Role Of Cardiolipin Remodeling In The Malignant Progression Of Breast Cancer

Jawaher H. Alkhamisy

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ROLE OF CARDIOLIPIN REMODELING IN THE MALIGNANT PROGRESSION OF BREAST CANCER

A Thesis
Presented in partial fulfillment of requirements for the degree of Master of Science in the Department of Biomolecular Science
The University of Mississippi

Jawaher Alkhamisy
December 2019
ABSTRACT

In spite of the current advances in both radiotherapy and surgical techniques as well as molecularly targeted therapies, over 90 percent of cancer mortality rate is due to metastases. Considering the pivotal role of metabolism in cellular physiology, there is no doubt that metabolic alterations are critical for metastatic progression.

Cardiolipin (CL), the signature phospholipid of mitochondria, is linked to a variety of vital mitochondrial processes, like oxidative phosphorylation. Cardiolipin is a unique dimeric phospholipid which contains four fatty acyl chains. Moreover, the CL remodeling process is believed to be responsible for generation of immature CL species that contribute to tumor growth progression. Abnormalities in CL composition and/or content have been discovered in various solid tumors. Cancer cells prefer saturated fatty acids over polyunsaturated fatty acids to avoid apoptosis triggered by CL peroxidation in mitochondria. However, little is known about the biochemical basis of the action of CL remodeling on breast cancer cell proliferation and metastasis. The effects of two potent phospholipase inhibitors which are bromoenol lactone [BEL], a calcium-independent phospholipase A2 inhibitor, and halopemide, a phospholipase D inhibitor, were examined on MDA-MB-231, MCF-7 cell lines and their corresponding bone metastasis BoM and MCF-7/BoM cell lines to study the role of CL remodeling on the progression of breast cancer and CL extraction method from breast cancer metastatic cells were established.
DEDICATION

This work is dedicated to my family and Hayat Alkhamisy for their continued support of my academic dream. To my great friends for all the kind words and encouraging pick me ups during this journey.
**LIST OF ABBREVIATIONS AND SYMBOLS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>CAFs</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>CDP-DAG</td>
<td>Cytidine diphosphate-diacylglycerol</td>
</tr>
<tr>
<td>CDS</td>
<td>Cytidine diphosphate-diacylglycerol synthase</td>
</tr>
<tr>
<td>CLS</td>
<td>Cardiolipin synthase</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine monophosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>DLCL</td>
<td>Dilyso-cardiolipin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>G3P</td>
<td>Glycero-3-phosphate</td>
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<tr>
<td>MLCL</td>
<td>Monolyso cardiolipin</td>
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<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PGP</td>
<td>Phosphatidylglycerol phosphate</td>
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<tr>
<td>PGPS</td>
<td>Phosphatidylglycerol phosphate synthase</td>
</tr>
<tr>
<td>PGPP</td>
<td>Phosphatidylglycerol phosphate phosphatase</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>TAZ</td>
<td>Tafazzin</td>
</tr>
<tr>
<td>MLCL-AT</td>
<td>Monolyso cardiolipin acyltransferase</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>TLCL</td>
<td>Tetralinoleoyl cardiolipin</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>BEL</td>
<td>Bromoenol lactone</td>
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<tr>
<td>iPLA2</td>
<td>Calcium independent phospholipase A2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>RTs</td>
<td>Retention times</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
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ACKNOWLEDGMENTS

I would like to acknowledge the Saudi Arabian Cultural Mission (SACM) and the University of Mississippi for the opportunity to fulfill my academic dream. My advisor, Dr. Dale G. Nagle for his continued support and my thesis committee, Dr. Yu-Dong Zhou, Dr. Marc Slattery, and Dr. Cole Stevens. Without your dedication and hard work in helping me to accomplish this great task, this would never have been a possibility.

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CHAPTER I: INTRODUCTION:

1.1. Breast Cancer

Other than skin cancer, breast cancer is the most common form of solid tumor in women with increasing mortality rate in America, Europe, Asia, and Africa.\(^1\) According to the National Cancer Institute, there will be 268,600 new cases of and the disease, among which 41,760 women will die in 2019.\(^2\)

These malignant tumors display discrete immunohistochemical profiles that are created by cancer cells within specific microenvironments, with unique intercellular cross talk which is linked to various clinical outcomes.\(^3\) Specifically, based on evidence from malignant breast tumor immunohistochemistry, breast cancer is divided into three major types, that include estrogen (ER\(^+\)) and progesterone (PR\(^+\)) receptor—positive, human epidermal growth factor receptor 2 overexpressing (HER2\(^+\)), and ER\(^-\), PR\(^-\), HER2\(^-\) or triple-negative breast cancers (TNBCs).
The previously described breast cancers have different prognoses (Figure 1). Estrogen (ER\textsuperscript{+}) and progesterone (PR\textsuperscript{+}) receptor–positive breast cancer is divided into two subtypes: luminal A including ER\textsuperscript{+} and/or PR\textsuperscript{+} and HER2\textsuperscript{-} breast cancer which is distinguished by low Ki-67 proliferation marker expression; and luminal B, which is ER\textsuperscript{+} and/or PR\textsuperscript{+}, HER2\textsuperscript{+} (or HER2\textsuperscript{-}) breast cancer that highly expresses Ki-67 and is associated with poor prognosis relative to luminal A-forms. The HER2\textsuperscript{+} expressing breast cancers are considered aggressive or malignant tumors that are associated with increased mortality and recurrence rate, in increased risk of metastasis progression. However, TNBCs account for about 12% of all breast cancers are the most heterogeneous subtype of breast cancer with the highest risk of recurrence and the shortest overall survival rates.\textsuperscript{3,4}
One of the significant characteristics of solid tumors is abnormal extrinsic microenvironments, including alterations in the extracellular matrix (ECM), blood and lymphatic tumor vessels, and non-cancerous stromal cells. These stromal cells are characterized by endothelial cells, immune cells, pericytes, activated adipocytes, cancer-associated fibroblasts (CAFs), and mesenchymal stem cells (MSCs), as well as epithelial cancer cells.5 As a result of interaction between tumor cells and their microenvironment components which act as metabolic ecosystems, proliferating tumor cells employ unique molecular mechanisms that range from intrinsic genetic mutations to extrinsic micro-environmental factor(s)-activated signaling pathways to modify their cellular metabolism.6-8

Metabolic plasticity is considered as a distinct hallmark of breast cancer cells. Notably, the primary source of energy in many breast cancer cells is aerobic glycolysis, called the Warburg Effect.8,9 This effect represents a metabolic phenotype of tumor cells that is associated with a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis for ATP production, even in the presence of relatively high oxygen tension, to simultaneously provide crucial building blocks required for rapid proliferation and growth.1 By contrast, experimental evidence also indicates that OXPHOS plays an important role in tumor growth and progression. Moreover, emerging evidence has demonstrated that cancer metabolism is not a static process, as tumor metabolism can be altered to compensate for cellular needs. The cellular requirements are monitored by the interactions between tumor and its microenvironmental crosstalk which produces a hybrid glycolysis/OXPHOS phenotype. This phenotype accelerates the metabolic plasticity of tumor cells, and is believed to contribute to metastasis and therapeutic-resistance.10
1.2. Breast Cancer Metastasis

In spite of current advances in radiotherapy, surgical techniques, and molecular-targeted therapies, over 90% of cancer mortality remains to be associated with secondary metastatic tumors. Secondary metastatic cancers are caused, in part, by progressive promotion of therapeutically resistant metastases that arise following primary therapies.\textsuperscript{11,12} These tumors develop resistance either by activating complimentary signaling pathways or by acquiring new mutations. Therefore, there is no current treatment option which can efficiently and selectivity curb the spread of many tumor cells to vital organs.\textsuperscript{12-14}

Research and clinical results have confirmed Paget’s century-old “seed and soil” hypothesis. This details that favorable interactions between metastatic tumor cells, the “seed,” and their organ-unique microenvironment, the “soil,” that generates a preferential pattern of organ-specific tumor metastasis (also known as “organotropism”). The invasion and intravasation of metastasis-initiating tumor cells into the vasculature, surviving circulation, and extravasation into distant target organs, form micro-metastases, and progress from a state of dormancy to outgrow into secondary lesions.\textsuperscript{11,13} While enormous number of tumor cells are released into circulation, only about 0.01% succeed in creating metastases. In breast cancer, secondary lesions are often formed in the lungs, bones, brain, and liver.\textsuperscript{12-14} The site-specific metastases vary in terms of evolution, growth rate, hormone receptors, immunogenicity, sensitivity to cytotoxic drugs, morbidity, and mortality. The interactions between tumor microenvironment and genetic mutation influence both organ tropism and metastatic propensity.\textsuperscript{15} Research has led to breakthroughs in regard to identifying the target organ-dependent gene expression profile signatures that are
predictive of breast cancer bone-, brain-, and lung-specific metastases.\textsuperscript{15-17} However, the connections between tumor metastasis and metabolism are less defined. Currently, metabolomic profiles indicate that the acquisition of a glycolytic phenotype is associated with rising metastatic potential.\textsuperscript{8} Considering the pivotal role of metabolism in cellular physiology, there is no doubt that metabolic alterations are critical for metastatic progression. Targeting metastasis-specific tumor metabolism represents a potentially straightforward means to more effectively curb metastasis.

\textit{Figure 2.} Tumor progression, growth and metastatic dissemination steps. The scheme illustrates different pathologically identified tumor progression stages; and various functions associated with metastatic spread. The functions linked to dissemination may be general metastasis mediators. In the purple box, subsequent functions are required for metastatic colonization which could be distinct to the micro-environmental factors of the various metastatic sites (adapted from Cold Spring Harb Symp Quant Biol 2005, 70, 149-58).\textsuperscript{15}
Mitochondria which act as energy-producing organelles are membrane-enclosed cellular structures, and their influence extends to a number of other cellular functions. Mitochondria have a unique structure that includes two membranes. The first membrane is a smooth outer mitochondrial membrane (OMM) and the second is an inner mitochondrial membrane (IMM), composed of many invaginations called cristae. The bioenergetics of mitochondria primarily depends on the inner mitochondrial membrane (IMM) physiology that hosts the respiratory system and phosphorylation redox complexes. The complexes that carry out the oxidative phosphorylation are considered to function as a highly efficient concerted energy-generating process. In this process, the energy produced from nutrient oxidation is used to drive ATP generation. Further, mitochondrial oxidative phosphorylation is responsible for approximately 90% of cellular oxygen consumption and provide more than 80% of the energy needed for cellular metabolism. Moreover, mitochondria have other functions in the cell which include the maintenance of cellular redox balance, the housing of important biosynthetic pathways, the modulation of calcium signaling, and in the regulation of cell death.

Mitochondria are not static entities and can be extremely dynamic. Mitochondria require a source of membrane lipids and a supply of specific proteins to be coordinated and adjusted in order to achieve physiological and biochemical demands. The lipids of the mitochondrial membranes are involved in many processes, such as energy production, protein biogenesis, membrane fusion, and apoptosis. Further, spatially defined lipid distribution may also influence mitochondrial processes, such as fission, fusion, or the membrane plane protein topology. Cardiolipin, the
signature phospholipid of mitochondria is the perfect example of the crucial role of membrane lipids in mitochondria function. Cardiolipin is linked to a variety of vital mitochondrial processes, like oxidative phosphorylation, apoptosis, and to the assembly and functionality of mitochondrial membrane proteins. Alterations in the CL pool is associated with many life-threatening mitochondrial dysfunction-related diseases, such as Barth Syndrome, cancer, diabetes, and other metabolic diseases.\textsuperscript{18,24}
1.4. Cardiolipin Metabolism

1.4.1. Cardiolipin Structure

Cardiolipins are a unique class of anionic phospholipids, and which predominantly, or nearly exclusively, are found in mitochondrial membranes, and are mainly localized in the mitochondrial inner membrane.\textsuperscript{18,24} Each CL molecular species (1,3-diphosphatidyl-sn-glycerol) is composed of a dimer of two phosphatidyl moieties, three distinct glycerol moieties with three stereogenic centers, and four fatty acyl chains.\textsuperscript{25,26}

\textit{Figure 3.} The unique conical structure of cardiolipin promotes curvature in lipid membranes. (A) Phosphatidylcholine chemical structure (PC). (B) Cardiolipin (CL). chemical structure (C) Cardiolipin exerts lateral pressure in a lipid bilayer to induce a negative curvature (adapted from Cell Mol Life Sci 2008, 65, (16), 2493-506.).\textsuperscript{24}
1.4.2. Cardiolipin Biosynthesis and Remodeling

The synthesis of the vast majority of mitochondrial membrane lipids occurs in the endoplasmic reticulum. Contrary to this, de novo CL biosynthesis exclusively takes place in the mitochondrial inner membrane (Figure. 3). The mitochondrial CDP-DAG synthase, an enzyme that catalyzes the first reaction of CL biosynthesis, converts mitochondrial phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG). Phosphatidylglycerophosphate synthase 1 (Pgs1) transfers the CDP-DAG phosphatidyl group to glycerol-3-phosphate to generate phosphatidylglycerolphosphate (PGP). The PGP phosphatase catalyzes the subsequent PGP dephosphorylation to phosphatidylglycerol (PG). The final reaction of de novo CL synthesis is catalyzed by CL synthase (Crd1) which is responsible for adding a phosphatidyl group from CDP-DAG to PG. The result produces CLs that contain two phosphatidyl groups that are connected by a glycerol molecule.

The cardiolipin remodeling processes is characterized by the removal and replacement one acyl chains with other acyl chains. Cardiolipin remodeling plays a vital role in creating symmetrical CL with characteristic fatty acid residues following de novo synthesis because the enzymes involved in de novo CL synthesis do not exhibit acyl chain-substitution specificity. There are two mechanisms by which CL remodeling can occur. In the two-step mechanism, CL is remodeled through the deacylation-reacylation cycle in which phospholipid is deacylated by phospholipases to form lysophospholipid. Then, acyltransferases or transacylases catalyze the addition of acyl groups that derive from acyl-CoA or neighboring phospholipids to the lysophospholipid. In this process, phospholipases deacylate CL and monolyso-CL (MLCL) are reacylated to CL by either acyltransferase or transacylase. However, in one-step CL remodeling
reaction, acyl groups are exchanged between adjacent phospholipids in a single transacylase-catalyzed step. During this single step, tafazzin, the transacylase remodeling CL, catalyzes CL remodels through only transacylation, in which the deacylation of phospholipids is not required.29,31

Figure 4. Model of CL biosynthesis and remodeling in pediatric heart failure: Acyl-CoA, acyl-coenzyme A; CL, cardiolipin; CDP-DAG, cytidinediphosphate-diacylglycerol; CDS, CDP-DAG synthase; CLS, CL synthase; CMP, cytidinemonophosphate; CoA-SH, coenzyme A (unconjugated); CTP, cytidinetriphosphate; DLCL, Dilyso-CL; FFA, free fatty acid; G3P, glycerol-3-phosphate; MLCL, monolyso-CL; MLCL-AT, MLCL acyltransferase; PG, phosphatidylglycerol; PGP, PG phosphate; PGPS, PG-P synthase; PGPP, PG-P phosphatase; Pi, inorganic phosphate; PLA2, phospholipase A2; TAZ, tafazzin (adapted from Am J Physiol Cell Physiol 2007, 292, (1), C33-44.).31
1.5. Mitochondrial Cardiolipin and signaling during Apoptosis

Apoptosis is a controlled process of cell death to maintain the homeostasis of cell populations in tissues; and occurs as a defense mechanism in damaged cells or in immune reactions. There are a number of reports indicating that during apoptosis, many alterations in mitochondrial lipid content and distribution occur. Among these alternations, in mouse liver mitochondria, an increase in monolysocardiolipin (MLCL).

Cardiolipin is highly vulnerable to oxidative damage not just for its high content of unsaturated fatty acids, but also for its location which is near the site of ROS. Mitochondrial H$_2$O$_2$ can oxidize cardiolipin; however, in the presence of cyt c, this oxidation is significantly enhanced. The dramatic enhancement of the protein’s peroxidase activity is due to the interaction of cyt c with cardiolipin which promotes cyt c unfolding. The native cyt c is a compact tertiary structure with a coordination of its heme iron to Met80 and His18. This is reported that native cyt c shows very low peroxidase activity which results from its hexacoordinated iron. There exists substantial unfolding of cyt c according to the reports which have examined cyt c and cardiolipin liposomes. The unfolding of cyt c can disrupt the Met80 ligation and exposes the heme iron to H$_2$O$_2$. There are other researchers who report deep penetration of one or two acyl chains of cardiolipin into the hydrophobic core of cyt c. This can also lose the Met80-Fe axial bond. The cardiolipin oxidation produces kinks in the acyl chain. This oxidation disturbs cardiolipin microdomains on the IMM and also causes curvature loss (Figure 5). In addition, cyt c is detached from the IMM due to the disruption of super complexes. All in all, this mitochondrial respiration inhibits which sets the stage for apoptosis. There is a synergy between oxidized cardiolipin and
Ca\(^{2+}\) which make the mitochondrial permeability transition (MPT) pore open. It is believed that the identity of the MPT pore is remains elusive, but non-specific which allows any molecules <1.5 kDa have free passage. There are several parts, including the voltage-dependent anion channel, the adenine nucleotide translocator and the F\(_{0}\)F\(_{1}\)-ATP. The permeability barrier of the IMM is disrupted by mitochondrial permeability transition. Therefore, there is a collapse of the mitochondrial potential and uncoupling of oxidative. Additionally, cyt c and other proapoptotic proteins are released into the cytosol as a result if which the caspase cascade and cell death are triggered by apoptosis. Necrosis can make cell death die due to severe depletion of ATP. This is interesting to note that in IMM-OMM contact sites, CL and phosphatidylethanolamine (PE) are predominate.\(^{18,24}\) The two phospholipids are non-bilayer lipids. These characteristics help them to adopt an inverted hexagonal phase (HII) when there are no membrane constraints. Although the lipids are to some extent speculative, at the contact sites, it is assumed that, non-bilayer lipids could be involved in membrane fusion. The involvement fosters CL access to the cytosolic face of OMM. where it would have direct interaction with t-Bid. This offers docking site for this active form of the pro-apoptotic protein Bid which is considered as the first step in triggering the action of Bax and Bak (other pro-apoptotic Bcl-2 proteins). This leads to OMM permeabilization. According to more recent reports, there is an involvement of CL-enriched contact sites in apoptosis-associated events. In the current research study, there is a hypothetic model proposing a contact sites act as activating platforms (raft like microdomains). Within these platforms, caspase-8 is anchored which cleaves its substrate (Bid). The T-Bid is the resulting product which is then recruited to promote Bak/Bax oligomerization. Membrane defects would be created due to the segregation of these domains from the rest of the OMM which fosters apoptogenic factor leakage.\(^{18,24}\)
Figure 5. Cardiolipin microdomains and peroxidation. Cardiolipin microdomains is destabilized by cardiolipin peroxidation on the inner mitochondrial membrane (IMM) and disrupts supercomplexes. Cardiolipin (CL) is sensitively vulnerable to oxidative damage for the high amounts of unsaturated fatty acids. The structure of cardiolipin (CLOOH) is altered by peroxidation of the acyl chains. As the result of which cardiolipin is prevented from aggregating into microdomains or rafts on the IMM. Cristae curvatures is abolished due to the breakdown of cardiolipin rafts which results in the disruption in the organization of respiratory complexes into higher order supercomplexes. The affinity for cyt (C) is also reduced by peroxidation of cardiolipin which makes the setting ready for the stage for cyt c to release into the cytosol and apoptosis. IMS: intermembrane space (adapted from Cell Mol Life Sci 2008, 65, (16), 2493-506.).
1.6. Cardiolipin Remodeling in Tumor Cell Survival

Metabolic reprogramming in cancer involves a process of unique alterations in mitochondrial membrane composition. Results from analysis of the inner membrane lipid composition in the mitochondria from various tumors indicate an increased cholesterol content and alterations in the relative content of various phospholipids species. In addition, changes in lipid acyl chain components are also observed. In general, the mitochondria phospholipids coming from tumor cells are shorter and have fewer unsaturated acyl chains compared with those from normal cells. These alterations of the lipid composition in mitochondrial membranes are believed to impact the mitochondrial phenotype which contributes to the Warburg Effect, and a decreased susceptibility to apoptotic stimuli. Dynamic processes in the altered mitochondrial membrane composition of cancer cells may be responsible for the susceptibility reduction in the respiratory chain activity; a relationship that has already been established for CL. On the other hand, remodeling of the mitochondrial membrane lipid composition can also lead to rise in mitochondrial membrane potential ($\Delta \Psi$) which is observed in some cancer cells.

Recently, CL has captivated considerable interest in cancer research. For example, a shotgun lipidomics study focused on determining CL in mitochondria from subcutaneous brain tumors, a stem cell tumor, an astrocytoma, an ependymoblastoma, and two microgliomas provided evidence that indicates that there is an alteration in CL content and composition in tumors. Deformities in CL biosynthesis and remodeling processes may cause abnormalities which include an abundance of immature molecular species along with the alteration of mature fully formed CL species. Overall, in terms of cancer, the data regarding these mitochondrial phospholipid, is
somewhat divergent; ranging from the CL content reduction in brain tumors and the appearance of immature molecular species. as well as a CL content reduction in rhabdomyosarcoma cells. In a peritoneal carcinoma rat model an increase in CL content and CL-fatty acid remodeling was observed, while no change in total CL was measured in a separate mouse strain with high propensity to form spontaneous gliomas.

In regard to chemotherapeutic-resistance phenotype, various studies on MCF-7 human breast cancer cells have explained an increase in CL content (expressed in the percentage of cell total phospholipids) in doxorubicin- and cisplatin-resistant cancer cells compared to drug-sensitive parental cells.

Oxidative stress has been connected to tumor initiation and progression. Production of reactive oxygen species (ROS) including hydrogen peroxide and superoxide from the mitochondrial electron transport chain (ETC) causes oxidative stress to the tumor cells. During the rapid proliferation, tumor cells develop sophisticated systems to counterbalance the oxidative stress. As mitochondria-associated phospholipids, CLs are the principal targets of ROS attack. In the most mammalian tissues, tetralinoleoylcardiolipin (TLCL) is the major CL that contains four linoleic acid (LA, 18:2, ω-6) chains. Research findings point out that CL peroxidation was involved in apoptosis via the release of proapoptotic factors like the cytochrome c (cyt c) from mitochondria into the cytosol. The Huiyong Yin and co-workers used a non-targeted metabolomic approach in hepatocellular carcinoma HCC tumor tissues. Lower levels of polyunsaturated fatty acids (PUFAs) like LA were observed. Other studies demonstrated that PUFAs are capable of inducing apoptosis through increasing intracellular products of lipid peroxidation. Another
study had employed a lipidomic approach to systematically profile the lipid signatures in HCC and adjacent noncancerous tissues and observed a gradual reduction of TLCL and total CL in tumor tissues during HCC progression. Moreover, CL oxidation products gradually decreased in tumor tissues. In the same study, a significant restoration of apoptotic sensitivity was observed with the treatment of sorafenib (Nexavar) or staurosporine, which are the standard treatment for late stage HCC patients, after Huh7, HepG2 and LM3 liver cancer cell lines had been incubated with TLCL liposome. Similar finding with in vivo results, greater amounts of oxidized CL were observed in the mitochondria of TLCL liposome stimulated apoptotic Huh7 cells. These in vivo and in vitro findings provide evidence highlighting the significance of reprogrammed CL metabolism in apoptosis in the context of oxidative stress associated tumor progression. Seminal work stated by Kagan and co-workers, established that oxidation of CL by the cyt c/CL complex was required to trigger intrinsic apoptotic pathway.\textsuperscript{40,41} Later studies demonstrated that enrichment of CL and CL peroxidation caused increased cell apoptosis.\textsuperscript{37} Cancer cells prefer saturated fatty acids over PUFAs, such as LA, to avoid apoptosis triggered by CL peroxidation in mitochondria. Consistent with this, the main oxidation products coming from TLCL oxidation, were all decreased in tumor tissues. Hence, decreased levels of TLCL and CL allowed tumor cells to evade apoptosis through attenuating cardiolipin oxidation. Furthermore, major abnormalities in CL content or/and composition were also found in mouse brain tumor, which was associated with dramatic reduction in ETC activities.\textsuperscript{34} The CL content was significantly lower in some brain tumors. The molecular species of CL either contains predominantly shorter chain saturated or monounsaturated FAs which are indicative of immature CL, or contain predominantly longer chain PUFAs that are indicative of mature CL.\textsuperscript{37} Free radical-induced lipid peroxidation has been associated with many human diseases including cancer. Robust ROS generation is a common mechanism for various
anticancer drugs to kill cancer cells, such as arsenic trioxide,\textsuperscript{42} doxorubicin, sorafenib, and \textit{N}-(4-hydroxyphenyl) retinamide (4-HPR, fenretinide). However, only limited reports considered the crucial roles played by the lipid peroxidation products produced by ROS. Previous studies indicated that some PUFAs, such docosahexaenoic acid (DHA), could sensitize various tumor cells such as breast cancer, cervical cancer, and ovarian cancer to ROS-inducing anticancer agents.\textsuperscript{39} The cytotoxic effect of combined treatment was because of induction of apoptosis, preceded by increased generation of intracellular lipid peroxidation products. Also, a recent in vivo study showed that ketogenic diet which is high in LAs, and low in protein and carbohydrates enhanced radio-chemotherapy responses in lung cancer xenografts through a mechanism that involved increased oxidative stress. Ketogenic diets may enrich mitochondrial CL with LA and help them to be more sensitive to cell death stimuli. Consequently, these results demonstrated that high PUFAs diet, and arachidonic acid (AA) could serve as a potent adjuvant therapy in the treatment of cancers by a mechanism linking apoptotic cell death to mitochondrial lipid peroxidation and oxidative stress. Many studies have demonstrated that sorafenib induced apoptosis correlated with the levels of TLCL and its oxidation, indicating that incorporation of linoleic acid into cardiolipin of tumor cells may sensitize the killing of malignant cells by standard treatment with sorafenib in HCC.\textsuperscript{36}
1.7. Conclusions

Due to the severity, prevalence, and overall impact of women’s breast cancer, there is a high urgent need for the exploration of innovative and effective therapeutic options. The modern and advanced treatment options have been proven themselves to be ineffectiveness in curing several metastatic forms of breast cancer. In addition, there is a close contribution between the metastases of breast tumors on one hand and the high mortality and morbidity rates on the other hand. Due to this high rate, there have been continuous attempts to investigate the cause of this metastatic potential. Cardiolipin involves in mitochondrial function, apoptosis and also it has strict association with mitochondria membrane proteins. This has made several therapies to function as potential agents who may succeed; while, the agents targeting other cellular processes fail. In other words, the membrane-lipid therapies are considered as a potentially useful approach which can treat cancer and other common pathologies. The membrane-lipid therapies treatment can be either on their own or as adjuvants when they are combined with other chemotherapeutic agents.
CHAPTER II: CARDIOLIPIN PROFILE IN METASTATIC BREAST CANCER CELLS

2.1. Introduction

In an attempt to investigate the effect of cardiolipin profile on the metastatic potential of breast cancer cells, the effects of two differentially targeted phospholipase inhibitors were evaluated in two pairs of breast cancer cell lines. Cell viability assays were performed in order to examine the effects of each inhibitor on the proliferation/viability of each cell line. The first inhibitor which is commonly called ‘bromoenol lactone’ (BEL, \( E-6\)-(bromethylene)-tetrahydro-3-(1-naphthyl)-2H-pyran-2-one) serves as an inhibitor of calcium independent phospholipase A2 (iPLA2). It is an irreversible, covalent inhibitor of iPLA. Moreover, it has displayed a 1000-fold selectivity for iPLA2 in compare to cPLA2 and sPLA2. Thus, BEL is considered a selective inhibitor of this enzyme as well as is commonly used to inhibit iPLA2 in cellular systems. BEL is usually existed as a racemate. However, the \((R)\)- and \((S)\)-enantiomers of BEL have distinct enzyme inhibitory properties. \((S)\)-BEL inhibited iPLA2\(\beta\) 10-fold more potently than \((R)\)-BEL enantiomer. While \((R)\)-BEL inhibited iPLA2\(\gamma\) almost 10-fold more potently than \((S)\)-BEL. The iPLA2 family of phospholipases has functions in membrane phospholipid remodeling. The second one was halopemide which used as an inhibitor of phospholipase D (PLD). Phospholipase D plays a pivotal role in the generation of phosphatidic acid (PA) and choline through the hydrolysis of phosphatidylcholine (PC).
Furthermore, it functions in altering cell morphology and growth. This production of PA and choline is crucial in beginning many lipids signaling pathways mediated by PA and other cellular messengers such as DAG22. After performing cell viability/proliferation experiments for each breast cancer cell line with the various phospholipase inhibitors,

mitochondria were isolated from the different cell cultures and liquid chromatography-mass spectrometry was performed in order to analyze their cardiolipin profiles. The cell lines selected were MDA-MB-231, BoM, MCF-7, and MCF-7-BoM. MDA-MB-231 is a widely studied triple-negative breast cancer (TNBC) cell line. The BoM cell lines are bone metastatic subclones derived from the MDA-MB-231 cell line. To the contrary, The MCF-7 possesses various genetic abnormalities with high amplification. Similarly, The MCF-7-BoM is a bone metastatic variant of the MCF-7 breast cancer cell line.
2.2. Materials and Methods

2.2.1. Cell viability assay

Cell culture was performed for each cell line before the cell viability assay was performed. Cells were maintained in RPMI-1640 medium with L-glutamine (Gibco), supplemented with 10% (v/v) fetal calf serum (FCS, Hyclone) and 50 units mL-1 penicillin and 50 μg mL-1 streptomycin (Sigma) (referred to as 'culture media'), at 37°C in a humidified environment (95% Air and 5% CO2). For each passage, the cells were detached with 1 mL Trypsin-EDTA (0.25%, Gibco) per 100 mm plate following removal of the conditioned media. After 5 min incubation at 37°C, culture media (9 mL per 1 mL trypsin) was added to neutralize the trypsin. The cell-containing media were transferred to a 15 mL centrifuge tube. The tubes were centrifuged at 600 rpm for 5 minutes (Jouan C412) and the supernatant removed. The cell pellet was re-suspended in culture media, the cell number counted on a hemocytometer, and the cells diluted to desired density. For cell viability/proliferation assay, cells were seed at the density of 1x10^6 cells/mL in a volume of 100 μL per well into 96-well plates and incubated for 24 h before the addition of test compounds. The stock solutions were then prepared for the inhibitors.

Because BEL is stable at maximum concentration of 25 mg/L, the stock solution was prepared to be 20 mg/L. This concentration along with the inhibitor’s molecular weight (317.18 g/mole) was used to calculate the molarity as 63 x 10^4 μM. To simplify the calculations, 60 mM was used. This yielded 5 mg dissolved in 262.7 μL of DMSO for the initial stock solution. Then prepared 6 mM for the stock solution by dissolving 50 of 60 mM in 450 μL of DMSO. A 1:10
dilution was performed in order to provide samples with the desired concentrations (60, 30, 10, 3, 1, and 0.3 μM) from the stock solution.

Next, the stock solution for halopemide was prepared. Halopemide is stable at a maximum concentration of 10 g/L. This concentration along with the MW of halopemide (416.88 g/mole) was used to calculate the molarity as 20 M. This yielded 5 mg halopemide dissolved in 599.7 μL of DMSO for the initial stock solution. Then prepared 6 mM for the stock solution by dissolving 150 μL of 20 mM in 350 μl of DMSO. A 1:10 dilution was once more performed to create samples with concentrations of (60, 30, 10, 3, 1, and 0.3 μM). It was not more than 60 μM because it contained 0.9% DMSO. Malignant cells cannot tolerate 1% DMSO. The working solutions were 2X of the final concentration. The working solutions (100 μL) were added to the wells with 100 μL conditioned media at the time of seeding and incubation for 48 hrs. Additionally, a media control was included in the 96-well plate as well as 10 μM cycloheximide (CHX). For fixing the cells, 100 μL of media was removed and 100 μL of 20% TCA was added to each well. The cells were incubated at 4°C for one hour. Then, the plates were washed with tap water four times. Each plate was left to air dry at room temperature. Next, to stain the cells, 80 μL of Sulforhodamine B (SRB) (0.4% w/v in 1% acetic acid) was added to the cells at room temperature for 10 minutes. After washing with 1% acetic acid three times, 100 μL of 10 mM Tris base solution was added to each well. The plates were placed on a shaker to extract the dye and the absorbance at 490-650 nm measured on a plate reader.

The percent control that each inhibitor had on the proliferation of each cell line was determined using OD<sub>490</sub> values (background at 650 nm subtracted) Data were normalized to the
media control and % Inhibition of cell viability and proliferation calculated using the formula: % Inhibition = 1 – OD\text{compound}/OD\text{media} Data shown are average +/- standard deviation, calculated using one-way ANOVA (Prism 8.2.1).

2.2.2. Clonogenic Assay

From each cell line, the cells were trypsinized using 0.25% Trypsin-EDTA solution and counted using a hemocytometer. Into a 6-well plate, 300 cells were seeded in each well in 2 mL of 10% FCS RPMI with P/S. To make sure that the cells were spread equally, the plates were put in plate shaker for 1 min. After an overnight incubation, 1ml from the media was removed and 1ml of the prepared compound, which were 2X of the final concentration, was added. After 24 hours, the media was aspirated, the cells washed with X1 DPBS, and then 2 mL of 10% FCS RPMI were added with P/S. The media were changed every 2-3 days. After 14 days, the cells were fixed using 1 mL of 0.1% crystal violet (0.5 g Crystal Violet (0.05% w/v), 27 mL 37% Formaldehyde (1%), 10 mL Methanol (1%), 100 mL 10X PBS (1X), and 863 dH\text{2}O to 1 L). Pictures were taken and analyzed using Image J software. A cluster with at least 50 cells were considered as a colony.
2.2.3. Energy Phenotypes Assay Using Seahorse

Seahorse XF Cell Energy Phenotype Test (Agilent Technologies) was performed according to the manufacturer’s protocol for the Seahorse XF-96 instrument. The test measures the utilization of each pathway- mitochondrial respiration and glycolysis. First under the starting medium conditions (Baseline Phenotype) and then upon injection of a stressor mix that induces an energy demand (Stressed Phenotype). The Seahorse XF Cell Energy Phenotype Test Report Generator plots these data on the Energy Map and displays the Metabolic Potential of the cells.

Briefly, MDA-MB-231 and MCF-7 cells (ATCC) were maintained in DMEM/High Glucose media (Hyclone) supplemented with FCS (10%, w/v) or supplemented with Charcoal Stripped Fetal Bovine Serum (Hyclone), and penicillin (50 units mL\(^{-1}\)) and streptomycin (50 µg mL\(^{-1}\)). Exponentially grown cells were seeded at the density of 6000 cells/well in a volume of 80 µL into XFe96-well cell culture plates. An Agilent Seahorse XFp Sensor Cartridge was hydrated overnight in sterile ddH\(_2\)O at 37 °C in a non-CO\(_2\) incubator overnight, and switched to the Agilent Seahorse XF Calibrant the next day. After an overnight incubation, the cells were washed in pre-warmed XF assay media (DMEM basal media supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, adjusted to pH 7.4). Cells were then maintained in 180 µL/well of XF assay media at 37 °C for 1 h in the absence of CO\(_2\). Oligomycin and FCCP were combined in a single tube to create a 10x solution (10 µM each). The stressor mix (20 µL) was added into port A of the sensor plate. The program was run at baseline for 3 cycles (3 min mix; 0 min wait; 3 min measure) and control/experimental for 3 cycles (3 min mix; 0 min wait; 3 min
measure). Data sets were analyzed using Seahorse XF Cell Energy Phenotype report generator and GraphPad Prism software.

2.2.4. Cardiolipin Extraction

A cardiolipin extraction was performed from whole-cell using 20 x10^6 cells and 60x10^6 cells/ cell lines. The cell pellet was first suspended in a disposable borosilicate glass tube (16 × 100 mm) with 1.00 mL deionized water. Then, it was sonicated for 10 s (2 s/cycle × 5 cycles) on ice using a tip sonicator to produce cell homogenate. Cell homogenates were transferred into a disposable borosilicate glass tube (13 × 100 mm).

Next, 10.0 µL of working solution (77.8 nM) of tetramyristyl-CL (1,1′,2,2′-tetratetradecanoyl cardiolipin, Avanti Polar Lipids), internal standard (IS) solution was added; 1.50 mL of chloroform/methanol (1:1, v/v) was added. Then, the mixture was vigorously shaken for 2 min and placed in ice for 15 minutes, followed by centrifugation at 10 °C and 2000 × g for 5 minutes. The lower organic layer was transferred to a borosilicate glass tube (13 × 100 mm); while, the remaining protein disk and the upper aqueous layer were extracted again with 1.50 mL chloroform/methanol (2:1, v/v). Two organic layers were combined and centrifuged at 10 °C and 2000 × g for 5 min.

The supernatant was transferred to a new borosilicate glass tube (13 × 100 mm) and evaporated at 35 °C to dryness under a stream of Argon. The residue was dissolved in 100 µL of the reconstitution solution and subjected to LC-MS/MS analysis.
2.2.6. Mass Spectrometry

Liquid chromatography-mass spectrometry (UPLC-MS) was performed on the cardiolipin sample and the breast cancer cell lines in order to detect cardiolipin and the standard within the cells. Waters Acquity UPLC system, Waters Xevo G2-S QToF MS was the instrument used to perform mass spectrometry analysis.

Table 1: Chromatographic conditions for CL analysis:

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow</th>
<th>% iPrOH/acetoni trile +0.05% FA</th>
<th>% 20 mM NH4Ac + 5% MeOH, pH 9</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.3</td>
<td>75.0</td>
<td>25.0</td>
<td>6</td>
</tr>
<tr>
<td>8.00</td>
<td>0.3</td>
<td>100.0</td>
<td>0.0</td>
<td>6</td>
</tr>
<tr>
<td>12.00</td>
<td>0.3</td>
<td>100.0</td>
<td>0.0</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2: UPLC-MS parameters for CL analysis

<table>
<thead>
<tr>
<th>MS Scan</th>
<th>50-1800 Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI MS</td>
<td>Negative mode</td>
</tr>
<tr>
<td>Capillary</td>
<td>2.8 kV</td>
</tr>
<tr>
<td>Cone</td>
<td>12</td>
</tr>
<tr>
<td>Source temperature</td>
<td>80 C</td>
</tr>
<tr>
<td>Desolvation temperature</td>
<td>480 C</td>
</tr>
<tr>
<td>Desolvation Gas Flow</td>
<td>1000 L/hr</td>
</tr>
<tr>
<td>Cone Gas Flow</td>
<td>50 L/hr</td>
</tr>
<tr>
<td>Collision Low Energy</td>
<td>20 eV</td>
</tr>
<tr>
<td>Collision Energy Elevated</td>
<td>30-50 eV</td>
</tr>
<tr>
<td>MS Lockspray</td>
<td>Leucine-enkephalin at MS 554.2615, The lock mass at a concentration of 5 µg/mL and flow rate of 10 µL/min</td>
</tr>
</tbody>
</table>
2.2.7. Molecular Networking

Cardiolipin (C14:0) and bovine heart Cl raw files were converted to the mzXML format using msconvert (ProteoWizard version 3.0.10141) for further processing. Then, the data were uploaded to Uploading data for MassIVE repository of GNPS using the default parameters (MassIVE Accession is MSV000083087). After Molecular Networking workflow was completed utilizing GNPS, analysis was done within the web interface. The GNPS web interface provides an easy and quick way to perform initial analysis of the data, particularly if it is required that the MS\(^2\) spectra of the nodes/clusters/networks generated be viewed by the Molecular Networking workflow. Lastly, the data were downloaded into Cytoscape (3.7.0) for visualization.

The molecular networking parameter which created using the online workflow at GNPS. The data was filtered through excluding all MS/MS peaks within ± 17 Da of the parent m/z. MS/MS spectra were window filtered by selecting only the top 6 peaks in the ± 50Da window throughout the spectrum. Then, data was clustered with MS-Cluster with a precursor mass tolerance of 2.0 Da and MS/MS fragment ion tolerance of 0.5 Da to form consensus spectra. Further, consensus spectra that contained less than two spectra were discarded. After that, the network was created where edges have a cosine score more than 0.7 and above six matched peaks. Additional edges between two nodes were kept in the network if each of the nodes come out in each other's respective top 10 most similar nodes. Then, the spectra in the network were searched against GNPS' spectral libraries. The library spectra were filtered in a similar way as the input data. Finally, all matches retained between network and library spectra were had to have a score more than 0.7 and at least six matched peaks.
2.2.8. Data

Data comparison was performed on cell viability data with one-way ANOVA with concentration of compound as the fixed factor, followed by Bonferroni post hoc analyses using GraphPad Prism 8.2.1 version. Two independent experiments for each cell line and \( n = 3 \) for each. Differences between data sets were considered statistically significant when \( p < 0.05 \). Because the two independent experiments were performed in different days, Two-way ANOVA test with concentration of compound and time as the fixed factors, was used to determine if there are any significant effects of the time as a predictor on the % of inhibition between the two experiments. Using R version 3.5.1, the concentration of the compound, time of the study, and their interactions were run. The triplicate raw data of each experiment were used in statistics after log transformation were done. The data was normally distributed.
2.3. Results

2.3.1. Cell viability results

The effects of CL remodeling alteration introduced by phospholipase inhibitors on cell proliferation/viability were examined in a panel of established human breast cancer cell lines. The Concentration-response studies of Bel and halopemide on cell proliferation were established to find out the concentrations that inhibit enzymatic activities, without pronounced cytotoxicity to use it in the following studies.

Following 48 h of compound treatment, all compounds affected cell proliferation/viability to a certain extent. The bromoenol lactone (BEL) showed a more potent effect than halopemide in all cell lines. More significant inhibitory activity was observed in the TNBC MDA-MB-231-derived bone metastatic BoM1833 (BoM) and MDA-MB-231in comparison to the ER+/PR+/HER2− MCF-7 (MCF-7)-derived subclones MCF-7-BoM when the cells were treated with halopemide (Figure 6).

The protein synthesis inhibitor cycloheximide (CHX, 10 µM) was used as a positive control which resulted in 69 ± 2, 70 ± 1, 60 ± 2, and 58 ± 4 % of inhibition to the control in MDA-MB-231, BoM, MCF-7, and MCF-7/BoM cell lines, respectively.
Figure 6. Concentration-response results of Bel and halopemide on cell proliferation/viability. MDA-MB-231, BoM, MCF-7 and MCF-7-BoM cells were exposed to Bel and halopemide at the concentrations of 0.3, 1, 3, 10, 30 and 60 µM. After 48 h, cell viability was determined and presented as “% Inhibition” of the media control. Data shown are average +/- standard deviation. Two separate experiments, n=3 for each experiment. X axis is log (concentration). There was no significant effect for the time and its interaction with the concentration (2-way ANOVA: F_{1,28}= 0.927, P= 0.35). Then, one-way ANOVA was run in prism 8.2.1. with concentration of compound as the fixed factor.

Since the logarithmic axis plots the logarithms of the values, it is only possible to plot positive values on logarithmic axes. There is no place to put 0.0 or negative values. The control results are 0 ± 5, 0 ± 6, 0 ± 5, and 0 ± 3 in MCF-7/BoM, MCF-7, BoM, and MDA-MB-231 respectively because it is not showing in the graph.
Table 3: IC$_{50}$ values and 95% confidence interval (CI) of bromoenol lactone (BEL) in breast cancer cell lines

<table>
<thead>
<tr>
<th>Bromoenol Lactone</th>
<th>MCF-7/BoM</th>
<th>MCF-7</th>
<th>BoM</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ (M)</td>
<td>2.07x10$^{-5}$</td>
<td>2.36x10$^{-5}$</td>
<td>2.75x10$^{-5}$</td>
<td>2.67x10$^{-5}$</td>
</tr>
<tr>
<td>95% CI (M)</td>
<td>1.12x10$^{-5}$–3.02x10$^{-5}$</td>
<td>1.36x10$^{-5}$–3.36x10$^{-5}$</td>
<td>6.78x10$^{-6}$–4.81x10$^{-5}$</td>
<td>1.32x10$^{-5}$–4.02x10$^{-5}$</td>
</tr>
</tbody>
</table>

Table 4: IC$_{50}$ values and 95% confidence interval (CI) of halopemide in breast cancer cell lines

<table>
<thead>
<tr>
<th>Halopemide</th>
<th>MCF-7/BoM</th>
<th>MCF-7</th>
<th>BoM</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$</td>
<td>2.49x10$^{-5}$</td>
<td>2.04x10$^{-5}$</td>
<td>2.46x10$^{-5}$</td>
<td>9.24x10$^{-6}$</td>
</tr>
<tr>
<td>95% CI</td>
<td>3.53x10$^{-6}$–4.64x10$^{-5}$</td>
<td>3.19x10$^{-6}$–3.76x10$^{-5}$</td>
<td>2.27x10$^{-6}$–5.18x10$^{-5}$</td>
<td>3.25x10$^{-6}$–1.52x10$^{-5}$</td>
</tr>
</tbody>
</table>

Table 3 and Table 4 offer a vivid analysis of the two inhibitors on the cell lines. At a confidence interval of 95%, similar results to Figure 6 are indicated. Bromoenol lactone has significantly different but potent changes as opposed to halopemide.

After the viability and proliferation study were performed and the IC$_{50}$ values were established, 10 μM concentration of BEL and halopemide were chosen for the following studies which have enzymatic inhibitory activity with less cytotoxicity effect.
2.3.2. Clonogenic assay results

Clonogenic assays were performed to assess the ability of phospholipase inhibitors on the colony-forming ability of single of BoM cell lines. Figure 7 Cells seeded at low density were exposed to test compounds (bromoenol lactone and halopemide each at 1, 10 μM, and cycloheximide 10 μM) for 24 h. The conditioned media were replaced with growth media. After a ten-day period, the cells were fixed and stained. The positive control CHX blocked colony formation. However, Bromoenol lactone and halopemide 10 μM shows significant reduction in the cells ability to create colony in MDA-MB-231 and BoM comparing to theirs effect on MCF-7-BoM. Moreover, Colony suppressing activity of 1 μM concentration of both Bel and halopemide compared to 10 μM were less pronounced. The cells demonstrated at different levels of exposure highlighted how phospholipase inhibitors influence the cancer cells in colony formation.
Figure 7. Clonogenic survival results. Cells seeded at low density were exposed to compounds (Bromoenol lactone, halopemide each at 1, 10 µM, and cycloheximide 10 µM) for 24 hrs. Images shown are crystal violet-stained colonies from media control and compound treated wells.
2.3.3. The Seahorse XF Cell Energy Phenotype results

The Seahorse XF Cell Energy Phenotype Test is used to generate a holistic, functional view of the metabolic phenotype of your cells. Definitive features were drawn through the use of energy-producing pathways, thereby creating the best cell mitochondrial respiration and glycolysis process.

This preliminary experiment showed the trend in to major energy producing pathways of the cell mitochondrial respiration and glycolysis; measures mitochondrial respiration and glycolysis under baseline and stressed conditions, to reveal the three key parameters of cell energy metabolism: Baseline Phenotype, Stressed Phenotype, and Metabolic Potential. There were two sets of cells. First set, cells were cultured in 10% fetal bovine serum (FBS) including control and treated cells with either 30 μM Bel or 10 μM halopemide. Second set, cells were cultured in Charcoal Stripped Fetal Bovine Serum (CS-FBS) which is stripped of its fatty acids.
Figure 8. Energy phenotypes of MDA-MB-231 and MCF-7 cells. Each cell line was cultured in media supplemented with either regular FBS or charcoal stripped CS-FBS then exposed to BEL at 30 µM and halopemide at 10 µM concentrations, using OCR and ECAR in baseline and stressed test. A) OCR in baseline and stressed test for MDA-MB-231 cells. B) OCR in baseline and stressed test for MCF-7 cells. C) ECAR in baseline and stressed test for MDA-MB-231. C) ECAR in baseline and stressed test for MCF-7 cells. Regular FBS or CS-FBS
For the MDA-MB-231 cells Oxygen Consumption Rates, baseline is more than stressed compared only in control in OCR compared with that in ECAR. In other words, comparing OCR and ECAR, baseline is less than stressed for CS-FBS-control (C Control), 30 Bel, CS-FBS-30 Bel (C 30 Bel), 10 halopemide, and CS-FBS-10 halopemide (C 10 halopemide). Considering MCF-7-oxygen consumption Rates, the baseline is less than stressed in all factors in both OCR and ECAR. Also, it is clear that the difference between the baseline and the stressed is the most significant in ECAR MCF-7-Oxygen Consumption Rates compared with that of all others. In comparison with MCF-7, MDA-MB-231 shows significant increase in ECAR rate in both baseline and stressed in both control and with the C 30 μM Bel. while there was significant reduction in MCF-7 cell line C10 halopemide. In MDA-231-XF Cell Energy Phenotype, there is a decrease in C Control, 30 Bel, C 30 Bel, 10 halopemide, and C 10 halopemide, except for Control. In MCF-7-XF Cell Energy Phenotype, there is a more significant decrease in C Control, 30 Bel, C 30 Bel, 10 halopemide, and C 10 halopemide as well as Control compared with those in MDA-231-XF Cell Energy Phenotype. Among all, 30 Bel shows the most drastic decrease in MCF-7-XF Cell Energy Phenotype.
Figure 9. Energy phenotypes graph of MDA-MB-231 and MCF-7 cells. Each cell lines had cultured in supplemented media with either Regular FBS or CS-FBS, then the cells were exposed to Bel at 30 µM and halopemide at 10 µM concentration.

The MCF-7 cell showed the same pattern which moves from quiescent toward energetic phenotype but no significant results compared to the control while MDA-MB-231 cells show a significant movement under stressed. More importantly, the cells that had been maintained in media supplemented with CS-FBS showing dramatic reduction in energy phenotype compared with cells that had been cultured in media with regular FBS.
2.3.4. CL extraction results

The MS results generated from breast cancer cell lines CL extraction, using 60x10^6 cells of MCF-7/BoM, and 20x10^6 cells of MCF-7, BoM and MDA-231, did display the peak for the cardiolipin internal standard with relative high recovery from MCF-7/BoM in compare to the rest of the cell lines (Figure 10). The same result presented in in Figure 11 showing the chromatogram of IS extracted from the cells. More importantly, three different species of cardiolipin were extracted from MCF-7/BoM and MDA-231 cells.
Figure 10. Abundance/intensity of CL Internal standard (C14:0), which has 1239.8862 m/z [M-H], and the extracted CL, from BoM, MCF-7, MCF-7-BoM, and MDA-MB-231 cell lines, (C18:2) which has 1447.9051 m/z.

Figure 11: Chromatogram of the internal standard CL alone and the extracted IS from MCF-7/BoM, MCF-7, BoM, and MDA-231.
Figure 12. Mass spectrum of the molecular CL (72:5) and (70:5) or CL (70:20) which had 1454.0134 and 1425.9695 m/z, respectively.

The extracted CL annotation was based on manual dereplication by comparing the predicted molecular formulae against multiple public or commercially available databases. The tetralinoleoylcardiolipin, 1447.9 m/z, had been extracted with very low abundance. In addition to a relative higher abundance CL (72:5) and (70:5) which had 1454.0134 and 1425.9695 m/z, respectively (Figure 12). Table 5 shows the fatty acid composition of each CL molecular species. These results confirm what was discussed above that the breast cancer cells shift the cardiolipin remodeling toward having less unsaturated species of CL.
Table 5: The possible fatty acid composition for each molecular species and the mass spectra in [M-H]⁻

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>[M-H]⁻ m/z</th>
<th>Possible fatty acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL(72:8)</td>
<td>1447.9</td>
<td>(C₁₈:2)₄ (C₁₆:1)/₁/(C₁₈:₁)₁/(C₁₈:₂)₁/(C₂₀:₄)₁</td>
</tr>
<tr>
<td>CL(70:5)</td>
<td>1425.9</td>
<td>(C₁₆:₀)₁/(C₁₈:₁)₁/(C₁₈:₂)₂ (C₁₆:₁)₁/(C₁₈:₁)₂/(C₁₈:₂)₁</td>
</tr>
<tr>
<td>CL(72:5)</td>
<td>1454.01</td>
<td>(C₁₈:₁)₃/(C₁₈:₂)₁</td>
</tr>
</tbody>
</table>

2.3.5. Molecular Networking of the standards

The MS/MS (MS²) data that generated from UPLC-MS of CL standards were uploaded to the publicly accessible Global Natural Product Social Molecular Networking (GNPS) platform (http://gnps.ucsd.edu), which was analyzed according to the molecular networking (MN) online workflow. To visualize the resulting networks, Software Cytoscape (Version 3.7.0) was used.

According to Olivon F. group, the MS Cluster cannot differentiate between the chromatograph of well-resolved isomers as retention times are not considered. Annotation with predicted chemical formulas is also semi quantification and not implemented is only based on the number of MS² scans. In spite of valuable outputs, some improvements are still required to generate unambiguous GNPS molecular networks. The MS Cluster algorithm implemented on the Global Natural Product Social Molecular Networking platform was developed for the identification of redundant spectra from the same molecule and their replacement with a single averaged spectrum. Based on the premise that the occurrence of truly the molecular eluting at different retention times (RTs) in liquid chromatography (LC) is rather uncommon, MS-Cluster developers did not integrate RT information for restricted clustering over limited RT ranges.
Moreover, the GNPS quantification system simply depends on the number of MS² spectra detected for each precursor ion. This method is highly dependent on the data-dependent acquisition mode parameters, such as the number of precursor ions per scan, ion intensity threshold, and exclusion rules. For that, GNPS quantification only leads to a rough approximation of relative or absolute abundances. Subsequently, the parent ions at m/z 1239.8, 1447.9, and 1449.9 (Figure 13), showed that isomers eluted at different RT were well separated in different nodes having the same parent ions. Cardiolipin’s (14:0)₄ RT are 3.94, 3.36, 3.22, and 3.18 min. The bovine heart cardiolipin’s (18:2)₄ RT are 3.89, 3.63 min while (18:2)/ (18:1) CL’s RT are 4.15, and 3.7 min. In Table 6 the fatty acid composition of each standard cardiolipin were displayed.
Figure 13. Molecular cluster observed from CL standards. Numbers within the nodes indicate parent ions. Edge thickness represents the cosine similarity between nodes as well as the chemical structures of each compound (MassIVE Accession is MSV000083087)

Table 6: Cardiolipin standards fatty acyl chain composition and the mass [M-H]- of each cardiolipin species

<table>
<thead>
<tr>
<th></th>
<th>Fatty acid composition</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine heart CL</td>
<td>(C_{18:2})_4</td>
<td>1447.9</td>
</tr>
<tr>
<td></td>
<td>(C_{18:2})<em>3/(C</em>{18:1})_1</td>
<td>1449.9</td>
</tr>
<tr>
<td>Synthetic CL (IS)</td>
<td>(C_{14:0})_4</td>
<td>1239.8</td>
</tr>
</tbody>
</table>
2.4. Discussion

This proposed research focuses on the mitochondrial membrane phospholipid CL. Changes in CL abundance and composition can lead to mitochondrial dysfunction, metabolic abnormalities, and altered apoptotic response. Our objective is to identify the CL profiles associated with treatment response and to enhance drug sensitivity in metastatic breast cancer cells by altering CL side chain length/unsaturation. To alter CL composition by suppressing CL remodeling, breast cancer cell lines were exposed to two differentially targeted phospholipase inhibitors. First, to set up a method for detecting changes in cardiolipin composition within the cell lines. Liquid chromatography-mass spectrometry (LC-MS) was performed on each cell line cardiolipin extracts and cardiolipin standards. The main target of GNPS is to be an open-access knowledge base for community-wide organization as well as sharing of raw, identified or processed tandem mass (MS/MS) spectrometry data. It assists in discovery and identification throughout the entire life cycle of data; starting from initial data acquisition/analysis to post publication. For that, GNPS is considered a data-driven platform for the storage, analysis, and knowledge dissemination of MS/MS spectra that allows community sharing of raw spectra, continuous annotation of deposited data, and collaborative curation of reference spectra. Currently, GNPS is the only public infrastructure that allows molecular networking. The molecular networks (MN) visualization in GNPS represents each spectrum as a node, and spectrum-to-spectrum alignments as connections (edges) between nodes. Nodes can be supplemented with metadata, including dereplication matches or knowledge that is provided by the user, for instance origin of product, abundance, hydrophobicity or biochemical activity, which can be reflected in the color or size of a node. To visualize the map of related molecules by either a molecular network online at GNPS or exported
for analysis in Cytoscape.\textsuperscript{43} However, as one of the drawbacks of MN the GNPS quantification system simply depends on the number of MS\textsuperscript{2} spectra detected for each precursor ion. Moreover, MS-Cluster developers did not integrate RT information for restricted clustering over limited RT ranges.\textsuperscript{44}

Noticeably, the MS-Cluster shows as parent ions of the CL standards at m/z 1239.8, 1447.9, and 1449.9 (Figure 13), indicated well separated nodes share the same parent ions and connected with edge represents the similarity, 0.99 cosine in 1239.8 MN, and in the bovine heart molecular net working the highest cosine score is 0.91 where the lowest one is 0.83. The isomers eluted at different RT is displayed as distinct node carried the same parent ion number. Preprocess the data before MN analysis might be an option to improve the analysis workflow in order to keep the MS-Cluster option disabled.

The phospholipase inhibitors used in the research combined two sets of data that required One-way ANOVA to analyze. The experiments used a cell line and n=3 to assess the different inhibitors and their effects on the compounds. A combination of CHX, bromoenol lactone (BEL), and halopemide in determining the cell lines was significant in the process. The main aim of the research is to measure the best inhibitor that can be used in remodeling the alteration on cell proliferation or viability while being examined for human breast cancer cell lines. The cell lines were significantly measured for the measured inhibitory activity that was premised in the research outcomes. Breast cancer cell lines used in the research were TNBC MDA-MB-231-derived bone metastatic BoM1833 (BoM) and MDA-MB-231in comparison to the ER\textsuperscript{+}/PR\textsuperscript{+}/HER2\textsuperscript{−} MCF-7 (MCF-7)-derived subclones MCF-7-BoM. Each was analyzed differently with the reagents to
highlight differences that could be drawn from the specific inhibitors. Results showed that the bromoenol lactone inhibitor had a more potent effect than halopemide in all the cell lines. In examining the potential for cancer, specific inhibitors used are potent for making the right decisions and developing perfect methods to align with the set hypothesis being tested. Although halopemide and bromoenol lactone were effective methods, the overall potent changes were observable on BEL. Halopemide is believed to be an effective inhibitor in most of the tests. However, from the research, BEL has more potent changes that are pertinent in defining the contextual measures needed. Figure 6 presents differences in the cell lines with the two inhibitors. TNBC MDA-MB-231-derived bone metastatic BoM1833 (BoM) and MDA-MB-231 in comparison to the ER+/PR+/HER2− MCF-7 (MCF-7)-derived subclones MCF-7-BoM were used to accentuate the specific patterns necessary in confounding the changes. The research highlights variability invisibility among the cell lines when exposed to each inhibitor. BEL does not have clear indications in comparison to the halopemide table. While being exposed to concentrations of 0.3, 1, 3, 10, 30, and 60 µM, there were differences in the contextual evidence indicated.

Table 3 and Table 4 offer a vivid analysis of the two inhibitors on the cell lines. At a confidence interval of 95%, similar results to Figure 6 are indicated. Bromoenol lactone has significantly different but potent changes as opposed to halopemide. The distinctive features highlight the use of each method as an inhibitor in research for breast cancer. Various results and compounding attributes are generally developed and mapped through the patterns, and practical ways indicated. The data gives a vivid description and difference among the specific values listed in the cell lines. Tabulated data used is easy to track and contextualize in drawing proper inferences from the results given. The MCF-7/BoM, MCF-7, BoM, MDA-MB-231 data sets indicate critical
patterns that need to be analyzed when pulling on the specific inhibitors indicated. Every paradigm has explicitly been contextualized in the research tools mapping out accurate results significant in the research. Inhibition patterns are reflective of the cell lines and pertinent features processed on each experiment. Every experiment is specifically drawn and authenticated through mirroring the ultimate features indicated, thereby underlying the needs perpetuated in the system accordingly.

In cancer research, as evident from the research collected on the two inhibitors, Bromoenol lactone offers the best chance of measuring the cancer lines. Changes and any potent attribute that characterizes the individual features can be easily analyzed and drawn from the research tools, and context indicated accordingly. In research, the specific features are essential in mapping out all the patterns from the data collected. Values for every element and the overall tools created to highlight the need to develop specific intervals and methods necessary in building a sturdy construct essential for change accordingly.

Clonogenic assay results were also used in assessing the ability of phospholipase inhibitors on the colony-forming ability of single BoM cell lines. The process used different methods of analysis and drawing on the CHX inhibitor in comparison to the BEL and halopemide inhibitors. Using positive control through the CHX inhibitors, data on individual patterns were used to assess CHX colony formation. The specific data sets were drawn with a particular contextual model being used in addressed different features as individually pronounced. Each element was critically integrated, thereby modeling the main methods and analysis of effective measures needed accordingly. The conditions were perfect in understanding different concentrates and drawing on specific patterns that were categorized in the research process. Results indicated that positive control CHX blocked colony formation. On the flip side, however, BEL and halopemide showed
a significant reduction in cells' ability to create a colony on the cell lines used. The cells
demonstrated at different levels of exposure highlighted how inhibitors influence the cancer cells
in colony formation. Figure 2 Cells seeded at low density were exposed to compounds (Bromoenol
lactone, halopemide each at 1, 10 µM, and cycloheximide 10 µM) for 24 hrs highlighted the
significant differences that can be drawn. At each level, the results were remarkable, especially
with positive control of the inhibitors. The three inhibitors used in the research were critical in
outlining differences in cell formation for cancer cell lines with different concentrations. It is
imperative to note that increasing the frequency from 1 µM to 10 µM, in halopemide and BEL led
to an increase in the level of colony formation. Different dynamics were potent in actualizing the
main measures and identifying appropriate tools necessary in acting as blockers in the experiment.
Understanding the dynamics indicated helped in achieving the processes and defining critical
features that were necessary for developing specific methods and controls needed in the systems
accordingly. Every construct was informed on the measures and techniques used by the research
to draw on the inhibitors and actualize the systems created. Definitive features were necessarily
highlighting the need to address the questions raised through the use of two inhibitors in the
experiment.

Seahorse XF Energy Phenotype results were used as a functional test to generate a holistic,
practical view of the metabolic phenotype of the cells. Definitive features were drawn through the
use of energy-producing pathways, thereby creating the best cell mitochondrial respiration and
glycolysis process. Understanding the glycolysis processes through the baseline and stressed
conditions helped in addressing different parameters used in the process. Baseline phenotype
highlighted phenotype and metabolic potential were the main models used in treating the cells and
effective measures used. Different sets of trials were conducted in a controlled environment to analyze the role of inhibitors in the process.

In comparing OCR and EACR in the test results indicates significant changes and positions that are critical in underpinning the main trends in the process. While analyzing the two under the baseline levels, the baseline is less than stressed in all factors. Using the cell lines to assess the different inhibitors and control processes help in actualizing the main features and accentuating the patterns indicated accordingly. Significant changes and models that can be drawn from the OCR and EACR help in further establishing a conclusion on halopemide and BEL. The control rates indicate the differences in stress levels for BEL and halopemide. The significant difference is drawn with ten halopemide showing a reduction in stress for the MCF-7 cell line. The dynamics are similar in C-control, however, for the cells in the process for halopemide as well as BEL. Individual factors are drawn from the premise indicated mapping out contextual basis and controls necessary in underlining a drastic decrease in the MCF-7-XF cell energy phenotype. Distinguishing features are drawn through close examination of the factors and drawing on underlying effective measures needed in the processes indicated. Reduction in energy phenotype is reminiscent of changes that were significant on the individual processes mapped accordingly. Every dynamic is potent and actualized through the means that have been significantly indicated in the procedures defined. Underlining different methods and integrating better patterns help in outlining differences in the processes and controls meant accordingly. Every aspect has been controlled through culturing of the supplemented media and helps in continually developing the best patterns essential in the research. In collecting data for breast cancer, a split has existed, especially in defining the role of inhibitors in the OCR model. The study helps in underscoring the
differences and actualizing specific features necessary in the processes accordingly. Objective analysis and examination of data help in outlining significant features that are essential in modeling specific patterns needed. The overall method is informed on the premise that each dynamic can be significantly contextualized and data collected through the measures indicated in the process.

The cardiolipin extraction method also helped in underlining and defining all the main techniques used in the research, and identifying different parts essential in the process helped in creating a map that can be traced and used to collect information on the process defined in the long-run. The phospholipase inhibitors used in the process have been appropriately modeled to help in drawing the perfect results and data. The information collected highlights different parts that need to be characterized and contextualized when examining the role of inhibitors in breast cancer research and treatment plans. Functional approaches have been significantly developed in the research outlining pertinent features and content necessary in making the best decisions essential in the research process.

Phospholipase inhibitors that have been used in the treatment of different conditions are characterized in the process. Evidence from the research highlights the primary constructs necessary in defining the main features and changes specified in the treatment process. Through assessing the reaction of different breast cancer cell lines, individual treatment options can be modeled from the research collected - every dynamic point to functional sets that are essential in outlining a positive method required. Halopemide and BEL inhibitors give an excellent control model inherent in marking specific changes in the treatment process. Mapping different patterns and changes in cell lines help in actualizing significant measures needed. Every process
characterizes the changes that are evident from mapping out every method and inculcating certain standards required. The two inhibitors have a considerable influence on the patterns and controls indicated accordingly. Halopemide and BEL are inhibitors that have been documented for their effects on cancer cell lines. Individual measures are necessary for defining the contextual approaches essential for fixing the cells. Metastatic cells were used in the research to assess the effect of inhibition in the processes indicated. Setting the cells was difficult, underscoring the need for research on every paradigm indicated. The use of different cell lines that lead to breast cancer was essential and effectively matched the main contextual processes indicated. Actualizing the specific systems and patterns helped in creating healthy controls and procedures that were necessary for cultivating positive outcomes in the research process accordingly. Incubating the cells to different degrees of temperature helped in addressing various measures that were necessary for the procedure. The overall method was properly correlated to derive the right results for the research. Research tools that were used underpinned the need to actualize and develop the specific measures needed in the study. An analysis of halopemide and BEL impact on cardiolipin modification was critical in defining the contextual methods required in the study. Identifying and mapping the role of inhibitors in the processes helped in outlining different changes and mapping out the specific patterns needed. Analysis through ANOVA helped in creating a proper control process for different control groups used in assessing the impact of the inhibitors on cardiolipin remodeling. Each inhibitor had a series of various tests that were used to examine different parts as essential in underlining the role of every element indicated accordingly.

Further research is necessary on the CL remodeling alteration by the two inhibitors to increase the level of efficiency and draw on specific parts that can be used in cancer treatment.
More still needs to be done in addressing the cancer problem, but inhibition cardiolipin remodeling by phospholipase inhibitors of cancer cells provides a platform that can be used in fighting the condition. Drawing on the research, different parts have been defined to outline the main measures needed in the long-run accordingly.
APPENDIX:

3.1. Glossary

**Oxygen Consumption Rate (OCR):** The rate of oxygen concentration decreases in the assay medium. The OCR is a measure of the mitochondrial respiration rate of cells. **Extracellular Acidification Rate (ECAR):** The rate proton concentration (or decrease in pH) increase in the assay medium. ECAR is a measure of the glycolysis rate of the cells. **Baseline phenotype:** OCR and ECAR of cells at starting assay conditions (specifically, in the presence of non-limiting quantity of substrates). **Stressed phenotype:** OCR and ECAR of cells under an induced energy demand (specifically, in the presence of stressor compounds). **Metabolic potential:** Percentage increase of stressed OCR over baseline OCR, and stressed ECAR over baseline ECAR. Metabolic Potential is the measure of cells' ability to meet an energy demand via respiration and glycolysis. **Oligomycin** inhibits ATP production by mitochondria and causes a compensatory increase in the rate of glycolysis as the cells attempt to meet their energy demands via the glycolytic pathway. **FCCP** depolarizes the mitochondrial membrane, and drives oxygen consumption rates higher as the mitochondria attempt to restore the mitochondrial membrane potential.


32. Petrosillo, G.; Ruggiero, F. M.; Pistolese, M.; Paradies, G., Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from


46.  Henkels, K. M.; Boivin, G. P.; Dudley, E. S.; Berberich, S. J.; Gomez-Cambronero, J.,


VITA

Jawaher Hamad H Alkhamisy

EDUCATION
University of Mississippi, BioMolecular Science, Oxford, MS 1/17-present
  • Master’s degree in Biomolecular Science
  • Rho Chi Honor Society
  • Expected to graduate 30/12/19

University of Pennsylvania, English Language Programs, Philadelphia, PA 5/15-11/16
  • Intensive academic English language training for graduate studies

King Abdul-Aziz University, School of pharmacy, Jeddah, Saudi Arabia, 9/05-6/11
  • Pharm. D in Pharmacy, (GPA 4.57/5),
  • Full University Scholarship for studying MS, PHD in pharmacognosy (granted 3/2015)

WORK EXPERIENCE
Demonstrator 3/12-Present
Natural Products and Alternative Medicine Department, King Abdul-Aziz University, Jeddah, Saudi Arabia
  • Taught two laboratory courses: Natural Products and Phytochemistry for almost 3 years.
  • Prepared the laboratories for students that included prepared samples, chemical reagents, and solutions for use; cleaned and calibrated equipment, did the experiments to make sure they worked.
  • Assisted students during structured labs with handling samples, maintaining order in lab, and assisting students with lab techniques.
  • Held 10 office hours/week to meet students and help them in those courses.
  • Collaborated with professor to create rubrics, midterm, final exam, grade student work.
  • Did inventory of chemicals, glassware and instruments of phytochemistry and natural products labs, then worked on the request forms to equip the lab for the following year.
  • Monitored safety procedures
  • Trained new teaching assistants and lab technicians.
  • Ran a workshop: “My Real Experience and Pharmacy” Under the supervisor of Prof. Nadia Sukkar
Pharmacist
Pharmacy, King Abdulaziz Hospital, Jeddah, Saudi Arabia 1/12-3/12

- Reviewed prescription order to determine accuracy and suitability
- Filled prescription orders and label with patient information dosage recommendations and safety precautions that should be taken
- Recorded all relevant patient information to be cross-referenced with medical records to ensure safety and accuracy of prescriptions
- Provided consultation for patients and nurses.
- Prepared solutions and infusions for operations and procedures throughout the hospital
- Researched the positive and negative effects of different medications on hospital patients
- Accepted and recorded shipments of pharmacy supplies then organized and stocked them

Lab Technician
Quality Control Dept, Al Jamjoom Pharmaceutical Factory, Jeddah, Saudi Arabia 11/11-1/12

- Followed safety regulations and quality control procedures
- Adhered to SOP’s, procedures and work instructions
- Cleaned, calibrated and worked on Friability Tester, Tablet Hardness Tester, Disintegration Tester, Tablet Dissolution Tester, Melting Point Apparatus, Room Dehumidifier / Inspection Tables

RESEARCH EXPERIENCE
Pharmacognosy Division, Biomolecular Science Department, University of Mississippi 1/17-12/19

- Thesis Research on Role of Cardiolipin Remodeling in the Malignant Progression of Breast Cancer
- Using two different type of phospholipase inhibitors to alter mitochondrial cardiolipin profiles. Finding IC50 and MIC by establishing concentration response curve.
- CL Profiles Targeted Disruption of CL Homeostasis
- Cardiolipin extracted from MDA231, BoM, MCF7 and MCF7-BoM
- The lipidomic CL analysis performed by the established Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry protocols
- Upload it to GNPS
- Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu)
- Molecular networking using cytoscape

Natural Products and Alternative Medicine Department, School of Pharmacy, King Abdul Aziz University, Jeddah, Saudi Arabia 10/14-4/15

- “Chromatographic Analysis of Rumex Nervosus” supervised by Prof. Nadia Sukkar • Prepared extract, did TLC to check the presence of flavonoids, used mass spectrometry to identify the structures of these compounds and helped in writing the paper.
- “Lycopene, A Strong Natural Antioxidant, Sources, Pharmacological Action” supervised by Prof. Nagwa Alshar • 4/14-9/14
Extracted the Lycopene from various tomato paste types, used uv-vis spectroscopy to determine concentration in each type and helped in writing the paper.

**TRAINING EXPERIENCES**

**Intern**
King Abdulaziz Hospital, Jeddah, Saudi Arabia
- Spent a month in each department of the hospital such as Infectious Disease, Cardiology, Nephrology, ICU, Ambulatory Care
- Attended morning rounds in the unit with the supervisor physicians and discussed patients’ profiles and their medications
- Presented case studies every week for pharmacy and medicine students and staff such as Management of Congestive Heart Failure and Management of ESRD (Diabetic Nephropathy) on regular dialysis session and Management of Acidosis
- Journal Club presentation each month such as Management of Hepatitis C, Congestive Heart Failure " Rationale, design, and methods for the Transplant-Eligible MAnagement of Congestive Heart Failure (TMAC) trial: A multicenter clinical outcomes trial using nesiritide for TMAC, Managing diabetes in hemodialysis patients: observation and recommendation
- Checked patient information to be cross-referenced with medical records to ensure safety and accuracy of prescriptions
- Prepared Drug Evaluation monograph for Rivaroxaban (Xarelto®), which resulted in the Administration buying the medicine from the company based on my monograph

**Summer Trainee**
Pharmacy of King Abdul-Aziz Hospital & Oncology Center, Jeddah, Saudi Arabia
- Trained on how to prepare and deal with chemotherapy
- Attended morning round in ACU and nephro dialysis unit with the supervisor physicians and discussed patient's profiles and their medications
- Presented case study every week.
- Trained in the pharmacy how to review prescription order to determine accuracy and suitability and fill prescription orders and label with patient information dosage recommendations and safety precautions that should be taken

**Graduation Project (Pharma.D)**
- Recent Advances in The Management of Coronary Artery Disease
  It is review paper of the latest treatment options for Coronary Artery Disease

**VOLUNTEER EXPERIENCE**
King Abdulaziz University Hospital, poor non-Saudi people pharmacy
- Recruited funds from wealthy donors and CEOs of large companies for the pharmacy and paying for urgent patients’ treatment such as cancer and nephro dialysis patients.
• Distributed boxes in public area such universities and malls and encouraged people to donate unneeded medication to poor
• Collected, verified, checked the validity, registered the medication then arranged the on shelf.
• Dispensed the medications to patients and consulted them.

OTHER TRAINING AND EDUCATION

• Maine Biotechnology and drug discovery, Pharmacy College, KAU 11/10/14
• symbiosis and marine microbial natural products Pharmacy College, KAU 11/12/14
• Maine Cyanobacteria in drug discovery Pharmacy College, KAU 11/11/14
• Application of Pharmacokinetics in Clinical Practice at Intercontinental Hotel, Jeddah, KSA 5/11/11
• VIII Saudi Scientific Hematology Society Congress, Crown Plaza Hotel, Jeddah 12/16-17/09
• Medication Errors and Patients Safety, International Medical Center, Jeddah 4/27-28/09
• The 2nd Diabetes Mellitus and Endocrinology, King Abdul-Aziz Hospital & Oncology Center 8/26-27/08