COMPARISON OF STANDARD TREATMENTS OF BREAST CANCER WITH
NEW EXPERIMENTAL TREATMENTS TARGETING CAVEOLIN-1

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JENNIFER STETLER

Dr. Brenda Peculis, Thesis Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the [thesis or dissertation] entitled

**COMPARISON OF STANDARD TREATMENTS OF BREAST CANCER WITH NEW EXPERIMENTAL TREATMENTS TARGETING CAVEOLIN-1**

presented by Jenniffer Stetler,

a candidate for the degree of [master of sciences, biochemistry],

and hereby certify that, in their opinion, it is worthy of acceptance.

______________________________
Professor Brenda Peculis

______________________________
Professor Mark Hannink

______________________________
Professor Salman Hyder
DEDICATION

I would like to dedicate this manuscript to Emmanuel, my parents Mike and Becky Stetler, sisters and brother-in-law Nikky, Melissa, and Casey, nieces and nephews, and friends Drew, Bassem, Ashutosh, Cynthia, Amber, Rachael, Danielle, and Irene for their support and editing.
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LIST OF ABBREVIATIONS

BC- Breast cancer
TN- Triple negative
Tam- Tamoxifen
Cav- Caveolin
HER2, HER2/neu- Human epidermal growth factor receptor 2
CK5/6- Cytokeratin 5/6
TP53- Tumor suppressor protein 53 or p53
EGFR- Epidermal growth factor receptor
GATA3- GATA binding protein 3
BRCA- Breast cancer type 1 susceptibility protein
CK14- Cytokeratin 14
CK17- Cytokeratin 17
KIT- Proto-oncogene and also called CD117 and mast/stem growth factor receptor (SCFR)
APC/C- Anaphase-promoting complex (APC) and also called Cdh1
ADC- Antibody drug complex
ABSTRACT

Breast cancer is a complex disease at both an anatomical and molecular levels. The complexity of the disease is a source of difficulty in identifying and treating the cancer, since varying subtypes of cancer respond differently to current treatments. The first chapter will present an overview of breast cancer and explain in more detail the subtypes of breast cancer, current treatments, and development of resistance. The more aggressive breast cancer subtypes are tamoxifen (TAM)-resistant and triple negative (TN) breast cancer. The current therapies and treatments are proving ineffective at treating these cancer subtypes. So the question becomes what is effective against TN and TAM-resistant breast cancer? Chapter two presents a discussion of caveolin proteins as a possible therapeutic target. Caveolin proteins are unique therapeutic targets that are located in the cell membrane and endoplasmic reticulum (ER). Also, the caveolin proteins act as platforms for several signaling pathways to control cellular signaling. Chapter three explores the use of compounds and antibody-drug complexes (ADC) to target caveolin proteins as potential treatments for TN and TAM-resistant breast cancer. This thesis describes potential therapies for TAM-resistant and TN breast cancer that use the caveolin proteins and caveolae lipid rafts in tumor cells.
Chapter 1: Standard treatments of breast cancer

I. Epidemiology and stages of breast cancer

Breast cancer is the one of the most prevalent cancers in women in the United States with one in eight women developing breast cancer in their lifetime. In 2012, there were about 227,000 new cases of breast cancer in women in the United States. The median age range of diagnosis is 60-69. Women over 50 often present with localized breast cancer that is not metastatic. However, in younger women from 20 to 35 years of age, who account for about 20% of breast cancers, there is a higher incidence of metastatic breast cancer. The breast cancers in older and younger patients are vastly different (1). Localized and metastatic cancers respond differently to treatment, therefore, it is important to create effective treatments for each type of cancer.

The survival rate of women with localized breast cancer has increased by 23% from 1975 to 2001, while the survival rate of women with metastatic breast cancer has decreased by 23%. The failure to detect metastatic breast cancers at an early stage contributes to the differences in the survival rates (1, 2). In addition, the metastatic cancers are more invasive and unresponsive to treatments (1-5).

A. Stages of breast cancer

Early stages of breast cancer are stages 1 or 2 (1). Stage 1 breast cancers are defined by the presence of a tumor that is 2 centimeters or less in size, with or without attachment of the tumor to muscle, with no palpable axillary lymph nodes, and no
metastasis. Stage 2 breast cancers are defined by the presence of a tumor between 2 to 5 centimeters, with or without attachment of tumor to muscle, with or without palpable metastasis in the axillary lymph nodes, and no distant metastasis (4). Increased success in treating early stage cancers is due to improvements in treatments of chemotherapy and hormone antagonist therapy (1).

The late stages of breast cancer are stages 3 and 4 (1-4). Stage 3 breast cancers are defined by the presence of a tumor larger than 5 centimeters, with or without attachment of the tumor to muscle, with fixed axillary lymph nodes, and no distant metastasis or secondary sites of metastasis. Stage 4 breast cancers are defined by the presence of a tumor that can be any size with metastasis in axillary, supra-, and infraclavicular lymph nodes, and as well as distant metastasis in other tissues such as bone, lung, and brain (4). Patients with late stage and metastatic cancers have a low survival rate (1-4).

II. Anatomy of the breast

The breast is composed of anatomical parts including the milk ducts, lobes, and lobules. The milk ducts are located underneath the surface of the skin at the nipple, where they connect to milk-producing glands. The glands and ducts are surrounded by supportive connective tissue made up of fat and fibrous material (6-8, 10, figure 1.1). The lobes are made of smaller sections called lobules. The lobules of the breast tissue are made of alveoli, which are clusters of grape-like structures that contain lactocytes or mammary secretory epithelial cells. In addition, the breast is composed of glandular and
adipose tissue held by the loose framework of fibers called the Cooper’s ligaments (8).

The anatomy of the normal breast is the framework for the anatomical classification of breast cancer.

![Gross anatomy of the breast. Figure reproduced with permission from reference 8.](image)

**Figure 1.1** Gross anatomy of the breast. Figure reproduced with permission from reference 8.

### III. Anatomical classification of breast cancer

There are two main types of breast cancer described by the location within the breast. These are the ductal and lobular. Ductal carcinoma is one of the two main types of breast cancer (7). In ductal carcinoma, the cancerous cells grow in the milk ducts. The more specific stages of ductal carcinoma include ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). Ductal carcinoma in situ is a cancer growing in the lining of the milk ducts, while the cancer that spreads out from milk ducts is invasive ductal carcinoma (7, 10, 12, table 1.1).

The other main type of breast cancer is lobular carcinoma. This cancer begins in the lobes and lobules. Lobular carcinoma is broken down into the more specific stages of cancer such as lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (IVC).
Cancer localized to the breast lobule is called a lobular carcinoma in situ (LCIS) while cancerous cells that have spread from the lobules is called invasive lobular carcinoma (ILC) (7, 10, 12, 13-15 table 1.1).

<table>
<thead>
<tr>
<th>Type of breast cancer</th>
<th>Prevalence</th>
<th>Cancer site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal carcinoma</td>
<td>73%</td>
<td>Milk or breast ducts</td>
</tr>
<tr>
<td>Ductal carcinoma in situ (DCIS)</td>
<td>15%</td>
<td>Cancer grows only on the lining of milk ducts.</td>
</tr>
<tr>
<td>Invasive ductal carcinoma (IDC)</td>
<td>65-85%</td>
<td>Cancer grows on milk ducts and spreads to breast tissue.</td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>12%</td>
<td>Lobes or lobules of breast</td>
</tr>
<tr>
<td>Lobular carcinoma in situ (LCIS)</td>
<td>Unknown</td>
<td>Cancer grows only in lobes of breast.</td>
</tr>
<tr>
<td>Invasive lobular carcinoma (ILC)</td>
<td>10-15%</td>
<td>Cancer grows in lobes and spreads to breast tissue.</td>
</tr>
</tbody>
</table>

Table 1.1 Classification of common breast cancers. Prevalence percentages are approximate thus do not add to 100% (7, 10, 12).

IV. Stroma-epithelial interactions in breast carcinoma

Breast carcinomas develop within a complex microenvironment containing normal cells. Some of the normal cells in the microenvironment include epithelial, immune, inflammatory, endothelial, adipocytes, fibroblasts, and bone marrow-derived cells (13-15, figure 1.2). The microenvironment allows for the growth and progression of tumors.
Figure 1.2 Microenvironment of breast cancer. Some of the cell types present include fibroblasts, myofibroblasts, neoplastic epithelial cells, immune cells, endothelial cells, and mesenchymal stem cells. In the microenvironment, the origins of the cancer begin from the epithelial with the aid of other cells such as myofibroblasts, stroma, and macrophages to continue growth and invasiveness of the tumor. Figure reproduced with permission from reference 14.

A. How epithelial cancer originates?

Tumor and cancer are two different terms that have very distinct meanings and need to be defined here. Tumor refers to a mass of cells that can be malignant or benign and cancer refers to a mass of malignant cells (7).

1. Genetic factors

The accumulation of genetic and epigenetic changes causes the development of a carcinoma cell (13-20). The accumulation of mutations occurs at oncogenes and tumor
suppressor genes (16-20). Mutations at these sites cause disruption of growth and cell
regulation circuitry, which results in uncontrolled cell proliferation and tumor formation.

2. Epigenetic factors

The epigenetic mechanisms also influence cancer growth. The epigenetic mechanisms include covalent histone modification such as methylation, phosphorylation, SUMOylation, ubiquitination, acetylation, and neddylation. The addition of a covalent modification can cause the increased expression of proto-oncogenes or reduced expression of tumor suppressor genes. In addition, epigenetic changes can effect CpG islands of chromatin by methylation of cytosine. The result of the CpG methylation is the silencing of tumor suppressor genes (21-24).

3. Two-hit theory

One theory of how cancer develops is the two-hit theory. The theory states that two mutations are necessary for the occurrence of cancer. The theory pertains to familial cancer or cancers that are heritable such as retinoblastoma and breast cancer caused from BRCA 1 or 2 in which inactivation of both alleles of a tumor suppressor must occur. The two-hit theory is not applicable to all types of cancer, but all cancers of epithelial origin can be described with the driver theory (16-19, 25).

4. Driver theory

The driver theory describes the origin of carcinogenesis in epithelial cells. Epithelial cells start as a homogenous population. Any cell can acquire a mutation at
random at some set frequency. This initial mutation is a driver mutation if the mutation affects the rate of cell growth or cell division. A passenger mutation is mutation that does not enhance the phenotype of the cancer (16-20).

There are differences between normal and cancerous cells in accumulating mutations, but the accumulation of a driver mutation causes tumor formation. The mutated cell in the epithelial cell population creates a heterogeneous population. The mutation allows proliferation of the cell containing the mutation. An example is the ability to sustain growth without exogenous growth factors. Once the mutated epithelial cells have demonstrated some preferential advantage, then their progeny are able to continue to grow and proliferate at a faster rate than the surrounding normal cells. As the mutated cell undergoes more cell proliferation, additional mutations called passenger mutation(s) occur and accumulate (16-20).

The next step is clonal expansion of the mutated cells. The clonal expansion phase allows the tumor to grow and penetrate across the basement membrane. Once the tumor penetrates the basement membrane, then the cancerous growth invades into the surrounding stromal cells (16-20). The invasion of epithelia cells into surrounding tissues depends on the development of a complex microenvironment, in which a variety of cells aid in the tumor growth.

**B. Stroma-epithelia microenvironment**

The microenvironment of a tumor is composed of a complex variety of cells that aid in tumor formation. Some of the cells present in the microenvironment include
epithelial, stoma, macrophages, and fibroblasts, and their growth is aided by paracrine factors.

1. **Fibroblasts, stromal, and macrophages**

Stromal, macrophages, and fibroblasts aid in tumor growth. The roles of stromal cells in normal breast tissue are to aid in duct formation and regulate the responsiveness of epithelial cells to the hormones estrogen and progesterone. In addition, in normal breast tissue, stromal cells control the polarity of epithelial cells (10). The loss of epithelial cell polarity leads to an increase in cell proliferation and tumorigenesis (10, 13-15, figure 1.2).

In a tumor, the fibroblast cells change into myofibroblast cells. Myofibroblast cells aid growth of epithelial cells by providing growth factors and cytokines. There are three ways to transform fibroblast cells into myofibroblast cells. The first is transformation of fibroblasts to myofibroblasts in tumor stroma. The second is the use of progenitor cells like fibrocytes and bone-marrow-derived mesenchymal stem cells (MSCs) to create myofibroblasts. A third way to create myofibroblasts is by the differentiation of other cell types such as epithelial, endothelial, adipocyte, or pericyte into a myofibroblast (13-15, figure 1.2).

Another cell type in the tumor microenvironment is macrophages. A major role of the macrophages is to increase tumor invasiveness through TNF-α dependent up-regulation of matrix metalloproteinase (MMPs). In addition, macrophages induce cyclooxygenase-2 (COX-2) expression through interleukin 1 – beta (IL-1β) signaling. The
tumor-associated macrophages increase the tumor invasiveness and metastatic abilities of epithelial cells through activation of EFGR signaling in the epithelia cells (13-15). The growth of the epithelial cells also relies on paracrine roles of stroma, fibroblast, and macrophage cells to grow and expand (13-15, figure 1.2).

2. **Paracrine signaling**

Paracrine signaling describes cell-to-cell communication using secreteable signaling molecules to influence surrounding cells. The binding of a secreted factor to its cognate receptor will initiate signaling pathways in the surrounding cells (9, 10, 13-15). The paracrine functions of stromal cells, fibroblasts, and macrophages aid the growth and invasiveness of epithelial cells. Stromal cells use paracrine signaling to create a microenvironment that provides an abundance of growth factors and cytokines which help the growth of the tumor. Myofibroblasts secrete stromal derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF-A), and MMPs to the epithelial cells. Secretions of these factors promote cell proliferation, angiogenesis, extracellular degradation, and inflammation (15). Macrophages use paracrine signaling to secrete epidermal growth factor (EGF) for cell invasion by epithelia carcinoma cells and the epithelia cells secrete colony stimulating factor 1 (CSF-1) to increase expression of the growth factor EGF by macrophages (9). Paracrine signaling is an integral aspect of tumor growth.

V. **Molecular subtyping of breast carcinoma**
The molecular subtyping of breast cancer allows cancer to be characterized by the presence or the absence of hormone and growth factor receptors (7, 11).

<table>
<thead>
<tr>
<th>Type of breast cancer</th>
<th>Molecular subtypes</th>
<th>Prevalence</th>
<th>Age Range of Diagnose</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A subtype</td>
<td>ER+, PR+, and HER2/neu-</td>
<td>62%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B subtype</td>
<td>ER+, PR+, and HER2/neu+</td>
<td>50%</td>
<td>50-54</td>
<td></td>
</tr>
<tr>
<td>Her-2 positive</td>
<td>ER-, PR-, and HER2/neu+</td>
<td>12%</td>
<td>50-54</td>
<td></td>
</tr>
<tr>
<td>Basal-like (triple negative)</td>
<td>ER-, PR-, HER2/neu-, CK5/6+, and EGFR+</td>
<td>10%</td>
<td>47-70</td>
<td>More prevalent in African Americans</td>
</tr>
<tr>
<td>Normal breast-like</td>
<td>ER-, PR-, and HER2/neu-</td>
<td>21%</td>
<td>20-39</td>
<td></td>
</tr>
<tr>
<td>Inflammatory breast cancer (IBC)</td>
<td>ER-, PR-, and HER2/neu-/+</td>
<td>21%</td>
<td>43-72</td>
<td>More prevalent in African Americans</td>
</tr>
</tbody>
</table>

Table 1.2 Molecular subtypes of breast cancer (6, 7, 12, 26-33). -/+ indicates some patients present with receptor while other patients do no present with receptor.

A. Molecular markers

The three frequently used molecular markers for breast cancer diagnoses are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) also known as neu (7). The importance of these molecular markers is due to their roles in promoting proliferation of breast cancer cells (7, table 1.2). Each molecular marker has a complex role in normal breast biology and in breast cancer.

B. Luminal breast cancer
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Luminal breast cancers are positive for estrogen and progesterone receptors. Luminal breast cancer has two distinct forms called type A and type B. Luminal type A is negative for the growth factor receptor HER-2 and luminal type B is positive for HER-2. Other differences between the type A and type B are that A has mutations in the phosphatidylinositol-3-OH kinase (PI3K) pathways with the TP53 tumor suppressor intact, while, in type B the TP53 tumor suppressor is mutated. Mutation of TP53 in type B may contribute to the aggressive nature of this cancer. Also, in luminal breast cancers there is the disruption of another tumor suppressor gene, retinoblastoma (RB1), which can occur in both types of luminal cancer, but is more prevalent in type B (6, 7, 26-28, table 1.2).

C. HER-2 positive

HER-2 positive breast cancer is negative for the hormone receptors estrogen and progesterone, while the growth factor receptor HER-2 is overexpressed in this subtype of breast cancer (6, 7, 26-28, table 1.2). In addition, in HER-2 positive breast cancer there are mutations in tumor suppressor genes TP53 and GATA3. The overexpression of the growth factor receptor and lack of tumor suppressors leads to a more aggressive cancer (28, 30).

D. Basal-like or Triple Negative (TN)

Basal-like or triple negative breast cancer is a type of cancer that is negative for ER, PR, and HER-2 (6, 7, 26-30, table 1.2). Triple negative breast cancers have mutations in the tumor suppressors TP53, RB1, and breast cancer type 1 susceptibility protein.
(BRCA 1), which is involved in DNA repair (28-30). The BRCA-deficient tumors in TN cancer rely on the error-prone non-homologous end joining (NHEJ) and single-strand annealing (SSA) for the repair of double strand breaks in DNA, rather than using homology-directed repair (HDR) (29). In addition, other diagnostic markers define the TN breast cancer. Some of the diagnostic markers include CK14, CK17, KIT, laminin, collagen type XVII, calponin I, and caveolin-1 and -2. The diagnosis of triple-negative cancer is not definitive. There are several subtypes of triple negative breast cancer, which vary in treatment and outcome. Diagnostic markers can start to describe the biology of the subtypes of the triple negative breast cancer (2, 26-30).

E. Normal breast-like cancer

Normal breast-like cancer is a subtype of triple negative breast cancer. The cancer lacks ER, PR, and HER-2. This cancer can be easily confused with triple negative breast cancer since the same molecular markers are used diagnose this cancer. However, the main difference between normal breast-like and triple negative cancer is the presence of cytokeratin (CK) 5/6 in the normal breast-like cancer (2, 25-30, table 1.2).

F. Inflammatory breast cancer (IBC)

Inflammatory breast cancer (IBC) is a rare, aggressive type of cancer that accounts for about 1 to 5 percent of all cases of breast cancer. This cancer affects younger women and is typically diagnosed in the metastatic state. Inflammatory breast cancer grows rapidly, due to the high percent of cells found in the S-phase, the synthesis
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phase, and the presence of mutations in p53. In addition, IBC lacks ER and PR; and HER-2 in IBC can be either present or absent (33, table 1.2).

VI. Standard treatments for breast cancer

Standard treatments for breast cancer are effective in treating early stage breast cancer and non-aggressive breast cancers. The treatments for early, non-aggressive breast cancer include surgery, radiation, chemotherapy, and hormone antagonist therapy (1, 4, 35). Each treatment provides a unique approach to killing the cancerous cells in the mammary tissue. Each treatment has benefits and side effects.

The types of treatments used for early and late breast cancer are different. As shown in figure 1.3, most of early stage breast cancer patients, underwent surgery to remove cancerous mammary tissue by breast-conserving surgery (BCS) or a full mastectomy. In addition to surgery, the patients underwent other treatments such as radiation or chemotherapy or both. The most common treatment for early stage breast cancer was to use BCS in combination with radiation, while the most common treatment for late stage breast cancer was to use a combination of a mastectomy, radiation, and chemotherapy (figure 1.3). The more advanced breast cancer patients use a combination of therapies to treat their cancer. Those therapies include surgery, radiation, hormone antagonist therapy, and chemotherapy (1, 4, 34).
Figure 1.3 Female breast cancer treatment patterns by stage from 2008; and BCS: breast-conserving surgery; RT, radiation therapy; chemo, chemotherapy (may include common targeted therapies). Percentage is the amount of the population of the data bases National Cancer Data Base (NCDB) and SEER-medicate linked and do not sum to 100% due to rounding. Figure reproduced with permission from reference 1.

A. Surgery

The standard procedure for treating early breast cancer is the use of surgery with or without radiation and chemotherapy. Breast conserving surgery (BCS) with radiation is a standard procedure for cancers with localized tumors less than 3-4 centimeters in size; typically these types of tumors are at either stage 1 or 2 breast cancer. Another surgical procedure is mastectomy, which removes the entire breast. After the mastectomy, the patient will undergo radiation, which reduces local reoccurrence of tumor growth (28, 35).

B. Chemotherapy

Chemotherapy is whole body exposure of chemical compounds to target actively growing cells. These compounds include anthracycline antibiotics, taxanes, alkylating
agents, anti-metabolites, anti-mitotic inhibitors, antibodies, and other drugs like etoposide and gemcitabine. The treatment of early stages breast cancer is successful with current chemotherapies, while late stages of breast cancer have not been as successful with current chemotherapies (4, 34, 35).

1. Anthracycline antibiotics

The most common chemotherapy for treating early stages of breast cancer includes anthracycline antibiotics. Anthracycline antibiotics come from the bacterium Streptomyces peucetius var. caesius (39). Some of the antibiotics include mitomycin, mitoxantrone, bisantrene, doxorubicin, epirubicin, and vinorelbine. The long-term usage of these anthracycline antibiotics can have side effects including cardiac dysfunction, induction of myelodysplasia, and acute leukemia. There are three general, but different modes of action for the antracycline antibiotics. They can disrupt DNA repair in the tumor by intercalating into DNA (reversible inclusion of a molecule between DNA base pairs), inhibit topoisomerases II, and produce free oxygen radicals that further damage DNA, proteins, and cell membrane (35). Some of the antibiotics act on other targets. Examples include doxorubicin and vinorelbine which act on p53 and MAPK signaling pathways (36-40).

2. Taxanes

Another commonly used group of chemical compounds for treating early stages of breast cancer is the taxanes. Taxanes include paclitaxel and docetaxel (4, 34). Taxane compounds were discovered in extracts from the Pacific Yew tree and are also
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synthesized (35). The taxane compounds are mitotic inhibitors that disrupt the microtubules essential for cellular division by stabilizing GDP-tubulin, causing a “frozen mitosis” (4, 34). Disruption of cell division leads to cell death.

3. Anti-mitotic agents

a. Vinca alkaloids

Anti-mitotic agents are compounds that disrupt mitosis and microtubules. Some of ant-mitotic agents include the vinca alkaloids and anti-mitotic inhibitors. The vinca alkaloids come from the Madagascar periwinkle (35). Vinca alkaloids include vincristine, vinorelbine, and vindesine. The mechanism for the vinca alkaloids is disrupting microtubule formation by disrupting tubulin polymerization and mitosis of the cell (34, 42, 43). Some of the side effects of vinca alkaloids include numbness or tingling in hands or feet, constipation, hair loss, lowered resistance to infection, bruising or bleeding, and anemia (35). The vinca alkaloids are just some of the anti-mitotic agents.

b. Anti-mitotic inhibitors

Anti-mitotic inhibitors act on specific proteins for polo and aurora kinases. The mechanism for how anti-mitotic inhibitors function is not well understood. The debate centers on whether the anti-mitotic compounds work solely on microtubule assembly or kinase checkpoints of mitosis or a combination of these.

Aurora kinases have three isoforms and are proteins A, B, C. Each Aurora kinase has a specific role in mitosis, with Aurora-A required for spindle assembly and Aurora-B
required to phosphorylate histone H3, and causing chromosome segregation and cytokinesis (41, 44). Some of the inhibitors of aurora kinases include tozasertib, PHA-739358, AS703569, AT9283, SNS-314, PF-03814735, MLN8054, MLN8237, and AZD1152 (45).

Polo-like kinases have four isoforms and are Plk1, Plk2, Plk3, and Plk4. Plk1 has mitotic roles in centrosome maturation, spindle assembly, chromosome segregation, cytokinesis, activation of the APC/C, and activation of the kinase cdk1 (44). The inhibitors of polo-like kinase include BI 2536, BI 6727, GSK461364, ON 019190.Na, HMN-214, ZK-thiazolidinone, NMS-1, CYC-800, DAP-81, and LC-445 (46).

These anti-mitotic inhibitors have been tested in clinical trials. In general, these inhibitors are not as effective in disrupting mitotic functions of cancerous cells in patients when compared to the taxanes and vinca alkaloids (41, 44).

4. Alkylating and Alkylating-like agents

Alkylating and Alkylating-like agents are treatments that add an alky group to guanine base at the nitrogen at position seventh in DNA. The first alkylating agent was nitrogen mustard and used in chemical warfare as mustard gas in World War II. Other mustard compounds applied at lower doses to treat breast cancer. These include ifosfamide, cyclophosphamide, melphalan, thiotepa, and chlorambucil. These alkylating agents disrupt tumor growth by inhibiting DNA replication and crosslinking guanine bases. The effects of alkylating agents are non-specific as they disrupt DNA replication in both cancerous and healthy cells, leading to toxicity issues. The side effects of alkylating
agents include a reduction of red and white blood cells, and platelets, and cause hemorrhagic cysts in ovary (34, 47).

Alkylating-like agents are platinum-containing compounds such as cisplatin and carboplatin (34, 47). These alkylating-like agents can disrupt DNA replication by crosslinking platinum to the guanine on opposite DNA strands via the nitrogen at position seven (47). The alkylating and alkylating-like agents have shortcomings in treating cancer since the presence of the enzyme O-methyl-guanine-DNA methyltransferase (MGMT) is required. The cross-linking of DNA is inhibited by MGMT. Epigenetic silencing of the MGMT promoter limits production of the enzyme, and can result in the tumor becoming more responsive to alkylating agents (48). The complex relationship between MGMT and the alkylating and alkylating-like agents can complicate treatment and limit the effectiveness of the treatments.

5. Anti-metabolites

Most of the anti-metabolites disrupt the synthesis of nucleic acids by competing with a normal substrate for a required enzyme. Anti-metabolite compounds include methotrexate and 5-fluorouracil (5-FU). In a cell, the enzyme converts dihydrofolic acid (folic acid) to tetrahydrofolic acid, which is required for purine and pyrimidine biosynthesis. The antimetabolite methotrexate binds dihydrofolate reductase (DHFR) prevents synthesis of essential DNA building blocks. Methotrexate bound to DHFR is highly toxic, teratogenic, and immunosuppressive. In addition, methotrexate may cause
other side effects such as ulcers, decreased white blood cell count, infections, and
dizziness.

Thymidylate synthetase is required for a conversion of dUMP to dTMP, which is
phosphorylated to dTTP and used for DNA synthesis. Anti-metabolite 5-FU irreversibly
binds thymidylate synthetase to inhibit pyrimidine biosynthesis. The therapeutic use of
5-fluorouracil is limited since the compound is highly toxic (34).

6. Etoposide

Etoposide is a topoisomerase inhibitor. This compound came from
Podophyllotoxin, a plant alkaloid. Etoposide disrupts DNA replication by forming a
ternary complex with DNA and topoisomerase II, which causes the DNA to break and
subsequently the cell to die. Some of the side effects of etoposide include hair loss,
constipation, and immunosuppression of bone marrow and white and red blood cells
and platelets (49).

7. Gemcitabine

Gemcitabine is a nucleoside analog that can replace cytidine during DNA
replication. The result of inserting this analogy is replication arrest, which leads to
inhibition of all cell growth including tumors. Some of the side effects of gemcitabine
include fever, fatigue, vomiting, flu-like symptoms, hair loss, mouth sores, and poor
appetite (50).

8. Antibodies
A new approach in targeting breast cancer is the use of monoclonal antibodies to disrupt specific cellular and molecular functions. Some of the antibodies in current use in clinical trials include Trastuzumab and Pertuzumab. Both antibodies target the growth factor receptor, HER-2 (51, 52). The mechanism, by which trastuzumab binds HER’s-2, causes an up regulation of p27, a protein that inhibits cell proliferation. The effects of trastuzumab are limited to HER-2 positive breast cancer since the cancer has an overexpression of the HER-2 receptor. The side effects of trastuzumab include diarrhea and palmar-plantar erythrodysesthesia (51, 52).

Pertuzumab, a very new antibody just approved for clinical trials, is the first agent to be termed as a "HER dimerization inhibitor". The mechanism by which pertuzumab acts is though binding HER-2 receptor and blocking HER-2 dependent signaling to cause a cessation of tumor growth (52).

VII. Hormone antagonist therapy

Another approach to treating estrogen receptor positive breast cancer is hormone antagonist therapy (34, 56). About 70% of breast cancers are estrogen positive breast cancer. Hormone antagonist therapy takes advantage of cells responding to estrogen signaling and targets a majority of breast cancer. Examples of hormone antagonist therapy include tamoxifen and other selective estrogen receptor modulator (SERM) (56).

A. Estrogen Signaling
The estrogen receptors (ERs) are members of the nuclear receptor superfamily. There are two forms: ER-α and ER-β (34, 56). ER-α and ER-β have distinct functions and tissue expression. ER-α induces proliferation and is found predominantly in breast epithelial cells, while ER-β inhibits proliferation in breast epithelial and stromal cells (56-58). Both ER-α and ER-β induce cellular changes through genomic and non-genomic effect (34, 56-58, 59).

The classic model of estrogen signaling begins at the nucleus. After estrogen diffuses across the plasma membrane, it binds to either ER-α or ER-β in the cytoplasm. The complex of estrogen bound to the ERs will enter the nucleus and recruit adaptor proteins. The adaptor proteins (including the activator protein (AP1) or the specificity protein (SP1)), interact with specific DNA sequences, referred to as estrogen response elements (EREs). This recruits the co-regulatory proteins that can be activators or repressors of the Pol-II dependent transcription (57, figure 1.4).

Non-genomic estrogen responses occur at the plasma membrane through binding of estrogen to plasma membrane-localized ERs. This activates several signaling pathways including tyrosine kinase, Ca2+, phosphoinositide 3-kinase (PI3K), nitric oxide (NO), and mitogen-activated protein kinase (MAPK) pathways. Activating these pathways may affect global transcription (57, 60, figure 1.4).
Figure 1.4 Estrogen signaling pathways at the plasma membrane. i) classical pathway, ii) non-genomic estrogen signaling pathway, and iii) non-ER membrane-associated estrogen-binding proteins (EBPs), which thought to initiate cellular responses. See text for more details. Figure reproduced with permission from reference 57.

B. Selective estrogen receptor modulator (SERM)

The selective estrogen receptor modulator (SERM) compounds have both classical and non-genomic modes of action. The SERM compounds can act as both agonist and antagonist. The SERM compounds effect multiple organs that include bone, uterine, ovaries, brain, and pituitary gland. The complex of estrogen-estrogen receptors bind adaptor proteins at an ERE and become activator or inhibitors based on the co-regulatory proteins that binds. Estrogen receptors adopt unique conformations make them activating or inhibitor as induced by the co-regulatory proteins. For example, a co-activator like SRC1 will bind to the agonist form of the estrogen receptor to activate
transcription, while the co-repressor like SMRT will bind to the antagonist form of the estrogen receptor to inhibit transcription (57, figure 1.5).

**Figure 1.5** SERMs as agonist and antagonist. The SERM binds the estrogen receptors (ERs) and competes with estrogen for the receptors. Once the SERMs binds the ERs there is a recruitment of co-regulator proteins that can be either activators or inhibitors. The recruitment of co-activator to the SERM-ER complex will cause the agonist form of the ER and result in transcription, while the recruitment of an inhibitor will cause the antagonist form of the ER and result in no transcription. Figure reproduced with permission from reference 57.

**C. Tamoxifen**

Tamoxifen is a SERM used to treat estrogen receptor positive breast cancer. The compound’s anti-estrogenic properties work by disrupting both the genomic and non-genomic estrogen signaling pathways. Tamoxifen competes with estrogen for binding to the estrogen receptors at the plasma membrane (57). This result in a decreased estrogen response since tamoxifen binds to the estrogen receptors and inhibits estrogen signaling. Long-term use of tamoxifen can be deleterious because of side effects, which
may result from tissues deprived of estrogen. The side effects include the development of venous thromboembolism, increased risk for endometrial cancer, and earlier onset of menopause (56, 61, 62).

VIII. Other treatments for breast cancer

A. Aromatase inhibitors

Another treatment for breast cancer is aromatase inhibitors. The aromatase inhibitors disrupt estrogen production by blocking the enzyme encoded by the gene CYP19, which controls the aromatization step in the production of estrogen. Aromatase inhibitors such as anastrazole, letrozole, or exemestane are effective in treating early stages of breast cancer, which are induced to grow by the presence of estrogen. Patients taking aromatase inhibitors have lower incidences of venous thromboembolism and endometrial cancer than those who are taking tamoxifen. Some of the side effects of aromatase inhibitors include increase bone fractures and joint pain (34, 56, 62, 63).

B. Poly (ADP-ribose) polymerase (PARP) inhibitor

Another therapy to use in treating breast cancer is the poly (ADP-ribose) polymerase (PARP) inhibitor. The PARP inhibitors disrupt homologous recombination and are typically used in breast cancer with BRCA 1 or 2 mutations (34). Clinical trials of the PARP inhibitors AZD2281 and ABT-888 have been successful in inducing DNA-damage more effectively than any other chemotherapeutic drugs, such as cisplatin and gemcitabine. PARP inhibitors have lower toxicity and milder side effects than tamoxifen
and aromatase inhibitors. Side effects of PARP inhibitors include fatigue, nausea, vomiting, and anemia (34, 56).

IX. Standard treatments fail in more aggressive breast cancers

While the original cancer cells due to respond to tamoxifen and aromatase inhibitors, resistance to these drugs develops over time (4, 56). The growth of the cancer cell is no longer inhibited and metastases develop. There are subtypes of breast cancer that are more aggressive and more frequently diagnosed in the metastatic state, including triple negative and tamoxifen-resistant. The development of resistance to treatment occurs when cellular signaling pathways are altered in the cancerous cells. Resistance to a compound occurs after the tumor is treated. The mechanism for how resistance develops can be specific for the cancer (64).

A. Tamoxifen-resistant breast cancer

For the past twenty years, tamoxifen (TAM) has been the gold standard of hormone antagonist therapy in estrogen receptor positive breast cancer. The development of resistance occurs after the treatment of tamoxifen in women with estrogen receptor positive breast cancer. This type of cancer is prevalent in post-menopausal women. In this type of cancer, the estrogen receptor is present even after the development of resistance to tamoxifen, and the estrogen receptors (ERs) aid in the resistance mechanism. The development of resistance is a complex mechanism that utilizes growth factor receptors and estrogen receptors. After the treatment of tamoxifen, the TAM binds to the ERs causing an increase in the crosstalk between the
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ERs and the growth factor receptors, HER-2 and epidermal growth factor receptor (EGFR). This interaction between the ERs and the growth factor receptor overrides the effects of tamoxifen and allows tumor growth. As a result, tamoxifen-resistance can make treating this type of breast cancer extremely difficult and allow the cancer to progress into advanced, metastatic breast cancer (MBC) (9, 61-65).

X. SUMMARY

Current treatments are not effective on the later stages of breast cancer (1). The more aggressive, metastatic, breast cancers (tamoxifen-resistant and triple negative), are a growing problem (1, 6, 64). The triple negative breast cancer affects younger women and African-Americans in the US, while the tamoxifen-resistant breast cancer affects older post-menopausal women (1, 6, 64, 67). Women who present with triple negative cancer have larger tumors and high recurrence rates, which shorten the median survival rate when compared to cancers that are positive for estrogen and progesterone receptors (68).

A more effective way to target cancer would require the identification of new proteins that are markers for resistance and metastatic breast cancer, and new tools to destroy those cells (7, 11, 26, 27, 33, 51, 64, 66, 67). When thinking of future markers, there is a need to understand that breast cancer is a complex disease with an inconsistent classification system. Cancers are classified based on anatomical position of the tumor and/or the molecular composition of the tumor (1-5, 10). The classification dictates the choice of treatment for the cancer (2-5, 10). In the next chapter, I will
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discuss a possible therapeutic target for the more aggressive breast cancers by using the caveolin proteins, previously identified but not well characterized as molecular marker for breast cancer.
Chapter 2: Potential use of caveolin-1 (cav-1) in breast cancer

I. Properties of the caveolin-1 protein

Caveolins (cav) are transmembrane proteins with unique structural and functional properties. The role of caveolin protein in the membrane is to provide a platform for several signaling pathways. The unique properties of the caveolin proteins suggest a potential therapeutic target for more aggressive breast cancers such as triple negative and tamoxifen-resistant (69-73).

A. Basic biology of caveolin proteins

The caveolin proteins are present in both mammary epithelial and stromal cells (2, 3). The basic functions and structure of the domains will begin to describe how the caveolin protein can act as tumor suppressor in breast cancer. There are three forms of caveolin proteins called caveolin-1, -2, and -3. Functions of caveolin proteins will be discussed below.

B. Structure of caveolin proteins

The caveolin proteins are a unique transmembrane protein. Most transmembrane proteins span the membrane with protein domains on both cytoplasmic and extracellular faces. The caveolin proteins enter and exit only the cytoplasmic face of the plasma membrane. The helix-break-helix motif in caveolin forms a horseshoe loop structure, which not only keeps both termini on the same face of the membrane, but gives rise to curvature of the caveolin proteins (69-77, figure 2.1).
II. Functional domains of caveolin-1 protein

The caveolin-1 protein has multiple domains that include the oligomerization domain, the amino-terminal membrane attachment domain (N-MAD) also known as the caveolin scaffolding domain (CSD), the transmembrane domain, and the carboxyl-terminal membrane attachment domain (C-MAD) (74, 78, figure 2.2).
Figure 2.2 Figure shows the structural domains of a dimer of caveolin protein inserted in the plasma membrane. Text will discuss individual domains in more detail. Figure reproduced from reference 74 with permission.

A. Oligomerization domain

The oligomerization domain spans residues 61 to 101. The caveolin proteins create oligomers of 14-16 caveolin-1 molecules in the Golgi apparatus (73, 74). This complex translocates from the trans-Golgi to the plasma membrane. The oligomers of caveolin-1 and caveolin-2 form homo- and hetero-oligomers and self-associate via carboxyl terminal interactions to form a larger network of caveolin proteins (not shown) (78-80).

B. N-MAD/caveolae scaffolding domain (CSD)
The twenty amino acid from residues 81 to 101 comprise the amino terminal membrane attachment domain (N-MAD) also known as the caveolae scaffolding domain (CSD). A binding motif of aromatic amino acids, named the caveolin binding motif (CBM), mediates protein-protein interaction between the caveolin-1 and signaling proteins that contain one of these sequences ΩxΩxxxΩxxΩ, ΩxxxΩxxΩ, or ΩxΩxxxxΩxxΩ where Ω is phenylalanine, tyrosine, or tryptophan residue and x being any other amino acid residue (68, 73, 74, 83, 84, 92, 93, figure 2.2).

**C. Transmembrane domain**

The 32 hydrophobic amino acids from residues 102 to 134 comprise the transmembrane domain. This domain initiates caveolae assembly in the endoplasmic reticulum (ER) machinery (76, 77, 81). The two alpha helices that comprise the helix-break-helix motif in addition to this transmembrane domain form the unique horseshoe loop structure that causes the two termini to face the cytoplasm.

**D. C-MAD**

The 15 amino acids from 135 to 150 comprise the carboxyl terminal membrane attachment domain (C-MAD). The C-MAD domain contains the sequence for directing caveolin proteins to the cis-Golgi apparatus for further sorting and packing (74, figure 2.2).

**E. Palmitoyl groups**
Caveolin proteins are post-translationally modified to add fatty acids on cysteine residues in the C-MAD domain (see figure 2.2). These help target the caveolin oligomers to the plasma membrane by increasing the hydrophobicity of the protein (73, 74, figure 2.2).

III. Caveolin gene family

There are three genes in the caveolin family, known as caveolin 1, 2, and 3. Caveolin-3 is only expressed in smooth muscle cells. Caveolin-1 and -2 are expressed in a variety of cells including endothelial cells, adipocytes, fibroblasts, stroma, cancer cell lines Ishikawa, cultured human endometrial cancer cells (CHEC), MCF-7/TAMR, and MDA-MB-231 (64, 70-73, 82, 83).

There are three separate genes encoding the caveolin 1, 2, and 3 proteins (73). Genes for cav-1 and cav-2 are on chromosome 7 at the q31.1 region and adjacent to the D7S522 locus, while cav-3 is on chromosome 3. Figure 2.3 is a schematic representation of the relative positions at the caveolin gene locus for chromosome 7 (23).

Caveolin-1 has two isoforms called α and β. The α isoform contains residues 1-178. The β isoform contains residues 32-178 and results from initiating translation at methionine at position 32. Caveolin-2 has three isoforms called α, β, and γ. The caveolin-2 α isoform is full length, while the β and γ isoforms are truncated variants from alternative splicing (see figure 2.3). The specific function of each isoform is neither known nor fully understood (74).
Figure 2.3 Organization of caveolin genes and proteins. The isoforms for each caveolin-1 protein are indicated as well as size of the isoform.

IV. Biology of caveolae lipid rafts
Caveolae lipid rafts are comprised of caveolin 1 and 2 proteins plus lipids concentrated in specialized areas of the plasma membrane. Caveolae lipid rafts occupy a space on the plasma membrane over a distance of 50 to 100 nanometers (69-73). The caveolin oligomers create 4 to 6 nm spheres of caveolin-1 homo-oligomers. These spheres interact to form nonlinear polymers of about 25 nm. The larger particles interact with stabilizers, such as glycosphingolipids and cholesterol, to generate the caveolae lipid raft (73, 74). The exact mechanism for assembly is not known (69-80).

A. Composition of caveolae lipid rafts

1. Cholesterol

Cholesterol is a structural component of lipid rafts and regulates caveolin-1 expression at the transcriptional level. Cholesterol negatively regulates SREBP-1 (steroid regulatory binding elements). The absence of the negative transcription factor SREBP-1 allows cav-1 to be transcribed. Thus, an increase in cholesterol leads to increase in the expression of caveolin-1 (74-79, 84, 85). In the next chapter, we will show ways to take advantage of this correlation in the treatment of cancer.

2. Glycosphingolipids and glycosylophosphatidylinositol-anchored proteins

Caveolae contain glycosphingolipids (sphingomyleins and gangliosides) and other lipids including ceramide, diacylglycerol, and phosphatidylinositol diphosphate. The lipid components of caveolae lipid rafts can interact with signaling proteins. The relatively
high concentration of signaling lipids in the caveolae lipid rafts help regulate signaling (71-75, 83, 84).

3. Signaling proteins

The “caveolae signaling hypothesis” proposes that signaling molecules are held in an inactive state at the cytoplasmic face of the plasma membrane via protein-protein interactions. These interactions occur between caveolin CSD and the hydrophobic sequence CBM of the signaling molecules. The CBM sequence adopts a variety of structures depending on the binding protein partner. The structural flexibility of this consensus sequence can potentially explain how the caveolin proteins can discriminate between signaling proteins. Signaling molecules become active once released from caveolin. Thus binding to caveolin proteins negatively regulates the signaling partners. These signaling proteins include receptors, small, and large molecules, including epidermal growth factor (EGF), EGFR, platelet derived growth factor (PDGF), tyrosine kinases, H-Ras, Patched, and estrogen receptor (71-74, 83, 84, 86, 88-92, 93, 94, figure 2.4).

Figure 2.4 Figure showw the organization of a typical caveolae lipid rafts. It demonstrates interactions between caveolin proteins and cholesterol, and their relationships with other components including signaling partners and receptors. Figure reproduced from reference 74 with permission.
V. Functions of caveolae lipid rafts

The caveolae lipid rafts are involved in potocytosis, cholesterol trafficking, and signal transduction. They can transport cholesterol, signal molecules, and receptors (77, 78).

A. Potocytosis

Potocytosis is a receptor-mediated endocytosis that transports small molecules across the plasma membrane by the caveolae lipid rafts. Potocytosis differs from endocytosis by the use of caveolae lipid rafts instead of clathrin-coated vesicles. The use of potocytosis establishes temporal and spatial control of small molecule transported across the plasma membrane, and enables small molecules to be delivered quickly into the cytoplasm. A well-known example of this is folate (23, 74, 84, 85).

B. Cholesterol trafficking

Cholesterol is a vital structural component of the caveolae lipid rafts, which contain 20% more cholesterol than the plasma membrane. In addition, cholesterol is a circulating component in blood. The caveolae lipid rafts capture free cholesterol from the blood or the plasma membrane and release it into the cytoplasm. The exact mechanism of this pathway is not entirely mapped out, but it is thought to use a complicated cycle called the Caveolae to Endoplasmic Reticulum to Golgi Apparatus (CERGA) pathway. The CERGA pathway recycles the caveolae vesicles from the plasma membrane to the endoplasmic reticulum and then to the Golgi apparatus. The
oligomers of the caveolin proteins are transported back to the plasma membrane to form caveolae lipid rafts (74, 84, 85, figure 2.5).

Figure 2.5 Caveolae to Endoplasmic Reticulum to Golgi Apparatus (CERGA) pathway. The recycling pathway of cholesterol and caveolin proteins from the caveolae lipid rafts at the plasma membrane to the endoplasmic reticulum, then to the Golgi apparatus. Cholesterol is exocytosed as HDL particles, while the caveolin proteins are recycled thru the Golgi apparatus to the plasma membrane. Caveolin proteins are transported back to the plasma membrane. Cholesterol molecules represented by blue triangles, while caveolin are red arches.

As represented in figure 2.5 caveolae lipid rafts remove “free” cholesterol from the cell exterior and transport it to the trans-Golgi network. The cholesterol is converted to high-density lipoprotein (HDL) particles, which are exocytosed from the plasma membrane to circulate in the blood. Thus, caveolae regulate extracellular trafficking of cholesterol (84, 85, figure 2.5).
Chapter 2 Potential use of caveolin-1 (cav-1) in breast cancer

C. Signal transduction

Control of signal transduction is a major role of caveolae lipid rafts. The caveolae scaffolding domain (CSD) and caveolae binding motif (CBM) play a role in signaling (74, 84, 85). Caveolae lipid rafts interact with specific proteins carrying an unique hydrophobic sequence (see page 31). Some of the proteins include epidermal growth factor receptor (EGFR), Fas, Ras-MAP kinase, Fyn, Akt, and Src (23, 70-72, 86-91, figure 2.4). The interactions with pathways will be explained in further detail below.

VI. Lack of caveolin proteins

When caveolae lipid rafts are disrupted either due to absence of caveolin or excess of cholesterol altered intracellular signaling will occur. The functional consequence of losing caveolin proteins has been examined in both cell culture and whole animal models. In cell culture work vesicular trafficking is disrupted. In whole animal models this can result in development of specific disease phenotypes including
diabetes and more aggressive tumors in mice (73, 83, 84, 92, 93).

**Figure 2.6** Disruption of signaling in caveolae lipid rafts shows no interaction between signaling partners and caveolin-1 and -2 proteins. Figure reproduced from reference 74 with permission.

**A. Cell culture models**

Caveolae lipid rafts act as a signaling conduit for many pathways to regulate cellular growth, immune response, and apoptosis (74). Caveolin binds signaling proteins and holds them in an inactive state at the plasma membrane. The loss of caveolin proteins leads to disruption of caveolae lipid rafts and results in constitutive (or absence of) signaling by the binding partners, since they are no longer tethered (11, 71-79, 83, 84, 92, 93). Tissue culture cell lines examined include 3T3-L1 adipocytes, mouse embryonic fibroblast (MEFs), and Chinese hamster ovary (CHO)-K2. The cell work has shown the role signaling for the caveolin proteins.

**1. Lipid homeostasis**

The 3T3-L1 adipocytes and CHO-K2 cell work indicate that caveolin proteins are crucial in the transportation of cholesterol and fatty acids from the Golgi apparatus to the plasma membrane. Loss of caveolin-1 causes cholesterol and fatty acids to accumulate in the Golgi apparatus and is not transported to the plasma membrane (see figure 2.5).

**2. Insulin resistance**
Insulin receptor is the one example that is down regulated by caveolin protein depletion. Normally the insulin binds the insulin receptor, which is bound by caveolin protein and results in a stimulation of Akt and GSK-3 β pathways. When caveolin proteins are depleted insulin binds, but do not activate the Akt and GSK-3 β pathways, which results in the development of insulin resistance. The loss of caveolin-1 in mouse embryonic fibroblast (MEF) disrupts insulin signaling. A mutation in the hydrophobic sequence of the CBM of the insulin receptor causes insulin resistance (74, 83, 84).

**B. Whole animal models**

The whole animal models have demonstrated that caveolin proteins are not essential for life, but deficiency or elimination results in phenotypes. Caveolin deficient mice are viable, but demonstrate complications resulting from pulmonary fibrosis, hypertension, and cardiac hypertrophy (74, 83, 84). The caveolin deficient mice are fertile in the absence of caveolae lipid rafts because they do have functioning classic estrogen signaling pathway.

**1. Lipid regulation**

In healthy rodents on a high fat diet the lipids are broken down into cholesterol, free fatty acids, HDL, and LDL, which are eventually removed from the serum by vesicular trafficking. Lipid regulation in caveolin-1 deficient mice is altered. The caveolin-1 deficient mice have elevated fatty acids and triglycerides in their serum. The phenotype of the cav-1 deficient mice is exacerbated with high fat diets, since the
impaired vesicular trafficking eventually leads to the development of type II diabetes (74, 83, 84).

2. Tumor suppressor

The loss of caveolin protein is correlated with fast-growing, more aggressive tumors (2, 3, 93, 97, 98). Deletion in chromosome 7 including the q31 locus where cav-1 and -2 reside are observed in many cancer such as breast, prostate, ovarian, colon, and renal cell carcinomas (23, 74, 97). About 94% of the triple negative breast cancer patients lack caveolin-1, thus caveolin-1 can be considered a tumor suppressor in breast carcinogenesis (3, 11, 32, 91, 94-97).

a. Growth factors

Epidermal growth factor receptor (EGFR), interacts with caveolin-1 proteins at the plasma membrane. Normally, interactions between the growth factor receptors and caveolin-1 proteins are inhibitory and decrease signaling efficiency to the cytoplasm and nucleus. The loss of caveolin-1 allows for unchecked signaling from the growth factors. This overstimulation contributes to tumor initiation and growth (2, 3, 71-74, 83, 84, 90-93).

b. Estrogen receptors

Both ER-α and ER-β interact with caveolin-1 in the caveolae lipid rafts. Estrogen receptors are inhibited by caveolin-1 proteins and decrease the proliferative effects of
the hormone. The loss of caveolae lipid rafts results in decreased non-genomic estrogen signaling (11, 58, 59, 91, 95, 96).

**VII. Caveolin-1 in breast cancer**

The roles of caveolin-1 have been studied at length under normal cellular conditions. Recently caveolin-1 has come under scrutiny as a potential therapeutic target for breast cancer in part because it can act as a negative regulator of growth and tumorigenesis (2, 3, 91, 98).

**A. Caveolin-1 regulates its signaling partners**

A main role function for caveolae lipid rafts is regulation of signaling partners (11, 32, 74). Since the caveolin proteins bind and negatively regulate some of its binding partners can be thought of as a sink for signaling molecules tethering them in an inactive state at the plasma membrane (74). The caveolin-1 and-2 act as tumor suppressors and the lack of these proteins can lead to more aggressive subtypes of breast cancer, such as triple negative and tamoxifen-resistant breast cancer (2, 3, 8, 11, 59).

**B. Immunohistochemistry of TN breast cancer**

As shown in figure 2.7, the patients who have caveolin-1 present in biopsies from TN breast cancer tend to have good survival rates ten years after treatment. However, patient who lack caveolin-1 in biopsies from TN breast cancer have a lower survival rate. The major differences occur in the first 5-year survival rates: patients with triple
negative breast cancer patients who are caveolin-1 positive have a 75.5% survival rate, while the patients lacking caveolin-1 have a 10% survival rate. What is not obvious from the plot is that when the biopsy lacked caveolin-1 the breast cancer tended to be more metastatic and reoccur more readily than those with caveolin-1 (2, 3, 6, 11, 32, 59, 74, 98, 99, figure 2.7). Caveolin-1 is to be a tumor suppressor, at least in patients with triple negative breast cancer.

**Figure 2.7** The effect of caveolin on the outcome of breast cancer in terms of survival. Immunohistochemistry in tumors from triple negative breast cancer patients lack caveolin-1 in stroma cells and have a lower survival rate. Figure reproduced from reference 3 with permission.

**VIII. Loss of caveolin-1 lead to tumorigenesis**

The loss of caveolin-1 in either the stromal or the epithelial cells of breast tissue can lead to tumorigenesis. Immunohistochemistry from biopsies in TN breast cancer can indicate whether stromal and epithelial cells have or lack caveolin-1. However, no information is currently known on about the paracrine signaling is involved with
caveolin proteins. Instead, there is information on the effects of epigenetics on caveolin proteins (2, 3, 23, 98, figure 2.7).

Epigenetic silencing of the caveolin genes can promote tumorigenesis. In the caveolae lipid rafts, a unique mixture of lipids and proteins, includes the sterol regulatory element binding proteins (SREBPs) and see pages 33-34. The SREBPS are transcription factors that control cholesterol and fatty acid biosynthetic pathways as well as transcription of the caveolin-1 gene. Methylation of the DNA encoding SREBPS and of caveolin-1 genes leads to silencing these genes and increased tumor growth. The epigenetic silencing is seen in triple negative breast cancer (31, 93).

IX. Caveolin-1 as a target for treating more aggressive breast cancers

In aggressive breast cancers, the caveolae lipid rafts are altered and this promotes tumor growth. In tamoxifen-resistant breast tumor cells, lipid rafts condense creating fewer, but larger signaling platforms. This concentration allows for enhanced responses by the growth factor receptors, which thrive in this type of cancer (29, 32, 64, 65). Triple negative breast cancer develops a more aggressive cancer by suppressing caveolin proteins from breast tissue (2, 3, 7, 26, 27, 59, 98).

A. Tamoxifen-resistant breast cancer

About 70% of breast cancer is estrogen receptor (ER) positive and 40% of ER+ breast cancer develops resistance to the endocrine therapy, such as tamoxifen (64).

1. Develop of resistance
Resistance to tamoxifen develops in estrogen receptor positive breast cancer and are triggered by a complex set of molecular responses (9, 27, 91, 94). The exact mechanism of tamoxifen-resistance is not defined, but a TAM-resistant mechanism is proposed in figure 2.8.

**Figure 2.8** The molecular model for tamoxifen-resistant breast cancer. At the membrane, tamoxifen or estrogen binds estrogen receptors in caveolae lipid rafts to initiate a response that triggers complexes with growth factor signaling molecules (such as insulin-like growth factor (IGF)-R1, insulin receptor substrate (IRS)-1, or phosphatidylinositol-3-OH kinase (PI3K)). These activate mitogen activated protein kinase (MAPK). ER-α receptor activates Src and a cascade of activating matrix metalloproteinase (MMP)-2 to cleave heparin-binding epidermal growth factor (Hb-EGF) from the membrane to bind and activate epidermal growth factor receptor (EGFR). EGFR results in the ultimate activation of MAPK and AKT, which in turn can phosphorylate and activate nuclear ER-α amplifying (AIB)1. AIB1 in the mammary tissue induce growth and survival of the tumor. Figure reproduced from reference 61 with permission.

The mechanism of TAM-resistance is thought to use membrane estrogen non-genomic effects in caveolae lipid rafts (27, 91, 94). Estrogen and tamoxifen compete to bind ER-α at the plasma membrane, but binding by the ERs in the caveolae lipid rafts is necessary to initiate downstream signaling. Cells that respond to TAM trigger a complex cascade of growth factors that include insulin-like growth factor (IGF)-R1, insulin
Chapter 2 Potential use of caveolin-1 (cav-1) in breast cancer

receptor substrate (IRS)-1, or phosphatidylinositol-3-OH kinase (PI3K). These growth factors will initiate other signaling pathway such as mitogen activated protein kinase (MAPK) or AKT cascade (62, 64, 74, 95, 96 figure 2.8)

ER-α also activates Src kinase. The activation of Src begins a cascade for activating other proteins, such as matrix metalloproteinase (MMP)-2. These cleave heparin-binding epidermal growth factor (Hb-EGF) from the membrane to bind and activate epidermal growth factor receptor (EGFR). In the cascade of signaling molecules, the receptor EGFR activates MAPK and AKT causing a nuclear population of ER-α to be phosphorylated. The activation of the protein (AIB) 1 in the mammary tissue promotes tumorigenesis, growth, and survival even in the presence of tamoxifen the anti-estrogenic endocrine treatment for breast cancer (9, 30, figure 2.8).

2. TAM-resistance requires enact caveolae lipid rafts

TAM-resistant breast cancer develops through altering caveolae lipid rafts. Some of the cell culture studies use a human breast cancer cell line which has become resistant to tamoxifen (MCF-7/TAMR cells). Treatment of these cells by filipin or methyl-β-cyclodextrin can render the cells sensitive to tamoxifen and allowing cell death to occur. These cell culture studies are a model for re-sensitizing breast cancer to tamoxifen (61, 64).

3. Current therapy
Current therapy for tamoxifen-resistant breast cancer involves surgery, radiation, and chemotherapy. However, the current therapy protocols have not been effective in treating the cancer, even in conjunction with the relatively new drugs, such as aromatase inhibitors (62).

A proposed way of restoring tamoxifen’s antagonist activity on tumor growth starts with blocking the growth factor receptor signaling pathways. Tamoxifen (TAM) bound to ER-α in the membrane activates EGFR, which triggers the signaling cascade that leads to tamoxifen resistance. The blocking of EGFR activation would prevent the activation of the MAPK and AKT. As the nuclear pool of ER-α is unable to be phosphorylated, the protein AIB1 will not be amplified in mammary tissue. The EGFR signaling pathway block leads to tamoxifen being able to disrupt tumor growth. A potential way to treat TAM-resistant breast cancer would be disruption of the signaling pathway by using compounds like filipin. The mechanism for how filipin disrupts caveolae lipid will be discussed further in chapter 3 (61, figure 2.9).
Figure 2.9 Disruption of TAM-resistant breast cancer by blocking the growth factor receptors EGFR. Tamoxifen (T) binds ER-α in the membrane to activate EGFR, which activates the signaling cascade for tamoxifen resistance. However, if EGFR is blocked would then prevent activation of MAPK and AKT and the phosphorylation of the nuclear pool of ER-α to amplify AIB1 in mammary tissue. The end result would be restoration of tamoxifen’s antagonistic activity on tumor growth. Figure reproduced from reference 61 with permission.

B. Triple negative breast cancer

1. In-vivo work

The triple negative cell line (MDA-MB-231 cells) is a human breast cancer cell line that has caveolin-1. In nude mice, there a co-injection of MDA-MB-231 cells with either a strain of immortalized stromal fibroblasts that have or alternatively do not have caveolin-1 produces different outcomes. The co-injection of cav-1 deficient stroma fibroblast with MDA-MB-231 increased cancerous growth and angiogenesis. After 2 weeks, the tumors are large and visible for harvest. The tumors lacking cav-1 are heavier and larger in weight and volume, have an increased angiogenesis, and are up-regulated for the metabolic markers such as PKM2 and LDH. The lack of caveolin-1 leads to unchecked growth and expansion of the tumor in the nude mice (81).

2. Current therapy

The current therapy for triple negative breast cancer is to use surgery, radiation, and chemotherapy, which are not very effective in treating this cancer (2, 3, 6, 7,26, 27, 59, 60, 66, 67, 68, 98, 99). With more knowledge about the signaling pathways used by TN breast cancer more viable and effective treatments may be identified.
X. SUMMARY

Caveolin proteins are transmembrane proteins unique in regards to their structure and function. The proteins have two main functional roles; one is as a negative regulator of signaling and the other as a tumor suppressor. Together these make caveolin a potential drug target for breast cancer. The tamoxifen-resistant and triple negative breast cancers use caveolae lipid rafts to alter signaling. The TAM-resistant breast cancer concentrates the caveolae lipid rafts into larger platforms, while TN breast cancer silences the caveolin genes. In either case, both alter caveolae lipid rafts in order to propagate cancerous growths in the tissues. Since the caveolin proteins are an unexplored treatment target for breast cancer, there is room for possible new applications. Chapter 3 will discuss four possible compounds for TAM-resistant breast cancer and treatment using antibody-drug conjugates for TN breast cancer.
Chapter 3: Potential therapies for tamoxifen-resistant and triple negative breast cancer

The breast cancer subtypes tamoxifen (TAM)-resistant and triple negative use the caveolae lipid rafts and lipid raft signaling in different ways to promote tumor growth. The TAM-resistant breast cancer amplifies the lipid raft-signaling by condensing components into fewer, larger rafts (64, figure 3.1). Triple negative breast cancer lacks the caveolin-1 and leads to alter signaling due to lack of caveolae lipid rafts (2, 3, 6, 7, 11, 26, 27, 64, 66-68, 99, figure 3.2). Any potential caveolin-based therapy for breast cancer must address the unique biology that exists with the caveolae lipid rafts. Described below are compounds and therapies that have potential use as a treatment for the TAM-resistant and triple negative breast cancer.
Chapter 3: Potential therapies for tamoxifen-resistant and triple negative breast cancer

**Figure 3.1** Caveolin-1 as target for TAM-resistant breast cancer. Caveolae lipid rafts are used extensively in TAM-resistant breast cancer and can decrease caveolae lipid rafts by cholesterol destabilizing agents. In the figure the compounds are positioned at areas of the cell they affect with filipin at caveolae lipid rafts, okadic acids at the CERGA pathway, nystatin, amphotericin B, and cholesterol oxidase at the plasma membrane and caveolae lipid rafts.

**Figure 3.2** Caveolin-1 as a target for triple negative breast. In triple negative breast cancer caveolin-1 is not expressed. The re-expression of the caveolin-1 proteins as a potential method for treating the cancer by use of antibody drug complex (ADC).

I. Therapies for tamoxifen-resistant breast cancer

Disrupting caveolae lipid rafts in the cellular membrane may identify potential therapies for tamoxifen-resistant breast cancer. One way to disrupt the caveolae lipid rafts is by disrupting cholesterol (62). The interactions between cholesterol and caveolin proteins are a key structural interaction for lipid raft integrity (84-86).
Chapter 3: Potential therapies for tamoxifen-resistant and triple negative breast cancer

A. Cholesterol destabilization in the membrane

Several compounds and products destabilize cholesterol in the membrane. The main method of destabilizing cholesterol in the caveolae lipid rafts is to bind cholesterol, which disrupts interactions between cholesterol and surrounding components (74). Some of the molecules that can disrupt cholesterol in the caveolae lipid rafts include filipin, nystain, and cholesterol oxidase (84-86).

1. Filipin

One potential therapy for tamoxifen-resistant breast cancer is filipin. Filipin is a compound from *Streptomyces filipinensis* that has antifungal and antibiotic properties (figure 3.3). Filipin can disrupt caveolae lipid rafts by directly binding cholesterol. Filipin causes disruption in the caveolae lipid rafts by binding to cholesterol, then disrupting the binding between cholesterol and caveolin proteins. The lack of cholesterol binding to caveolin protein causes instability in the caveolae lipid rafts and collapse of the caveolae. The disruption of caveolae lipid rafts will re-sensitize breast cancer to tamoxifen (62-64, 84-86, 100, 101).

The re-sensitization to TAM occurs on three levels. First, the growth factor receptors EGFR and HER-2 in the caveolae lipid rafts have disrupted signaling. Second, the downstream signaling of the kinases Akt and MAPK decreases. Thirdly, the disruption of nuclear protein AIB decreases to cause a halt in tumor growth and tamoxifen re-sensitization (62-64, 84-86, 100, 101, figure 2.9). Once the cell is re-
sensitized to tamoxifen, then antagonist hormone therapy will be effective against the cancer.

The use of filipin is novel and therefore limited to cell culture work. The exact dosage and delivery procedures would need to be estimated for mouse studies, but one can estimate that filipin should be given oral and the dosage should be similar to 3-50 mg/kg. A high dose of the filipin may cause toxicity in mouse studies (62-64). The use of this compound may elucidate possible alternative mechanisms for re-sensitizing the resistant breast cancer to TAM.

![Figure 3.3 Structure of filipin. Figure adapted from NCBI PubChem database with permission.](image)

2. **Nystatin and amphotericin B**

Another potential therapy to use for tamoxifen-resistant breast cancer is nystatin. Nystatin is an antibiotic that has strong antifungal effects and was discovered
in 1944 from the *Streptomyces noursei* (figure 3.4). The mechanism of action for nystatin is to create leaky cell membranes for small solutes. The practical applications of nystatin are a topical and oral antifungal medicine for skin, mucous membrane, and intestine (84, 85, 102).

**Figure 3.4** Structures of nystatin and amphotericin B. Figures adapted from NCBI PubChem database with permission.

Similar to nystatin, amphotericin B is another antibiotic made by *S. noursei*. Both compounds have antifungal properties and act on cell membrane. Nystatin and amphotericin B act like porins or pores to allow molecules to diffuse passively across the membrane. Both antibiotic compounds work exclusively on sterol components of the cell membrane. In the caveolae lipid rafts, the major sterol component is cholesterol. The interaction between cholesterol and caveolin proteins is the basis for the structural
Chapter 3: Potential therapies for tamoxifen-resistant and triple negative breast cancer

backbone of the cave-like appearance of caveolae in the lipid bilayer (70-73, 84, 85, 102). The introduction of either antibiotic nystatin or amphotericin B disrupts the interactions between cholesterol and caveolin proteins, destroying the caveolae (figure 3.4).

Nystatin has been used to treat people with topical and intestinal fungal infection. The dose of antibiotic is effective concentration at $1 \times 10^{11}$ mg/kg units with using topical cream or oral methods to deliver the antibiotic. The side effects and off-target effects of nystatin are not known because of the limited use in cell biology (102). Nystatin may be a potential drug to use for disrupting caveolae lipid raft cancer in TAM-resistant breast cancer.

3. **Okadaic acid**

Another therapy for tamoxifen resistant is okadaic acid. Okadaic acid is a cytotoxin from the marine sponges *Halichondria okadai* and *Halichondria malanodocia* and the algae group, dinoflagellates. Okadaic acid has several cellular activities, which include inhibition of the serine and threonine phosphatases 1, 2A, and 2B, and disruption of the CERGA pathway (84, 103, figure 2.5, see page 36).

Okadaic acid acts on two different components of the caveolae lipid rafts and lipid raft signaling. The pathway form caveolae to endoplasmic reticulum to Golgi apparatus (CERGA) is inhibited by okadaic acid. The CERGA pathway is a continual cyclic pathway for caveolin-1 to go from the plasma membrane, endoplasmic reticulum, Golgi
apparatus, and back up to the plasma membrane. The CERGA pathway acts as a recycling process for the caveolin proteins. Okadaic acid interrupts caveolae trafficking by preventing the CERGA pathway from internalizing vesicles (103). The CERGA pathway is regulated by a phosphatase required for internalization of caveolin proteins. The phosphatase inhibitor, okadaic acid, prevents internalization for caveolae lipid rafts and potocytosis (84, 103, 104, figure 2.4). Since okadaic acid directly inhibits phosphatases needed by the CERGA pathway it is not clear if the inhibition of CERGA pathway by okadaic acid is a direct or indirect effect.

Okadaic acid is very toxic to mammals with only nano-molar concentrations effecting the CERGA pathway (84, 103, figure 2.5, figure 3.5). The use of okadaic acid as a treatment for TAM-resistant breast cancer is possible, but the effective range for treatment will need to be tested.

![Structure of okadaic acid](image)

**Figure 3.5** Structure of okadaic acid. Figure reproduced from reference 103. Reprinted with permission from (Tachibana, K., Scheuer, PJ., Tsukitani, Y., Kikuchi, H., Engen, DV., Clardy, J., Gopichand, Y., and Schmitz, FJ. (1981). *Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria*. Journal of American of Chemical Society 103(9): 2469-2471.). Copyright (2013) American Chemical Society.

4. **Cholesterol oxidase**
Another potential therapy for tamoxifen-resistant breast cancer is cholesterol oxidase. Cholesterol oxidase is an enzyme that mediates the reaction of adding oxygen groups to any cholesterol moieties. The cholesterol moieties in the caveolae lipid rafts add stability to the caveolae lipid rafts. When cholesterol becomes oxidized, there is instability in the rafts and disruption of signaling. The use of this enzyme may seem impracticable in whole organisms because of the high potential for cytotoxic side effects (105). However, the potential use of cholesterol oxidase as a treatment for TAM-resistant breast cancer should be considered.

II. Therapies for triple Negative Breast Cancer

There is no effective treatment for this triple negative breast cancer, which affects younger women below the age of thirty-five (7, 26, 27). A proposed approach to therapy of triple negative breast cancer is the use of an antibody-drug conjugate (ADC) (51).

A. Antibody-drug conjugate (ADC)

The antibody-drug conjugate is a new therapeutic approach to improve chemotherapeutic agents’ cellular distribution by “tumor-targeting monoclonal antibodies”. The creation of an antibody-drug conjugate begins with a conjugation of an inactive prodrug to an antibody. The drug remains inactive until it localizes at the tumor site.
The ADC must contain an antibody that recognizes an epitope present on the external surface of the plasma membrane perhaps a membrane receptor unique to the cancer cell. Once at the tumor, the ADC binds to the surface of tumor cells and is internalized to release the drug at full potency (104). The use of prodrugs is to increase pharmaceutical properties such as solubility, chemical stability, absorption, permeability, and toxicity (106, 107 figure 3.7). The use of a prodrug for ADC additionally creates a stable treatment that can target an active drug only to a particular set of cells.

**Figure 3.6** Antibody-drug complex (ADC) basic concept. The ADC is combination of drug to antibody with a linker. The ADC is prodrug form until it binds the targeted cell and the drug be enzymatically cleaved into an active form.

The ADC can be used to target triple negative breast cancers using an epigenetic drug coupled to an antibody directed to specific epitope. The triple negative breast cancer does not have caveolin-1, ER, PR, or HER-2, but this subtype of breast cancer does overexpresses the EGFR on the cell surface. The proposed treatment is to combine procaine with an antibody to EGFR. The drug procaine is a DNA methyltransferase
inhibitor (DNMTi). The combination of the procaine and EGFR antibody can make drugs that can penetrate the triple negative breast cancer cells. The procaine will be linked to the EGFR antibody via an ester linkage. The use of ester link between procaine and the antibody will create a prodrug that will be activated upon contact of cells overexpressing EGFR (21, 51, 67, 106, 109, 110, figure 3.8). Procaine, once internalized, will target CpG Islands on the histones. Specificity in disrupting epigenetic silencing of several genes including caveolin genes will need to be verified. Once the caveolin genes are activated, caveolin proteins will allow the formation of caveolae lipid rafts (23). The reintroduction of several proteins that include ER, PR, HER-2, and caveolae will cause the triple negative breast cancer to be more manageable to treat.

The current ADC for HER-2 positive breast cancer, trastuzumab emtansine (T-DM1), has some side effects that include diarrhea and palmar-plantar erythrodysesthesia or redness, swelling of the hands and feet; the proposed ADC of procaine-EGFR may have similar side effects typically seen with other chemotherapeutic antibiotic drugs (51). The ADC of procaine-EGFR is a way to start thinking of how to create more effective treatments for triple negative breast cancer. Similar as the side effects of the proposed ADC of procaine to antibody for EGFR, there may exist toxicity associated to chemotherapeutic drug procaine or with the antibody. The use of antibody-drug complex is new treatment for breast cancer and should be explored more for advanced and solid cancers.
Figure 3.7 Structure of procaine. Figure reproduced from reference 110 with permission.

III. SUMMMARY

The creation of new therapies will ensure continued success in treating breast cancer. These proposed compounds and treatments can give an insight on how to utilize the caveolae lipid rafts as a drug target in treating tamoxifen-resistant and triple negative breast cancers. Increasing caveolae lipid rafts and lipid raft signaling promotes tumorigenesis in TAM-resistant breast cancer, while epigenetic silencing the caveolin genes and removing caveolae lipid rafts results in TN breast cancer. It makes sense to create drugs that correctly alter the levels of this biological component.
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VITA

Jenniffer Stetler was born in Pasenda, Texas in 1987. She obtained her Bachelor of Sciences in Animal Sciences and Biochemistry from University of Missouri-Columbia in 2010. Also, she earned General University Honors and Biochemistry Departmental Honors as an undergraduate. Jenniffer is a McNair Scholar as well as a Leadership Alliance alumni. In 2010, she entered the Biochemistry Department at the University of Missouri- Columbia and in May 2013 will be graduating with a Master’s of Science of Biochemistry.