AN UNCONVENTIONAL APPROACH
TO DIRECT TARGETING OF BREAST CANCER CELLS

A Thesis submitted in partial fulfillment of the requirements
for the Master of Science degree in Biology

by

Hurig Vahan Katchikian

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The Thesis of Hurig Vahan Katchikian is approved:

______________________________________  ___________________
Lisa R. Banner, Ph.D.  Date

______________________________________  ___________________
Ray L. Hong, Ph.D.  Date

______________________________________  ___________________
Dr. Steven B. Oppenheimer, Chair  Date

California State University, Northridge
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ABSTRACT

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A superior mechanism of detecting cancerous cells and delivering anti-cancer drugs to highly localized targets is currently lacking in the field of oncology. Unfortunately, chemotherapy is not a specialized method of delivering therapeutic reagents to the body, and is therefore toxic to cancerous and noncancerous cells alike. For this reason, it is imperative to seek alternative forms of treatment. Lectins, for example, act as the ideal ‘bait’ because they exhibit a high affinity towards carbohydrate-rich cellular surfaces. Transformed cells have altered surfaces that often express elevated levels of glycoprotein than do healthy cells, and readily bind to lectins. Unlike older lectin assays that are be costly and require fresh, robust cells with no guarantee of actually producing any viable results, our lab has developed a derivatized bead assay that allows for the rapid screening of cell surface markers. This assay involves the use of beads that are derivatized with over a hundred different molecules including various proteins other than only lectins, amino acids, and sugars, that will bind to carbohydrate-rich molecules in seconds to allow for rapid screening of various cell surface markers. Clustering or binding of these lectins to their preferred target cells helps differentiate
between cancerous and noncancerous cell lines. Furthermore, characterization of these cells will help determine specific surface markers that are unique for each type of cell. In this study, we test the efficacy of the lectin-bead assay in distinguishing cancerous (HTB-126) and healthy (HTB-125) breast cells from the same patient. Each cell line was cultured and subjected to the bead assay using beads derivatized with the following lectins: Wheat germ agglutinin (WGA), Lens culinaris agglutinin (LCA), Concanavalin A (ConA), and Poly L-lysine (PL) – an adhesive reagent rather than a lectin. Cell binding affinity to each lectin bead was also tested in the presence or absence of haptenic/nonhaptenic sugars. In order to determine cell viability in the presence of these lectins, each cell line was exposed to free lectins of different concentrations over 6, 12, 24, and 48 hour incubation periods. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay was performed to measure toxicity levels for each experimental group.

Histochemical results using the derivatized bead assay indicated that WGA and LCA preferentially bound to cancerous cell lines only, while ConA did not distinguish between cancerous and noncancerous cell lines. The cell viability assay suggested that WGA is a toxic inhibitor of cell proliferation for both HTB-126 and HTB-125 on a concentration-dependent basis. ConA reduced cell viability to a lesser degree than WGA, while LCA did not prove to be significantly detrimental to either cell line.

The major significance of these results is that the lectin bead assay can distinguish cancer from non-cancer breast cells depending on the type of lectin used. In addition, the ability of free lectins to kill cells in culture may offer a new approach in the development of more specific anti-cancer drug
CHAPTER 1: INTRODUCTION

Cancer is a global epidemic that is currently on the rise. It is estimated to affect over 1.5 million individuals in 2012 alone\(^1\). Of those effected, roughly one-third of the victims will succumb to the disease\(^1\). Surveillance Epidemiology and End Results (SEER) reports that although a significant decrease in incidence rates were observed at the turn of the new millennium, these numbers have gradually been climbing post-2009\(^1\).

Cancer can be attributed to a multitude of factors such as environmental agents, genetic components, viruses, and chromosomal mutations\(^2\)-\(^5\). Interestingly, 60 – 90% of cancers can be traced back to environmental cofactors, and should therefore be preventable. Malignant tumors develop as a mass in the body due to uncontrolled hyper-proliferation of damaged cells that have lost their ability to undergo apoptosis, or programmed cell death. Eventually, most debilitating cancers will metastasize from the primary site of infection and spread to distal locations in the body where they interfere with normal regulatory pathways of organs in these locations.\(^2\)

Breast cancer, in particular, is the main culprit in cancer death amongst women in the United States\(^6\). Cancers of the breast comprise a little over 15% of all cancers, claiming 17% of its victims lives\(^7\). In comparison to other cancers, breast cancer has been on the rise since 2005 after a substantial decline in incidence rates from 1999 to 2005\(^7\). Specifically, it is expected that 1 in every 8 women will develop breast cancer at some point in their lifetime\(^7\). Like many other cancers, breast cancer is a result of a combination of environmental and genetic factors\(^6\). Population studies suggest that descendants of foreign countries such as Japan who have migrated to the U.S. have increasingly become susceptible to developing breast cancer after just a few generations.
of exposure to their new environment. Hormonal fluctuations have been linked to breast cancer diagnoses, specifically an increase in urinary estrone and estradiol-to-estriol ratios in comparison to healthy women, as well as obesity and related dietary issues. More significant, however, is one’s genetic disposition to developing the disease. The discovery of the BRCA1 gene in 1994 served as the initial step in the movement towards the discovery of yet another critical genetic counterpart in the field of breast cancer – BRCA2. Regardless of what may be the cause of breast cancer, it is imperative to focus our efforts in developing alternative treatment options that are reliable and less invasive in order to improve preventive care, detection, and outcome.

**Objective**

Although there has been significant movement towards improving cancer screening and anticancer drug development, there exists paucity for a superior mechanism of detecting cancerous cells and delivering anti-cancer drugs to highly localized targets commercially. The most commonly used lectin associated approach for detection is to allow fluorescent (FITC) labeled lectins to bind to cancerous cells. This approach, however, must be carefully designed in order to reliably detect any fluorescent emission and requires several controls to validate any results, which can be time consuming and expensive. Similarly, older assays that utilized lectins are time-consuming and costly, and require fresh, robust cells with no guarantee of actually producing any viable results. As for treatment, chemotherapy is not a specialized method of delivering therapeutic reagents to the body, and is toxic to cancerous and noncancerous cells alike.
Our laboratory offers an alternative cost-effective approach to screening and detecting cancerous cells using a derivatized bead assay that allows for the rapid screening of hundreds of cells’ surfaces simultaneously. This assay involves the use of beads that are coated with over a hundred different molecules including lectins, various other proteins, amino acids, and sugars, that will agglutinate carbohydrate-rich molecules in minutes to allow for rapid screening of various cell surface markers at once. Clustering of these lectins to their preferred target cells will help differentiate between cancerous and noncancerous cell lines and provide more in-depth characterization of the cell’s surface. Furthermore, the derivatized bead assay has the potential to deliver drugs with adequate amounts of dosage to specific cancerous markers without causing any adverse effects to healthy, normal cells.

Previously, the lectin-bead assay was successfully applied to human colon cancer and noncancerous cells\textsuperscript{12,13}. The purpose of this study is to apply this novel technique to help distinguish cancerous from noncancerous breast cells. This multi-parameter approach will combine derivatized beads with lectins (Triticum vulgaris agglutinin or WGA); Lens culinaris agglutinin or LCA; and Concanavalin A or ConA) and haptenic/non-haptenic sugars in order to examine their interaction and effect (toxicity) on cancerous and healthy breast cells from the same patient. Specifically, our aims are to 1) delineate the binding properties of each lectin to cancerous and noncancerous mammary cells, and to 2) evaluate the toxic properties of lectins (if any) when bound to either cell line.

In summary, we will see if the cancer cells bind better to a specific lectin derivatized bead than do the non-cancer cells. If so, it is more likely that cancerous cells
will adhere to specific free lectins in culture rather than non-cancerous cells, and therefore be more susceptible to lectin toxicity. These findings will contribute to the development of anti-cancer drugs that can specifically bind to cancerous cells and induce cell death.
CHAPTER 2: LITERATURE REVIEW

Breasts are primarily comprised of adipose and fibrous tissue each consisting of 15 to 20 lobes. They are innervated by a series of blood and lymph vessels that make up a smaller component of the body’s entire immune system that is pertinent to fighting off infections. Several different types of breast cancers exist, some more prevalent than others [e.g. ductal carcinoma]. In most cases, the cancer originates in the lactiferous ducts. Depending on the severity of the cancer, the malignant cells will infiltrate other regions of the body (i.e. invasive ductal carcinoma) or remain confined to the breast as a mild neoplasm (i.e. ductal carcinoma in situ)\textsuperscript{14}. Certain types of breast cancer are relatively uncommon, such as inflammatory breast carcinoma, but still incur high mortality rates amongst their victims\textsuperscript{15}. Regardless of the type of breast cancer, the patient’s chances for survival are significantly improved and contingent upon early diagnosis\textsuperscript{16}.

Traditional breast examinations help reduce the risk secondary cancers from forming. The typical screening procedure includes breast examinations, mammograms, and if necessary, biopsies. Current research involved in optimizing screening procedures have found the use of fluorescent probes in conjunction with PET radiotracers a promising new approach to optical imaging of gliomas and breast tumors\textsuperscript{16}. However, determining the correct wavelength to use for each tumor is quite a feat for the undertaking and remains undecided. Unfortunately, many cases of breast cancer can go undetected, as most commonly occurs in the case of lobular carcinoma. For this reason, many researchers have redirected their efforts to developing more accurate methods for detecting cancerous masses by targeting the superficial features of a cancerous cell.
Use of Lectins for Cancer Therapy

The cell’s surface is its most influential feature, as it drives any interaction a cell is capable of making with another molecule. Cancer cell surfaces are dramatically altered in comparison to normal cell lines. Because the cell’s surface houses markers that initiate cell growth and regulates cell-cell interactions, it also plays a critical role in cellular proliferation, metastasis, and invasion. Alteration of the cell’s surface is what promotes uncontrolled cellular proliferation and an increase in ligands present for extracellular binding\(^2, 5, 17-18^\). The surface of a cancerous cell expresses high levels of glycoproteins, and, as indicated by its name, is very carbohydrate-rich. Lectins are proteins typically derived from plants that exhibit a high affinity toward carbohydrate-rich molecules, and as a result readily bind to these receptors. Specifically, they bind to certain sugars that are present in carbohydrates. Lectins have the ability to cross-link many receptors, which is why they tend to cluster together while binding to cancerous cells. Clumping occurs due to mobile surface receptors that are present on the surface of transformed cells, whereas healthy, normal cells exhibit mild, sporadic agglutination due to their immobile receptor sites.\(^2, 5\) This mobility can partly be attributed to the lack of cytoskeletal elements in malignant cells that are normal present in healthy cells. For this reason, cancer cells are ideal targets for agglutination by lectin binding, in comparison to healthy cells that may not be as carbohydrate-rich. This preferential binding process makes it feasible to distinguish cancerous cells from healthy ones. Furthermore, a lectin’s specificity can help differentiate between cell surface markers, and ultimately detect proteins that would make ideal therapeutic targets.
Studies have shown that although virtually all cells possess surface receptors that allow for lectin binding, altered or transformed cells are more prone to lectin-mediated binding than noncancerous cells. Tissues that have shown an aggressive interaction with lectins are prime suspects for malignancy, as Burres et al have suggested in their research. After assessing the binding of Helix pomatia agglutinin (HPA) in breast cancer cells, Schumacher et al. also suggest that altered glycosylation plays an important role in promoting lectin binding to their receptors. Lectins have also been used as markers in fluorescence imaging to detect tumors in a variety of cancers. For example, the use of certain lectins, such as Axinella corrugata 1, are proving to be useful diagnostic tools in the detection of neoplasms by attaching to tumorigenic cells that express increased levels of monocasscharides.

Typically, lectins are cultured with cells and grown in media for several hours in order to ensure adhesion to target cells and delivery to their appropriate receptors. Recently, the use of derivatized beads has gained popularity in lectin studies, as seen in several previously reported investigations. Our lab, however, has developed a novel form of the assay that makes it feasible to screen the binding interaction of an unlimited amount of compounds to produce rapid results. The beads are coated with over a hundred different surface molecules including sugars, amino acids, lectins and other proteins, that allow for rapid interaction with our cells of interest simply by mixing them together – a process that literally takes seconds to complete.

Contributing Factors and Mutations

As is the case for all cancers, breast cancer cells present an altered state of superficial markers and genes. Some hormonal imbalances and genetic alterations are
commonly found throughout all types of breast cancers, while others are unique to specific types of breast cancer and therefore make it feasible to distinguish between each type of disease. The most abundant hormonal receptor (HR) is the estrogen receptor (ER) and progesterone receptor (PR), found in nearly 70% of patients exhibiting growths in their breasts\textsuperscript{24}. Typically, HR+ patients that exhibit upregulated levels of estrogen and progesterone have an increased risk of metastasis, and inhibition of these hormones is crucial in preventing cancer proliferation. In an attempt to cease elevated levels of these hormones, their receptors are now the targets of novel anti-estrogen therapeutics\textsuperscript{24}. In addition to anti-estrogen therapy, concurrent administration of chemotherapy has shown to decrease the risk of late relapse in patients with HR+ breast cancer\textsuperscript{24}.

As most targeted therapies and cancer prevention have worked to suppress the expression of hormone receptors, there exists a paucity in treatment aimed towards other types of breast cancer, such as those that express mutations in the BRCA1 and BRCA2 genes. Many chemotherapeutic agents can effectively treat and eradicate malignant masses, but often times, tumors are resistant to these treatments and continue to proliferate. Recent studies suggest the importance of regulating tumor suppressor genes (TSGs) and that their expression is directly proportional to the sensitivity of cancer cells\textsuperscript{25}. For example, deregulation of tumor suppressor gene BRCA1 renders its incapable of DNA repair and mutagen-induced apoptosis, and inhibits the prevention of cell cycle checkpoints that enable the cell to function properly\textsuperscript{25}. Thus effectively targeting these suppressor genes in order to control their signaling pathway and ultimately manipulate tumor sensitivity is a viable alternative for patients who are resistant to traditional therapeutic treatment.
Traditional Treatments

Standard treatment often involves a combination of surgery, chemotherapy, and/or radiation. Invasive surgery is the initial step taken to remove as much of the mass as possible before it has the ability to metastasize. Surgically removing a tumor occasionally allows the body to fight off any residual infections caused by the cancer, and decreases the necessity of undergoing chemotherapy or radiation. Chemotherapy is the systemic delivery mechanism in treating cancer patients. Those undergoing chemotherapeutic treatment receive several doses of a concoction of chemical agents as part of their regimen, lasting anywhere from a few months to several years depending on the severity of the prognosis. The main advantage, unlike surgery and radiation therapy, is its ability to treat cancerous cells extensively throughout the body, rather than localizing in one region. Although chemotherapy is primarily responsible for curing and elongating the lives of millions of people diagnosed with cancer, it is not without its side effects. Out of every few thousand drugs that are tested for FDA approval, only a couple dozen are found to be safe and effective therapeutic agents. Mild symptoms include nausea and vomiting, diarrhea, constipation, hair loss, bone marrow depression, fatigue, stomatitis, and infections. Severe complications have been noted in several case studies.

The type of treatment a patient will receive is contingent on whether or not the breast cancer is invasive or not. In the case of most noninvasive breast carcinomas, such as ductal carcinoma in situ (DCIS), chemotherapy may not be necessary at all. Rather, a lumpectomy followed by a few rounds of radiation is usually sufficient in curing the cancer. For this reason, it is under debate whether or not DCIS should even be
categorized as a precursor for more invasive types of breast cancer since it presents itself as an intermediate between healthy and cancerous breasts\textsuperscript{14}. Interestingly, Virnig et al. reports that no conclusion could be drawn on the benefits of using either tamoxifen or raloxifene in reducing the incidence of DCIS and invasive breast cancers based on the findings of several different studies worldwide\textsuperscript{14}. Lobular breast carcinoma in situ (LCIS) is not as easily diagnosed. Likewise, treatment still remains controversial because it is difficult to predict if both breasts will become affected by the outcome of the other. Such circumstances require development of superior screening methods that will enable physicians to make choices better suited for their patient’s longevity.

Invasive breast carcinoma is far more involved and mandates the use of chemotherapy, such as tamoxifen and raloxifene. Patients suffering from hereditary breast cancers - such as those with defective hormone receptors, BRCA1/2 genes, or PALB2 - require a combination of drugs and (poly(ADP)-ribose polymerase) (PARP) inhibitors in order to effectively eradicate the cancerous cells while keeping the healthy ones intact\textsuperscript{8}. Even so, the use of drugs along with inhibitors remains inconclusive based on several long-term trials unable to prove their effectiveness\textsuperscript{8}. Inflammatory breast carcinoma (IBC) is a rare albeit devastating cancer that generally has poor prognosis. Long-term survivors attribute their longevity to extensive chemotherapeutic treatment\textsuperscript{15}. IBC patients exhibit increased levels of lymphangiogenesis and angiogenesis. Research has shown that targeting genes that are upregulated in patients leading to these symptoms can help increase survival rates in conjunction with traditional therapy.

Since chemotherapy functions by disrupting the cell cycle of any cell it comes into contact with, it is incapable of distinguishing mutated cells from healthy cells and is
therefore not limited to targeting malignant cell lines. Hypercalcemia (or even hypocalcemia), for example, is not uncommon in patients receiving chemotherapy\textsuperscript{27}. Under circumstances that one is diagnosed with a cancer that cannot be cured, chemotherapy is prescribed in order to attenuate the symptoms and pain a patient is experiencing. This type of chemotherapy is termed palliative, and is often considered the last resort for providing patients an improved quality of life\textsuperscript{28}.

Radiation therapy is a type of treatment that involves the used of high-energy penetrating beams to target the DNA in cancerous cells. The efficacy of radiation is dependent on how deep the rays can penetrate through the skin with minimal irritation\textsuperscript{26}. The outcome of radiation is similar to that of chemotherapy: shrinking of the tumor and prevention of metastasis. However, unlike chemotherapy, normal cells are not as prone to apoptosis due to the fact that their DNA is less susceptible to the effects of radiation and have higher rates of recovery in comparison to their mutated counterparts. Side effects are similar to those of chemotherapy, but vary depending on the location receiving the radiation. Symptoms include but are not limited to pain, swelling, development of rashes, decrease in blood count, nausea and upset stomach, weight loss, an under severe circumstances, hemorrhaging.

\textit{Experimental Treatments: The Direct Targeted Approach}

Despite the advances that have been made in cancer treatment over the past century, cancer cells have evolved several pathways to resist treatment and thrive\textsuperscript{30}. Drug resistance is and will remain to be a major issue that should be taken into consideration by those who intend on developing new methods of treatment. Oncogenic cells are frequently being transformed and can adapt to therapeutic agents rapidly. These cells
have developed highly sophisticated mechanisms that allow them to alter the cell cycle and evade apoptosis. For this reason, the health care field must consistently be developing new approaches to treating cancer. In essence, it is our responsibility to always be ‘one step ahead’ in order to ensure successful outcomes in patients receiving therapy.

Direct-targeted treatment has gained popularity over the past couple decades as a viable approach to treating breast cancer. Treatment is directed towards deregulated pathways involving the cell cycle, angiogenesis, cellular differentiation and apoptosis\textsuperscript{31}. For example, targeted treatments administer inhibitors to combat upregulated levels of HER2 with trastuzumab and lapatinib, or elevated levels of VEGF with bevacizumab as seen in DCIS patients. The specificity of this approach helps customize a treatment that caters to the patient’s needs based on their tumor and location. Although this approach would significantly minimize the amount of chemicals a patient is exposed to by directing treatment to certain areas of the body, they are commonly administered as a complementary mechanism with conventional chemotherapy in order to effectively improve survival rates\textsuperscript{31}.

Comparative studies have been performed between cancerous and noncancerous cells of the same tissue with hopes of delineating potential markers for cancer\textsuperscript{18, 32}. Schummer \textit{et al.} identified receptors of interest in ovarian and breast cancer cell lines by first using a ‘transcript-based’ discovery approach that required 5 separate phases of screening followed by a serum assay they developed. Unfortunately, the assay is only conducive to screening for ovarian cancer. Moreover, their initial approach is not only time consuming, but also requires high-quality preserved specimens that are difficult and
expensive to obtain. Other comparative studies have been performed to identify genes with elevated risks for developing cancer and delineate their significance in early detection and poor prognosis\textsuperscript{18}.

Derivatized beads have been used in previous studies to examine cell surface properties, but seldom have they focused on the cell binding interaction of potential therapeutic drugs and compared their association to that of regular, noncancerous cells of the same tissue. We propose to clearly distinguish any surface molecules present on cancerous breast cell lines from noncancerous cell lines of the same tissue by using a new method developed by our lab: the derivatized-bead assay. This novel approach allows for the binding of an infinite number of molecules (for example lectins) that exhibit a high affinity towards cell surface markers and suspends them on agarose beads for further scrutiny.\textsuperscript{24} Results are comparable to similar binding assays, such as immunofluorescence, but only take a fraction of the time to complete.

Previous studies have shown that lectins have a higher affinity towards cancerous cells due to their altered and more numerous surface receptors, specifically glycoproteins. However, a detailed description of these binding properties is not available, and therefore targeted drug therapy is limited. Furthermore, it is important to be able to distinguish between these types of cells in order to accurately deliver effective, targeted treatment to only cancerous cells. It is imperative to expand upon what little information is available regarding these cells and bead-binding techniques in order to develop drugs that bind efficiently to cancerous cells and exhibit low-toxicity towards normal, healthy cells.

To better understand the binding properties between lectins and their target cells, it is important to elucidate common patterns that are found amongst cancerous cells that
are lacking in healthy cells. Li et al. implemented the use of a multiplex bead assay to investigate the glycosylation patterns of serum proteins in an effort to determine useful biomarkers for targeted therapy in pancreatic cancer. They conclude that the bead assay is faster yet successful in identifying pancreatic cancer biomarkers, making it feasible to distinguish between malignant and healthy cells by using two different lectins$^{33}$. This research supports the use of incorporating lectins with the bead-binding assay in order to positively identify cancerous markers on the cells surface, but it does not discuss any potential toxicity that may be caused by the binding.

In this study we investigate the type of interactions that takes places between lectins and cells once they are bound. We examine the binding properties of lectin-agarose beads to cancerous and noncancerous breast cancer cells, as well as any toxicity the free lectins afflict onto the cancerous and noncancerous cells. Previously, students discovered that beads derived with various surface molecules such as proteins and sugars associated more with the malignant cells$^{12-13}$. Lectins that clustered more abundantly on cancerous cells also exhibited higher levels of toxicity towards these malignant neoplasms, while healthy cells were affected to a lesser degree. Expanding on this study with human mammary tissues gives us hope that this technique is not limited to treating a specific kind of cancer, but to a variety of ailments. Using this approach, the targeted cell can be abolished in a dosage dependent manner by selectively targeting mutated cells and minimizing the exposure of toxins to the rest of the body.
CHAPTER 3: METHODS AND MATERIALS

Cell lines

Two adherent cell lines, noncancerous (HTB-125/Hs 578Bst) and cancerous (HTB-126/Hs 578T) cells, were isolated from the same 74 years old Caucasian woman diagnosed with infiltrating ductal carcinoma of the breast. Cells were purchased from American Type Tissue Collection (ATCC, Manassas, VA) and cultured according to the recommended protocol under aseptic conditions. Briefly, cells were thawed immediately upon receipt and transferred to 15 cm$^2$ conical Falcon tubes containing complete growth media (CGM). Each cell line’s CGM was prepared as follows: 90% Dulbecco’s Modified Eagle’s Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 0.01 mg/ml bovine insulin for HTB-126; and Hybri-care medium (which is essentially modified DMEM, NCTC 125, and insulin), 1.5 g/l NaHCO$_3$, 10% FBS, and 30 ng/ml mouse EGF for HTB-125. Each cell line was centrifuged for 5 minutes at 125 x g and resuspended in their respective growth media prior to being transferred to 25 cm$^2$ T-flasks for incubation. Cells were incubated at 37°C and 5% CO$_2$ until they reached 70% confluency. Media renewal was maintained every 2 to 3 days.

Subculturing and Freezing Cells

Both cell lines were passaged upon reaching 70% confluency by the following method: First, the growth media was discarded and the monolayer was briefly washed with 0.25% Trypsin-EDTA to discard any remaining media. Next, cells were incubated for 5 minutes in at 37°C in 1mL Trypsin-EDTA solution to detach cells and create a single cell suspension. Upon detachment, 3 mL of CGM was added to each T-flask in
order to inhibit further Trypsin-EDTA activity. Cells from each flask were counted using a hemacytometer and assessed for viability using Trypan Blue exclusion prior to reseeding at a concentration of $1 \times 10^6$ cells/mL in new 25 cm$^2$ T-tubes. Both cell lines exhibited a viability of 95% or better. Cell media was renewed every 2-3 days and subsequently passaged once more into 75 cm$^2$ T-tubes. Cells were trypsonized as mentioned above and spun at 125 x g for 5 minutes after the addition of CGM. The supernatant was discarded and the cell pellet was resuspended in 2 mL fresh media and 2 mL of cryopreservation media, which consisted of 95% CGM and 5% DMSO. Cells were stored in 1mL aliquots of $1 \times 10^6$ in -80°C.

Histochemical Assay:

Fixing Cells

Cells were fixed in 4% formaldehyde with PBS prior to performing the lectin bead-binding assay. Cells from each cell line were counted and tested for viability using Trypan Blue exclusion. Each cell line was used only after demonstrating over 95% viability. From each cell line, 1mL aliquots containing $1.0 \times 10^6$ cells/mL were transferred to 15 mL conical Falcon Tubes and centrifuged for 3 minutes at 800 RPM (125 x g). The supernatant was removed and cell pellets were washed with PBS (without CaCl$_2$ and Mg Cl$_2$) and spun at 800 RPM (125 x g) for 3 minutes. This step was repeated three times prior to fixing the cells in 1mL of 4% formaldehyde at ambient temperature for at least 45 minutes. Afterwards, the formaldehyde was discarded and the pellet was washed with nanopure DI water and centrifuged at 800 RPM (125 x g) for 3 minutes.
three times prior to being resuspended in 1 mL of nanopure water for the last time. Fixed cells were stored at 4°C and used within 3 days of fixation.

*Lectin agarose beads*

Lectin beads were washed in order to remove any impurities and reduce the risk of any erroneous cellular interactions. The following lectins derivatized on 4% cross-linked agarose beads were obtained from Sigma Aldrich: Triticum vulgaris (Wheat Germ Agglutinin – WGA), Concanavalin A (ConA), Lens culinaris agglutinin (lentil – LCA). Poly L-lysine (PL) was used as a control (adhesive reagent) for noncancerous breast cells HTB-125. 100µl of each lectin was transferred to a 15 mL conical falcon tube containing 1mL nanopure DI water. Lectin beads were centrifuged thrice at 800 RPM (125 x g) for 3 minutes each with DI water and resuspended at a final volume of 100µl/mL. Washed lectin beads were stored at 4°C and used within no later than 3 days.

*Sugars*

Haptenic sugars are sugars that preferentially bind to lectins and subsequently prevent them from interacting with other molecules and cells. In the lectin-bead binding assay, N-acetyl-D-glucosamine was used as a haptenic sugar for WGA, and D-(+)-glucose and D-(+)-mannose were used for both ConA and LCA. L-(−)-fucose was used as a nonhaptenic sugar for all 3 lectins. Sugars were reconstituted to 0.2M for the assay.

*Bead-binding Assay*

Slides were prepared in triplicate for each lectin bead-cell-sugar combination. Each slide contained 40µl DI water (control), haptenic or nonhaptenic sugar, and 4µl of
lectin beads. The lectin-bead and sugar combination was swirled together with a toothpick for one minute in order to allow adequate interaction time. 4µl of cells were added to each slide and swirled again for another minute. Slides were observed at 100x and 200x total magnification to determine any positive binding between the cells and lectin beads. The slides were gently agitated and observed once more under the light microscope to ensure that the lectin-cell association was not disrupted as a result. Positive and negative bindings of cells to lectins were observed. Cells that were positively bound were counted and divided by the total number of cells available in each replicate per treatment group in order to determine the percentage of beads attached. Percentages were then averaged amongst the replicates per treatment group. Poly L-lysine slides were prepared in triplicate with 40µl DI water and noncancerous HTB-125 cells to serve as a positive control. Photographs were taken for each slide observed at 100x and 200x total magnification as evidence of interaction amongst the cells and lectin beads.

Cell Viability Assay:

Free Lectins

Wheat germ agglutinin, L. culinaris, and ConA were purchased from Sigma and reconstituted in sterilized PBS (without CaCl₂ and MgCl₂). Stock solutions of each lectin were prepared in serum-free DMEM or Hybri-care media and stored in -20°C until use.

MTT Assay

The MTT Cell Proliferation Assay (ATCC, Manassas, VA) is a colorimetric assay that tests the proliferation and viability of cells by reducing yellow tetrazolium MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble purple
formazan crystals. Reduction is directly correlated to the amount of enzymatic metabolism present in each sample and is quantified via spectrophotometric means. Moreover, the MTT assay provides a reliable method to ascertain toxicity levels of lectins that bound to the cancerous and noncancerous breast cells. It was performed in conjunction to Trypan blue exclusion because it is much more sensitive to cell viability. Unlike Trypan blue, it is a quantitative assay that can measure the toxicity levels of exogenous drugs and growth factors that have been added to the original cell culture.

The MTT reagent is light and contamination sensitive, and as a consequence was stored in the dark at 4°C in 2mL working aliquots. Extra care was also taken to ensure that all cell lines were free of any contamination, such as mycoplasma and bacteria, because their presence can produce false positives as a result of cleaving the tetrazolium ring of the MTT reagent. Cell lines were routinely checked under the microscope for any visible contamination and were discarded if any contamination was noticeable. If so, new cell lines were ordered or thawed from frozen stocks. Cells were seeded in 96 multi-well tissue culture-grade plates and incubated at 37°C and 5% CO₂. Twenty-four hours later, 10µl of MTT was added aseptically to each well and incubated for 4 more hours. The plates were then inspected under an inverted microscope for the formation of purple crystals. The final step involves the addition of 100µl of MTT Detergent and incubation at room temperature for 4 hours in order to lyse the cells and dissolve the crystals that had formed. Plates were read using an ELISA plate reader (Spectromax 190, Molecular Devices, Sunnyvale, CA) at 570nm with a reference wavelength of 690 nm. Absorbance readings are a direct indicator of cell viability. Thus, a decrease in metabolic rates would
be consistent with a reduction in cellular proliferation and produce low absorbance readings, whereas actively metabolizing would be indicative of high absorbance readings.

**Optimal Cells Count for the MTT Assay**

Prior to performing the assay, it was imperative to determine the optimum number of cells and incubation cell time to produce reliable absorbance readings. Cells from both cultures were counted and resuspended to $1 \times 10^6$ cells/mL. Serial dilutions were subsequently performed to yield cell concentrations of $5 \times 10^5$, $2.5 \times 10^5$, $1.25 \times 10^5$, $6.25 \times 10^4$, $3.125 \times 10^4$, $1.5 \times 10^4$, and $7.8 \times 10^3$ cells/mL. Each concentration was plated in triplicate and incubated under standard conditions for 24 hours. The MTT assay was performed as delineated above and absorbance values were compared to those of the control wells with cell growth media only. Absorbance rates were plotted against the aforementioned cell concentrations, producing a linear curve.

**Cellular Proliferation and Toxicity**

After the determination of the optimum cell concentration, cell viability was assessed as a result of toxicity levels induced by incubation with lectins at different concentrations over time. Fifty microliters of each cell line were transferred to 96-well plates and incubated overnight. Cells that were to be incubated for 24 and 48 hours were done so with the addition of lectins at a final concentration of 0.5, 1.0, 10.0, 50.0, and 100 µg/mL. Cells that were to be incubated for only 6 and 12 hours were done so with lectins at concentrations of 0.05, 0.25, 0.5, 5.0, 25.0 and 50.0 µg/mL. Lectin dilutions were performed with serum-free media. Wells that contained cells, MTT reagent, and serum-free media instead of lectins served as a negative control. Wells that contained
lectins, MTT reagent, and serum-free media served as blanks. All experimental treatments including controls and blanks were plated in triplicate. Cell viability was assessed at 6, 12, 24, and 48 hours to determine the affects of lectin toxicity on cancerous and noncancerous breast cells. Survival rates were calculated as percentage of cell viability as a function of each lectin at each concentration using the following formula: % Viability = \((\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}})\) \times 100, where “A” is the absorbance value and “sample” refers to the specific treatment group. Each experimental treatment was performed in triplicate at least twice to ensure reliable results.

**Statistical Analysis**

All statistical analyses were performed on Microsoft Excel 2011 and SYSTAT 13 and stated as means ± SEM. Results for the histochemical portion of the experiment were calculated using a one-way ANOVA to test if the lectin type affects the binding affinity of cells to the beads. A two-way ANOVA was used to compare the mean cell survival rate between cancerous and noncancerous breast cells and any effect due to the different concentrations for each cell line during the MTT assay. Significant results are reported as \(p < 0.05 (*)\) and \(0.001 (**)\).
CHAPTER 4: RESULTS

Histochemical Assay

Fixed cancerous (HTB-126) and noncancerous (HTB-125) cells that bound to lectins were counted in triplicate and averaged (Table 1 and Table 2, respectively). Binding results are reported as percentages of the means calculated by dividing the number of cells attached by total number of cells available (bound and unbound) for each lectin type per treatment (haptenic/nonhaptenic sugars vs. distilled water) and are reported as percentages of total cells viewed in focus.

Overall, control cancerous cells bound to all lectin beads with greater affinity (p<0.001) than their noncancerous counterparts (Figure 1A). Noncancerous breast cells did not bind to any lectin bead other than Concanavalin A, while positive binding was noted in cancerous cells to all 3 lectin beads. Cancerous cells in DI water showed a significant preference (p<0.05) in attachment to both ConA and L. culinaris in comparison to WGA (Figure 1B). However, in the presence of their respective haptenic sugars, even ConA and LCA showed a significant decrease (p<0.05) in attachment rates to cancerous cells (Figure 1C). Interestingly, the type of haptenic sugars applied did not contribute significantly to the inhibition between the cells and the beads (Table 1). Noncancerous (control) cell binding to WGA and LCA was negligible (6% in both cases). As mentioned above, even noncancerous HTB-125 cells bound efficiently to ConA lectin beads, with 96% of cancerous cells binding in comparison to 90% of noncancerous cells (Figure 1D). Poly L-lysine was used as a control for the noncancerous cells and exhibited 100% binding between the molecules. Cells bound to lectin beads results are clearly illustrated in Figure 2.A – F below.
Table 1. Results for histochemical assay with HTB-126

<table>
<thead>
<tr>
<th>Lectin Bead</th>
<th>Conditions</th>
<th>% positive binding results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>Nanopure Water</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>0.2M L-(-)-fucose</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-glucose</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-mannose</td>
<td>20%</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td>Nanopure Water</td>
<td>73%</td>
</tr>
<tr>
<td></td>
<td>0.2M L-(-)-fucose</td>
<td>77%</td>
</tr>
<tr>
<td></td>
<td>0.2M N-acetyl-D-glucosamine</td>
<td>1%</td>
</tr>
<tr>
<td>Lens Culinaris</td>
<td>Nanopure Water</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>0.2M L-(-)-fucose</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-glucose</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-mannose</td>
<td>14%</td>
</tr>
</tbody>
</table>

Percentage of cancerous cells bound to lectins in distilled water and with/without the presence of haptenic/nonhaptenic sugars. Slides were prepared in triplicate and percentages are calculated based on the number of bound beads divided by the total number of beads counted.

Table 2. Results for histochemical assay with HTB-125

<table>
<thead>
<tr>
<th>Lectin Bead</th>
<th>Conditions</th>
<th>% positive binding results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>Nanopure DI Water</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-glucose</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td>0.2M D-(+)-mannose</td>
<td>23%</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td>Nanopure DI Water</td>
<td>6%</td>
</tr>
<tr>
<td>Lens culinaris</td>
<td>Nanopure DI Water</td>
<td>6%</td>
</tr>
<tr>
<td>Poly L-lysine</td>
<td>Nanopure DI Water</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Percentage of noncancerous cells bound to lectins in distilled water or with/without the presence of haptenic sugars. Slides were prepared in triplicate and percentages are calculated based on the number of bound beads divided by the total number of beads counted.
Cancerous breast cells bind more efficiently to lectin beads than do noncancerous cells

A.

Cancerous cells preferentially bind to ConA and LCA

B.
Figure 1. (A) Cell binding of both cell lines to all 3 lectin bead types in DI water only. Cancerous HTB-126 cells bind significantly better to lectin beads in general than do noncancerous HTB-125 cells (** denote p<0.001). (B) Cancerous cell binding to each of the 3 lectin beads in DI water only: ConA, WGA, or LCA. Cancerous cells attached more significantly to Concanavalin A and L. culinaris lectin agarose beads than they do to Wheat Germ Agglutinin (* denotes p<0.05). (C) Sugar inhibition of cancerous cells binding to ConA and LCA lectin beads. The type of haptenic sugar does not significantly alter the bead binding abilities of cancerous cells. However, binding does significantly decrease for cancerous cells in the presence of any haptenic sugar (* denotes p<0.05). (D) Comparison of cancerous and noncancerous cells binding to lectin bead ConA in DI water only. Both cell types attached to ConA lectin beads with comparable efficacy in the presence of DI water only. All reported percentages are means of the 3 replicates per experimental treatment (DI water vs. haptenic sugars).
Figure 2. (A) Binding results of cancerous HTB-126 cells (smaller spheres) with WGA derivatized agarose beads (larger spheres) in distilled water with. Positive binding observed. (B) Binding results of cancerous HTB-126 cells with LCA derivatized beads in distilled water. Positive Binding observed. (C) Nonbinding results of cancerous HTB-126 cells with LCA in the presence of haptenic sugar D-(+)-glucose. Negative binding is observed. (D) Binding results of noncancerous HTB-125 cells with PL derivatized agarose beads in distilled water. Positive binding observed. Binding results for derivatized ConA agarose beads with cancerous HTB-126 (E) and noncancerous binding (F). Positive binding observed in both cell lines.
Cells Viability Assay

**Optimum Cell Count Determination**

It was necessary to determine the optimum cell concentration that would generate the strongest absorbance readings for the MTT assay. Absorbance readings were plotted against the different dilutions of HTB-126 concentrations. The appropriate absorbance would not only produce the strongest wavelength, but would also fall in the linear range of the graph. From the 8 different dilutions, $5 \times 10^5$ produced the strongest absorbance reading. As a result, cells were plated at a concentration of $5 \times 10^5$ cells/mL in each well for the actual MTT assay for both cell lines. Low absorbance readings are a consequence of inadequate incubation time after transferring cells to the 96-well plate and may be resolved by an additional over-night incubation prior to the addition of the MTT reagent.

![Graph showing the relationship between cell concentration and absorbance](image_url)

**Figure 3.** Determination of cell concentration to use in cell proliferation MTT assay for HTB-126 (cancerous breast cells). Strongest absorbance wavelength was observed at 500,000 cells/mL.
**MTT Assay**

All results are reported as percent viable cells in comparison to untreated control cells. Treatment groups contained 50 µL cancerous or noncancerous cells and 50 µL of a specific concentration of WGA, L. culinaris, or ConA in serum-free media (SFM) incubated over a certain period of time (6, 12, 24, and 48 hours). Control treatments contained 50 µL of either cancerous or noncancerous cells incubated in equal parts SFM. Blanks contained 50 µL of each lectin incubated in 50 µL of complete growth media. Graphs are represented as cell survival rates (as a percentage of controls) as a function of each treatment group. Variation between samples is indicated with standard error bars of the mean.

**Wheat Germ Agglutinin**

Incubation of cells with WGA for the initial 6 hours produced remarkable differences in cell viability between cancerous HTB-126 and noncancerous HTB-125 (p<0.001) at all concentrations (Figure 4A). Cancer cells show 96% viability at 0.05 µg/mL, 86% at 0.25 µg/mL, 82% at 0.5 µg/mL, 73% at 5.0 µg/mL, 22% at 25.0 µg/mL, and 20% viability at 50.0 µg/mL. Noncancerous cells exhibited a stimulatory effect of 120% and 125% (at 0.05 µg/mL and 0.25 µg/mL, respectively) but encountered a decline down to 54% at 50.0 µg/mL. Aside from differences between both cell lines, notable distinctions can also be made within each cell line at different concentrations regarding cell viability. For example, lectin concentrations played a critical role (p<0.001) in cell viability in both cell lines around 25.0 µg/mL (Figure 4A), at which a decrease in cell proliferation can be seen. Continuous exposure to WGA for 12, 24, and 48 hours did not
result in any significant differences in cell survival rate between the two cell lines. Although significant differences were not apparent in cell viability between cancerous and noncancerous cell lines at 12 and 24 hours, both incubation periods denoted significant decrease (p<0.05) at particular concentrations: 5.0 µg/mL for noncancerous cells and 0.5 µg/mL for cancerous cells for the 12-hour incubations; and from 1.0 µg/mL for noncancerous cells and 50.0 µg/mL for cancerous cells for 24-hour incubations (Figure 4B and 4C). Figure 4D suggests that cancerous and noncancerous breast cells are equally susceptible to high doses of WGA after a prolonged period of incubation time (48 hours), generating nearly identical graphs for both cell lines. Ultimately, both healthy and cancerous breast cancer cells succumb to concentrations of WGA 50 µg/mL and higher.

Concanavalin A

Cell survival of ConA exhibited similar cell viability trends to that of WGA but with much less significance between the cell lines. During the 6-hour incubation period, cells exposed to ConA showed significant differences (p<0.05) in cell viability trends between the two cell lines. However, the decrease was observed in cancerous cells instead of the cancerous cells, as seen in WGA. After an initial decrease in the viability of both cell lines from 0.05 µg/mL to 0.25 µg/mL, the inhibitory effects by ConA were alleviated at 0.25 µg/mL (albeit at a lower rate for noncancerous cell lines) prior to coming to tapering off around 67% for HTB-126 and 55% for HTB-126 by 50.0 µg/mL (Figure 4E). Negligible differences were observed during the 12 and 24-hour incubations as both cell lines were unaffected by the presence of lectins. AT 48-hour incubation, although cell production didn’t warrant any statistical significance between the two cell lines, it is important to note that after the initial stimulatory effect of the lectins on
cancerous and noncancerous cells, cancerous HTB-126 cell proliferation began to decrease from 50.0 µg/mL to 100.0 µg/mL (92% and 72%, respectively) while noncancerous HTB-125 cells remained highly viable (99% viability at 100 µg/mL) (Figure 4F).

**Lens culinaris**

Incubation with LCA lectins generally proved to be nontoxic to both cell lines, especially at the prolonged incubation period of 48-hours. The only significance (p<0.05) in cellular proliferation was noted between HTB-126 and HTB-125 during the 24-hour incubation period: cancerous cell lines from L. culinaris’ experience inhibition most significantly at 0.5, 10.0, and 100.0 µg/mL in comparison to the noncancerous cell lines that seem to cease their decline around 50 µg/mL (Figure 4G). There exists also a concentration-dependent significance (p<0.001) during the 12-hour incubation period at 0.25 µg/mL, at which both cell lines experience substantial cell death in a similar fashion after experiencing a stimulatory effect at 0.5 µg/mL. Regardless of their steep decline, both cell lines exist in equilibrium around 60-65% viability until 50 µg/mL (Figure 4H).
Figure 4A. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with WGA free lectins for 6 hours. Significant decrease in cell survival rate is observed between cancerous and noncancerous cells at all concentrations (** denotes p<0.001 between cell lines). Both cell lines show significant decline at 25 µg/mL (** and *** denote p<0.001 for cancerous and noncancerous cells, respectively).

Figure 4B. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with WGA free lectins for 12 hours. Significant decrease in cell survival rate is observed in both cell lines at a concentration of 0.5 µg/mL for cancerous cells and 5.0 µg/mL for noncancerous cells (¹ and ² denote p<0.05 for cancerous and noncancerous cells, respectively).
Figure 4C. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with WGA free lectins for 24 hours. Significant decrease in cell survival rate is observed in both cell lines at a concentration of 50.0 µg/mL for cancerous cells and 1.0 µg/mL for noncancerous cells (* and * denote p<0.05 for cancerous and noncancerous cells, respectively).

Figure 4D. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with WGA free lectins for 48 hours. No significant difference is observed between the two cell lines: both cell lines perish after prolonged exposure to WGA at higher concentrations. However, both cell lines experience significant decrease in cells at 10.0 µg/mL of WGA (*** and ** denote p<0.001 for cancerous and noncancerous cells, respectively).
Figure 4E. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with ConA free lectins for 6 hours. Healthy cells are significantly more susceptible to ConA lectins from 0.25 µg/mL to 0.5 µg/mL (* denotes p<0.05 between cell lines).

Figure 4F. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with ConA free lectins for 48 hours. Noncancerous breast cells retain 99% viability at a concentration of 100 µg/mL while cancerous cells decrease by 20% from 50 µg/mL to 100 µg/mL (92% to 72%, respectively), but no statistical significance is indicated.
Figure 4G. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with LCA free lectins for 24 hours. Cancerous cells were more sensitive to LCA’s inhibitory effects except at 1 µg/mL and 50 µg/mL and continued to decrease in viability at 100 µg/mL (* denotes p<0.05 between cell lines).

Figure 4H. Cell viability assay was assessed by MTT colorimetric assay. Results are expressed as percentages of controls representing 100% cell viability. Standard error of means are based on the average of samples per concentration and reported by standard error bars. Cell viability of cancerous and noncancerous cell lines after incubation with LCA free lectins for 12 hours. Both cell lines experience significant decrease in cell viability at 0.25 µg/mL but regain equilibrium by 50 µg/mL (** and *** denote p<0.001 for cancerous and noncancerous cells, respectively).
CHAPTER 5: CONCLUSION AND DISCUSSION

Interpreting the Results

The use of lectins in cancer research has emerged as a promising alternative for screening and treating metastatic growth. Many biochemical factors contribute to their resilience. Tumorigenic cells exist in great numbers because they have drastically reduced amounts of chalones (that typically inhibit mitosis) and, in some cases, cyclic AMP. Physically, transformed cells exhibit elevated levels of surface proteins that may act as receptors to ligands such as lectins. Furthermore, these cells often have incomplete sugar chains that may also help facilitate lectin binding. For example, Subhashini et al. noted in their study that WGA has a specific affinity towards the core portion of N-glycans of breast cancer sera, while ConA interaction with this region is much weaker. The altered state of a cancerous cell and its elevated levels of carbohydrate expression on its surface have made them desirable targets in the fight against cancer. The use of lectins has become a popular choice for elucidating the tumorigenic properties of cancerous cells. For instance, staining of oncogenic markers has allowed researchers to effectively map questionable neoplasms. Similarly, studies focusing on the binding properties of cancerous cells have given invaluable insight into the chemical characteristics and molecular makeup of these cells. Lectin beads in a variety of assays ranging from fluorescent labeling to antibody staining have shown to be effective in distinguishing between cancerous and noncancerous cells. Chen et al. describes their success in measuring the glycosylation response to ConA in sera from pancreatic cancer patients using this versatile approach. In our study, the derivatized bead-binding assay showed preferential binding of cancerous breast cells to ConA as well as to WGA and L.
culinaris. Noncancerous breast cells, however, also readily attached to ConA lectin beads with comparable results to their cancerous counterparts. The histochemical assay clearly depicts the inhibition of lectin-bead binding to cancerous cells in the presence of haptenic sugars. These binding properties are also evident in Subhashini’s study which utilized lectins in their lectin affinity chromatography (LAC) based assay to investigate the attachment properties of WGA, ConA, and RCA-I to human breast sera\textsuperscript{34}. These three lectins were immobilized onto three separate columns and subjected to human breast cancer and healthy breast sera. Subhashini found that the lectins bound readily to the differentially expressed glycoproteins due to breast cancer. These glycoproteins remained in the column in the presence of each lectin and were eluted only in the presence of their respective haptenic sugars, which prohibited any interaction between the lectins and their targets. Our bead-binding assay produced similar results, inhibiting lectin-to-cell binding in the presence of haptenic sugars. This inhibition was not contingent upon the type of haptenic sugar used, but more so by the presence of any haptenic sugar at all. These results further confirm those observed by Dresch \textit{et al.}, explaining a substantial decrease in agglutination of cancerous in the presence of several inhibitory sugars such as N-acetyl-D-mannosamine\textsuperscript{21}.

Several important conclusions can be drawn from each portion of our two-part study. Aside from noting the obvious interaction between cancerous cells and lectin-beads and the lack thereof between noncancerous cells (with the exception of ConA which interacted with both cell lines), it is also important to note that the cancerous breast cells have a stronger association with ConA and LCA beads in particular as opposed to WGA lectin-beads. Based on these interactions, one would assume that ConA and LCA
would have decreased their cell viability much more prominently than WGA during the MTT toxicity assay. However, this was not the case at all. WGA was much more detrimental to cellular proliferation for both cancerous and noncancerous cells, followed by ConA and then LCA.

Based on the toxicity assay, WGA was the most potent lectin in terms of deterring cell growth in both cancerous and noncancerous cells. This was most evident during the initial 6-hour incubation period at which there was a dramatic loss in viability in the cancerous cells compared to the noncancerous breast cells at all concentrations. Long-term incubation showed that this trend was only temporary, with the decline of noncancerous cells paralleling that of the cancerous HTB-126 cell line. These results indicate that long-term exposure (48-hours and longer) of both healthy and cancerous breast cells to WGA is disadvantageous to their viability, similar to what has been observed in colon cancer cells in previous studies\textsuperscript{12-13}. WGA recognizes the N-acetylglucosamine and sialic acid residues on the cancerous cell surface and is internalized upon binding to the appropriate receptors. This internalization is what induces its toxicity and results in cell apoptosis\textsuperscript{35}.

ConA incubation produced the most significant outcomes at the 6 and 48 hours incubation time frames. However, the initial 6-hour incubation was significantly more inhibitory to healthy cells than to the noncancerous cells. This does not come as a surprise considering the results from the bead-binding assay: >90% of both cell lines attached to ConA beads. As mentioned above, although both cell lines were quite robust until 10.0 µg/mL (~100% viability for both cell lines) during the 48-hour incubation period, it is important to note that cancerous cells declined by 15 – 20% at each of the
following concentrations (50.0 µg/mL and 100.0 µg/mL) while normal breast cells retained their 100% survival rate. This could possibly indicate that cancerous cells are more likely to perish in the presence of ConA at prolonged incubation times under higher concentrations as opposed to noncancerous breast cells that remain unaffected.

*L. culinaris* did not deter cell growth for either cell line during the 6 and 12-hour incubations. The only minor significance that was observed was the slight but gradual decrease in cancerous breast cells in comparison to the healthy cell line during the 24-hour time frame. Noncancerous cells retained 97% of their cells at a concentration of 100 µg/mL of LCA, while cancerous breast cells were reduced to 67% (about a third less than that of HTB-125). This trend did not continue during the 48-hour incubation period as cancerous cells had rebounded slightly, although to a lesser degree than that of the normal cell line.

The MTT assay also demonstrates the importance of lectin concentrations and how they effect cell line proliferation. For example, a closer look at the 48-hour incubation (Figure 4D) reveals that the inhibitory effects of WGA are mostly apparent at 10.0 µg/mL, at which cell viability decreased by 50% from 1.0 µg/mL. A similar trend is observed during the 48-hour incubation of cells with ConA showing a 20% loss of cancerous cells between 50.0 µg/mL and 100.0 µg/mL (Figure 4F). These results can help gauge the best dosage/concentration to use for a specific time intervals for a particular lectin.

Determining lectin toxicity is imperative in its application as a therapeutic treatment. Certain lectins do not exhibit significant levels of toxicity, and therefore do not alter a cell’s growth pattern (as in the case of LCA). Others, like WGA, are known to be
detrimental to certain types of cells, such as colon and breast cancers. For a lectin to be considered toxic, it must 1) be compatible with the superficial makeup (such as sugar chains and proteins) of their target cell in order to bind efficiently, and 2) be internalized to effectively eradicate its host\textsuperscript{2,35-36}. Toxic lectins have the ability to not only bind to their targets, but act as the drug themselves. Their binding specificity enables them to selectively attach to particular components of cancerous cells that are lacking in healthy cells, such as highly-glycosylated cell membranes. Upon binding to their targets, lectins can be internalized and subsequently release their cytotoxicity on their hosts. Camby et al. investigated the influence of lectins in modulating tumorigenic proliferation of human astrocytes. Their findings suggest that both ConA and WGA effectively antagonize transformed cellular growth in a dose-dependent manner\textsuperscript{37}. Alternatively, lectins that are considered nontoxic can be used as shuttling mechanisms to efficiently deliver drugs to specific targets. In order to do so, it is important to delineate the binding properties of each lectin to its host cell. For example, WGA will readily interact with any glycoproteins that express levels of N-acetyl-D-glucosamine in their plasma membrane, regardless of whether or not these are part of normal or cancerous cells\textsuperscript{38}.

Future Prospects

The importance of early detection and diagnosis for any type of cancer cannot be over-emphasized. As mentioned earlier, screening is the initial step in cancer prevention. Treatment is often times invasive and can incur detrimental side effects to the patient, as seen in chemotherapy patients. The underlying reason for this is due to that fact that anticarcinogenic drugs are not delivered in a localized manner. Chemotherapy is the standard mechanism used to deliver a combination of drugs that are toxic to any cells they come
into contact with, regardless if the cells are transformed or healthy. Several studies have documented chemotherapies adverse and long-term effects such as neuropathy, cardiovascular disease, and bone marrow depression, not to mention chemo-related deaths.\(^{27-28, 39-41}\)

The development of a safe, cost-effective, and rapid screening and drug transport methods are long overdue. The simplicity and accuracy of using derivatized beads coated with various types of lectins has the potential to readily replace other time-consuming, high-maintenance cancerous cell identification techniques that are in current use. Here, we have proposed an alternative method of cancer cell screening that may deliver the appropriate drug only to intended targets of interest while inhibiting any interaction with benign cells.

Aside from its clinical implications, patients that were fortunate enough to survive their battle with cancer suffer from long-term psychological and physiological complications. A study by Mulrooney *et al.* suggests that cancer survivors are at higher risk of developing a myriad of heart conditions in comparison to their healthy siblings due to prolonged exposure to chemotherapy and radiation as part of their normal course of treatment\(^4\). Survivors are often faced with cognitive challenges, with dramatic decreases in memory, attention, and processing of information\(^{40}\). Therefore, effective treatment is imperative for the well-being of patients not just physically, but mentally and emotionally as well. The development of drugs to antagonize cancerous cells is just the initiation of the treatment protocol. It is just as important to continue with the healing process as soon as a patient enters remission in order to ensure him or her a long and productive life. Although society is significantly advanced in terms of technology and
medicine, we have yet to see the rate of cancer subside. This may be due in part to an increase in global population and higher rates of survival. In correlation with this is the number of deaths per year that are traced back to chemotherapy. O’Brien et al. reports that of about 2000 patients that received chemotherapy between April and September of 2005, 161 (8.1%) of them didn’t survive more than a month. Applying these rates to all 1.5 million cases that were diagnosed just in 2012 would produce catastrophic outcomes. The need for a more specific, effective, and safe delivery of drugs is in need today to ensure a healthier, more productive world that will thrive tomorrow.

This study is performed on breast cancer cells, but is just as compatible with a plethora of different types of cancer cells. A superior mechanism of detecting cancerous cells and delivering anti-cancer drugs to highly localized targets is currently lacking in the field of oncology. Unfortunately, chemotherapy is not a specialized method of delivering therapeutic reagents to the body, and is therefore equally toxic to cancerous and noncancerous cells alike. The derivatized bead assay is a promising new approach to delivering drugs with adequate amounts of dosage to specific cancerous markers that have been thoroughly characterized without causing any adverse effects to healthy, normal cells. In the future, we hope to expand upon the application of the derivatized bead assay and implement its use as part of the regular standard for treating patients in a clinical setting with hopes of coming one step closer to reducing morbidity and mortality rates in our fight against cancer.
REFERENCES:


