UNDERSTANDING THE NATURE OF GLYCYRRHIZIC ACID IN BREAST CANCER TREATMENT

by

JESSICA S. HALL
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JESSICA S. HALL

APPROVED:

______________________________
Robert J. McKallip, PhD
Advisor

______________________________
Christy C. Bridges, PhD
Program Director

______________________________
Gretchen Bentz, PhD
Committee Member

______________________________
James Drummond, PhD
Committee Member

______________________________
Jean R. Sumner, MD
Dean, School of Medicine
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ABSTRACT

UNDERSTANDING THE NATURE OF GLYCIRRHIZIC ACID IN BREAST CANCER TREATMENT
By: JESSICA SHEREASE HALL
Under the direction of: DR. ROBERT J. MCKALLIP, PhD

Breast cancer (BC) is the second most common cancer in women with 1 in 8 women in the United States developing BC within their lifetimes. Of the numerous types of breast cancer, invasive ductal carcinoma (IDC) is the most common accounting for 80% of all breast cancers.

The use of chemotherapeutic drugs such as doxorubicin (DOX) improves the prognosis and survival of patients diagnosed with BC. Yet, many BC cells form a drug resistance leading to relapse and worsening of prognosis for the patient. We hypothesize that the alternative medicine, glycyrrhizic acid (GA) will lead to the induction of apoptosis in BC cells while sensitizing the cells in combination with first-line chemotherapeutic, DOX. The effects of treatment on BC cell growth was assessed and measured using TACS MTT Cell Proliferation Assay, Trypan Blue Dye Exclusion, DeadEnd™ Fluorometric TUNEL System, Annexin-V/PI-double staining, Western Blot, cellular production of reactive oxygen species (ROS) and the detection of mitochondrial membrane potential to
determine mitochondrial function. In the current study, treatment with GA led to decreases in cell proliferation and viability in addition to the induction of apoptosis. Our results also show that exposure to GA leads to increased ROS generation. Furthermore, we demonstrated that GA may be effective when used as co-treatment with DOX for BC treatment. Recommendations for further study involves illustrating the role and mechanism of hyaluronic acid (HA) on each cell line, investigating the usefulness of co-treatment with GA and DOX, examine the effects of ROS inhibitors on ROS generation and transition studies to focus on 3-D BC cell models.
CHAPTER 1

INTRODUCTION

**Breast Cancer Statistics and Risk Factors:**

Breast cancer (BC) is a worldwide public health dilemma as it is one of the most common tumors on the globe (Akram et al. 2017). It is the second most prominent cancer affecting women with 1 in 8 women in the United States developing BC within their lifetimes. Despite the scientific advancements in the medical field, metastatic breast cancer has poor prognosis (Sobhani et al. 2018). In 2020, 276,480 new cases of invasive breast cancer are expected to be diagnosed in women in the U.S., along with 48,530 new cases of non-invasive (in situ) breast cancer (Breastcancer.org 2020).

Common risk factors include race, biological sex, increasing age, history of breast conditions, family history, inherited genes, obesity, and exposure to radiation. BC is more common in the African-American community with African-American women under the age of 45 being more likely to die from BC than white women. Five to ten percent of all breast cancers are linked to gene mutations passed throughout generations including breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2), both of which have been attributed to an increase in the risk of BC (Mayo Clinic 2019). Ashkenazi Jewish women
have increased risk of developing BC because of a higher rate of BRCA mutations (Breastcancer.org 2020). Advancements made in the field of breast cancer have resulted in a tremendous increase in survival rate of BC patients; however, this is applicable only with early detection without metastasis (Jamdade et al. 2015).

In order to diagnose BC, mammograms, breast ultrasounds, breast MRIs and manual breast exams are a few of the methods commonly utilized. Post diagnosis, it is imperative to establish the stage of BC. Determination of the stage of BC is based upon the tumor size within the breast, lymph nodes affected in addition to any signs that may indicate that BC has invaded other organs within the body (National Breast Cancer Foundation 2020). Determination of the BC stage helps determine prognosis and the best method of treatment for a given patient.

**Types and Prevalence of Breast Cancer:**

All breast cancers start within the breast and can be either invasive or non-invasive. Tumor cells can be located within the ducts, lobules or in some cases, the tissue in between. Tumor cells also differ in their appearance under the microscope and other tumor characteristics such as hormone receptor status all of which affect prognosis (Susan G. Komen 2020).

The type of breast cancer is determined by the site of origin (lobules or milk ducts) in addition to its invasiveness which refers to whether the cancer has
spread to other parts of the body or not. The most common type of noninvasive breast cancer is ductal carcinoma in situ (DCIS), which starts within the milk ducts and is considered non-invasive because it has not spread beyond the milk duct into any normal surrounding breast tissue. There are numerous types of invasive breast cancer including invasive ductal carcinoma (IDC), invasive lobular carcinoma, tubular carcinoma, mucinous (colloid) carcinoma, carcinomas with medullary features and invasive papillary carcinoma (American Cancer Society 2020). Invasive lobular carcinoma (ILC) is the second most common type of invasive carcinoma accounting for 10% of all breast cancers. ILC starts within cells lining the milk glands or lobules of the breast that begin to proliferate through the wall of the lobules allowing the cells to spread to nearby lymph nodes and/or to other parts of the body. Of the numerous types of breast cancer, invasive ductal carcinoma (IDC) is the most common accounting for 80% of all breast cancers. IDC starts within the cells lining a duct and invades the surrounding breast tissue. Due to the prevalence of IDC, we focus on this type of BC for research purposes.

**MCF-7 versus MDA-MB-231 cell line:**

Due to the major impact breast cancer has on the population, it requires further elucidation at the molecular level in order to better understand the implications of various treatments. Cell lines can be used in many different
aspects of laboratory research, particularly, as in vitro models in cancer research (Burdall et al. 2003).

The MCF-7 cell line, derived at the Michigan Cancer Foundation in 1973 from a pleural effusion of a malignant breast cancer is an extensively studied model for hormone-dependent human breast cancer (Soule et al. 1973). MCF-7 cell line has estrogen receptors, and is dependent on estrogen for growth (Nohara, Wang and Spiegel 1998), which makes this cell line a suitable model for hormone therapy. These cells are very well characterized which allows its use to shed more light into breast cancer pathogenesis and treatment practices through reliable in vitro studies.

In contrast, the highly metastatic MDA-MB-231 cell line provides a model for human breast cancer, which exhibits an estrogen-independent state and does not express estrogen receptors (Cailleau et al. 1974). MDA-MB-231 is a type of triple-negative breast cancer (TNBC) defined by a lack of immunohistochemical expression of estrogen receptors (ER), progesterone receptors (PR) and HER2, accounting for approximately 15-20% of BC patients (Curigliano and Goldhirsch 2011; Penault-Llorca and Viale 2012). A large portion of patients with TNBC still do not respond to therapies and a number of studies are ongoing focusing on strategies to improve response rates to treatment.

Both MCF-7 and MDA-MB-231 were derived from human breast tumors, however, they differ in their proliferative potential. The MCF-7 cell line
originates from metastatic sites in adenocarcinoma, as a tumorigenic adherent breast epithelial cell line, whereas the MDA-MB-231 cell line is a tumorigenic metastatic breast cell line (Visagie, Mqoco and Joubert 2012). Tumors are usually formed by cells with varying phenotypes and metastatic potential (Pozo-Guisado et al. 2002). Due to diversity in cell types, it could be of interest to analyze the effects of glycyrrhizic acid (GA) and doxorubicin (DOX) on cellular proliferation and viability, determine if the inhibition on cellular proliferation is due to the induction of apoptosis and to examine if there is a relationship between the protective role of hyaluronic acid and GA-induced reactive oxygen species (ROS).

**Cell Cycle Machinery and Apoptosis:**

We have gained a wealth of knowledge pertaining to the heterogeneity of BC and have evolved a complex and multidisciplinary treatment approach to the disease after decades of basic and clinical trials research (Ganz and Goodwin 2015). Apoptosis or programmed cell death has been defined as distinct morphological characteristics and energy-dependent biochemical mechanisms leading to cell death (Elmore 2007). Failure of the initiation of apoptosis is a critical factor that leads to the formation of cancer (Kasibhatla and Tseng 2003). Consequently, prominent research continues to focus on the elucidation and analysis of cell cycle machinery in addition to different signaling pathways that control cell cycle arrest and apoptosis in order to manage the disease (Elmore 2007).
**Usefulness and Limitations of Current Therapeutics:**

Breast cancer is highly metastatic, leading to multiorgan dysfunction and a high mortality rate (Ertas et al. 2012; Novakovic et al. 2004), which has been reduced with scientific advances in medical treatment and early detection. Generally, treatment for BC is based on the type and stage of cancer, which commonly involves surgery, radiation therapy, chemotherapy and/or hormonal therapy. However, the adverse effects associated with these treatments may cause long-term complications such as critical alopecia, nephrotoxicity and hepatotoxicity, which may add to increased morbidity and mortality among survivors (Wu, William and Gulley 2007; Sopkova and Mechl 1986).

**Emerging Drug Resistance to Therapy:**

Drug resistance is a complex phenomenon involving several major mechanisms, such as, increased repair of DNA damage, decreased apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that reduce the capacity of cytotoxic agents to eradicate cancer cells (Kovalchuk 2008). Many BC cells form a drug resistance leading to relapse and worsening of prognosis for the patient. Due to the complexity of drug resistance, there has been a shift of attention in chemotherapy from the use of a single drug to multiple drugs for the management of cancers with the ultimate goal of regulating multifaceted
signaling processes critical for the survival of tumors via suppression or activation of multiple targets concurrently (Giordano and Petrelli 2008).

The discovery of novel compounds with low toxicity, which efficiently inhibits growth, migration and invasion is an important research area. Due to this, we explored utilizing both a chemotherapeutic drug, doxorubicin (DOX) and an alternative medicine, glycyrrhizic acid (GA) to examine their effects on breast cancer cell growth.

**Hyaluronic Acid (HA):**

Hyaluronic acid (HA) is a prevalent component of the normal human breast extracellular matrix and alterations in HA contribute to breast cancer cell growth and progression (Auviene et al. 2000; Götte and George 2006; Urakawa et al. 2011). HA in excess suppresses tumor growth in the absence of hyaluronidase (HYAL), therefore inhibition of HA degradation may be key in decreasing the advancement of the disease (Girish and Kemparaju 2007; Mcatee, Joseph, and Simpson 2014). Both the levels and size of HA play a role in tumor development and progression, however, the mechanisms remain poorly understood.

**Evidence linking HA to increased cellular proliferation:**
There is an urgent need for increased knowledge on the biology leading to breast cancer, which can lead to the design of increasingly accurate drugs against a patients’ specific molecular aberrations (Sobhani et al. 2018). Among the many targets is the fibroblast growth factor receptor (FGFR), which induces accumulation of hyaluronic acid (HA) within the extracellular matrix upon stimulation (Bohrer et al. 2013). FGFR-mediated HA accumulation requires activation of the signal transducer and activator of transcription 3 (STAT3) pathway, which regulates expression of hyaluronan synthase 2 (HAS-2) and subsequent HA synthesis contributing to breast cancer cell growth and progression (Bohrer et al. 2015).

**Implications of HAS-1, HAS-2, HAS-3:**

The levels of HA accumulate in BC, partly due to increased synthesis via hyaluronan synthases (HAS), which are associated with decreased survival and poor response to therapy (Auvinen et al. 2012; Karihtala et al. 2007). The biosynthesis of HA is regulated by the three isoenzymes hyaluronan synthase 1 (HAS-1), hyaluronan synthase 2 (HAS-2) and hyaluronan synthase 3 (HAS-3), all of which are integral plasma membrane proteins (Rilla et al. 2005). HAS-1 synthesizes low levels of high molecular weight (HMW) HA (~1x10^6 to 1x10^7 Da) (Spicer and McDonald 1998; Shyjan et al. 1996). HAS-2 produces relatively high amounts of HMW HA (~1x10^6 to 1x10^7 Da) and is involved in cellular proliferation, angiogenesis in addition to embryonic and cardiac development.
HAS-3 is the most biologically active HAS molecule and is known to contribute to the malignant phenotype in many different cell types while synthesizing LMW HA (~1x10^5 Da) (Spicer and Nguyen 1999; Spicer and McDonald 1998). Studies have shown that HAS-1 restores the metastatic potential of mouse mammary carcinoma mutant cells that have low HA production and low metastatic capability (Itano et al. 1999). While overexpression of HAS-2 and HAS-3 stimulates both tumorigenicity and tumor progression (Kosaki, Watanabe and Yamaguchi 1999; Liu et al. 2001), the roles of HA in cancer progression may differ according to HAS isoforms expressed, meaning cancer cells at different stages may differentially utilize the three HAS isoforms to maximize their survival (Stern 2008).

Hyaluronic Acid relationship with Tumor Formation and Inhibition:

HA degradation is even more abundant and rapid in malignant tissues (Menegazzi et al. 2008). According to Hanoux et al., HA degradation stimulated by treatment with exogenous HYAL promotes the MCF-7 cell growth (2018). Evidence has shown that the differences in size, high and low molecular weight HA, exhibit contrasting effects on cellular response (Girish and Kemparaju 2007). It has been reported that high molecular weight hyaluronan inhibits tumor formation, conversely, low molecular weight (LMW) forms have been shown to stimulate cancer cell migration and invasion possibly through differential interactions with its receptors, such as CD44 (Bohrer et al. 2013). The percentage
of LMW fragments of HA is greater in tumors and tumor patients than in normal patients (Kumar et al. 1989).

**CD44-receptor:**

CD44, commonly known as a homing cell adhesion molecule is a multi-structural cell molecule involved in cell-cell and cell-extracellular matrix communication regulating various signaling pathways while also acting as both a growth and arrest sensor, and inhibitor of angiogenesis and invasion in response to signals from the microenvironment (Ouhtit et al. 2018). Marhaba et al., reports that CD44 is known to promote BC progression and metastasis through the binding of CD44 to its major ligand HA, or to other ligands, including osteopontin, fibronectin, collagen IV, and laminin (Marhaba and Zöller 2003). CD44 cleavage, shedding, and elevated levels of soluble CD44 in the serum of patients is a marker of tumor burden and metastasis in several cancers (Senbanjo and Meenakshi 2017). Studies have shown that in high CD44 expressing breast cancer cells HA modulates tumor cell adhesion and motility and also increases the expression of its own receptor, CD44 (Herrera-Gayol and Jothy 2001). CD44 expression has been shown to be linked with both poor and favorable outcomes that suggests the dual nature of CD44 mediating breast tumor development (Ouhtit et al. 2018).
Figure 1. Dual nature of CD44 mediating breast tumor development. A, CD44 promotes tumor progression directly mediating signal transduction via activation by LMW HA, which upon binding recruits signaling mediators to the cytoplasmic tail of CD44, activating signaling pathways that promote cellular migration and invasion. B, CD44 inhibits tumor progression in response to extracellular cues, primarily binding to HMW HA. (Louderbough and Schroeder 2011).

**Hyaluronidase, HYAL-1 and HYAL-2:**

The hyaluronidase (HYAL) family of enzymes has been broadly implicated in various cancers, and have demonstrated potential for clinical utility as both biomarkers and therapeutic targets (Mcatee, Joseph, and Simpson 2014). The levels of HYAL, the enzymes responsible for the degradation of HA are variable in cancer; in some cases they are elevated and in others they are suppressed, relative to normal tissues (Stern 2008). The levels of how these enzymes are regulated remains to be unknown.
**Hyaluronidase (HYAL) inhibitors:**

Hyaluronidase (HYAL) inhibitors are powerful regulating agents involved in sustaining the balance between the anabolism and catabolism of HA (Girish and Kemparaju 2007). Studies available are suggesting that HA fragments will facilitate cancer progression, however, very little remains known about this class of inhibitors. A thorough understanding of the anabolic and catabolic systems for HA may provide breakthroughs into our current level of understanding of cancer progression in addition to novel opportunities for therapeutic intervention (Stern 2008).

**Doxorubicin:**

Doxorubicin (DOX) is one of the most important anti-cancer chemotherapeutic drugs derived from the bacterium *Streptomyces* and is beneficial in cancer treatment due to its ability to intercalate between base pairs in the DNA helix, thereby preventing DNA replication and ultimately inhibiting protein synthesis (National Center for Biotechnology Information 2020). In addition, doxorubicin is advantageous because it can inhibit the activity of the enzyme topoisomerase-II after inserting itself into the DNA structure, which prevents cellular reproduction (Yang, Kemp and Henikoff 2014).
The use of chemotherapeutic drugs, such as doxorubicin, improves the prognosis and survival of patients diagnosed with BC. Doxorubicin belongs to a class of compounds named anthracyclines and is one of the first-line treatments for BC, still, adverse side effects and drug resistance remains a barrier restricting its clinical application (Zhang et al. 2019). Growing evidence supports the idea that this drug can diminish the quality of patients’ life during and after doxorubicin treatment due to the toxicity associated with the heart, brain, kidney and liver which complicates cancer treatment limiting its therapeutic doses (Carvalho et al. 2009).
Glycyrrhizic Acid:

Although great efforts have been made through both basic and clinical trials research to design more potent drugs for the treatment of BC, the results remain unsatisfactory. In fact, some herbal drugs in alternative medicine have been suggested to be a better choice to improve the current therapeutic strategy.

Glycyrrhizic acid (GA) is a major active constituent of licorice root extracts, containing 8 hydroxyl groups and it is highly hydrophilic; therefore, it is water-soluble and is easily absorbed and metabolized by the digestive tract (Lin et al. 2017). It is a triterpene glycoside with numerous pharmacological effects including as anti-inflammatory, anti-viral, anti-tumor and hepatoprotective activities (Menegazzi et al. 2008). Studies have shown that GA may inhibit the migration and invasion of several types of cancer cells with high efficiency and
low toxicity (Smolarczyk et al. 2012; Kohlschütter et al. 2008). This may provide possibilities to combine GA with first-line chemotherapeutic drugs resulting in novel treatment with reduced adverse effects for patients.

Figure 4. Structure of Glycyrrhizic acid (National Center for Biotechnology Information 2020).

**Benefits of Combination Therapy:**

Due to toxicity associated with doxorubicin, it is imperative to find novel treatment strategies for the management of patients with breast cancer and to maintain focus on combinatorial therapies with the hopes of reducing the adverse effects associated with clinical dosages. Pre-clinical studies have shown that the combination of drugs has a better therapeutic effect on cancer than single agent therapy (Hostetler, Uchakina and McKallip 2017). This may be in
relation to the synergistic effect caused by diverse mechanisms of multiple drugs (Su et al. 2017). We will use the human adenocarcinoma breast cancer cell lines, MCF-7 and MDA-MB-231 as \textit{in vitro} models for studying the therapeutic effects of combination therapy utilizing both doxorubicin and glycyrrhizic acid.

\textbf{In conclusion:}

Accelerating progress against cancer requires both increased national investment in cancer research and the application of existing cancer control across all segments of the population (Siegel, Miller, and Jemal 2016). In order to address this global issue, we will focus on the following aims:

\textbf{Aim \#1:} Investigate the effect of GA on the breast cancer cell lines, MCF-7 and MDA-MB-231.

\textit{Hypothesis:} GA treatment will lead to the induction of apoptosis in BC cells.

\textbf{Aim \#2:} Investigate the abilities of GA to sensitize BC cells to conventional therapy.

\textit{Hypothesis:} GA treatment will sensitize the BC cells in combination with first-line chemotherapeutic, DOX.
CHAPTER 2

METHODOLOGY

Cell Culture:

The human adenocarcinoma breast cancer cell lines, estrogen-dependent MCF-7 (Catalog Number: HTB-22) and estrogen-independent MDA-MB-231 (Catalog Number: HTB-26) was obtained from the American Type and Culture Collection (ATCC). Both cell lines were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% fetal bovine serum, and 0.2% MycoZap Antibiotics (Lonza). The cells were maintained in a 5% CO₂ incubator at 37°C and passaged by 0.25% trypsin-EDTA every 2-3 days.

TACS MTT Cell Proliferation Assay:

Cell proliferation was analyzed using TACS MTT Cell Proliferation Assay (Trevigen, Inc., Minneapolis, MN), which detects the metabolic activity of cells. This protocol was also useful in determining the working concentrations of glycyrrhizic acid (GA) and doxorubicin (DOX) that would be the focus of each experiment proceeding forward. It was vital to determine the concentrations of
each treatment that would lead to cytotoxic effects in both the MCF-7 and MDA-
MB-231 cell lines.

A colorimetric assay with tetrazolium salt was utilized to assess the anti-
proliferative effects of treatment providing an indication of cell viability and
metabolic activity. Both the MCF-7 and MDA-MB-231 cell lines were seeded in
triplicate with a final volume of 100 µl at 2.5x10^5 cells/ml (or 2.5x10^4 cells/well)
in 96-well plates for 24 h to allow cell adherence. The cells were then treated
with variable concentrations of GA (0.5, 1.0 or 2.0 mM) and DOX (0.25, 0.5, 1 and
2 µM) for 24 and 48 h after which 10 µl of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,
5-diphenyltetrazolium bromide] (Catalog #: 4890-25-01) was added and then
incubated at 37°C for 4 hours. Post incubation, 100 µl of the MTT Detergent
Reagent (Catalog #: 4890-25-02) was added to each experimental group, which
was then covered and placed in the dark for 2-4 hours or overnight. After
dissolving the formazan crystals, a microplate reader (Multiscan FC, Thermo
Scientific) was used to measure optical density at a wavelength of 595 nm.
Finally, the data were reported as means ± standard deviations for all
experiments with more than three replicates, and statistically analyzed.

**Trypan Blue Dye Exclusion to assess cell viability:**

To examine the effect of treatment on cell viability, each sample was
harvested and counted utilizing the Trypan Blue Exclusion Method. The purpose
of this assay is to quantify the cells present in suspension based on the principle
that the cell membranes of live cells remain intact which excludes dyes, such as
trypan blue, whereas necrotic or dead cells do not. First, we added equal parts of
0.4% trypan blue dye (Lonza) and our cell suspension (example: 10 µl of cells to
10 µl trypan blue dye) to the 96-well plate and mixed by slowly pipetting up and
down. With the coverslip in place, we added 10 µl of the cell suspension by
placing the tip of the pipette at the notch of the hematocytometer. We then
placed the hematocytometer on the stage of the light microscope (Zeiss
Invertoskop 40C) and counted all cells in each large square in each corner and
calculated the number of viable cells.

**Cell death measurement by DeadEnd™ Fluorometric TUNEL System followed
by Flow Cytometry analysis:**

To determine if the inhibition of cellular proliferation by GA was due to
the induction of apoptosis, a terminal deoxynucleotide transferase mediated
fluorescein-dUTP nick-end-labeled (TUNEL) assay by Promega (Catalog #: G3250,
Madison, WI) was utilized. Both cell lines were seeded at initial cell density of
2.5x10⁵ cells/mL in a 24-well plate. Cells from each experimental condition were
harvested by trypsinization (0.25% trypsin-EDTA) followed by centrifugation at
200g for 5 minutes and washed by PBS twice. After discarding the supernatant,
we added 500 µl of 1% paraformaldehyde allowing incubation for 20 minutes on
ice. Then, we proceeded to centrifuge each sample at 300 g for 5 minutes at 4°C
followed by removing the supernatant and resuspending the cells in 1 mL of phosphate-buffered saline (PBS). We then, permeabilized the cells by adding 500 µl of 70% ethanol, incubating the samples at -20°C overnight. The following day, we centrifuged the cells at 300g for 10 mins, and washed by PBS three consecutive times followed by resuspending each sample in 40 µl equilibration buffer, incubating for 5 minutes at room temperature. Next, we prepared our rTdT incubation buffer (22.5 µl equilibration buffer + 2.5 µl nucleotide mix + 0.5 µl rTdT enzyme per reaction). After incubation in equilibration buffer, we centrifuged the cell samples at 300g for 5 minutes, removed the supernatant and resuspended each sample in 25 µl of rTdT incubation buffer. Lastly, we incubated each sample in a water bath for 1 hour at 37°C protecting from direct sunlight, followed by termination of each reaction by adding 400 µl of 20 mM EDTA to each sample. Each sample was analyzed by flow cytometry using FACS Aria II flow cytometer (BD Sciences, San Jose, CA).

**Cell death detection by Annexin-V/PI-double staining followed by Flow Cytometry analysis:**

To further analyze apoptosis, TACS Annexin V-FITC (Trevigen, Inc.) assay was used. MDA-MB-231 cell line was seeded at initial cell density of 2.5x10^5 cells/mL in a 24-well plate. Cells from each experimental condition were harvested by trypsinization (0.25% trypsin-EDTA) followed by centrifugation at 300g for 5 minutes, resuspended in 1 mL PBS, twice. We prepared 400 µl 1X
binding buffer (Catalog #: 4830-01-2) for each sample in addition to preparation of 100 µl Annexin V incubation reagent (Catalog #: 4830-01-1) for all samples excluding both positive and negative controls. We proceeded to resuspend samples in Annexin V incubation reagent followed by incubation for 15 minutes in the dark at room temperature. Lastly, we added 400 µl 1X binding buffer to each sample, which was analyzed by flow cytometry.

**Western Blot to assess PARP activity:**

Apoptosis is depicted by activation of caspase-3-associated proteins leading to cleavage of poly (ADP-ribose) polymerase (PARP). To determine whether GA induced apoptosis, protein expression was measured by western blotting. The amount of PARP protein within cells can provide a general overview of cell health and viability. Cell lines, MCF-7 and MDA-MB-231 were seeded at 2.5x10^5 cells/mL in 24-well plates utilizing 4 wells per treatment group for both 24 and 48 hour experiments. Cells from each experimental condition were harvested by trypsinization (0.25% trypsin-EDTA). Each sample was rinsed with cold PBS, twice, and lysed with 500 µl of RIPA buffer with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1% phosphatase inhibitor cocktail 2 and incubated for 30 minutes. After samples were transferred to microcentrifuge tubes, they were centrifuged for 15 minutes. To analyze protein content, a Bradford assay was performed. Once protein content was analyzed, each sample (~12 µg/µL proteins per well) were electrophoresed using SDS
polyacrylamide gels, and then transferred onto a PVDF membrane via the TransBlot Turbo Transfer System (BioRad). Next, 5% milk in TBST (TBS with 0.05% Tween 20) (Bio-Rad, Hercules, CA, USA) was utilized to block the membranes for 1 hour at room temperature, and subsequently incubated overnight in the presence of the primary antibody directed against β-actin or PARP (1:1000) (Cell Signaling Technology, Danvers, MA, USA) at 4°C. Afterwards, the membranes were washed with TBST for 10 mins, three consecutive times. Horseradish peroxidase-conjugated (HRP)-linked secondary antibody (1.5 μl per 5 mL TBS) was incubated for 1 hour at room temperature followed by washing with TBST, three times. Following washing, bands were visualized with the chemiluminescent detection reagent, WesternBright™ Peroxide and WesternBright™ ECL (Advansta) using the ChemiDoc Touch Imaging System (BioRad).

**Cellular production of reactive oxygen species (ROS):**

To quantify GA-induced reactive oxygen species (ROS), the detection of intracellular ROS protocol was used. Both cell lines were seeded at initial cell density of 2.5x10^5 cells/mL in a 24-well plate. Cells from each experimental condition were harvested by trypsinization (0.25% trypsin-EDTA) followed by collection in 5 mL round bottom tube by centrifugation at 200g for 5 minutes at room temperature. Each cell pellet was washed with 4 mL PBS, centrifuged at 200g for 5 minutes at room temperature and resuspended in PBS with 20 μM of
2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (Invitrogen Molecular Probes) by gently pipetting up and down. Each cell sample was then incubated in a 5% CO$_2$ incubator at 37°C for 45 minutes. Finally, propidium iodide (PI) (0.5 µl/0.5 mL tube) was added to each sample and the cells were analyzed by flow cytometry.

**Detection of Mitochondrial Membrane Potential to determine mitochondrial function:**

The depletion of mitochondrial membrane potential (ΔΨm) suggests the loss of mitochondrial membrane integrity, which reflects the initiation of a proapoptotic signal. Within this assay, the cell-permeant, green-fluorescent, lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$) (Invitrogen) was used which accumulates in the mitochondria due to their large negative membrane potential which allows it to be monitored and analyzed via flow cytometry.

The MCF-7 and MDA-MB-231 cell lines were seeded at a density of 2.5x10$^5$ cells/mL in a 24-well plate for 24 h to allow cell adherence. Following 24 h treatment, we prepared the DiOC$_6$ solution with a final concentration of 400 nM (Stock = 100 mM) and carefully removed 100 µl of supernatant from each well, then added 100 µl of 400 nM DiOC$_6$. The plate was placed in the 5% CO$_2$ incubator at 37°C for 30 minutes. We then harvested the cells by trypsinization (0.25% trypsin-EDTA) followed by centrifugation at 200g for 5 minutes and
washed in cold PBS. The cell pellet was resuspended in PBS for analysis using flow cytometry.

**Data Analysis:**

Statistical analysis performed with the Data analysis ToolPak Excel Add-in.

Student’s t-test was used to determine statistical significance with an asterisk denoting p<0.05.
CHAPTER 3

RESULTS

Glycyrrhizic acid (GA) and doxorubicin (DOX) treatment reduces cell proliferation and viability in MCF-7 and MDA-MB-231 BC cells *in vitro*. Experiments were conducted to determine whether GA treatment in combination with DOX affect MCF-7 and MDA-MB-231 BC cell growth.

*Induction of cell death by GA in MCF-7 cells.*

MCF-7 cells were treated with various concentrations of GA (0.5, 1.0 or 2.0 mM) for 24 and 48 h. The cytotoxicity of GA was measured by TACS MTT Cell Proliferation assay which demonstrates significant reduction on cell proliferation at concentrations of GA >0.5 mM following 24 and 48 h treatment (Figure 1a and 1b). Cell viability was determined using trypan blue dye exclusion. The results show that MCF-7 cell viability was significantly reduced at GA concentrations >1 mM following 24 h treatment (Figure 2a) and >0.5mM following 48 h treatment (Figure 2b). After 24 h treatment, cell viability was significantly reduced from $3.0 \times 10^6$ to $2.17 \times 10^6$ to $1.83 \times 10^6$ following treatment with 1 mM and 2 mM GA respectively. In contrast, after 48 h treatment, cell viability was significantly reduced from $1.01 \times 10^6$ to $6.48 \times 10^5$ to $6.72 \times 10^5$ to $5.76 \times 10^5$ following treatment.
with 0.5 mM, 1 mM and 2 mM GA respectively suggesting that MCF-7 cells are sensitive to GA treatment.

Figure 1a. Glycyrrhizic acid treatment leads to significant decreases on cell proliferation in MCF-7 BC cells in vitro. MCF-7 cells were cultured for 24 h in the presence of GA (0.5, 1.0 or 2.0 mM). Asterisks denote statistical significance (*p<0.05, **p<0.01, compared with the untreated control, n=4).
Figure 1b. Glycyrrhizic acid treatment leads to significant decreases on cell proliferation in MCF-7 BC cells \textit{in vitro}. MCF-7 cells were cultured for 48 h in the presence of GA (0.5, 1.0 or 2.0 mM). Asterisks denote statistical significance (*$p<0.05$, **$p<0.01$, compared with the untreated control, n=4).

![Box plot of cell viability vs GA concentration](image)

Figure 2a. Glycyrrhizic acid treatment leads to significant decreases on cell viability in MCF-7 BC cells \textit{in vitro}. MCF-7 cells were cultured for 24 h in the presence of GA (0.5, 1.0 or 2.0 mM). Cell viability was determined by trypan blue dye exclusion. Asterisks denote statistical significance (*$p<0.05$, **$p<0.01$, compared with the untreated control, n=5).
Figure 2b. Glycyrrhizic acid treatment leads to significant decreases on cell viability in MCF-7 BC cells *in vitro*. MCF-7 cells were cultured for 48 h in the presence of GA (0.5, 1.0 or 2.0 mM). Cell viability was determined by trypan blue dye exclusion. Asterisks denote statistical significance (*p<0.05, **p<0.01, compared with the untreated control, n=5).

**Glycyrrhizic acid induces apoptosis in MCF-7 BC cells in vitro.**

To determine if the inhibition on cell proliferation and viability on MCF-7 BC cells by GA was due to the induction of apoptosis, the cells were treated with various concentrations of GA (0.5, 1.0 or 2.0 mM) for 48 h. The percentage of cells undergoing apoptosis was determined by TUNEL (Figure 3A-B) and confirmed by examining PARP cleavage (Figure 3C). GA treatment resulted in increased DNA fragmentation with 20.5% and 24.1% in 0.5 mM and 1 mM GA respectively compared to control (17.5%) (Figure 3B). Analysis of PARP cleavage revealed that a dose of 1 mM GA resulted in a large shift in the normalized ratio of cleaved-PARP/uncleaved-PARP from 0 in the control to 26.8 in the GA treated
groups (Figure 3D). There was a decrease in cleaved PARP at 2 mM GA which we attribute to obliterating the cells at that concentration. MCF-7 BC cells were also assayed for loss of mitochondrial membrane potential (Figure 3E). The data was consistent with the TUNEL assay showing a loss of mitochondrial membrane potential in the GA treated groups with 13.8%, 15.9% and 16.2% in 0.5 mM, 1 mM and 2 mM GA respectively, compared to control (11.4%). The data confirms that treatment of MCF-7 BC cells with GA results in the induction of apoptosis.
Figure 3. Treatment with GA enhances levels of apoptosis in vitro. The effect on the induction of apoptosis was determined by TUNEL staining (A, B). The induction of apoptosis in MCF-7 cells was confirmed by analyzing PARP cleavage (C, D). In addition, the effect on mitochondrial membrane potential as examined (E).

**Exposure of MCF-7 cells to GA leads to increased levels of ROS.**

Literature suggests that GA may kill tumor cells through the generation of reactive oxygen species (ROS). To explore this mechanism, MCF-7 BC cells were treated with GA (0.5, 1.0 or 2.0 mM) for 48 h and then examined for changes in intracellular ROS by flow cytometric analysis. Results show that the control exhibited 6.6% of cells expressing ROS compared to GA treated groups where we
observe increases in ROS generation (Figure 4A-B). Results also show that cells following GA treatment showed higher 2,7’-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence compared with that in the normal control group which refers to the average of all cells expressing ROS (Figure 4C).

Figure 4. Exposure of MCF-7 BC cells leads to increased levels of ROS. The effect of GA treatment on intracellular ROS levels was determined by treating DCFDA-labeled MCF-7 cells with various concentrations of GA (0.5, 1.0 or 2.0 mM) for 48 h and determining ROS levels by flow cytometric analysis (A-B). (C) The graph depicts the DCFDA fluorescence intensity.

**Induction of cell death by GA in MDA-MB-231 BC cells.**

MDA-MB-231 BC cells were treated under various concentrations of GA (1.0 or 2.0 mM) for 24 and 48 h. The cytotoxic effect of GA was measured by the MTT Cell Proliferation assay. Results show no significant decreases on cell proliferation following 24 h GA treatment (Figure 5a), however, there are
significant decreases on cell proliferation at concentrations of GA >1 mM following 48 h treatment (Figure 5b). Cell viability was assessed via trypan blue dye exclusion. Results show that MDA-MB-231 BC cell viability was significantly reduced at concentrations of GA >1 mM following both 24 and 48 h treatment (Figure 6a and 6b). Following 24 h GA treatment, cell viability was significantly reduced from 2.6x10^6 to 2.0x10^6 to 1.47x10^6 following treatment with 1 mM and 2 mM GA respectively. In contrast, following 48 h GA treatment, cell viability was significantly reduced from 2.64x10^6 to 1.77x10^6 to 8.4x10^5 following treatment with 1 mM and 2 mM GA respectively suggesting that MDA-MB-231 cells are also sensitive to GA treatment.

Figure 5a. Glycyrrhizic acid treatment does not lead to significant decreases on cellular proliferation in MDA-MB-231 BC cells in vitro. MDA-MB-231 cells were cultured for 24 h in the presence of GA (1.0 or 2.0 mM). Asterisks denote statistical significance (*p<0.05, **p<0.01, compared with the untreated control, n=3).
Figure 5b. Glycyrrhizic acid treatment leads to significant decreases on cellular proliferation in MDA-MB-231 BC cells \textit{in vitro}. MDA-MB-231 cells were cultured for 48 h in the presence of GA (1.0 or 2.0 mM). Asterisks denote statistical significance (*$p<0.05$, **$p<0.01$, compared with the untreated control, n=5).

Figure 6a. Glycyrrhizic acid treatment leads to significant decreases in cell viability in MDA-MB-231 BC cells \textit{in vitro}. MDA-MB-231 cells were cultured for 24 h in the presence of GA (1.0 or 2.0 mM). Cell viability was determined by trypan blue dye
exclusion. Asterisks denote statistical significance (*p<0.05, **p<0.01, compared with the untreated control, n=3).

**Figure 6b.** Glycyrrhizic acid treatment leads to significant decreases in cell viability in MDA-MB-231 BC cells *in vitro*. MDA-MB-231 cells were cultured for 48 h in the presence of GA (1.0 or 2.0 mM). Cell viability was determined by trypan blue dye exclusion. Asterisks denote statistical significance (*p<0.05, **p<0.01, compared with the untreated control, n=3).

**Glycyrrhizic acid induces apoptosis in MDA-MB-231 BC cells in vitro.**

To determine whether GA led to reduced cell proliferation and viability via induction of apoptosis, MDA-MB-231 cells were treated with various concentrations of GA (1.0 or 2.0 mM) for 48 h. The percentage of cells undergoing apoptosis was determined by Annexin V/PI staining (Figure 7A) and confirmed by detecting PARP cleavage (Figure 7D). Annexin V/PI staining demonstrated a dose-related increase in the percentage of apoptotic tumor cells following GA treatment. There was a modest increase in the early apoptotic cell
population from the control (2.0%) to 1 mM GA (2.3%) and a much greater shift at 2 mM (5.1%), additionally there was a greater increase in the late apoptotic cell population from the control (3.1%) to a shift seen at 2 mM (8.6%) (Figure 7A-B). GA reduced the percentage of viable MDA-MB-231 BC cells from 94.3% to 83.5% in a dose-dependent manner (Figure 7C). Analysis of PARP cleavage revealed that a dose of 2 mM GA resulted in a greater increase in the normalized ratio of c-PARP/PARP from 0.0 in the control to 2.8533 in the 2 mM GA treated group (Figure 7E). The MDA-MB-231 BC cell line was also analyzed to examine the loss of mitochondrial membrane potential (Figure 7F). Results shown a slight increase in the loss of mitochondrial membrane potential in the control (2.7%) to 3.2% and 4.2% in the 1 mM and 2 mM GA treated group respectively. This data confirms that treatment of the MDA-MB-231 cells with GA results in the induction of apoptosis.
A. Ctrl 1 mM 2 mM

[Graph showing flow cytometry plots with annexin V and propidium iodide (PI) staining for each concentration, indicating different cell populations (Annexin-/PI-, Annexin-/PI+, Annexin+/PI-, Annexin+/PI+).]

B. Graph showing cell population (%) distribution across different GA (mM) concentrations, with early and late apoptosis curves.

C. Bar chart illustrating the percentage of cells (% G A (mM)) across different GA (mM) concentrations, with categories for dead, healthy, early apoptosis, and late apoptosis.
Figure 7. Treatment with GA enhances levels of apoptosis in vitro. (A) The effect on the induction of apoptosis was determined by Annexin V/PI double-staining. (B) Represents the increase in the percentage of both early and late apoptosis occurring following GA treatment. (C) Results show the decrease in the percentage of viable/healthy cells following GA treatment. The induction of apoptosis in MDA-MB-231 cells was confirmed by analyzing PARP cleavage (D, E). In addition, the effect on mitochondrial membrane potential is examined (F).

**Induction of cell death by combination therapy in MCF-7 cells.**

MCF-7 cells were treated in the presence of doxorubicin (0.25, 0.5, 1.0 or 2.0 µM) alone and in combination with 1 mM GA for 24 and 48 h. Cell viability
was determined using trypan blue dye exclusion. Results show significant
decrease in cell viability when treated with 0.5, 1.0 and 2.0 µM DOX in addition
to significant decreases in each treatment group following both 24 and 48 h
combination therapy compared to control (Figure 8A-B). Results show significant
decreases in viability with the addition of 1 mM GA to 0.5 µM DOX (Figure 8A).
Following 48 h GA treatment, significant decreases were observed in all DOX
treated groups with the addition of 1 mM GA (Figure 8B). MTT assay was also
performed which showed similar results as shown below.

Figure 8A-B. Treatment with glycyrrhizic acid in combination with doxorubicin
leads to a significant decrease on cell viability in MCF-7 BC cells in vitro. MCF-7
cells were cultured for 24 and 48 h in the presence of DOX (0, 0.25, 0.5, 1.0 or 2.0
µM) and 1 mM GA. Asterisks denote statistical significance (*p<0.05, **p<0.01,
compared with the untreated control, n=3).

*Induction of cell death by combination therapy in MDA-MB-231 cells.*
The MDA-MB-231 BC cells were treated with DOX (0.25, 0.5, 1.0 or 2.0 µM) and 1 mM GA for 24 and 48 h. After 24 h treatment, cell viability results show significant decrease within each treatment group compared to control (Figure 9A-B). Results also demonstrate significant decreases in cell viability with the addition of 1 mM GA to 0.25 and 2 µM DOX following 24 h treatment (Figure 9A). In contrast, each group showed a significant decrease in cell viability with the exception of the treatment groups with 0.25 and 0.5 µM DOX alone when compared to control, following 48 h treatment (Figure 9B). Following 48 h treatment, each DOX treated group with the addition of 1 mM GA showed significant decreases in viability (Figure 9B). MTT assays were also performed demonstrating very similar results as shown below.

Figure 9A-B. Treatment with glycyrrhizinic acid in combination with doxorubicin reduces cellular proliferation in MDA-MB-231 BC cells in vitro. MDA-MB-231 cells were cultured for 24 and 48 h in the presence of DOX (0, 0.25, 0.5, 1.0 or 2.0 µM)
Exposure of MDA-MB-231 cells to GA and DOX leads to increased levels of ROS.

To examine ROS generation with combination therapy, MDA-MB-231 cells were treated with DOX (0.25, 0.5, 1.0 or 2.0 µM) alone and in combination with 1 mM GA. The MDA-MB-231 cell line was then examined for changes in intracellular ROS by flow cytometric analysis. Results show slight increases in intracellular ROS generation with the addition of 1 mM GA (Figure 10).

Figure 10. Exposure of MDA-MB-231 cells to combination therapy leads to slight increases of intracellular ROS. The effect of co-treatment on intracellular ROS levels was determined by treating DCFDA-labeled MDA-MB-231 cells with various concentrations of DOX (0.25, 0.5, 1.0 OR 2.0 µM) and 1 mM GA for 48 h with determination of ROS levels by flow cytometric analysis.

Co-treatment of MDA-MB-231 with GA and DOX enhances levels of apoptosis in vitro.
To analyze the initiation of apoptosis and cell death, we examined mitochondrial integrity or the loss of mitochondrial membrane potential via the Mitochondrial Membrane Potential Assay. Cells, treated with Ga and/or DOX, were stained with a lipophilic dye 3,3’-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>) which accumulates in the mitochondria allowing us to monitor mitochondrial membrane potential using flow cytometry. Results show significant increases in the percentage of cells losing mitochondrial membrane potential (Figure 11).

Figure 11. Treatment with GA and DOX enhances levels of apoptosis <i>in vitro</i>. The effect on mitochondrial membrane potential is examined.
CHAPTER 4

DISCUSSION

In the current study, we examined the effects of GA on breast cancer cell growth utilizing two different breast cancer cell lines, MCF-7 and MDA-MB-231. Treatment with GA led to decreases in cell proliferation and viability in addition to the induction of apoptosis in human BC in vitro. Our results also show that exposure to GA leads to increased ROS generation. Furthermore, we demonstrated that GA may be effective when used as a co-treatment with DOX for treating BC.

Despite scientific advances, emerging resistance to therapy and relapse pose a major threat in breast cancer treatment. Natural products of herbal origin have received increasing attention in recent years and have filled the void for the discovery of novel cancer therapeutic agents (Sharma et al. 2012) while exhibiting minimal side effects. Glycyrrhizic acid (GA), the major active ingredient extracted from the licorice root shows great potential for anti-cancer treatment. According to Xitong et al., besides the well-known anti-inflammatory and hepatoprotective function of GA, it has been reported to possess inhibiting effects on diverse cancers such as leukemia, malignant glioma, colon cancer and lung cancer. The occurrence and development of cancers have been shown to be
inhibited by GA through inducing targeted apoptotic pathways in cancer cells (Chuech et al. 2012). Cell metastasis in cancer tissues were also controlled (Khan et al., 2013). There are also multiple derivatives of GA, such as, glycyrrhetic acid which is active against human hepatoma, promyelotic leukemia, and stomach cancer cell lines in vitro (Hibasami et al. 2006) and glycyrrhetinic acid which was found to reduce the proliferation rate of prostate cancer cells (Hawthorne and Gallagher 2008).

There are a limited number of reports of GA being effective against breast cancer cell growth with no reports on the effectiveness of co-treatment for breast cancer using DOX in addition to GA. Previous evidence suggested that the derivative, glycyrrhetinic acid (GRA), induced apoptosis in MCF-7 cells. That study observed that GRA has considerable antiproliferative effects on the MCF-7 cell line and initiated steps involved in apoptosis, including caspase activation, loss of mitochondrial membrane potential, upregulation of Bax/Bcl2 ratio, and the induction of pro-apoptotic protein Bim (Sharma et al. 2012). A previous study also demonstrated that glycyrrhizic acid (GA) is cytotoxic to human breast cancer cell line MDA-MB-231, which appears to involve promotion of apoptosis, autophagy and ROS generation via mitochondria (Lin et al. 2017). Our findings that GA effectively reduces BC cell proliferation in vitro provides additional support for earlier findings in the possible use of GA in the treatment of BC in addition to chemotherapeutic, DOX.
PARP belongs to a family of nuclear enzymes which helps repair DNA when it becomes damaged through UV exposure, radiation or other environmental substances. PARP activation has been shown to result in specific programmed cell death pathways involving NAD+/ATP depletion, loss of mitochondrial membrane potential ($\Delta \Psi_m$), and the release of apoptosis inducing factor (Morales et al. 2014). GA-induced cancer cell apoptosis has been well-documented to occur via a mitochondrial pathway (Lin et al. 2017). In our present study, we observed decreased levels of mitochondrial membrane potential in the MCF-7 (Figure 3E) and MDA-MB-231 (Figure 7F) cell lines. Additionally, GA-induced ROS generation, and co-treatment with GA and DOX decreases proliferation and cell viability within the MCF-7 cell line. Furthermore, co-treatment with GA and DOX were observed to decrease both cell proliferation and viability, increase ROS generation and loss of mitochondrial membrane potential within the MDA-MB-231 cell line.

While the mechanism of GA activity against tumors remains under investigation, previous work showed exposure to GA led to the induction of apoptosis in a number of cancer cell types. A previous study showed that glycyrrhizin did not activate capase-3 or -8 in prostate cancer cell lines LNCaP and DU-145 supporting previous studies where glycyrrhizin failed to induce caspase activity in HLE and KATO III cells, yet was able to induce apoptosis in those cells (Thirugnanam et al. 2008). GA has also been shown to increase ROS
levels, reduced the mitochondrial membrane potential and stimulated caspase-3 activity in WEHI-3 leukemia cells in vitro (Chueh et al. 2012).

Evidence in our present study show that GA induces ROS generation in the MCF-7 cell line while also increasing ROS generation within the MDA-MB-231 cell line as co-treatment with chemotherapeutic, DOX. As demonstrated by Chuech et al., we also found that GA causes significant increases in DNA fragmentation and mitochondrial membrane potential (ΔΨm), permeabilization.

Single agent therapy tends to have lower response rates and provides less durable disease control than do combinatorial regimens. Combination therapy is the most appropriate for most patients in the case of breast cancer to circumvent emerging drug resistance and to induce less toxicity. The most common features of combination therapy include a) drugs achieve significant responses in mono-therapy b) drugs are administered at the highest tolerable doses c) drugs have unique mechanisms of action to obtain synergistic responses and d) alternate mechanisms of cellular resistance development (Hostetler, Uchakina and McKallip, 2017). GA is an active constituent of the licorice root that has been shown to inhibit the migration and invasion of several types of cancer cells with high efficiency and low toxicity. Furthermore, DOX is a well-known chemotherapeutic for improving prognosis and survival of breast cancer patients with known side effects. The current study shows co-treatment using both GA and DOX produced a synergistic response likely attributed to different
mechanisms of activity. Co-treatment could produce a more durable response with less potential for drug resistance; however, further investigation is warranted.

The overall purpose of combination therapy is to produce a durable synergistic response that reduces the development of resistance and does not increase cytotoxicity on normal cells (Hostetler, Uchakina and McKallip, 2017). The present study begins examining GA as a candidate for breast cancer treatment to combine with first-line chemotherapeutic, doxorubicin, in order to decrease side effects and combat resistance to current therapy. Future studies of GA and its effects on breast cancer cells are warranted, in addition to further investigations on the effectiveness of combination therapy.
CHAPTER 5

CONCLUSIONS

In conclusion, the present study demonstrates that GA induced apoptotic cell death and inhibited BC cell growth, increases the loss of mitochondrial membrane potential ($\Delta \Psi_m$) and induces PARP-dependent cell death *in vitro* in both cell lines, MCF-7 and MDA-MB-231. We also began to focus on co-treatment with GA and DOX, which may provide a synergistic response in BC treatment. Our findings suggest that GA and DOX warrant further investigations as a promising therapeutic drug for the treatment of human breast cancer.
CHAPTER 6

FUTURE DIRECTIONS

Although this study shines a light onto the effects of GA on the BC cell lines, MCF-7 and MDA-MB-231, it is necessary to explore the mechanisms and potential future directions.

Our BC cells have unique characteristics of producing their own hyaluronic acid (HA) and literature suggests that alterations in HA via the enzyme hyaluronidase (HYAL) contribute to BC cell growth and progression. With preliminary data characterizing GA as a hyaluronidase inhibitor, it is vital to take the data we have gathered and focus on HA metabolism and GA-induced apoptosis which we can do by illustrating the role and mechanism of HA on each cell line. We hypothesize that modulation of HA metabolism (production and cleavage) by BC provides a protective environment preventing cells from therapy induced cell death/apoptosis.

With limited data on the usefulness of co-treatment with GA and DOX, it is important to explore combination therapy as our goal is to produce a durable synergistic response that reduces the development of resistance without increasing cytotoxicity on normal cells.
Evidence in our study shows an increase in ROS generation. To explore mechanisms, it is vital to investigate the usefulness of ROS inhibitors, namely, NAC to examine if it would lead to significant reduction in GA-induced cell death suggesting that oxidative stress may play a role.

It is also important to investigate treatment in additional cell lines to verify its effectiveness across all BC types. Lastly, we should transition studies to focus on 3-D BC cell models, including both spheroids and organoids.
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