THE INFLUENCE OF HYALURONIC ACID METABOLISM ON THE DEVELOPMENT OF CHEMORESISTANCE IN 3D BREAST CANCER CELL MODELS

By

VIOLET IYAHEN

A Thesis Submitted to the Faculty

of Mercer University School of Medicine

Department of Biomedical Sciences

in Partial Fulfillment of the

Requirements for the Degree

MASTER OF SCIENCE IN BIOMEDICAL SCIENCES

Macon, GA

June 2021

THE INFLUENCE OF HYALURONIC ACID METABOLISM ON THE DEVELOPMENT OF CHEMORESISTANCE IN 3D BREAST CANCER CELL MODELS

By

VIOLET IYAHEN

APPROVALS:

_____

Robert J. McKallip, Ph.D. Date
Advisor/Principal Investigator

_____

Christy C. Bridges, Ph.D. Date
Program Director

_____

Gretchen Bentz, Ph.D. Date
Committee Member

_____

Pamela Cook, Ph.D. Date
Committee Member

_____

Jean R. Sumner, M.D. Date
Dean, School of Medicine

ACKNOWLEDGEMENTS

I am very grateful to my advisor and principal investigator, Dr. R. McKallip, for accepting me as his student and guiding me through this project. And thank you to my committee members, Dr. G. Bentz and Dr. P. Cook, for their support throughout my research. Thank you to Dr. Olga Uchakina for helping me expand my knowledge and skills in the laboratory. Lastly, thank you to my fellow graduate classmate and lab mate, John D. Christianson.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

ABSTRACT

By: VIOLET IYAHEN

THE INFLUENCE OF HYALURONIC ACID METABOLISM ON THE DEVELOPMENT OF CHEMORESISTANCE IN 3D BREAST CANCER CELL MODELS

Under the Direction of DR. ROBERT MCKALLIP, Ph.D

Breast cancer is the most diagnosed malignancy in the world. Of the various subtypes of breast cancer cells, the ductal carcinoma is responsible for 70% to 80% of worldwide breast cancer diagnoses. Current treatments for breast cancer include hormonal therapy, surgery, radiation therapy, and chemotherapy. Unfortunately, many breast cancer tumors become resistant to these therapies overtime prompting the need for new targeted therapies. It is becoming increasingly clear that the tumor microenvironment plays a critical role in tumor cell survival and development of resistance. In particular, the CD44 receptor and its ligand hyaluronic acid (HA) have been implicated in a number of processes related to tumor progression and survival. HA is a major component of the extracellular matrix (ECM) in both normal and abnormal tissues. In this current study we focused on examining the role of HA metabolism on the resistance of breast cancers to the commonly used chemotherapeutic agent doxorubicin (dox). Specifically, we examined the influence of HA in the ductal carcinoma cell line, MCF-7 using both a 2D and 3D tumor model. The results from this study supported previous findings that 3D MCF-7 cultured cells are resistant to doxorubicin treatment when compared to 2D models and further established the novel finding that cells grown in 3D models have increased gene expression of hyaluronic acid synthase.

Consequently, 4-methylumbelliferone (4MU) was chosen due to its inhibiting mechanism during HA synthesis. For this reason, 4MU's effect was vital to our hypothesis regarding modifications of HA content to augment the strength of low concentrations of dox on tumor cells. MCF-7 cells were grown and cultured two-dimensionally and three-dimensionally using unique methods and specialized plates. Assays were used to quantify and contrast the HA content in each culture along with the specific enzymes responsible for HA's anabolic and catabolic processes. Then, overall cellular proliferation was measured after administering dox and 4MU separately followed by combination treatments of both. Results revealed an increase in HA synthase enzymes in the 3D cultures; however, overall HA concentration was lower when compared to the 2D cultures. Cellular proliferation was repeatedly measured, and on average, 3D cultures were more resistant to individual treatments of dox and 4MU. Similar results were seen when combination treatments were administered, and cellular proliferation did not decrease in the 3D groups.

CHAPTER 1

INTRODUCTION

Breast Cancer and MCF-7 Cells

Today, breast cancer takes its throne as the most diagnosed cancer in the world and the most diagnosed cancer among the female gender in almost every region of the world. Breast cancer topped the charts with 2.26 million diagnoses alone in 2020 while lung cancer followed closely behind (Ferlay, 2021). In total, breast cancer comprised roughly 25% of all cancer diagnoses in women, with equivalent numbers found in both less developed and more developed countries. Since 2012, the annual number of breast cancer diagnoses has nearly doubled to today's statistic while maintaining a terrifyingly higher mortality rate than the other types of cancers diagnosed amongst women (Akram, 2017). The world's need for effective and efficient treatments will never cease because the demand for a cure will continuously rise paralleling the cancer's incidence and mortality rates. Meticulous studies using tissue models have shown to be the main contributors defining the prognosis of breast cancer thus far. However, as cellular mechanisms continue to evolve and as technological advancements continue to rise, humanity will be in constant need of more accurate, in vivo research models to further polish the prognosis.

It is because of recent studies that we understand breast cancer to be a myriad of molecularly distinct tumors stemming from epithelial cells of breast tissue as opposed to a single ailment (COMŞA, 2015). Epidemiological data reveal that the ductal carcinoma is the most reported tumor type among all breast cancer cases. Notably, the ductal carcinoma comprises roughly 80% of all breast cancer diagnoses worldwide. For this reason, it was important to

choose a cell line like the adenocarcinoma line titled the Michigan Cancer Foundation-7 (MCF-7) cells that could represent much of the targeted patient population. In general, MCF-7 cells are luminal-A molecular subtypes that have estrogen receptors (ER-positive) and progesterone receptors (PR-positive). In ideal conditions, MCF-7 cells have the capacity to migrate and invade; however, they normally do not (COMŞA, 2015). The MCF-7 cell line has helped uncover much of what we know of breast cancer today especially regarding its anti-chemotherapeutic properties. However, the necessity of a more in vivo-like model still persists in order to continue investigating and predicting clinical results with more accuracy.

2D and 3D Cellular Models

Developing relevant and predictive models is crucial for increasing the translational potential of basic research. Furthermore, models that accurately mimic the *in vivo* structures and processes help hone our understanding of general cellular biology including tissue morphology, disease processes, drug delivery and reaction, protein production, and tissue engineering (Kapalczynska, 2016). Especially when investigating drug therapies, it is important to understand that approval of oncology drugs for clinical use has the lowest likelihood at 7% when compared to other new drug trials. This is thought to be due in part to a failure of 2D cell culture models to faithfully mimic the behavior of *in vivo* tumors. Thus, it is hoped that implementation of three-dimensional models, which are seen as more realistic and relevant models to predict *in vivo* drug responses, will lead to increased successes in these expensive and long-term neoplastic studies (Imamura,2014).

Three-dimensional (3D) cell cultures are becoming highly regarded as more appropriate *in vitro* models that more accurately mirror the naturally complex life cycle of various cancerous tumors and their surrounding microenvironments. Culturing cells three-dimensionally provides another platform for studying human diseases in whole, when compared to their original two-dimensional (2D) culture. The 3D cultures emulate the extraordinary properties of cell-cell and cell-environment interactions that are vital for studying cellular niches. 3D cultures are thought to produce more representative quantitative data unveiling many contributing factors in a tumor microenvironment. 3D cultures also enhance the results of studies that look at drug therapy development and screening and strengthen the results of investigations that look at tumor-immune cell interactions (Sasmita, 2018). Naturally, MCF-7 cells are adherent cells. Normally, uncompromised cells attach to the surface of their container, a tissue culture treated polystyrene plate, and proliferate out as a monolayer. But the adherent property of the cells directly influences their behavior in culture, as well as their responses to drug therapy. In comparison, the 3D cultures are freely floating, multicellular aggregates with secure cell-to-cell bonds and the absence of a lumen. Due to their physical shape and structure, cellular aggregates are thought to more accurately predict tumor behavior as well as resistance.

2D cultures have been instrumental in the numerous research experiments and findings that have contributed to much of the information that is referenced today. This model has served as a more cost-efficient alternative due to its relatively low maintenance that is followed by testing with functional assays (Kapalczynska, 2016). However, the disadvantages of monolayer cultures heavily outweigh its few advantages. A few factors of monolayer cultures have rendered them as less sufficient models, and overtime, these limitations have proven to be problematic for

the generation of results that are relevant to the behavior of *in vivo* tumors. For example, adherent cells easily engage in cell-cell linkage but lack the unique cell-extracellular environment interactions that are found in the body. Monolayer cells also lose their polarity, which further alters their response to various stimuli. And lastly, in addition to the loss of their original morphology, 2D cultures have easy and direct access to the unlimited nutrients in their media (Kapalczynska, 2016). Many of these limitations act as confounding factors that alter the behavior of 2D cells, ultimately overestimating or underestimating the responses compared to what are observed from *in vivo* tumors.

In contrast, 3D cultures utilize an additional dimension to further characterize cell interactions that are not present in monolayer models. These 3D cultures can preserve the cells' original morphology, polarity, phenotype, and proliferating behavior. They can also better simulate the variable access to oxygen and nutrients that are observed in original tumor conditions. Consequently, 3D cultures have been used to study the connection between their structures and the delay in drug delivery and efficacy, radiation treatment outcomes, their lumen presence and absence, the increased concentration of specific components in the tumor habitat, anoikis results, and the effects of hypoxic surroundings (Kapalczynska, 2016). Moreover, these findings also reveal information that further elucidates the initiation and progression of malignant tumors along with the invasion and metastatic processes.

There are three types of 3D cultures, 1) suspension culture on nonadherent plates, 2) cultures in concentrated mediums or gel-like substances, and 3) cultures on scaffolds. In each type, round aggregates of cells or 'spheroids' are formed as the cells come together and attach through their cell-cell linkages. Proliferating and metabolically active cells are seen on the

peripheral, while the presence of a core depends on the origin of cells. Lumens can form through targeted apoptosis, like with pancreatic cells, or the spheroids can contain a center filled with cells. The combination of a necrotic core with proliferating peripheral cells is more commonly seen when culturing spheroids from MCF-7 cells (Zhang, 2016). Each type of culture possesses its own disadvantages; however, the advantages and potential use are believed to outweigh its drawbacks as a newer culture model. Particularly, the suspension culture on nonadherent plates successfully aids research by being the simplest and most cost-effective of the current three. Overall, the different forms of 3D cultures allow for selection of a model that best suits a study. Currently, 2D models are still the most used model; however, with increasing popularity in the scientific community, 3D models may have the potential to help reveal much that is yet to be discovered about cancer.

Breast Cancer Treatments and Doxorubicin

Currently, the best outcome and survival rate of any cancer results from early detection and effective ensuing treatment. It is the identification of the type of cancer, the tumor size, and the spread that reveals the characteristic stage of that cancer, and subsequently, the best course of treatment to sustain life. There are various strategies of management such as hormonal therapy, radiation therapy, chemotherapy, targeted therapy, and surgery (Akram 2017). Combination therapies including chemotherapy, sometimes in conjunction with radiation therapy, are a popular route that can produce therapeutic results for many cancer patients.

Chemotherapies are almost always prescribed for breast cancer patients. Some oncological drugs are effective because of their many modes of action, for example, the well-

known, potent chemical doxorubicin with its three different mechanisms of action. Doxorubicin (dox) is an anthracycline antibiotic derived from the Streptomyces *peucetius* bacterium (Meredith, 2016). It is a robust chemical that causes growth arrest or cellular death by apoptosis, senescence, autophagy, and necrosis. Its general methods of action include intercalating DNA, inhibiting topoisomerase II, and creating reactive oxygen species resulting in free-radical damage. Due to its potency, it is regularly included in chemotherapeutic regimens for breast cancer patients. However, dox is nonselective to cancer cells, which results in its cytotoxic effects on all cells, normal and abnormal. It is noted that the heart, liver, brain, and kidneys are the organs most affected by doxorubicin. Moreover, the toxic side effects from this drug can take place immediately or take many years to present (Meredith, 2016). Examples of unpleasant adverse effects of dox include but are not limited to serious heart arrhythmias, shortness of breath, edema, nausea and vomiting, hair loss, oral sores, and skin pigment change. Though the drug is effective, these unpleasant aftereffects are highly motivating factors to continue studying dox for ways to make it more manageable and combining it with other therapies in order to keep its dosage low.

Aside from their adverse effects, another key problem regarding chemotherapeutic drugs is cancer cell resistance. Existing drugs are seeing a slow, but gradual increase in the development of cellular resistance over time. Resistance can be due to acquired mechanisms after drug exposure, for example, an increase in efflux pumps reducing intracellular drug concentrations. Or resistance can stem from natural factors before drug exposure, for example, resistance due to the tumor's 3D structure and ECM interactions (Lovitt, 2018). Despite the widespread popularity of dox due to its potent effects, there has been growing interest in

combining dox with other agents to keep its concentrations low and avoid the cellular resistance seen in single treatment regimens. Combination therapies allow for the use of two or more drugs at lower doses but with equal efficacy and decreased toxicity compared with the use of either drug alone at higher doses. However, successfully studying the activity and resistance of any drug candidate will require representative models that accurately depict the elements of tumors and their unique interactions in a three-dimensional arrangement (Lovitt, 2018). Only with appropriate models will we be able to thoroughly study and predict the anticancer properties of these agents.

The Extracellular Matrix and Hyaluronic Acid as a Potential Target

The extracellular matrix (ECM), composed of multidomain macromolecules, serves many roles that simultaneously influence various biochemical reactions. Its diverse roles include serving as a physical scaffold for tissue formation and acting as a signal transducer prompting the cells when to grow, migrate, and differentiate. Regardless of the type of cell, and whether it is normal or abnormal, the extracellular matrix continues to serve as the extremely vital regulator of cellular behavior and thus, homeostasis. The ECM in conjunction with infiltrating immune cells, increased vasculature, connective tissue, and other stromal cells makes up the tumor microenvironment. And overtime, the ECM has become the topic of interest in diverse neoplastic studies. Malignant cells are theorized to have dynamic and complex ECMs disseminating signals that result in changes characterized to be the hallmarks of cancer development. The increased signals prompt for malignant survival and proliferation by cueing for excess growth hormone release, evasion of growth suppression, death resistance, increased angiogenesis, and immune

system evasion all benefiting the tumor microenvironment (Pickup, 2014). As a regulator of homeostasis, especially for active tumor development, the ECM additionally contains macromolecules in excess including a crucial sugar molecule known as hyaluronan that could serve as new, potential chemotherapeutic targets.

Hyaluronan, popularly known as hyaluronic acid (HA), is a vital component of the ECM that was discovered in 1934 from bovine vitreous humor. It is a large molecular weight glycosaminoglycan and the only non-sulfated glycosaminoglycan found in nature. HA is synthesized on the inner plasma membrane by three different hyaluronic acid synthase enzymes named HAS1, HAS2, and HAS3. It is created when an HAS polymerizes disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid by ß-(1-3)-glucuronidic bonds (Price, 2018). HAS2 is specifically known for synthesizing larger molecular weight polymers followed by HAS1, and then HAS3 is known for synthesizing the smaller polymers. Regardless of size, HA continues to have significant effects in any microenvironment for example, the very low molecular mass oligomers of HA have been shown to still interfere with growth inhibition and stimulate angiogenesis, thus continuing to support abnormal cellular proliferation. In addition to the three HA synthases, HA is broken down by a family of three different catalytic enzymes: the hyaluronidases HYAL1, HYAL2, and HYAL3. These endogenous enzymes fragment the HA macromolecule by hydrolyzing the ß-(1-4)-hexosaminidic bond. It is known that HYAL2 yields the high molecular weight HA polymers, while HYAL1 further yields the comparatively smaller oligosaccharide polymers (Price, 2018).

In normal tissues, HA helps with important processes such as embryogenesis, tissue regeneration, and wound healing by augmenting cellular proliferation, migration, and adhesion.

Similarly, HA has been hypothesized to aid in the initiation, progression, metastasis, and therapy resistance of abnormal cells, known in this paper as cancer cells. HA, residing in the extracellular matrix, binds to various cell surface receptors including a distinct surface receptor termed cluster of differentiation 44 (CD44). CD44 receptors are another topic of interest in other neoplastic studies because they are consistently expressed by breast carcinoma cells and are characterized as a cancer stem cell marker. They are also necessary in HA's signaling cascades. HA is continuously produced and exported by the abnormal cells in a positive feedback loop mechanism. Binding of the receptor by HA allows the carcinoma cells to firmly adhere to the ECM while HA initiates complex signaling cascades intracellularly to affect cytokine and chemokine release and expression ultimately producing and exporting more HA to continue the process.

In vivo studies in the past have observed increased HA concentration of various molecular weights during periods of cellular and organismal stressed conditions. In fact, it has been proposed that HA levels within a tumor microenvironment could possibly serve as an indicator for poor prognosis. These studies have connected the overexpression of HA synthases and hyaluronidases by the cells rooted in the tumor microenvironment to the heightened continuation of tumor growth and epithelial-to-mesenchymal cell transition, both of which directly increase malignancy mass. The increased malignancy proportionally increases the relapse and mortality rate of breast cancer. It has also been theorized that inhibiting HA synthases will result in the reduction of tumor growth directly decreasing malignancy. More specifically, studies have noted that preventing the HA-CD44 complex, by any means, discourages early tumor formation for carcinoma cells (Witschen et al, 2020).

4-Methylumbelliferone (4MU)

Hyaluronic acid has been identified as a potential contributor to numerous pro-tumorigenic effects stemming from abnormal cells. Additionally, an HA dense environment has been linked to many cellular benefits including maintaining homeostasis, serving as structural foundation, and increasing proliferation through continual release of growth factors and cytokines. Other postulations bring attention to the positive connection between tumor aggressiveness and drug resistance in correlation with stromal HA concentration (Nagy, 2015). These and additional theories help isolate HA as a plausible candidate for targeted therapy against cancer cells.

4-methylumbelliferone (4MU) is umbelliferone, a derivative of coumarin with a hydroxyl group attached to its seventh carbon with an additional methyl group added onto the fourth carbon. 4MU is thought to have two known methods of action. Most commonly, 4MU is known for its inhibiting effects on HA synthesis. 4MU also directly interferes with HAS' substrates. Nonetheless, its methods are still being investigated in experimental studies. However, some trials have described 4MU's effects as contrasting HA's fundamental benefits. For instance, 4MU has been linked to tumor cell growth arrest and apoptosis. It has also been found to suppress angiogenesis which is vital for tumor cells to metastasize and invade (Nagy, 2015). Ultimately, 4MU is still being studied for its potential value as a therapeutic agent. Specifically, further studies with newer models could reveal its newfound benefit as an adjunctive agent in breast cancer therapy.

Hypothesis

In this study, an effective protocol was established to successfully culture 3D aggregates. Multiple MCF-7 spheroids were grown to manipulate their extracellular environment, and different trials were carried out to discover if changes to HA content in the microenvironment would affect the growth of the cells and their chemoresistance.

The overarching hypothesis is that external modifications of hyaluronic acid, by inhibiting production, will negatively impact the growth of MCF-7 cells in the developed 3D cultures while simultaneously augmenting the effects of the chemotherapeutic agent doxorubicin.

CHAPTER 2

METHODS

Cell Culture

The human adenocarcinoma Michigan Cancer Foundation 7 cells (MCF-7) (Catalog Number: HTB-22) were used for the entire duration of the experiment. More specifically, the hormone-dependent, ER-positive ad PR-positive MCF-7 cell line that is classified into the luminal A subtype (COMŞA, 2015). They were retrieved from the American Type and Culture Collection (ATCC). The cells are continuously cultured in Corning's Dulbecco's modified Eagle's medium (DMEM) mixed with 10% fetal bovine serum (FBS) and 0.2% of MycoZap Antibiotics (Lonza). The medium was additionally supplemented with 4.5 g/L glucose, L-glutamine and sodium pyruvate. Each day, the cells were incubated in a 5% CO_2 incubator maintained at 37°C. Media was changed out twice weekly.

Developing 2D Cultures

Cells that were frozen in DMEM supplemented with FBS were thawed and allowed to adhere to the bottom surface of a 50 mL canted neck tissue culture flask filled with 5 mL of fresh DMEM. Once adhered, the cells were allowed 48 hours to grow and multiply before being transferred to a larger (75 cm^2) vented cap flask holding 12-13 mL DMEM to continue the process. In order to transfer the cells, the current media was poured out and the cells were rinsed with phosphate-buffered saline (PBS) that did not contain calcium or magnesium. Then 3 mL of Cellgro's 0.25% trypsin + 2.21 Mm EDTA (in HBSS) was added. Cells were incubated with trypsin for 3 to 5 minutes. Afterwards, 7 mL of DMEM was added to stop trypsin, totaling 10

mL in flask. 7 mL of cells were centrifuged 200xg for 5 minutes then counted using Trypan Blue and a hemocytometer. 3 mL of cells were left in the original flask with additional fresh media and incubated. Calculations were completed in order to initially seed 2500 cells/50 µL of DMEM in a 96-well, flat-bottom plate (Microtest^{TM}). The cell count was later increased to 6000 cells/50 µL of DMEM in each well or $1.2x10^5$ cells total per 1 mL of DMEM. Cells were then incubated for 48 hours to adhere and further grow.

Developing 3D Spheroids by the Hanging-Drop Method

Methylcellulose (MC) Stock Preparation

The MC stock was prepared. Added 3 mL of 3% MC (R&D Systems) to 6 mL of DMEM to have a 1% stock solution. Then made a 0.24% MC stock in 2 mL by adding 480 µL of 1% and 1,520 µL of DMEM.

Hanging-Drop Protocol (Adapted from Zhang, 2016)

Cells were rinsed with PBS and trypsinized. They were then centrifuged and immersed in 3 to 5 mL DMEM for counting. 1 mL, approximately $3x10^5$ cells, was taken and centrifuged again. Supernatant was poured out. 600 µL of 1% MC stock and 1.9 mL DMEM were added to the cellular pellet totaling 2.5 mL of 0.24% MC + DMEM cellular mix. After complete mixing, 25 µL drops (containing 3,000 cells) were slowly pipetted onto the inside of a 48-well plate lid. 100 to 300 µL of PBS were added to each of the 48 wells to preserve moisture. The lid was carefully turned over and attached to the plate. The filled plate was incubated for 48 hours. Drops remained on the lid due to the viscosity of MC.

Harvesting Spheroids Cultured by the Hanging-Drop Method

The lid was carefully detached from the plate. A pipet and wide-opening tips were used to carefully and slowly collect each droplet. One at a time, they were transferred into a round-bottom, 96-well plate. Each 25 µL drop will have one spheroid aggregate.

Developing 3D Spheroids Using Ultra-Low Attachment Surface Plates (Adapted from Strathearn & Rothenberg, 2012)

The same method for developing 2D cultures was used. After calculations, 6000 cells in 50 µL of DMEM were directly seeded in each well of the round-bottom, 96-well plate (Corning). The filled plate was incubated for 48 hours to allow cells to aggregate.

Treatment Preparations Using Doxorubicin and 4-Methylumbelliferone

Dox

100 µM stock of dox was initially used to create a 16 µM dox solution diluted in DMEM. The 16 µM dox solution was then further diluted four times to create the final concentrations of 0.25 µM, 0.50 µM, 1.0 µM, 2.0 µM, and 4.0 µM in DMEM. 25 µL of each treatment concentration was added to three designated wells. And 25 µL of DMEM was added to single treatment groups totaling 50 µL of treatments dispensed to each well. 100 µL was the final volume in each well after treatments. 50 µL of DMEM alone was added to three wells to serve as the 0 µM control group. Wells that were assigned combination treatments received 25 µL of dox and 25 µL of 4MU to reach the final volume of 100 µL. In 3D plates, treatments were pipetted on the side of the wells in order to not disturb spheroid aggregates.

4MU

400 Mm stock of 4MU was used to create an 8 Mm 4MU solution diluted in dimethyl sulfoxide (DMSO) from Corning. The 8 Mm 4MU solution was then further diluted two times to create the final concentrations of 0.50 Mm, 1.0 Mm, and 2.0 Mm in DMSO. 25 µL of each treatment concentration was added to three designated wells. 25 µL of DMEM was added to single treatment groups totaling 50 µL of treatments dispensed to each well. 100 µL was the final volume in each well after treatments. Wells that were assigned combination treatments received 25 µL of 4MU and 25 µL of dox to reach the final volume of 100 µL. In 3D plates, treatments were pipetted on the side of the wells in order to not disturb spheroid aggregates.

DMSO was used as the vehicle control for 4MU. 2.0 Mm of DMSO was created using the same volume that was taken from the 8 Mm 4MU stock but from DMSO and mixed with DMEM. Initially, the 2.0 Mm DMSO stock was further diluted in DMEM to treat cells with 0.50 Mm, 1.0 Mm, and 2.0 Mm concentrations of DMSO. But later on, the two lowest concentrations were removed. Designated cells were only treated with 2.0 Mm DMSO.

MTT Assay

The protocol for TACS® MTT cell proliferation assay was followed. A control of media only was prepared in three wells totaling 100 µL each. After treatment, plates were incubated. After designated incubation time, 10 µL of MTT reagent was added to every well and incubated for 4 hours. Then 100 µL of MTT detergent was added to every well. The plate was wrapped in foil and placed in a dark, ambient area up to 4 hours or overnight. Afterwards, the absorbance of every well was measured at 570 nm in a plate reader.

RNA Purification and PCR

RNA was isolated from cell samples following the Thermo Scientific kit protocol. Samples were spun in a micro centrifuge, and purity was measured using a nanodrop spectrophotometer. Afterwards, the purified RNA samples were put into a gradient thermal cycler (MJ Mini) to produce cDNA. PCR was carried out using SYBR green and specific human primers for *has* 2, 3, *hyal* 1, 2, 3, and two housekeeping primers (18S & *gapdh*). Samples were plated in duplicates and real time PCR performed (Applied Biosystems).

ELISA

An ELISA assay was carried out using the DuoSet ELISA kit protocol (R&D Systems) for measuring hyaluronan. Supernatant samples were collected from 2D and 3D cultures and individually labelled. All samples were diluted two times using reagent diluent. The filled plate was placed in a microplate reader to be shaken and read at 570 nm.

DNA Isolation and Protein Quantification

Cellular samples were collected from 2D and 3D cultures. For 2D cultures, supernatant was collected from each well first. The wells were then washed with PBS and incubated with 50 µL of trypsin individually for one minute or less. 100 µL of DMEM was thoroughly mixed into each well to stop trypsin. A pipette was used to collect the 150 µL solution from each well and centrifuged inside tubes. The 2D cellular pellet was then accessible. For 3D cultures, supernatant and spheroids were collected and centrifuged in tubes. Afterwards, supernatant was collected,

and the spheroid pellet became accessible. DNA isolation was carried out following the DNeasy Blood and Tissue Kit's purification protocol (Qiagen).

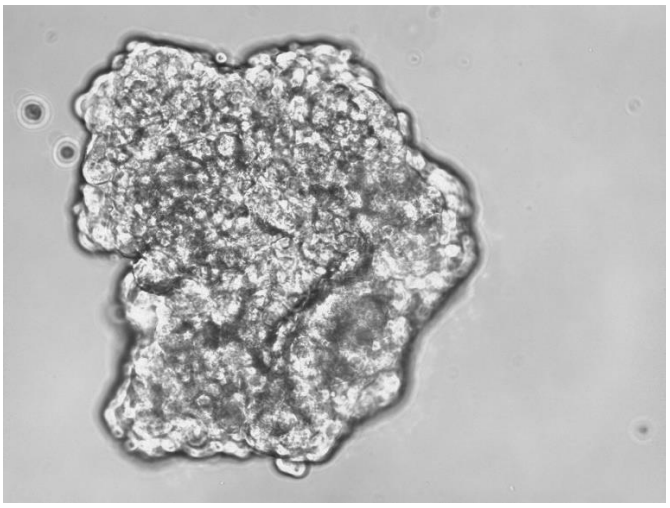
Protein isolation was carried out using cellular samples resuspended in 1 mL of RIPA buffer mixed with 10 µL of PIC 1 and 10 µL of PIC 2. Samples were incubated in ice for 30 minutes in a dark area. Then, they were centrifuged at high speed for 10 minutes. A coomassie protein assay (Pierce Biotechnology) was completed per protocol in order to quantify protein content in the two cultures.

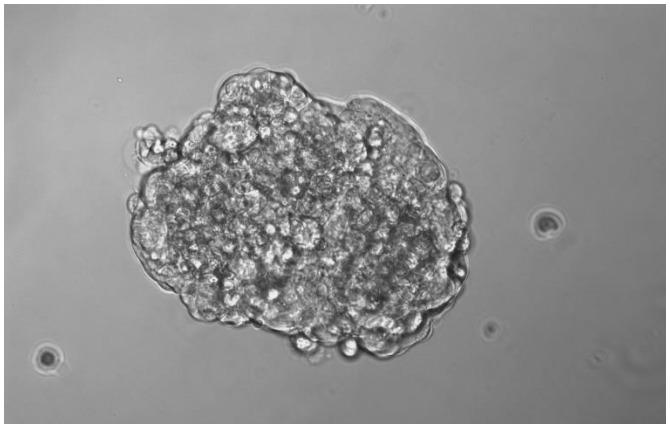
CHAPTER 3

RESULTS

3D Spheroid Cultures

Two separate methods were used to successfully culture viable spheroids from the original 2D MCF-7 cell line. They are known as the hanging drop method and the spheroid protocol adapted to include ultra-low attachment surface plates (Corning). Microscope images were taken in order to further compare the aggregates. The spheroid protocol was determined to be an easier culturing method, which produced larger spheroids compared to the hanging drop method that required an additional agent and direct handling of the aggregates.





Figures 1A and 1B. MCF-7 spheroids cultured using the hanging-drop method. They were seeded at 3000 cells/drop using methylcellulose and cultured as inverted 25 µL drops. They were allowed 48 hours to culture and aggregate before being transferred to another plate for the treatment process. Images were captured using a cell imaging microscope (Floyd Evos). Original image.

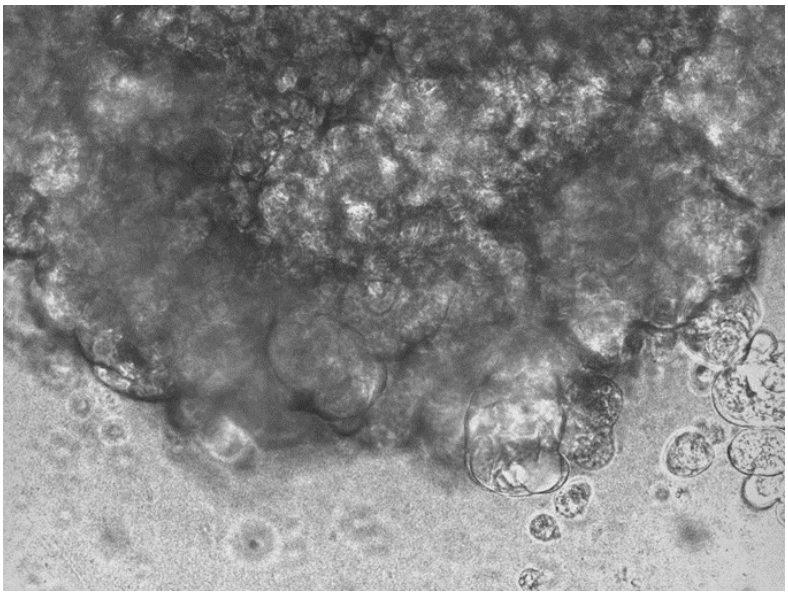
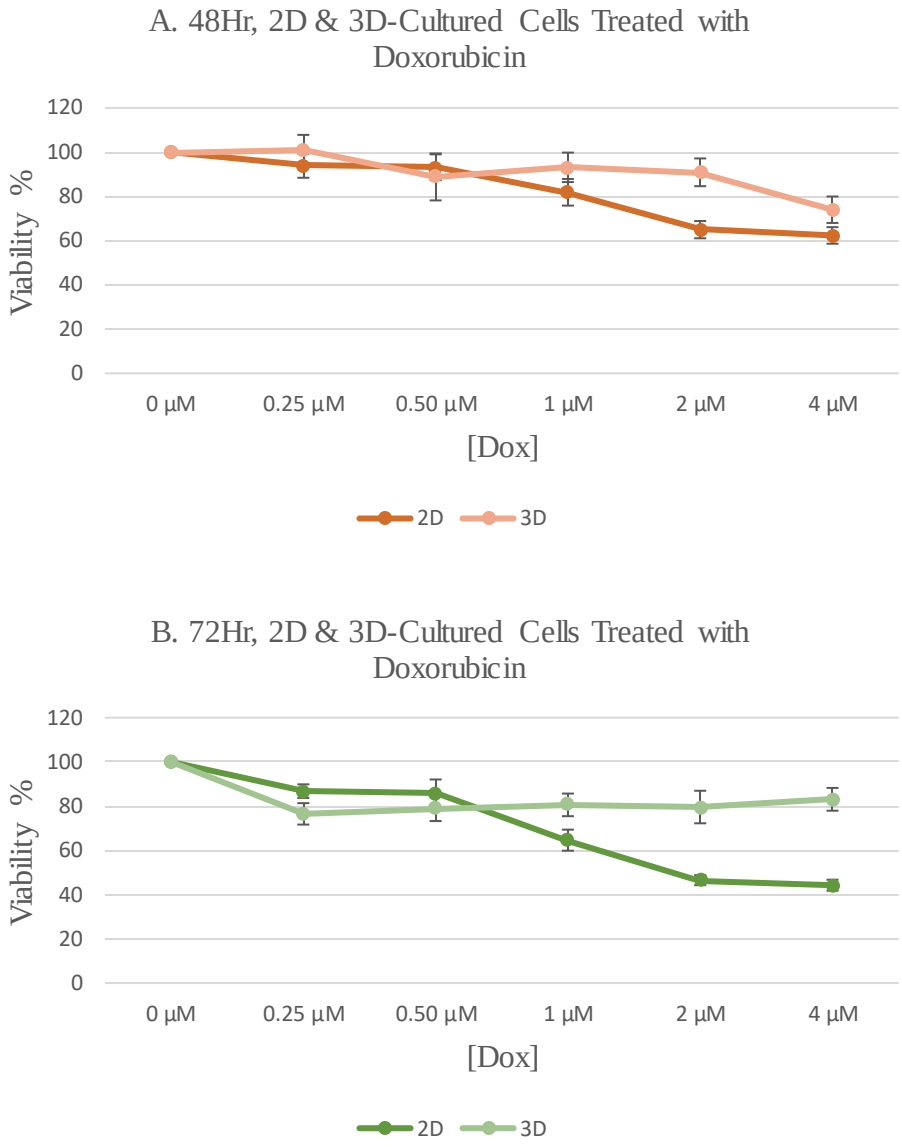


Figure 2. MCF-7 spheroid created using an ultra, low-attachment Corning plate. This spheroid was seeded at 6000 cells/50 µL and allowed 48 hours to aggregate before proceeding to treatment. Seeding number was increased due to the success of fully aggregated spheroids. Image was captured using a cell imaging microscope (Floyd Evos). Original image.

Effect of the Single Agent Doxorubicin on Cell Proliferation/Viability

Fundamentally, it was important to observe the direct effect that doxorubicin had on cellular proliferation in general. 2D and 3D cultures were prepared for 48 hour and 72 hour measurements and read using an MTT proliferation assay. The cells were treated with five increasing concentrations of doxorubicin and compared to a control group treated with DMEM media only. In the 2D cultures, there was a visible decrease in cellular proliferation as the dox concentration increased up to 4µM. Average 2D cellular viability was reduced to as low as 60% after the first 48 hours and 40% after 72 hours. In contrast, average cellular viability for the dox treated 3D groups dropped to as low as 75% after the first 48 hours and 80% after 72 hours.



Figures 3A and 3B. Doxorubicin toxicity. A line graph displaying the toxicity outcome of doxorubicin on cellular viability after 48 & 72 hours. Data presented exhibits the numerical mean of each concentration group and replicated in at least three separate trials where cells were plated and treated in triplicate using equal volumes. The error bars represent the standard error of each mean. Original image. This figure is presented in color.

Hyaluronic Acid Production in 2D vs. 3D MCF-7 Models

HA is speculated to play a role in multiple biochemical reactions in a tumor microenvironment ultimately benefitting cancer cell growth and malignancy. Accordingly, HA synthase gene expression was measured using real time SYBR green PCR to quantify the concentration of synthase enzymes in 2D models compared to 3D models. After running PCR, the 3D spheroids showed an average increased expression for each HA synthase enzyme compared to the 2D models. The 2D models' average synthase quantity was programmed as the baseline for each enzyme. Accordingly, the 3D models have heightened enzyme activity for synthesizing HA.

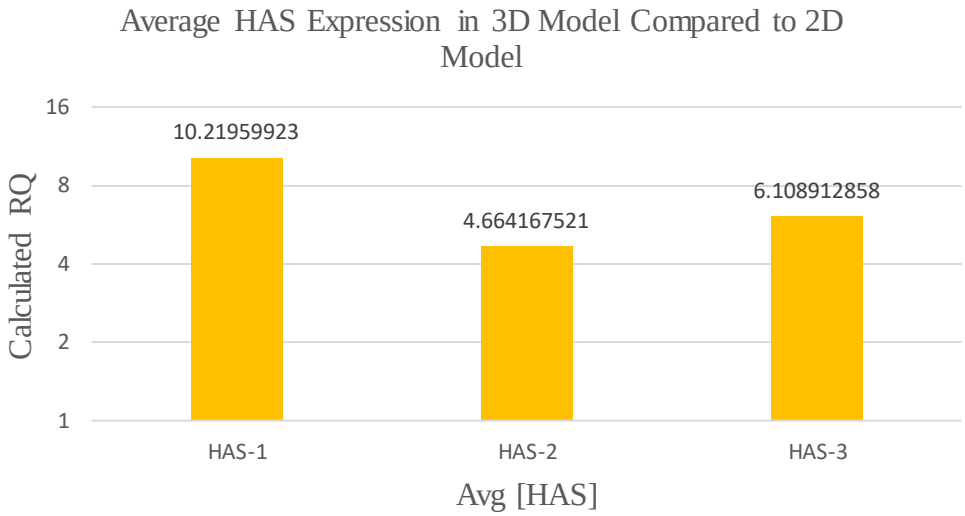


Figure 4. HA Synthase expression in 3D model. This bar chart exhibits results that measure an increase in the average enzyme expression for the individual HA synthase enzymes HAS 1, 2, & 3 in untreated 3D cultures compared to the average expression measured in untreated 2D cultures set as the baseline for each enzyme type. Original image. This figure is presented in color.

Hyaluronic Acid Concentration Quantified in Two Models

4-methylumbelliferone was introduced to this study due to its inhibiting effects during HA synthesis. First, overall environmental HA was measured. Cellular supernatant was collected for an HA-ELISA to measure the starting HA concentration in a 3D environment compared to a 2D environment. Although the 3D spheroids have increased expression of synthase enzymes, the 2D cultures were shown to have an overall higher HA concentration shown by an ELISA. Looking at the 2D model, there was a higher mean HA quantity present after the first 24 hours compared to the 3D. After 48 hours, the 2D model's HA quantity rose significantly from 32 to 52 ng/mL, whereas the3D model's HA quantity stayed precisely at 21 ng/mL after 24 and 48 hours.

MCF7 cells are adherent cells and grow two-dimensionally over a flat surface. Notably, the soluble HA content can effectively be measured in the supernatant as it is released into the extracellular environment. However, spheroids have been discovered to have a core with contents distinct from the outer domain. Therefore, the suspected true 3D HA content is hypothesized to be enclosed in the core in high quantities as opposed to being released out into the environment. The small amount that was detected in the 3D cultures may therefore represent the HA made and released by only the spheroids' peripheral cells. This is one potential explanation for the increased HA detected in the 2D cultures compared to the 3D cultures.

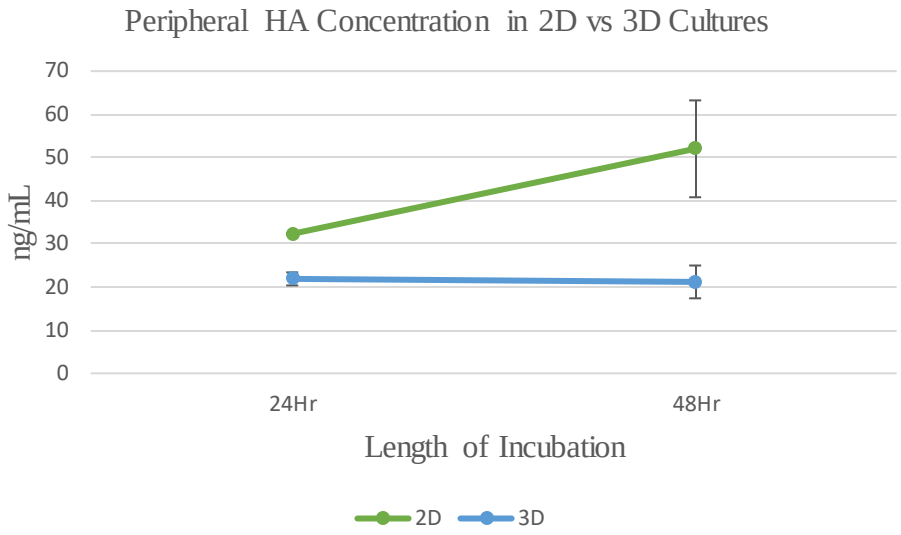


Figure 5. Initial HA concentration in 2D vs 3D models. This line graph exhibits the mean HA concentration, in ng/mL, of two distinct cellular models after the first 24 and 48 hours of incubation with DMEM media only. Cells were plated and treated in triplicate using equal volumes. The error bars represent the standard error of each mean. Original image. This figure is presented in color.

Furthermore, additional supernatant was collected immediately after 3D cultures were treated with 4MU. The treated groups were compared to the supernatant of an untreated group that was cultured in DMEM media only at the same final volume. After the first 24 hours of incubation, 4MU significantly decreased the HA quantity to as low as 30 ng/mL compared to the 52 ng/mL that was measured in the untreated group. This data helps show that 4MU does have an immediate inhibiting effect on the HA quantity that it comes into contact with like the aggregates' environmental HA.

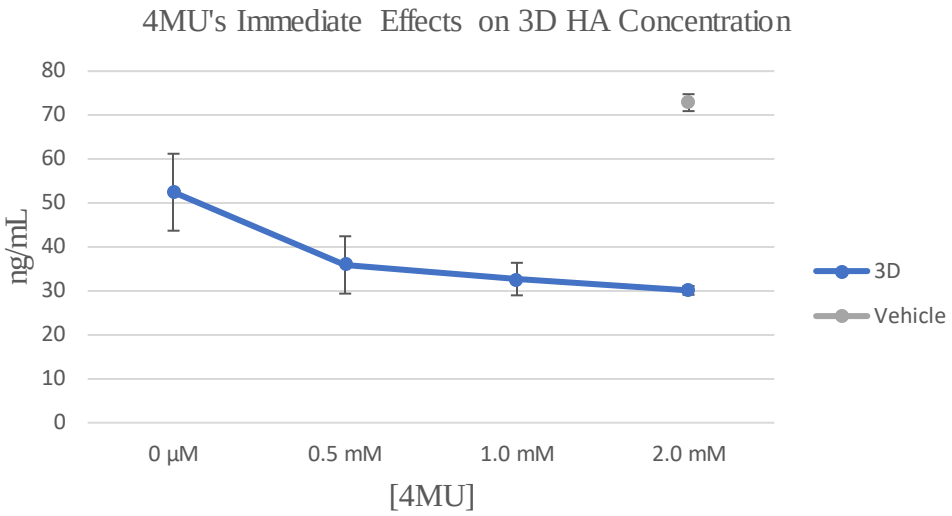
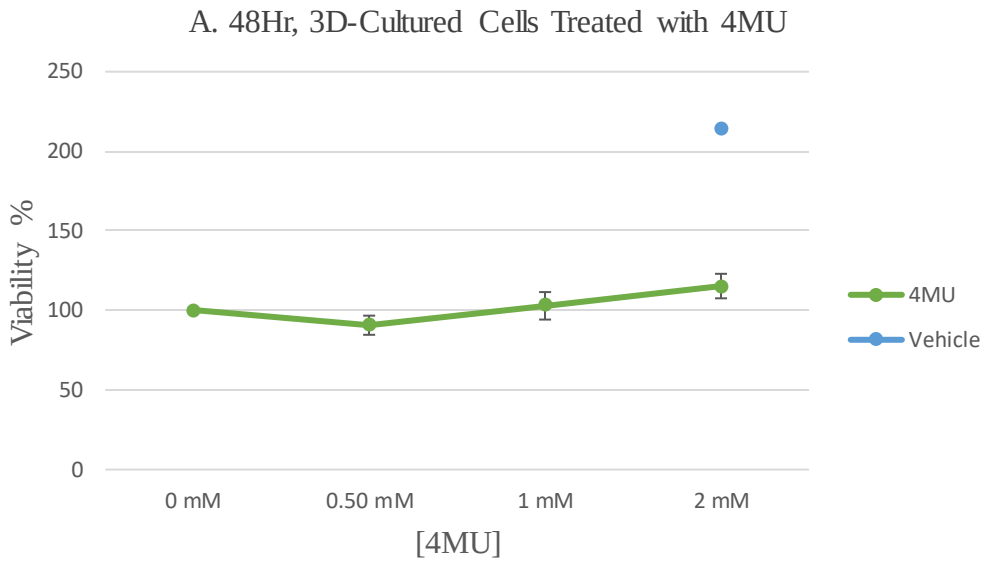


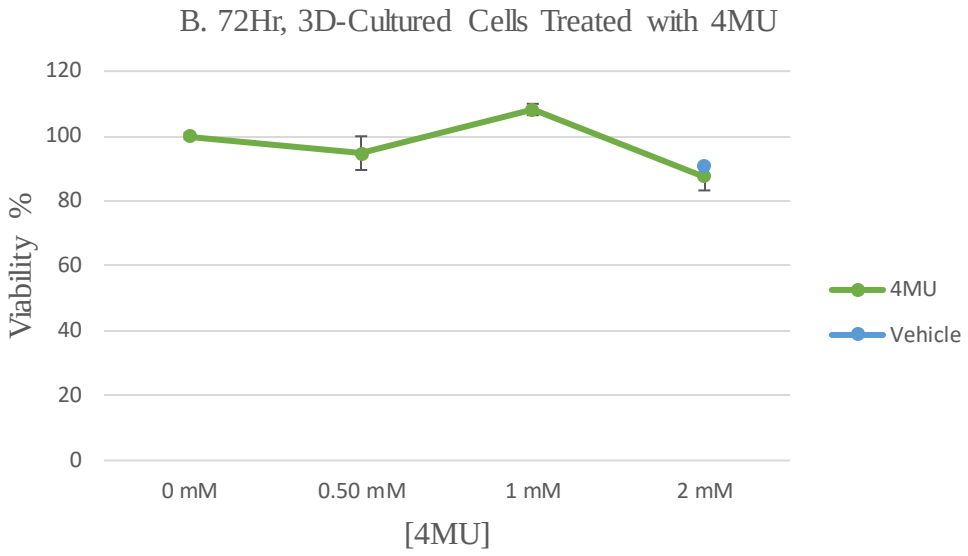
Figure 6. 4MU's initial effect on HA content in the supernatant of 3D spheroids. This line graph displays the immediate inhibiting effects of 4MU on 3D cultures after 24 hours. The data points represent the mean quantity of HA at each increasing concentration of 4MU administered in mM quantities. Cells were plated and treated in triplicate using equal volumes. Additionally, the vehicle represents 2.0 mM DMSO created as a control using the same volume as 2.0 mM 4MU. The error bars represent the standard error of each mean. Original image. This figure is presented in color.

Effect of a Hyaluronic Acid Synthesis Inhibitor on MCF-7 Cell Viability in the 3D Model

Subsequently, cellular viability for each treated group was measured to observe the outcome of HA synthesis inhibition on the actual cells. An MTT proliferation assay was carried out at the 48 hour and 72 hour timepoints on the 3D cultures. The cells were treated with three increasing concentrations of 4MU and compared to a control group treated with DMEM media only. After administering the treatment, the 3D cultures responded differently showing a less prominent trend line. Average cellular viability for the 4MU treated 3D groups only dropped to 87% at both time points. Despite being incubated with 4MU for 2-3 days, the cells of the

spheroids were still thriving. The 13% reduction in viability of the 4MU-treated 3D cultures is theorized to be attributed to the death of the peripheral cells due to their reduced HA synthesis over the 48 and 72 hour time spans. Although 4MU was shown to have reduced the average peripheral HA concentration, it did not significantly impact the mean cellular viability.



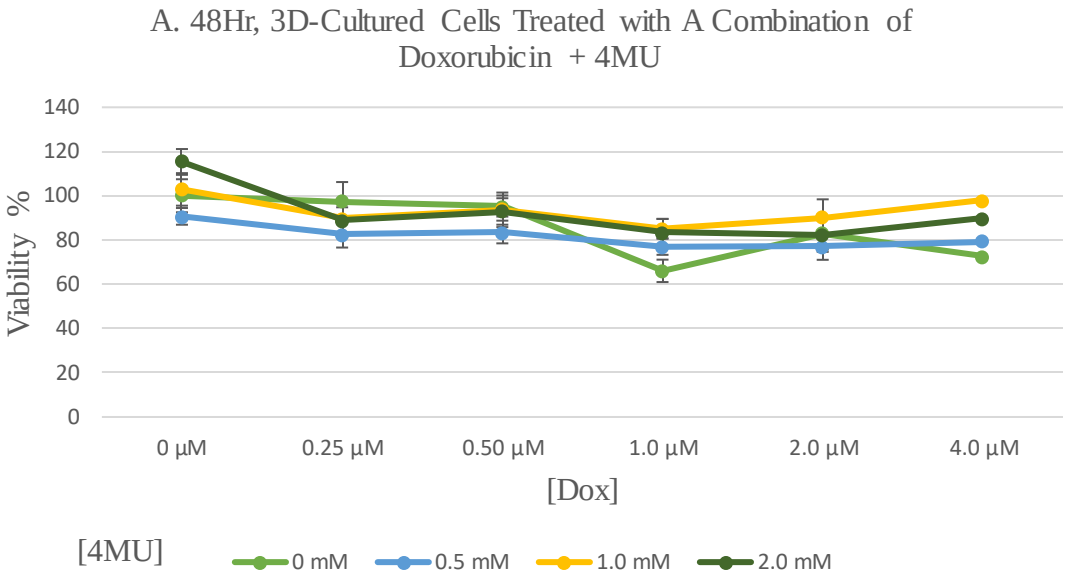


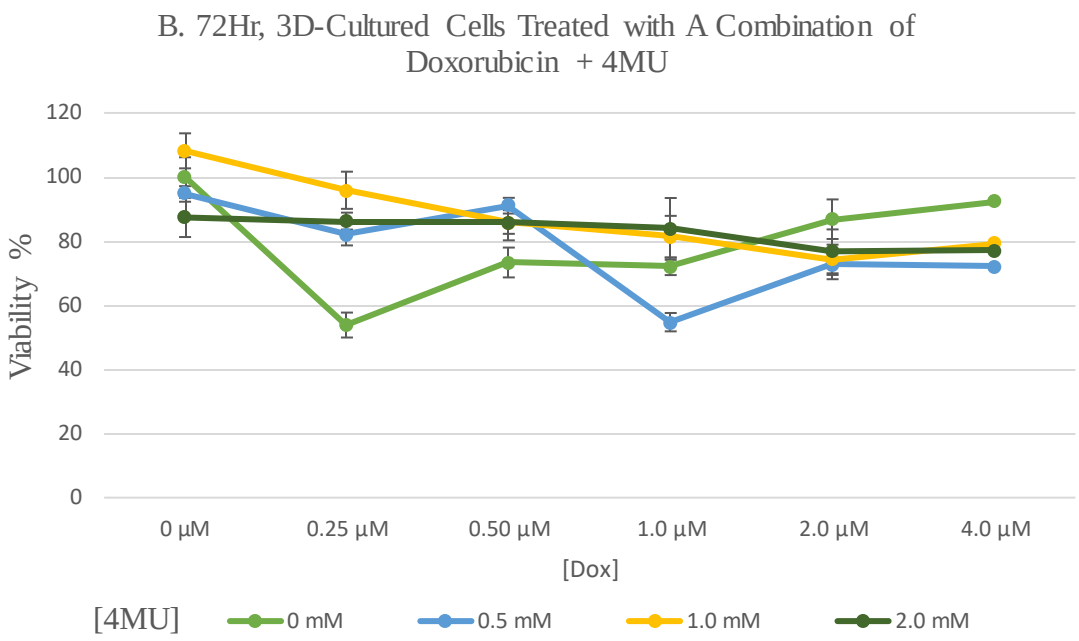
Figures 7A and 7B. Spheroid viability after incubation with 4MU. Line graphs exhibiting cellular viability of 3D cultures after 48 & 72 hours of incubation with increasing mM quantities of 4MU. Data presented displays the numerical mean of each concentration group replicated in at least three separate trials where cells were plated and treated in triplicate using equal volumes. The vehicle represents 2.0 mM DMSO created as a control using the same volume as 2.0 mM 4MU. The error bars represent the standard error of each mean. Original image. This figure is presented in color.

Combination Treatment Outcomes

Lastly, cells cultured three-dimensionally were treated with both dox and 4MU separately and in combination of the two chemicals. Cells treated with one agent were given additional µL of DMEM media to reach the 100 µL final volume in each well. Cells treated with both drugs were given equal volumes of the chemicals while mixing different concentrations per well. Cellular viability was measured using an MTT proliferation assay after 48 and 72 hours of incubation. Data from the 3D combination treatments displayed an indistinct pattern where the

trend line showed no clear direction. Combination therapy with Doxorubicin and 4MU at the concentrations tested did not lead to significant reduction of MCF-7 viability in the 3D model.





Figures 8A and 8B. Combination treatment of 4MU and dox on MCF-7 cells. Line graphs displaying the viability percentages of cells 48 & 72 hours after being treated with doxorubicin in combination with 4MU. Data presented exhibits the numerical mean of each concentration group replicated in at least three separate trials where cells were plated and treated in triplicate using equal volumes. The error bars represent the standard error of each mean. Original image. This figure is presented in color.

CHAPTER 4

DISCUSSION

Throughout the study, experiments were completed to assess the effect of doxorubicin, 4MU, and combination therapy using both doxorubicin and 4MU on breast cancer cells using a 3D culture system, which more realistically models in vivo tumors. Methods were replicated and modified in order to successfully culture viable spheroids as the 3D models. At different time points, data were collected using both the conventional 2D model and the novel 3D model. The results showed that the 3D models were more resistant to the individual and combined chemical agents as evidenced by a minimal reduction of cellular viability compared to the reduction quantified in the 2D models. One plausible hypothesis was that the environmental HA concentration could be hindering drug delivery and distribution to the spheroids. Additionally, the amount of chemotherapeutic agent that does reach the aggregates may only be affecting the peripheral cells as the internal cells are left unaffected. Furthermore, the 3D cultures had heightened synthase enzyme expression; however, the 2D models had a higher detectable HA content. It was theorized that this finding could be due to the spheroidal shape of the 3D models housing its true HA content in its core.

Establishing a new scientific model is necessary as newer drugs entering the market are not showing the anticipated effects on natural tumors that were originally observed on 2D cell lines. Our findings reported here using 3D culture models not only unveil hyaluronic acid as a potential chemotherapeutic target to help make cells more susceptible to current drugs but also revealed new directions exploring the tumor microenvironment that have the potential to yield life changing results in neoplastic studies.

The data presented in this study helped identify challenges and construct new directions that could potentially introduce a novel combination therapy for breast cancer cells involving their HA supply. First, it would be ideal to find a method of measuring the cell count in 3D cultures in order to normalize all data collected. This would allow for increased accuracy and efficiency when comparing data between the models. Second, studying the results of adding high and/or low molecular quantities of HA could allow for observations that describe HA's complete effect related to its availability to abnormal cells. Third, there is interest in looking at methods of growing HA three-dimensionally to possibly alter its role in chemoresistance. Lastly, finding ways to image or dissect the spheroids to study their inner core could reveal knowledge about their true HA content and benefits. Further research into these hypotheses could result in the breakthrough needed to improve and advance personalized medicine for the most diagnosed and most fatal cancer afflicting women all around the world.

REFERENCES

Akram, M., Iqbal, M., Daniyal, M., & Khan, A. U. (2017). Awareness and current knowledge of breast cancer. *Biological Research, 50*(1). https://doi.org/10.1186/s40659-017-0140-9

Comşa, S., Cîmpean, A. M., & Raica, M. (2015). The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *Anticancer Research, 35*(6), 3147-3154. https://ar.iiarjournals.org/content/35/6/3147#sec-3

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2021). Cancer statistics for the year 2020: An overview. *International Journal of Cancer*. https://doi.org/10.1002/ijc.33588

Imamura, Y., Mukohara, T., Shimono, Y., Funakoshi, Y., Chayahara, N., Toyoda, M., Kiyota, N., Takao, S., Kono, S., Nakatsura, T., & Minami, H. (2015). Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncology Reports, 33*(4), 1837-1843. https://doi.org/10.3892/or.2015.3767

Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, L., & Lamperska, K. (2018). 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. *Archives of Medical Science, 14*(4), 910–919. https://dx.doi.org/10.5114%2Faoms.2016.63743

Lovitt, C. J., Shelper, T. B., & Avery, V. M. (2018). Doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. *BMC Cancer, 18*(1). https://doi.org/10.1186/s12885-017-3953-6

Meredith, A., & Dass, C. R. (2016). Increasing role of the cancer chemotherapeutic doxorubicin in cellular metabolism. *Journal of Pharmacy and Pharmacology, 68*(6), 729-741. https://doi.org/10.1111/jphp.12539

Nagy, N., Kuipers, H. F., Frymoyer, A. R., Ishak, H. D., Bollyky, J. B., Wight, T. N., & Bollyky, P. L. (2015). 4-Methylumbelliferone treatment and Hyaluronan inhibition as a therapeutic strategy in inflammation, autoimmunity, and cancer. *Frontiers in Immunology, 6*. https://doi.org/10.3389/fimmu.2015.00123

Pickup, M. W., Mouw, J. K., & Weaver, V. M. (2014). The extracellular matrix modulates the hallmarks of cancer. *EMBO reports*, *15*(12), 1243-1253. https://doi.org/10.15252/embr.201439246

Price, Z., Lokman, N., & Ricciardelli, C. (2018). Differing roles of Hyaluronan molecular weight on cancer cell behavior and chemotherapy resistance. *Cancers*, *10*(12), 482. https://doi.org/10.3390/cancers10120482

Witschen, P. M., Chaffee, T. S., Brady, N. J., Huggins, D. N., Knutson, T. P., LaRue, R. S., Munro, S. A., Tiegs, L., McCarthy, J. B., Nelson, A. C., & Schwertfeger, K. L. (2020). Tumor cell associated Hyaluronan-CD44 signaling promotes pro-tumor inflammation in breast cancer. *Cancers*, *12*(5), 1325. https://doi.org/10.3390/cancers12051325

Zhang, W., Li, C., Baguley, B. C., Zhou, F., Zhou, W., Shaw, J. P., Wang, Z., Wu, Z., & Liu, J. (2016). Optimization of the formation of embedded multicellular spheroids of MCF-7 cells: How to reliably produce a biomimetic 3D model. *Analytical Biochemistry*, *515*, 47-54. https://doi.org/10.1016/j.ab.2016.10.004