting in cancer cells. In 1995, the first glucose-conjugated anticancer drug, glufosfamide, was synthesized and evaluated. Glufosfamide was designed explicitly as a cancer specific chemotherapeutic agent to enhance the cytotoxic effects and selectivity of ifosfamide, a DNA alkylating agent. Moreover, Liu et al. attached paclitaxel, an FDA approved chemotherapeutic agent for ovarian, breast, and other cancers, to 2’-glucopyranose to form paclitaxel prodrugs. These prodrugs showed enhanced activity toward glucose transporters as well as enhanced water solubility. Clinically, the radiolabeled glucose analog, 2-deoxy-2-(^18F)fluoro-D-glucose (^18F-FDG) has been used to visualize tumors that have metastasized due to elevated glucose uptake in cancer compared to normal cells, further emphasizing the importance of sugars and their derivatives in oncology.

Glucose transporter proteins (GLUTs) mediate the facilitated transport of glucose across the plasma membrane. GLUT1, a uniporter protein, is found on both the surface of the plasma membrane and blood brain barrier to mediate basal intracellular and extracellular glucose transport. GLUT1 overexpression is a defining characteristic in many cancer cells.
indicating an increase in malignant cell proliferation.\textsuperscript{144} For example, breast cancer cells have a 2.8-fold over-expression of GLUT1, which correlates to the dysfunctional metabolism of the Warburg effect.\textsuperscript{145}

DCA is a glycolytic inhibitor that acts as a pyruvate mimic to inhibit PDK that reverses the Warburg effect by switching cellular metabolism to oxidative phosphorylation from glycolysis. Because of the defective mitochondrial integrity in cancer cells due to the hypoxic environment, DCA is more selectively cytotoxic toward cancer cells.\textsuperscript{128} Therefore, enhancing mitochondrial targeting of anticancer agents with glycolytic inhibitors, such as DCA can be an effective strategy. Combining DCA with 2-Deoxy-D-glucose or other glycolytic inhibitors may be an extremely effective strategy to inhibiting cell proliferation. Dual targeting of the Warburg effect via GLUTs and glycolytic metabolites, such as the pyruvate mimic, DCA can further enhance DCA’s targeting to cancer cells.

6.2 Materials

Ethanolamine, dichloroacetic acid anhydride (DCA-A), triethylamine (TEA), and dichloromethane were purchased from Sigma Aldrich (St. Louis, MO). 2,3,4,6-Tetra-O-acetyl-beta-D-glucopyranosyl 2,2,2-trichloroacetimidate was purchased from TCI (Portland, OR). Normal breast epithelial (MCF-10A), estrogen receptor positive breast cancer (MCF-7), and triple negative breast cancer (MDA-MB 231) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The CellTiter 96\textregistered AQueous One Solution Proliferation assay kit with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] reagent was purchased from Promega (Madison, WI). The LDH Cytotoxicity Detection Kit was purchased from Takara (Shiga, Japan). Deionized water for all the experiments was obtained through a Millipore Milli Q
water purification system (Millipore Corp., Danvers, MA). All other chemicals were of analytical grades and used as obtained from the suppliers.

6.3 Methods

6.3.1 Cell Culture

Complete growth media for estrogen receptor positive breast cancer cells (MCF-7): Dulbecco’s Modified Eagle Medium (DMEM, Gibco) as base medium; 10% final volume of fetal bovine serum (FBS, ATCC); 1% final volume of 200mM L-Glutamine, 10mM non-essential amino acid (NEAA), and penicillin/streptomycin antibiotic, respectively (Gibco); and 70% final volume of 10µg/mL of bovine pancreas insulin (Sigma Aldrich). Complete growth media for triple negative breast cancer cells (MD-MBA 231): L-15 base media (ATCC) and 10% final volume of FBS. Complete growth media for normal breast epithelial cells (MCF-10A): Dulbecco’s Modified Eagle Medium F-12 (DMEM-F12) was used as base medium (Gibco); 5% final volume of horse serum (Invitrogen); 20ng/mL of 100µg/mL stock of epidermal growth factor (EGF, Invitrogen); 0.5mg/mL of 1mg/mL stock of hydrocortisone (Sigma Aldrich); 100ng/mL of 1mg/mL stock of bovine pancreas insulin (Sigma Aldrich); and 1% of final volume of penicillin/streptomycin antibiotic (Gibco). All three cell lines were cultured and maintained at 37°C in 5% CO₂ humidified atmosphere. Culture media was changed every 1-2 days until cells reached confluence.

6.3.2 Cell Viability Assay (MTS Assay)

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega) was used to analyze cell viability. To perform the cell viability (MTS) assay, cells were seeded to 96-well plates in 100µL of serum free medium at a density of 5x10³ cells/well for each of the three cell lines (MCF-7, MD-MBA 231, MCF-10A) for 24h. The cells were treated with 100µL of G-DCA prodrug, native DCA (Sigma Aldrich), prodrug physical
mixture, and physical mixture of 2-Deoxy-D-Glucose and native DCA (Sigma Aldrich) samples in the concentration range of 50-10,000µM. Treated cell were incubated for 24h at 37°C with 5% CO₂ and 95% relative humidity. The amount of formazan MTS assay product formed was determined by adding 20µL of MTS reagent to the culture wells. The plate was then further incubated for 4 h at 37°C in a humidified, 5% CO₂ atmosphere. The amount of formazan MTS assay product formed was determined by adding 20µL of MTS reagent to the culture wells. The plate was then further incubated for 4 h at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance was measured at 490 nm using a BioRad microplate reader (BioRad Hercules, CA, USA). Cells treated with medium and 1% Triton-X-100 were used as negative and positive controls, respectively. The percent (%) cell viability was determined using the following equation:

\[
\text{Cell Viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100 \quad \text{(Equation 6.1)}
\]

The absorbance represents the amount of formazan product formed in viable cells. All experiments were conducted in triplicates and cytotoxicity results were rated according to ISO-standard 10993-5 as non-cytotoxic (cell viability higher than 75%), slightly cytotoxic (cell viability ranging from 50% to 75%), moderately cytotoxic (cell viability ranging from 25% to 50%), and severely cytotoxic (cell viability lower than 25%).

6.3.3 Cell Cytotoxicity Assay via Lactate Dehydrogenase (LDH Assay)

Lactate dehydrogenase (LDH) is released from cells when membrane integrity is compromised. LDH levels can be measured extracellularly from the culture supernatant. In the LDH colorimetric assay, LDH is released into the cell culture media when cellular membrane integrity is compromised. LDH reduces NAD⁺ to NADH and H⁺ via the oxidation of lactate to pyruvate. The tetrazolium salt is protonated leading to the red color of
the formazan salt. MCF-10, MCF-7 and MDA-MB 231 cells were seeded into a 96-well plate at a density of \(1 \times 10^3\) cells/well in 100\(\mu\)L in DMEM media (Gibco) containing 10% FBS (ATCC). The cells were treated with 100\(\mu\)L G-DCA prodrug, PE-DCA and G-DCA (1:1 prodrug mixture), physical mixture of 2-Deoxy-D-Glucose and native DCA, and native DCA (Sigma Aldrich) samples in the concentration range of 50-10,000\(\mu\)M. Treated cells were incubated for 24h at 37\(^\circ\)C with 5% CO\(_2\) and 95% relative humidity. Extracellular LDH release was measured from the culture supernatant quantified via the LDH assay kit (Takara Bio Inc). Cells treated with medium and 1% Triton-X-100 were used as negative and positive controls, respectively. Absorbance was read at 490nm using the BioRad microplate reader (BioRad Hercules, CA, USA). The percent extracellular LDH release was expressed via the following formula:

\[
LDH\,release\,(\%) = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100
\]

(Equation 6.2)

6.3.4 Statistical Analysis

All experiments were performed at least in triplicate (n=3) and the results were expressed as mean ± standard error (SE) to compare mean values. ANOVA and pos-hoc Tukey tests were employed to compare mean values within each treatment group. A p value of \(\leq 0.0001\) is considered as statistically significant.

6.4 Results and Discussion

All experiments were conducted in triplicates and cytotoxicity results are rated according to ISO-standard 10993-5 as non-cytotoxic (cell viability higher than 75%), slightly cytotoxic (cell viability ranging from 50% to 75%), moderately cytotoxic (cell viability ranging from 25% to 50%), and severely cytotoxic (cell viability lower than 25%). MTTRaw diad.
cell viability data on normal breast epithelial cells (MCF-10A) indicate that after 24 hr treatment, the conjugated prodrugs, prodrug physical mixture, as well as DCA, are non-cytotoxic on normal breast epithelial cells (Figure 5.1). It is noted that DCA is mildly cytotoxic on normal cells. Because of the implication of enhanced targeting efficiency of the novel DCA prodrugs on cancer cells as compared to native DCA, the prodrug physical mixture was tested as well. The prodrug physical mixture is a 1:1 ratio of PE-DCA and G-DCA (Figure 6.1). As shown, in Figure 5.1, the prodrug mixture is non-cytotoxic on normal breast epithelial at the denoted concentration range.

![Figure 6.1: Physical Mixture and Prodrug Physical Mixture significance](image)

MTT viability and LDH cytotoxicity data comparing G-DCA to native DCA and PE-DCA show that on MCF-7 cells, G-DCA is moderately cytotoxic which can be attributed to the metabolic profile of ER+ breast cancers. (Figures 6.2 and 6.3). Despite higher glucose uptake, cancer cells’ dysfunctional metabolism causes them to switch from glycolysis oxidation to that of lactate fermentation for sustainability even in oxygen environments. Cancer cells, like ER+, move cellular glucose to adjacent metabolic pathways to generate
metabolites for proliferation, such as nucleotides, and fatty acids.\textsuperscript{147} ER+ breast cancers, as well as TNBCs, have enhanced induction of GLUT1 transporters. GLUT transporters allow glucose to enter the cells and GLUT1, can be overexpressed in breast cancer by 42 to 90\%.\textsuperscript{147} ER+ breast cancer cells have a higher reliance on mitochondrial oxidative phosphorylation as compared to TNBC cells suggesting that the mitochondria of ER+ breast cancer cells are more functional than that of other cancer cell types.\textsuperscript{148} Because the G-DCA prodrug has glucose conjugation, it can be assumed that it is shunted into the mitochondria of cancer cells via GLUT transporters in which the prodrug can cause the reversal of the Warburg effect to decrease cell proliferation via DCA. ER+ breast cancer cells have a propensity for oxidative phosphorylation and therefore the G-DCA prodrug can enhance mitochondrial function in the cancer cells further promoting cell death pyruvate entry into the Krebs cycle.

Figure 6.2: MTT cell viability of ER+ (MCF-7) cell line treated with PE-DCA, G-DCA, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant
Figure 6.3: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with PE-DCA, G-DCA, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Like PE-DCA, as compared to the prodrug physical mixture, G-DCA is more effective in cancer killing, but there are discrepancies between the MTT and LDH data for the prodrug physical mixture (Figures 6.4 and 6.5). Cell viability is higher for the MTT assay as compared to the LDH assay for the prodrug physical mixture. This can be attributed to DCA being a metabolic enhancer and its mechanism of action as a pyruvate mimic causing the reversal of cancer cell metabolism from lactate fermentation to that of mitochondrial oxidative phosphorylation (LDH is inhibited).
Figure 6.4: MTT cell viability of ER+ (MCF-7) cell line treated with G-DCA, prodrug physical mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.05 is considered as statistically significant.

Figure 6.5: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with G-DCA, prodrug physical mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
The physical mixture differs from that of the prodrug physical mixture. In a paper by Pyaskovskaya et al., when the glycolytic inhibitors 2-Deoxy-D-glucose and DCA are used concomitantly in a 2:1 physical mixture ratio in the treatment of Lewis Lung Carcinoma, DCA had enhanced anti-tumor activity on mice. In comparison with the physical mixture, G-DCA is more effective in killing ER+ breast cancer cells (Figures 6.6 and 6.7). The physical mixture is not cytotoxic on ER+ breast cancer cells at the concentration range (Figure 6.6). The physical mixture and the prodrug physical mixture have similar cytotoxicities in ER+ breast cancer cells (Figures 6.8 and 6.9). The prodrugs are glycolytic inhibitors and can further cause the reversal of the Warburg effect to decrease cancer cell proliferation. MTT and LDH assays were also conducted on TNBCs. As compared to DCA, G-DCA is severely cytotoxic on TNBC cells further showing that the glucose conjugation enhances DCA activity and targeting (Figures 6.10 and 6.11).

Figure 6.6: MTT cell viability of ER+ (MCF-7) cell line treated with G-DCA, physical mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 6.7: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with G-DCA, physical mixture, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 6.8: MTT cell viability of ER+ (MCF-7) cell line treated with prodrug physical mixture and physical mixture for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 6.9: LDH cell cytotoxicity of ER+ (MCF-7) cell line treated with prodrug physical mixture and physical mixture for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 6.10: MTT cell viability of TNBC cells (MDA-MB 231) cell line treated with G-DCA, and Native-DCA for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Like that of ER+ breast cancer cells, the physical mixture and prodrug physical mixture have cytotoxic effects on TNBCs, but G-DCA is a better anti-cancer agent in comparison (Figures 6.12 and 6.13). CC$_{50}$ values for G-DCA on TNBC and ER+ breast cancer cell lines were calculated based on MTT data for the two cell lines (Figures 6.14 and 6.15). As previously mentioned, the CC$_{50}$ for native DCA is 20-30mM for breast cancer. By adding the 2-Phenoxyethylamine to DCA in a novel prodrug synthesis, CC$_{50}$ values are significantly lower in both cancerous cell lines further proving that G-DCA is a promising therapeutic agent and that DCA’s targeting is enhanced through the conjugation. CC$_{50}$ values range from 25-30 mM in aggressive breast cancers, such as TNBC, and is ~25 mM in ER+ breast cancers.$^{130}$ Table 6.1 compares the CC$_{50}$ values for G-DCA and PE-DCA in MCF-7 and MDA-MB 231 cell lines as compared to literature.
Figure 6.12: MTT cell viability of TNBC cells (MDA-MB 231) cell line treated with G-DCA, physical mixture, and prodrug physical mixture for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 6.13: LDH cell cytotoxicity of TNBC cells (MDA-MB 231) cell line treated with G-DCA, physical mixture, and prodrug physical mixture for 24 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 6.14: CC\textsubscript{50} of G-DCA on ER+ breast cancer cells (MCF-7)

Figure 6.15: CC\textsubscript{50} of G-DCA on TNBC cells (MDA-MB 231)

Table 6.1: CC\textsubscript{50} value comparison of PE-DCA and Native DCA on MDA-MB 231 and MCF-7 cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Native DCA</th>
<th>PE-DCA</th>
<th>G-DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB 231</td>
<td>25-30mM</td>
<td>14.72µM</td>
<td>16.22µM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>25mM</td>
<td>47.60µM</td>
<td>97.54µM</td>
</tr>
</tbody>
</table>
6.5 Conclusion

ER+ breast cancer cells can reprogram their metabolism based on the presence of glucose in the environment. In high glucose microenvironments, glycolysis is enhanced, and the Krebs cycle is repressed. Contrastingly, in low levels of glucose, Krebs cycle utilization is increased signaling the transition to oxidative phosphorylation. Optimal drug efficacy CC_{50} values are in the nM range, but there is considerable improvement of DCA targeting when using the DCA conjugated prodrugs (Table 6.1). Understanding the metabolic profiles of the cancerous cell lines can give insight on the enhanced mechanism of action of the DCA prodrugs as compared to native DCA. The above MTT and LDH cell analysis data has implied that G-DCA has better anti-cancer proliferation on ER+ breast cancer cells as compared to PE-DCA’s enhanced targeting to TNBC cells.

Glucocojugation can improve targeting and selectivity of anticancer therapeutics. Linking chemotherapeutics to a glucose or other sugars can be an effective strategy in targeting the Warburg effect, the cancer hallmark that defines how cancer cells grow through aerobic glycolysis. In the G-DCA chemical structure, the DCA is added to the C1 position, similar to the glucoconjugated drug glufosfamide (Figure 6.15). Substitution is stable at this carbon position as long as the oxygen is still attached. Moreover, when the R group is in the equatorial position, there will be enhanced GLUT1 recognition, leading to more glucose uptake. Glufosfamide is effective on ER+ breast cancer cells and when used concomitantly with docetaxel, can trigger pro-apoptotic pathways. Further metabolic analysis, such as intracellular ROS and autophagy induction will be reviewed in upcoming chapters to further understand the mechanism of action of G-DCA on different breast cancer subtypes.
Figure 6.16: (A) G-DCA and (B) Glufosfamide structures
CHAPTER 7
QUANTIFYING METABOLIC RESPONSE IN BREAST CANCER CELLS AFTER DCA PRODRUG TREATMENT

7.1 Rationale

The relationship between autophagy and reactive oxygen species (ROS) in cancer cells is highly complex and regulated. ROS describes active oxygen compounds, such as hydrogen peroxide that are produced during aerobic metabolism. Autophagy describes the cellular self-digestion process in which cells regulate homeostasis in response to oxidative stress via the degradation of organelles and macromolecules. Evidence has shown that upregulation of ROS can cause damage to macromolecules, such as DNA and impair mitochondrial membrane potential leading to apoptosis.

DCA inhibits cancer cell proliferation by inhibiting PDK causing increased activity of PDH reversing mitochondrial dysfunction, restoring mitochondrial membrane potential, and triggering pro-apoptotic pathways to trigger cell death. Because DCA inhibits glycolysis and reduces lactate accumulation via the reversal of the Warburg effect, the tumor microenvironment becomes destroyed. DCA induced autophagy occurs via the inhibition of the Akt-MTOR negative feedback pathway that regulates autophagy in response to reduced lactate levels and ROS production.

Because ER+ and TNBC cells have varying metabolic profiles quantifying the relationship between ROS in autophagy in the prodrugs G-DCA and PE-DCA can give insight into the mechanism of action of these two prodrugs and the metabolic profile of DCA in breast cancer cells.
7.2 Materials

PE-DCA and G-DCA were synthesized based on novel synthesis schema as mentioned in previous chapters. Normal breast epithelial (MCF-10A), estrogen receptor positive breast cancer (MCF-7), and triple negative breast cancer (MDA-MB 231) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The Autophagy Assay and DCFDA / H₂DCFDA - Cellular ROS Assay kits were purchased from Abcam (London, UK). Deionized water for all the experiments was obtained through a Millipore Milli Q water purification system (Millipore Corp., Danvers, MA). All other chemicals were of analytical grades and used as obtained from the suppliers.

7.3 Methods

7.3.1 Cell Culture

Complete growth media for estrogen receptor positive breast cancer cells (MCF-7): Dulbecco’s Modified Eagle Medium (DMEM, Gibco) as base medium; 10% final volume of fetal bovine serum (FBS, ATCC); 1% final volume of 200mM L-Glutamine, 10mM non-essential amino acid (NEAA), and penicillin/streptomycin antibiotic, respectively (Gibco); and 70% final volume of 10 µg/mL of bovine pancreas insulin (Sigma Aldrich). Complete growth media for TNBCs (MDA-MB 231): L-15 base media (ATCC) and 10% final volume of FBS. Complete growth media for normal breast epithelial cells (MCF-10A): Dulbecco’s Modified Eagle Medium F-12 (DMEM-F12) was used as base medium (Gibco); 5% final volume of horse serum (Invitrogen); 20 ng/mL of 100µg/mL stock of epidermal growth factor (EGF, Invitrogen); 0.5 mg/mL of 1mg/mL stock of hydrocortisone (Sigma Aldrich); 100ng/mL of 1mg/mL stock of bovine pancreas insulin (Sigma Aldrich); and 1% of final volume of penicillin/streptomycin antibiotic (Gibco). All three cell lines were cultured and
maintained at 37°C in 5% CO₂ humidified atmosphere. Culture media was changed every 1-2 days until cells reached confluence.

7.3.2 Reactive Oxygen Species Assay (DCFDA Cellular ROS)

Percent intracellular reactive oxygen species (ROS) were measured quantitatively, rather than qualitatively, in order to statistically validate the relationship between ROS and autophagy in cancer cells. The DCFDA Cellular ROS Detection Assay kit (Abcam) was used to quantitatively measure reactive oxygen species (ROS) in adherent cells using a microplate reader. The assay uses the reagent 2’,7’–dichlorofluorescin diacetate (DCFDA), a dye that measures intracellular H₂O₂ activity. After cellular uptake via passive diffusion, DCFDA is deacetylated by cellular esterases to a non-colorimetric compound, which is later oxidized by H₂O₂ into 2’, 7’–dichlorofluorescein (DCF). Cells were seeded to 96-well plates in 100 µL of serum free media at a density of 2.5x10⁴ cells/well for each of the cancer cell lines (MCF-7 and MDA-MB 231) for 24h. The media was removed and 1 x Buffer was added to each well (100 µL/well). The buffer is then removed after several minutes and 25 µM of DCFDA solution is added to each well (100 µL/well). Cells are incubated for 45 minutes at 37°C with 5% CO₂ and 95% relative humidity. DCFDA solution is removed by washing wells with 100 µL 1x buffer. Buffer is removed and cells are treated with 100 µL PE-DCA, G-DCA, prodrug physical mixture, and native DCA (Sigma Aldrich) drugs in the concentration range of the 1 mM, 5vM, and 10vM in serum free sodium pyruvate, low glucose, and high glucose media (Sigma Aldrich) to understand the metabolic differences between the three cell lines. Treated cells were incubated for 6h at 37°C with 5% CO₂ and 95% relative humidity. The DCF absorbance was measured at 519 nm using the BioRad microplate reader (BioRad Hercules, CA, USA). Cells treated with media and 50vµM tert-
butyl hydrogen peroxide (TBHP) solution were used as negative and positive controls, respectively. The positive control was diluted in media without phenol red. The percent (%) cellular ROS was determined using the following equation:

\[
ROS(\%) = \frac{Absorbance \ of \ sample - Absorbance \ of \ media \ control}{Absorbance \ of \ TBHP - Absorbance \ of \ media \ control} \times 100 \quad (Equation \ 7.1)
\]

7.3.3 Autophagy Detection Assay

Percent autophagic cells in the three cancer cell lines were calculated by measuring quantitatively the presence of autophagic/lysosomal vacuoles via the Autophagy Detection Kit (Abcam). There will be a color change present on vesicles produced during autophagy. Cells were seeded to 96-well plates in 100 µL of serum free media at a density of 1x10⁴ cells/well for each of the cancer cell lines (MCF-7 and MDA-MB 231) for 24 h. The media was removed and 1 x Buffer was added to each well (100µL/well). Buffer is removed and cells are treated with 100 µL PE-DCA, G-DCA, prodrug physical mixture, and native DCA (Sigma Aldrich) samples in the concentrations of 1mM, 5mM, and 10mM in serum free sodium pyruvate, low glucose, and high glucose media (Sigma Aldrich) to understand the metabolic differences between the three cell lines. Treated cells were incubated for 8h at 37 ºC with 5% CO₂ and 95% relative humidity. Microplate dual detection reagent is prepared by using 1 µL of green detection dye, 1 µL of nuclear stain using 1 mL of culture media with 5% FBS and without phenol red. This reagent is then added to each well (100 µL/well). Cells are incubated for 30 min at 37 ºC with 5% CO₂ and 95% relative humidity. The absorbance was measured at 461 nm using the BioRad microplate reader (BioRad Hercules, CA, USA). Cells treated with media and 50nM of autophagy inducer, rapamycin were used as negative and positive controls, respectively. The positive control was diluted serum free media
without phenol red. The percent (%) autophagic cells was determined using the following equation:

\[
\text{Autophagy} \, (\%) = \frac{\text{Absorbance of sample} - \text{Absorbance of media control}}{\text{Absorbance of rapamycin control}} \times 100
\]

(Equation 7.2)

7.3.4 Statistical Analysis

All experiments were performed at least in triplicate (n=3) and the results were expressed as mean ± standard error (SE) to compare mean values. ANOVA and pos-hoc Tukey tests were employed to compare mean values within each treatment group. A p value of \(\leq 0.001\) is considered as statistically significant.

7.4 Results and Discussion

ER+ breast cancer cells have a higher reliance on mitochondrial oxidative phosphorylation as compared to TNBC cells suggesting that the mitochondria of ER+ breast cancer cells are more functional than that of other cancer cell types. Intracellular ROS levels were detected via the DCFDA assay in various treatments: sodium pyruvate, DMEM high glucose (25mM), and DMEM low glucose (5.6mM) media without serum and phenol red in order to accurately quantify ROS species. Percent intracellular reactive oxygen species (ROS) were measured quantitatively, rather than qualitatively, in order to statistically infer the relationship between ROS and autophagy in cancer cells. The DCFDA Cellular ROS Detection Assay kit (Abcam) was used to quantitatively measure reactive oxygen species (ROS) in adherent cells using a microplate reader. The assay uses the reagent 2’,7’–dichlorofluorescin diacetate (DCFDA), a dye that measures intracellular \(\text{H}_2\text{O}_2\) activity. After cellular uptake via passive diffusion, DCFDA is deacetylated by cellular esterases to a non-colorimetric compound, which is later oxidized by \(\text{H}_2\text{O}_2\) into 2’, 7’–dichlorofluorescein.
DCF). DCA shifts metabolism from aerobic glycolysis to that of oxidative phosphorylation which can increase intracellular \( \text{H}_2\text{O}_2 \) levels as well as make the tumor microenvironment more basic, leading to a decrease in mitochondrial membrane potential and an increase in apoptotic proteins, like caspase 3 and 9.\(^{159}\)

In pyruvate media (absence of glucose), intracellular ROS species are increased when treated with PE-DCA, G-DCA, the prodrug physical mixture, as well as native DCA (Figure 7.1). Increased pyruvate entry into the mitochondria stimulates ROS production by hyperpolarizing the mitochondria and causing the conversion of pyruvate to glucose and the activation of the Krebs cycle and an increase of glucose uptake via GLUTs.\(^{160}\) There is increased intracellular ROS species at 1mM concentration as compared to the others which can be attributed to the CC\(_{50}\) values of G-DCA and PE-DCA being closer to 1 mM in ER+ breast cancer cells (PE-DCA: 47.60 \( \mu \text{M} \) and G-DCA: 97.54 \( \mu \text{M} \)). tert-Butyl hyperperoxide (t-BHP) is an organic peroxide and an inducer of oxidative stress used as the control. In pyruvate media, t-BHP does not show an increase in intracellular ROS because pyruvate can act as an antioxidant.\(^{161}\)

ROS can regulate autophagy in cancer cells via \( \text{H}_2\text{O}_2 \) activating delipidation causing nutrient starvation. Depending on the cancer stage, tumor, and tissue, autophagy can play a protective role by preventing ROS damage to DNA to inhibit tumorigenesis. In late stage cancer development, autophagy has a pro-tumorigenesis role because an increase in ROS in hypoxic tumor environments produce metabolites for cancer survival and proliferation.\(^{162}\) DCA can induce autophagy in colorectal, prostate, and esophageal cancers.\(^{155,163}\) The induction of autophagy and the reversal of the Warburg effect results in more pyruvate being shunted to the mitochondria for Krebs cycle activation and a reduction in lactate production.
causing the release of pro-apoptotic factors. Figure 7.2 shows elevated autophagosomes (autophagic cells) in PE-DCA and G-DCA treated cells at lower concentrations as compared to prodrug physical mixture implying that the prodrug physical mixture does not enhance targeting capabilities of DCA as compared to the prodrugs alone. The elevated ROS in the prodrug physical mixture treated cells may illicit a protective pro-tumorigenesis response as exemplified in MTT and LDH assay data. The two prodrugs may act on different and competing pathways that enhance cancer cell viability. The control of rapamycin acts an autophagy inducer by inhibiting the Ser/Thr protein kinase mammalian target of rapamycin (mTOR) that blocks signals for cell proliferation and growth.164

Figure 7.1: Measure of intracellular ROS of ER+ breast cancer (MCF-7) cells in pyruvate media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 7.2: Measure of percent autophagic cells of ER+ breast cancer (MCF-7) cells in pyruvate media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant

In elevated glucose media, intracellular ROS species are higher for PE-DCA, G-DCA, prodrug physical mixture, and DCA (Figure 7.3). The increase in ROS species relates to enhanced glucose uptake via GLUTs, suggesting improved uptake of the formulations into the cell. Contrastingly, despite elevated intracellular ROS in ER+ breast cancer cells in high glucose media, the percent autophagic cells at various treatment concentrations is low in the PE-DCA and G-DCA prodrug formulations (Figure 7.4). This suggests that at high glucose concentrations, glucose absorption in the cell increases and is used to produce NADPH via the pentose phosphate pathway (PPP) and the conversion of oxidized glutathione (GSSG) to the reduce form, GSH. This activity is a protective survival mechanism in cancer cells that allows cells to resist the oxidative stress promoted by an increase in intracellular ROS. The prodrug physical mixture has elevated autophagy induction as compared to DCA and the
novel prodrugs which can be explored by further understanding the mechanism of action in using the prodrugs concomitantly in cancer cells. Moreover, the increased levels of autophagic cells in media alone suggests that glucose can induce autophagy via the inhibition of mTOR.\textsuperscript{165}

Figure 7.3: Measure of intracellular ROS of ER+ breast cancer (MCF-7) cells in high glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Figure 7.4: Measure of percent autophagic cells of ER+ breast cancer (MCF-7) cells in high glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant

In low glucose media, intracellular ROS species are elevated for the drug formulations (Figure 7.5). Glucose deprivation promotes cell survival in cancer cells by increasing the hypoxic tumor microenvironment inducing ROS production by activating HIF1α. HIF1α regulates oxygen homeostasis in conditions of prolonged hypoxia. Percent autophagic cells is lower in low glucose media as compared to that of pyruvate media suggesting that in low glucose environments, autophagy is induced to support cell survival and proliferation (Figure 7.6).
Figure 7.5: Measure of intracellular ROS of ER+ breast cancer (MCF-7) cells in low glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 7.6: Measure of percent autophagic cells of ER+ breast cancer (MCF-7) cells in low glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
Similar assays were done in TNBC cells (MDA-MB 231) to illustrate the differences in the metabolic profile between breast cancer molecular subtypes. Glycolytic metabolism is correlated with tumor proliferation and using glycolytic inhibitors like DCA can reduce cancer cell growth.\textsuperscript{167} In cancer cells with more dysfunctional mitochondria, like that in TNBCs, lower oxidative phosphorylation can enhance aerobic glycolysis and cell proliferation.\textsuperscript{168}

In pyruvate media, intracellular ROS is higher in G-DCA treated cells as compared to other formulations because, based on MTT and LDH assay data on TNBC cells, G-DCA is moderately cytotoxic (\textbf{Figures 7.7, 5.7 and 5.8}). TNBC cells, as compared to ER+ breast cancer cells have elevated mitochondrial dysfunction leading to elevated aerobic glycolysis levels and enhanced production of ROS.\textsuperscript{137} TNBC cells are more readily able to uptake pyruvate and use it for lactate fermentation. Furthermore, PE-DCA has substantially lower intracellular ROS production in pyruvate media, as well as the prodrug physical mixture in comparison to G-DCA suggesting that G-DCA may be more selective for TNBC cells than that of PE-DCA (\textbf{Figure 7.7}). In comparing autophagic cells in low pyruvate, the prodrug formulations, when compared to native DCA, have higher production of autophagic cells (\textbf{Figure 7.8}). Overall, autophagy induction in TNBC cells is somewhat low in comparison to intracellular ROS suggesting that the increase in intracellular ROS may have a cancer cell protective mechanism and that TNBC cell death can trigger apoptotic pathways further revealing the complexities of autophagy in cancer cells.
Figure 7.7: Measure of intracellular ROS of TNBC breast cancer (MDA-MB 231) cells in pyruvate media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 7.8: Measure of percent autophagic cells of TNBC breast cancer (MDA-MB 231) cells in pyruvate media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
In high glucose media, intracellular ROS in G-DCA and PE-DCA treated cells are similar because of increased glucose uptake via GLUTs on the cell surface (Figure 7.9). DCA intracellular ROS is higher at 10 mM is higher than the other formulations at this concentration because DCA shows enhanced activity at high concentrations as attributed to its CC50 value (25-30 mM).\textsuperscript{169} Like that of pyruvate media treatment, the presence of autophagic cells is lower as compared to intracellular ROS as well as similar result in ER+ breast cancer cells. This can be associated with the intense mitochondrial dysfunction in TNBC cells. The percent autophagy in TNBC cells treated with formulations in high glucose media is somewhat low in comparison to intracellular ROS, suggesting that the increase in intracellular ROS may have a cancer cell protective mechanism and that TNBC cell death could be related to other apoptotic pathways further revealing the complexities of autophagy in cancer cells (Figure 7.10).

Figure 7.9: Measure of intracellular ROS of TNBC breast cancer (MDA-MB 231) cells in high glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.05 is considered as statistically significant
Figure 7.10: Measure of percent autophagic cells of TNBC breast cancer (MDA-MB 231) cells in high glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 7.11 shows increased intracellular ROS in TNBC cells upon treatment of PE-DCA at 1mM in glucose concentrations. PE-DCA shows increased cytotoxicity in TNBC cells as compared to ER+ breast cancer cells which can be attributed to the metabolic profile differences of the two cancers. Estrogens influence metabolic processes, such as glucose and lipid uptake via the expression of antagonistic effectors, AMP-activated kinase (AMPK) and peroxisome proliferator activated receptor (AKT).\textsuperscript{135} When AKT expression is increased as a response to estrogen levels, AMPK is activated acting as a sensor to reprogram metabolism.\textsuperscript{136} TNBC cells have both dysfunctional mitochondrial activity as well as an enhanced glycolytic metabolism.\textsuperscript{137} Similar to the other autophagic results in various media environments, TNBC cells treated with formulations in low glucose media is somewhat low in comparison to intracellular ROS, suggesting that the increase in intracellular ROS (Figure 7.11).
Figure 7.11: Measure of intracellular ROS of TNBC breast cancer (MDA-MB 231) cells in low glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 6 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.

Figure 7.12: Measure of % autophagic cells of TNBC breast cancer (MDA-MB 231) cells in low glucose media when treated with Native DCA, G-DCA, PE-DCA, and prodrug physical mixture for 8 h. Data represents mean ± SE. A p value of ≤0.0001 is considered as statistically significant.
7.5 Conclusion

It is noted that elevated DCA concentrations above 10mM are required to alter to metabolic activity, increase intracellular ROS species, and induce cancer death.\textsuperscript{170} Cell viability and intracellular ROS data show that the DCA prodrugs at low concentrations are cytotoxic on breast cancer cells and have the ability to increase ROS species in high glucose environments for both TNBC and ER+ breast cancer cell lines. In elevated glucose environments, DCA can induce apoptosis in response to elevated ROS levels. Because autophagy has various mechanistic avenues in cancer cells: pro-proliferative and anti-tumorigenic depending on the stage, grade, and tissue type of the cancer, cell death triggered by the DCA prodrugs may not directly related to autophagic pathways, but apoptosis. Our results indicate that the prodrugs are cytotoxic to TNBC and ER+ breast cancer cells based on LDH and MTT data mentioned in previous chapters but based on data of the presence of autophagic cells in various media environments, cell-death pathways may not directly correspond to autophagy. It is noted that autophagy can a pro-tumorigenic and pro-proliferation response in cancer cells as well as trigger apoptotic pathways. Autophagy can solicit survival in dormant and metastatic breast cancers further emphasizing the complexities of autophagic activation.\textsuperscript{169} The differences in the metabolic profiles of TNBC and ER+ breast cancer cells are illustrated in the uptake of the formulations in diverse media environments and the quantification of ROS and autophagosome species intracellularly.
CHAPTER 8
QUANTIFYING INTRACELLAR UPTAKE OF DICHLOROACETATE VIA LC-MS/MS

8.1 Rationale

DCA is a small molecule metabolite that acts as a pyruvate mimic causing the reversal of cancer cell metabolism or the Warburg effect via the inhibition of PDK by activating the PDH complex. This inhibition leads to a metabolic shift from aerobic glycolysis for energy production to the more energetically preferred process of oxidative phosphorylation as illustrated in normal cell metabolism. The process of oxidative phosphorylation, the major source of ATP production, occurs in the inner mitochondrial membrane in eukaryotic cells and DCA’s shift to this energetically favorable process, allows for an increase in mitochondrial metabolism by decreasing lactate levels and converting it to pyruvate.\textsuperscript{171}

The oral and intravenous administration of DCA increases glycolysis oxidation leading to enhanced acetyl-CoA levels for both Krebs Cycle and electron transport chain activation.\textsuperscript{105} Moreover, DCA increases the activity of p53, the master gene regulator via elevated mitochondrial reactive oxygen species levels (ROS), especially that of hydrogen peroxide. This results in decreased intracellular potassium levels causing activation of apoptosis by deactivating pro-proliferative factors.\textsuperscript{172} DCA’s mechanism of action on cancer cells causes decreased cell proliferation and angiogenesis and increased cancer cell death.

Measuring intracellular DCA uptake of the two formulations, G-DCA and PE-DCA via LC-MS/MS, gives a more comprehensive understanding of DCA metabolism and the specific mechanisms of action of the prodrugs on two very distinct cancer cell lines: ER+ (MCF-7) and triple negative (MDA-MB 231) breast cancer receptor types.
8.2 Materials

Normal breast epithelial (MCF-10A), estrogen receptor positive breast cancer (MCF-7), and triple negative breast cancer (MDA-MB 231) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). DCA was purchased from Sigma Aldrich (St. Louis, MO). PE-DCA and G-DCA were synthesized based on novel synthesis schema as mentioned in previous chapters.

8.3 Methods

8.3.1 Development of a LC-MS/MS method for quantification of DCA

LC-MS/MS was performed on an AB Sciex 3200 QTrap Spectrometer (Foster City, CA) coupled to a Shimadzu UFLC system (Columbia, MD) using electrospray ionization (ESI) in negative mode with Analyst v.1.4.2 software. 100µM of DCA stock solution were prepared in water and diluted to 10µM in 100% water with 0.1% formic acid which was then infused into mass spectrometer and different compound dependent parameters (Table 8.1) were optimized using Analyst quantitative optimization wizard. The most intense fragment (Q3) was selected for the final method. Source dependent parameters were optimized using flow injection analysis operation of Analyst quantitative optimization wizard using same 10µM standard. Given the polar nature of DCA, both hydrophilic interaction and reverse phase chromatography were assessed. Different analytical columns tested were: Atlantis HILIC (Waters, Milford, MA, USA) Silica column (150×2.1 mm, 5µm), Roco (Restek, State College, PA) C8 column (100×3.0 mm, 3µm) and Ascentis Express C18 (Supelco, Bellefonte, PA) column (100×2.1mm, 2.7 µm). Two ion-pairing reagents, N, N-dimethylhexylamine (Alfa Aesar, Haverhill, MA) and triethylamine (Sigma-Aldrich, St. Louis, MO) were tested in the mobile phase at different pH.\(^{173}\) Besides, both isocratic and gradient elution were tested.
Table 8.1: Mass spectrometric parameters for the most intense MS/MS transition of DCA and Chlorzoxazone in negative ESI mode detection.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent Ion (Q1)</th>
<th>Fragment Ion (Q3)</th>
<th>Delustering Potential (V)</th>
<th>Entrance Potential (V)</th>
<th>Collision Cell Entrance Potential (V)</th>
<th>Collision Energy (V)</th>
<th>Collision Cell Exit Potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA</td>
<td>127.1</td>
<td>83.0</td>
<td>-20.0</td>
<td>-8.5</td>
<td>-8.0</td>
<td>-14.0</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

*aGlobal method parameters were: source temperature 150 °C, curtain gas (CUR) 20, ion spray voltage (IS) -4500 V, Gas flow (GS1 and GS2) 50 (arbitrary units), collision associated dissociation (CAD) High.

8.3.2 Linearity, sensitivity, matrix effect and recovery study

Standard 5mM DCA was serially diluted in a step of 2 for 13 steps in water with 0.1% formic acid and analyzed by the developed method in triplicate to construct the standard curve (Figure 7.1). Limit of detection (LOD) and lower limit of quantification (LLOQ) was calculated using the following equations:

\[
LOD=\frac{3.3\times\text{Standard deviation of lowest concentration}}{\text{Slope of the standard curve}} \quad \text{(Equation 8.1)}
\]

\[
\text{LLOQ}=\frac{10\times\text{Standard deviation of the lowest concentration}}{\text{Slope of the standard curve}} \quad \text{(Equation 8.2)}
\]

Recovery and matrix effect were analyzed using ER+ breast cancer cell line (MCF-7, ATCC, Manassas, VA) extract spiked with DCA. Briefly, \(10^3\) breast cancer cells/mL were grown to confluence. The cells were washed 3 times with 100 µL PBS. Each time, after addition of PBS, the cells were left still for 5 mins, and then the supernatant was discarded. Finally, 100µL of cold PBS was added and the plate was kept in -80°C overnight. The next day, cells were extracted with 150µL cold acetonitrile, mixed well on ice and transferred to chilled Eppendorf tube. The extract was then centrifuged at 5000 rpm for 30 mins at 4°C. The supernatant was collected on a fresh chilled Eppendorf tube.
100µL of cold acetonitrile was added to cell wells, mixed well, and combined with pellets obtained from the 1<sup>st</sup> centrifugation, resuspended well. The cellular suspension was then centrifuged at 5000 rpm for 15 mins at 4 °C. The supernatant was collected and combined with the 1<sup>st</sup> supernatant. To this 100 µL of cold water was added, freeze dried under vacuum and reconstituted in 100µL water with 0.1% formic acid to prepare the matrix. Matrix was then spiked with DCA and filtered using solid phase extraction protocol with low protein binding durapore (PVDF) (Milex R-GV, Japan). Similarly, the blank matrix was filtered, and the extract was spiked with DCA. These two matrices, spiked before and after the solid phase extraction, were analyzed by the developed method to calculate the recovery:

\[
\text{Recovery of DCA} = \frac{\text{area unit of DCA before spike}}{\text{area unit of DCA after spike}} \times 100
\]  

(Equation 8.3)
To check the matrix effect, the extracted matrix was spiked with three different concentration of DCA (3µM, 100µM and 3000µM). Similarly, these concentrations of DCA were prepared in water with 0.1% formic acid. These samples were analyzed by the developed method. Matrix effect was by:

\[
\text{Matrix effect} = 1 - \frac{\text{area of DCA in matrix}}{\text{area of DCA in water}} \times 100^{174} \quad \text{(Equation 8.4)}
\]

8.3.3 DCA uptake study using LC-MS/MS

Cells were seeded to 96-well plates in 100µL of serum free medium at a density of 1x10^3 cells/well for each of the three cell lines (MCF-7, MDA-MB 231, MCF-10A) for 24h. The cells were treated with 100µL of G-DCA, PE-DCA, and native DCA (Sigma Aldrich, St. Louis, MO) samples at 1mM, 5mM, and 10mM concentrations. At 2 hr, 10 hr, and 24 hr, the wells were washed 3 times with 1mL PBS. The samples were then processed as described before, freeze dried under vacuum and reconstituted in 200µL water-acetonitrile (50:50, v/v) with 0.1% formic acid for LC-MS analysis.

8.3.4 Statistical Analysis

All experiments were performed at least in triplicate (n=3) and the results were expressed as mean ± standard deviation (SD). Student t-test was applied to compare mean values. And a p value of ≤0.05 is considered as statistically significant.

8.4 Results and Discussion

To determine the LC-MS/MS chromatogram of DCA, different solvent systems at various pH in both reverse phase and hydrophilic interaction chromatography were tested and the best chromatogram (Figure 8.2) was obtained with a isocratic flow of water with 0.05% triethylamine and 0.15% formic acid for 7 min with a Ascentis Express (Supelco) C18 column (100×2.1 mm, 2.7 µm). The pH of the mobile phase was 2.75 and the flow rate was 0.28
mL/min. Average retention time for DCA was 3.04 minutes with a standard error of 0.005 min calculated from injection of 40 samples during constructing standard curve.

Figure 8.2: LC-MS/MS chromatogram of 1 mM DCA

The LC-MS/MS method was validated via LOD and LLOQ. Limit of detection (LOD) and lower limit of quantification (LLOQ) for DCA were calculated as 0.78µM and 2.3µM from the standard curve (R² = 0.9958). Recovery and matrix effect were calculated using the formula described in the method section. Overall recovery was found to be 109.19% with a standard error of 0.85%. Recovery at three different concentrations were in acceptable range (Table 8.2).

Table 8.2: Matrix effect analysis of DCA in breast cancer cell extract at three different concentration

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Matrix Effect (%ME) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.38 ± 1.24</td>
</tr>
<tr>
<td>100</td>
<td>7.94 ± 0.89</td>
</tr>
<tr>
<td>3000</td>
<td>2.97 ± 0.28</td>
</tr>
</tbody>
</table>
The intracellular DCA uptake study with G-DCA, PE-DCA, and native DCA was performed using the following cell lines: normal breast epithelial (MCF-10A), ER+ breast cancer (MCF-7) and triple negative breast cancer (MDA-MB 231). There is increased intracellular DCA uptake in normal breast epithelial cells (MCF-10A) when treated with native DCA at various concentrations as compared to the G-DCA and PE-DCA formulations further emphasizing that the prodrug conjugations enhance DCA targeting to cancer cells (Figure 8.3 A-C). Fortunately, native-DCA is not cytotoxic on normal breast epithelial cells as shown in literature and confirmed in Figure 5.1 suggesting that DCA’s mechanism of action in inducing cell death is only activated in cancerous cells due to mitochondrial dysfunction variation between breast cancer molecular subtypes. Moreover, DCA uptake of native DCA in ER+ breast cancer cells are negligible and very limited for TNBC cells at various concentrations suggesting inefficiency of DCA targeting in breast cancer cells (Figure 8.3A).

DCA uptake was measured in normal and breast cancer cells that treated with PE-DCA at the same concentration range of native DCA (Figure 8.3B). PE-DCA is higher in TNBC cells as compared to ER+ breast cancer cells and normal breast epithelial cells illustrating that not only the 2-phenoxyethylamine conjugation to DCA optimizes DCA targeting efficiency, but PE-DCA has a similar mechanistic approach like other phenoxyethanamine derivatives. Like other phenoxyethanamine derivatives, like DPPE, PE-DCA has an affinity for the antiestrogen receptor binding site (AEBS) in the cellular microsomes of cytochrome P450 enzymes and P-glycoprotein leading to efflux inhibition and decreased cancer cell proliferation.\textsuperscript{116-117} AEBS is highly expressed in TNBCs compared to ER+ breast cancers due to a lack of estrogen receptors, which suggests that the PE-DCA
A prodrug may have therapeutic capabilities for the aggressive TNBC.\textsuperscript{118} Uptake of PE-DCA in MCF-10A cells increases as concentration of the drug increases more than that of the prodrug formulations illustrating that although PE conjugation to DCA makes DCA more cancer cell targeted, there is still a need to improve PE-DCA’s specificity toward cancer cells. MTT cell viability shows that at the concentration range of 250-10,000µM, PE-DCA is not cytotoxic on normal breast cancer epithelial cells (Figure 5.1). Intracellular DCA uptake on cell lines treated with G-DCA shows that G-DCA has increased uptake in TNBC cells at 1mM, (Figure 8.3C) which can be associated with the CC\textsubscript{50} value for G-DCA being 16.22µM (Figure 5.17). Similar to PE-DCA uptake in normal breast epithelial cells, G-DCA’s specificity needs to be improved to be more cancer cell targeted.

In comparing the three formulations, DCA intracellular uptake in all three cell lines is somewhat higher in comparison to prodrug uptake in cancerous cell lines, but native DCA uptake in normal cells is exponentially higher, further proving the need for specific targeting to cancer cells. Moreover, in ER+ breast cancer cell lines, intracellular DCA uptake is low despite prodrug treatment. In earlier chapters we proposed that G-DCA gets shunted into the ER+ breast cancer cells via GLUTs, since they are overexpressed in breast cancer cells in order to maintain energy and metabolites to support cell survival. Intracellular uptake results for MCF-7 cells suggest that there is a need for more specific targeting to accommodate the fluctuating metabolic reprogramming defining ER+ breast cancer cells.
Figure 8.3: DCA uptake in MCF-10A, MCF-7, and MD-MBA 231 cell lines for different formulations: (A) Native-DCA, (B) PE-DCA, (C) G-DCA
8.5 Conclusion

A LC-MS method was successfully developed and validated to determine intracellular DCA uptake in various cells line: MDA-MB 231, MCF-7, and MCF-10A. Results showed that the DCA prodrugs are more cell targeted to cancer cells than normal breast epithelial tissues. DCA, G-DCA, and PE-DCA show elevated levels of intracellular DCA uptake normal breast epithelial cells illustrating a need to optimize G-DCA and PE-DCA targeting efficiency. MTT and LDH viability and cytotoxicity data have shown that DCA and its prodrugs are not cytotoxic on normal cells. Because DCA is a small molecule it can diffuse through cellular membranes easily which can describe how easily DCA and its related prodrugs are able to get into normal cells at such a high rate. In comparison, normal breast cancer cells have functional mitochondria as compared to their cancerous counterparts. The mitochondria in TNBCs are more dysfunctional than ER+ breast cancers which can explain discrepancies in DCA uptake when cells are treated with PE-DCA and G-DCA prodrugs.
9.1 Summary

Two novel DCA prodrugs, PE-DCA and G-DCA were synthesized at high yields (>80%). Based on physicochemical analysis, such as XRD and SEM, the two prodrugs, are distinctively different from that of native DCA indicating that the prodrugs are novel structures. As illustrated via MTT and LDH data, G-DCA and PE-DCA are more cancer cell targeted than that of native DCA as indicated by low CC$_{50}$ values as compared to native DCA. G-DCA has implications of being cytotoxic to both TNBC and ER+ breast cancer cells, while PE-DCA shows elevated cytotoxicity induction on TNBCs. Furthermore, a 2-DG and DCA physical mixture does not show cytotoxicity on breast cancer cells indicating the importance of the conjugation synthesis reactions in G-DCA and PE-DCA formation.

The metabolic profiles of TNBC and ER+ breast cancer cells were explored via intracellular ROS and autophagosome studies in different media environments. TNBC cells have both dysfunctional mitochondrial activity, as well as an enhanced glycolytic metabolism. TNBC cells also have increased glucose uptake leading to elevated cell proliferation rates and increased sensitivity to glycolytic inhibitors. Therefore, enhancing DCA’s glycolytic inhibitory function by the conjugation synthesis of G-DCA and PE-DCA can make DCA more cell targeted. TNBC cells rely on glycolysis compared to other molecular subtypes of breast cancer even under normoxic conditions due to compromised mitochondrial respiration. MCF-7 cell lines are able to modulate between aerobic glycolysis and mitochondrial oxidative phosphorylation metabolic pathways depending on glucose concentration in the tumor microenvironment. In high glucose availability, the Krebs cycle
is inhibited. Cell viability and intracellular ROS data show that the DCA prodrugs at low concentrations are cytotoxic to breast cancer cells and can increase ROS species in high glucose environments for both TNBC and ER+ breast cancer cell lines. In elevated glucose environments, DCA can induce apoptosis in response to elevated ROS levels. Because autophagy has various mechanistic avenues in cancer cells: pro-proliferative and anti-tumorigenic depending on the stage, grade, and tissue type of the cancer, cell death triggered by the DCA prodrugs may not be directly related to autophagic pathways, but instead, apoptosis. Our results indicate that the prodrugs are cytotoxic to TNBC and ER+ breast cancer cells based on LDH and MTT data mentioned in previous chapters but based on data of the presence of autophagic cells in various media environments, cell-death pathways may not directly correspond to autophagy. It is noted that autophagy can trigger a pro-tumorigenic and pro-proliferation response in cancer cells as well as trigger apoptotic pathways. Autophagy can solicit survival in dormant and metastatic breast cancers further emphasizing the complexities of autophagic activation. 

Intracellular uptake was quantified via LC/MS and validated to determine intracellular DCA uptake in various cells line: MDA-MB 231, MCF-7, and MCF-10A. Results showed that the DCA prodrugs are more cell targeted to cancer cells than normal breast epithelial tissues. DCA, G-DCA, and PE-DCA show elevated levels of intracellular DCA uptake in normal breast epithelial cells, illustrating a need to optimize G-DCA and PE-DCA targeting efficiency. MTT and LDH viability and cytotoxicity data have shown that DCA and its prodrugs are not cytotoxic to normal cells. Because DCA is a small molecule, it can diffuse through cellular membranes easily which can describe how easily DCA and its related prodrugs are able to get into normal cells at such a high rate. In comparison, normal
breast cancer cells have functional mitochondria as compared to their cancerous counterparts. The mitochondria in TNBCs are more dysfunctional that of ER+ breast cancers which can explain discrepancies in DCA uptake when cells are treated with PE-DCA and G-DCA prodrugs.

9.2 Future Perspectives

Both physicochemical and biological analysis of the two prodrugs, PE-DCA and G-DCA as compared to native DCA, have indicated that through the conjugation synthesis reactions of DCA with that of PE and glucose, respectively, has improved the targeting and cancer killing capabilities of DCA. The increase of intracellular reactive oxygen species accumulation after treating ER+ and TNBC cell lines with the novel prodrugs, indicates that there is a reversal of the Warburg effect. To further prove this theory, it would be necessary to analyze mitochondrial membrane potential at various concentrations to illustrate the enhanced mitochondrial function of cells treated with the two prodrugs as compared to the dysfunction exhibited when cells are treated with native DCA. Moreover, qualitative analysis of Intracellular ROS and autophagosomes can be used to visually illustrate the relationship between autophagy induction and ROS.

Furthermore, drug efficacy can be optimized by lowering the IC$_{50}$ of the prodrugs to the nanomolar range, although this value is now in the millimolar range, significantly lower than the millimolar concentration of DCA. To further enhance DCA’s breast cancer chemotherapeutic properties and address its strong neurotoxicity, toxicity studies using the two prodrugs compared to native DCA should be performed. Because solubility has changed within the two prodrugs as compared to DCA, suggesting that both G-DCA and PE-DCA are more hydrophobic than DCA alone, additional solubility studies can be performed to further
ascertain the physicochemical properties of the two prodrugs. Computer modelling to determine the mechanism of action of G-DCA and PE-DCA can improve targeting, delivery approaches, and specificity on apoptotic pathway activation. In vivo efficiency of the two prodrugs should be explored using various metabolic and cytotoxic assays similar to this in vitro dissertation work.
APPENDIX

Abbreviations

2DG: 2-Deoxy-D-Glucose
DCA: Dichloroacetate
DCA-A: Dichloroacetic anhydride
ER+: Estrogen receptor positive
G-DCA: Glucose dichloroacetamide
MAPK: Mitogen-Activated Protein Kinase
MMP: Mitochondrial Membrane Potential
PDH: Pyruvate dehydrogenase
PDK: Pyruvate kinase
PR+: Progesterone receptor positive
PE: 2-Phenoxyethylamine
PE-DCA: Phenoxyethyl dichloroacetamide
Rb: Retinoblastoma
ROS: Reactive Oxygen Species
SEM: Scanning Electron Microscopy
TNBC: Triple Negative Breast Cancer
XRD: X-Ray Diffraction
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VITA

Danielle Thomas was born January 4, 1988 in Los Angeles, CA. She graduated as class valedictorian from St. Mary’s Academy (Inglewood, CA) in 2006. In 2010, she earned her Bachelor of Arts in Comparative Literature with an emphasis in Russian and English Literature from the University of Pennsylvania in Philadelphia, PA. Upon graduation, she pursued post-baccalaureate work in biomedical sciences at St. Joseph’s University (Philadelphia, PA) until 2011. While there she worked as a surgical assistant under Dr. Marek Ma at the University of Pennsylvania studying axonal degeneration.

She moved to Las Vegas, NV in 2011 to pursue a career in secondary education. In 2014, she earned a Bachelor of Science in Biology with a Chemistry minor from Nevada State College (Henderson, NV). While there she was a science, Spanish, and standardized test tutor for various students from elementary to healthcare professional in the Las Vegas valley. In 2016, she earned a Master’s in science in Cell & Molecular Biology from the UMKC School of Biological Sciences. She started her doctoral education Fall 2016 at the UMKC School of Pharmacy with a primary discipline in Pharmaceutical Sciences and a co-discipline in Molecular Biology & Biochemistry. During her graduate tenure, she was in various student organizations, such as the American Association of Pharmaceutical Scientists (AAPS) UMKC Chapter, as well as a winner of various awards and scholarships, such as UMKC Three Minute Thesis Grand Prize winner in 2018 and 2019. Upon competition of the doctoral degree requirements, Ms. Thomas will pursue a career in breast cancer research and academia.