Phytochemical and Synthetic AHR Ligands as Novel Therapy for Refractory Breast Cancer

Jonathan V. Wooten

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Phytochemical and Synthetic AHR Ligands as Novel Therapy for Refractory Breast Cancer

by

Jonathan V. Wooten

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Pharmacology

May 2022
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

Eileen Brantley, Associate Professor of Pharmacology

Carlos A. Casiano, Professor of Microbiology and Molecular Genetics and Biochemistry

Daisy De Leon, Professor of Physiology

Susanne B. Montgomery, Professor of Social Work and Social Ecology

Charles Wang, Professor of Microbiology
ACKNOWLEDGEMENTS

First and foremost, I thank God for bringing me through to this point in my academic career. Looking back on my life, it’s hard to ignore the design and placement of events and achievements that led me to this moment. Even in the most difficult of moments, from the struggles of course work to the failures of experiments, He gave me the mental peace and the strength to move forward.

I thank Dr. Eileen Brantley, my P.I. and mentor for allowing me the opportunity to work in your lab, for your mentorship and guidance throughout the program, and for the creative freedom to pursue my own research passions.

I also thank my committee members, Dr. Carlos Casiano, Dr. Daisy De Leon, Dr. Susanne Montgomery, and Dr. Charles Wang for your intellectual guidance and support throughout this journey. My experience in this program would not be the same without the IMSD community. To Dr. Marino De Leon, Nannette Nevaes, Lorena Salto, and Daniela Soto, your pleasant conversations and check-ins were always appreciated, especially on my worst days. Special thanks to Ann Bradshaw, you believed in me before I even got to know you. Thank you for your prayers and encouragement every time I visited your office. The department is not the same without you but I wish you well in your future endeavors. I also want to thank Dr. Ubaldo Soto for the midnight, thought-provoking discussions and guidance throughout the years. My presentation style and critical thinking has improved greatly because of our talks.

This achievement would not have been possible without a good support system. To my classmates, Hameed Alenazi, Ivana Alicea-Polcano, Evgeny Chirshev, Greisha Ortiz-Hernandez, Jerry Jovanni, Zeb Masongo, Ezinne Aja, the “Mutsa’s” George
Mukosera, Janviere Kabagwira, and Mutsawashe Mukosera, Julia Kim, Karina Mayagoitia, Mary Beth Yu, Nicole Mavingire and Tiantian Liu, thank you for making this journey worthwhile and enjoyable.

To Dr. and Mrs. Wiafe, Dr. Adwoa Wiafe, Amma Wiafe, Michael, Wiafe, Aantuu Mengistu, Dr. Loren Hall, and Larry Salas, thank you for adopting me into your family. You have truly been my home away from home. To my friends who have been with me before it all began, J Baws, Big Meth, Gabo, and Big Smoke, those conversations that had me laughing for five mins straight were always right when I needed them.

Finally, I want to thank my family, Karen Wooten, Joe Wooten, and Dr. David Wooten for your constant love, care and support. You can’t convince me that all of your prayers didn’t single handedly put this degree in my hands. You are all my biggest supporters and I pray that God continues to bless you immensely.

I wish I had more pages to express my gratitude to everyone who has helped me on this journey. For those not mentioned here, you know you mean a lot to me. I am grateful for those who have touched my life in one way or the other. God is good!
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<tr>
<td>5F203</td>
<td>2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole</td>
</tr>
<tr>
<td>AE</td>
<td>Aqueous Extract</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl Hydrocarbon Receptor</td>
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<tr>
<td>AhRKnockout</td>
<td>AhR Knockout</td>
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<tr>
<td>A-NHEJ</td>
<td>Alternative Non-Homologous End-Joining</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine Orange</td>
</tr>
<tr>
<td>BAK1</td>
<td>BCL2 Antagonist/Killer 1</td>
</tr>
<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast Cancer Susceptibility Gene 1/2</td>
</tr>
<tr>
<td>C-NHEJ</td>
<td>Classical Non-Homologous End-Joining</td>
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<tr>
<td>CTL</td>
<td>Control</td>
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<tr>
<td>CYP1A1</td>
<td>Cytochrome P450 1A1 gene</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Cytochrome P450 1A2 gene</td>
</tr>
<tr>
<td>CYGB</td>
<td>Cytoglobin</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA Damage Response</td>
</tr>
<tr>
<td>DDS</td>
<td>Double Strand Breaks</td>
</tr>
<tr>
<td>DIM</td>
<td>3,3' Diindolylmethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTS</td>
<td>Dibenzyl Trisulfide</td>
</tr>
<tr>
<td>EE</td>
<td>Ethanolic Extract</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>GADD45a</td>
<td>Growth Arrest and DNA damage Inducible Alpha</td>
</tr>
<tr>
<td>GHW</td>
<td>Guinea Hen Weed</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>gBRCAm</td>
<td>Germline Mutations in BRCA</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>LMP</td>
<td>Lysosomal Membrane Permeabilization</td>
</tr>
<tr>
<td>LTA</td>
<td>Lymphotoxin-alpha</td>
</tr>
<tr>
<td>MMR</td>
<td>DNA Mixed Match Repair</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>P. alliacea</td>
<td>Petiveria alliacea L.</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PARPi</td>
<td>PARP Inhibitor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>RSF</td>
<td>Relapse-Free Survival</td>
</tr>
<tr>
<td>SSB</td>
<td>Single Strand Breaks</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
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<tr>
<td>TCDD</td>
<td>Tetrachloro-Dibenzo-Dioxin</td>
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<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>Z-VAD</td>
<td>Z-VAD-FMK</td>
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Patients with triple-negative breast cancer (TNBC) possess tumors that lack estrogen receptor, progesterone receptor, and human growth factor receptor 2 expression. Such patients have lower survival rates than those diagnosed with other breast cancer subtypes in part, due to the scarcity of targeted therapy for TNBC. In addition to chemotherapy, patients with TNBC often consume natural remedies, which has led us and others to evaluate the ability of plant isolates to confer anticancer and chemopreventive actions. Poly-ADP ribose polymerase (PARP) is an enzyme that promotes DNA single-strand break repair. PARP inhibitor olaparib exploits DNA repair enzyme deficiency in tumors to demonstrate anticancer activity. Emerging evidence suggests an inverse relationship between PARP and aryl hydrocarbon receptor (AhR) signaling activation due to a negative feedback mechanism between these pathways. Putative AhR agonist Dibenzy1 trisulfide (DTS) is derived from Petiveria alliacea, a perennial shrub that grows in tropical regions of the world. AhR agonist 3,3’-diindolylmethane (DIM), a major metabolite of indole-3-carbinol found in cruciferous vegetables confers anticancer and chemopreventive actions. This manuscript denotes an intensive investigation of the chemotherapeutic and chemopreventive actions of DTS
and DIM. We previously revealed that synthetic AhR agonist 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) induces the expression of putative tumor suppressor cytoglobin (CYGB) in TNBC cells and xenografts. Here, we demonstrate that DTS confers potent anticancer activity in TNBC cells in a caspase-independent manner. Furthermore, DIM and 5F 203 suppress TNBC cell migration, proliferation and enhance olaparib anticancer activity in TNBC cells. Notably, 5F 203 and to a lesser extent DIM bind to the CYGB promoter and induce CYGB in an AhR-dependent fashion to mediate anticancer actions in TNBC cells. Taken together, plant isolates represent promising agents to treat refractory forms of breast cancer and a diet rich in cruciferous vegetables promotes the anticancer actions of PARP inhibitors.
CHAPTER ONE

INTRODUCTION

An Introduction to Breast Cancer

Life and integrity are synonymous with one another. Life cannot be maintained without genetic integrity and integrity cannot exist without life. Deoxyribonucleic acid (DNA) is a self-replicating material that is present in nearly all living organisms as the main constituent of chromosomes and carrier of genetic information. DNA can also be classified as the fundamental and distinctive characteristics or qualities of someone or something. As such, DNA's integrity and stability are essential to life. DNA, however, can be damaged and/or compromised and if left unrepaired, mutations, diseases, and ultimately death are ensured. Cancer is one of the most prominent diseases resulting from compromised DNA integrity. The oldest documented case of cancer dates back to ancient Egypt from an ancient Egyptian textbook called the Edwin Smith Papyrus. It describes eight cases of tumors of the breast.[1] Since then, research has been dedicated to understanding the mechanisms behind the cause of cancer as well as methods for its cure.

Breast cancer (BC) is defined as uncontrollable cell growth of breast cells.[2, 3] While the definition of BC may be simple, understanding what BC is in its entirety is a complex, seemingly never-ending investigation. BC is the most frequently diagnosed cancer among women worldwide.[4] It is estimated that over 287,000 women will be diagnosed with BC in 2022.[5] Although extremely rare, men can also develop BC. In
fact, male BC represents approximately 1% of cancers that occur in men and approximately 1% of all BCs worldwide.\[6\]

The female breast is macroscopically divided into two main parts, 1) the glandular component and 2) the fatty and fibrous or connective tissue.\[7\] The glandular tissue include the lobules and ducts which function to produce milk and carry the milk to the nipple, respectively. The fatty and connective tissue help make up and support the breast.

While BC can begin in different parts of the breast, most BCs begin in the ducts or lobules.\[8\] Ductal carcinoma in situ (DCIS) is characterized by abnormal ductal epithelial cells that have yet to invade the myoepithelial cells that line the lower membrane of the ducts.\[9\] Conversely, while DCIS is noninvasive, it can progress to invasive breast cancer. BC that has spread or invaded into the surrounding breast tissue is referred to as invasive BC. Invasive BCs are histologically categorized into two major types: invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC).\[10\] IDC is a nonspecific invasive carcinoma belonging to the epithelial class of tumor and is the most common type of BC, accounting for approximately 75% of all BC cases.\[11, 12\] IDCs originate in a milk duct in the breast, eventually breaking though the wall of the duct and invading the nearby breast tissues.\[10\] Invasive lobular breast cancer (ILC) is the second most common type of invasive breast cancer and accounts for 5-10% of all BC cases.\[13\] ILCs originate in the lobules and, like IDC, can metastasize to other parts of the body. ILC occurs more frequently in older women and is distinct from other BC subtypes in its clinical, radiographic and histopathology features.\[13, 14\] Furthermore, ILC shows distinct signaling in pathways essential for breast cancer growth and proliferation.
compared to IDC, including WNT4 signaling in response to estrogen stimulus or blockade.[12, 15, 16]

Breast Cancer Subtypes

Through comprehensive gene expression profiling of large sets of tumors by multiple independent groups and technologies, six molecular subtypes of breast cancer have been identified: normal breast-like, luminal A, luminal B, human epidermal growth factor 2 overexpressing (HER2+/ER-, ErbB2+), basal-like/triple negative, and Claudin-low.[17-21] Furthermore, luminal A, luminal B, HER2+, and triple negative subtypes are widely addressed as the major BC subtypes. To date, two methods can determine the subtype of BC: gene-based assays and IHC-based markers.[22] BC subtypes are classified based on the expression level of endocrine receptors, proliferative genes, oncogenes, estrogen receptor (ER), progesterone receptor (PR) HER2, and Ki-67 (a proliferation index marker).[23] Tumor size, tumor grade, and nodal status are other methods used to identify subtypes.

Normal breast-like tumors, as the name suggests, falls under a category of tumor samples that are predominantly composed of normal breast tissue and not tumor tissue.[24] Normal breast-like tumors show low tumor cellularity (<50%) when examined pathologically.[25, 26] Luminal breast cancers are highly heterogeneous and comprise different histologies, gene-expression profiles and mutational patterns with varying clinical courses and responses to systemic treatment.[27] Luminal A and luminal B BCs are the two main ER-positive BC subtypes. The luminal A subtype has been shown to have better outcomes than other BC subtypes across many datasets of patients with
early breast cancer, including several clinical trials (TransATAC, GEICAM9906, CALGB9741, ABCSG08, NCIC-CTG MA.5, NCIC-CTG MA.12), where patients were given various adjuvant systemic treatments.\textsuperscript{28} Compared to luminal A tumors, luminal B tumors are characterized as having lower levels of ER expression, lower or no PR expression, higher tumor grade, higher expression of proliferation-related genes, and active growth factor receptor (GFR) signaling pathways including the PI3K/AKT/mTOR pathway.\textsuperscript{18, 27, 29} HER2 is a growth-promoting protein expressed at high levels in 30% of breast tumors.\textsuperscript{30, 31} Additionally, HER2 is mutated in 10-20% of breast cancers where it’s expression is amplified—allowing breast cells to grow and divide uncontrollably. These breast cancers are referred to as HER2-overexpressing. Compared to breast cancers that are HER2-negative, HER2-overexpressing breast cancers are more aggressive and prone to metastasis leading to relapse.\textsuperscript{32}

**Triple Negative Breast Cancer**

Triple negative/basal-like and Claudin-low tumors are ER negative, PR negative, and HER2 negative and carry a poor prognosis.\textsuperscript{24} Claudin-low tumors, in particular, are characterized by low expression of genes involved in tight junctions and cell-cell adhesions including claudins 3, 4 and 7, occludin and E cadherin.\textsuperscript{33} Furthermore, these tumors are enriched with unique biologic properties that are linked to mammary stem cells (MaSCs), epithelial mesenchymal transition (EMT), and display features of tumor initiating cells (TICs or cancer stem cells).\textsuperscript{34-36} While, triple negative and basal-like cancers lack the expression of ER, PR, and HER2, the difference between them is

1
disputed, with triple negativity in clinical practice providing a more practical and routinely applicable classification.[33]

Specifically, studies have further divided triple negative tumors into several groups: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). BL1 is heavily enriched in cell cycle and cell division processes while the BL2 subtype has unique gene ontologies that involve growth factor signaling. The IM subtype includes genes involved in immune cell processes. M subtypes display a variety of unique gene ontologies heavily enriched in cell motility, ER membrane protein complex (ECM) receptor initiation, and cell differentiation pathways. MSL shares similar genes with the M subtype but also contains genes that play a role in growth factor signaling and has low expression of Claudin 3, 4, and 7. LAR subtypes are ER negative but display luminal gene expression patterns.[37, 38]

Notably, normal breast-like tumors lack the expression of ER, PR, and HER2 and can also be classified as triple-negative. However, they are not considered to be basal-like cancers as they are negative for the basal-like markers, epidermal growth factor receptor (EGFR) and cytokeratin 5 (CK5).[33] Interestingly, the majority of basal-like breast cancers lack or display low levels of ER, PR and HER2, suggesting that there are some basal-like tumors that do express the ER or HER2. As such, it is important to not consider triple-negative and basal tumors as synonymous. Triple negative breast cancers (TNBCs) account for 10%-20% of all breast cancers and are more common in younger patients and women of West African ancestry.[39] Compared to other BC subtypes, these tumors are larger in size, are of a higher grade, have lymph node
involvement at diagnosis, are biologically more aggressive, and have higher recurrence rates and carry poorer prognosis.\textsuperscript{[40, 41]} Additionally, less than 30\% of women with metastatic TNBC survive 5 years.\textsuperscript{[37, 41]}

Ki-67 is a proliferation index marker that has been found to correlate significantly with young age, large tumors, positive lymph nodes, negative ER/PR, tumor suppressor p53\textsuperscript{[42]} overexpression, and positive HER2. Furthermore, a higher Ki-67 index correlates with a poorer prognosis and early recurrence and a lower Ki-67 index correlates with a better prognosis and late recurrence.\textsuperscript{[43]} Taken together, the major subtypes of BC can be summarized and categorized as follows: Luminal A (ER and/or PR-positive, HER2-negative Ki67 $\leq$ 14\%), Luminal B (ER and/or PR-positive, HER2-negative, Ki67 >14\%), HER2 overexpressing (ER and/or PR-negative, HER2-positive, Ki67 $\leq$ 14\% or $>14\%$), and Triple negative (ER-negative, PR-negative, HER2-negative, Ki67 $\leq$ 14\% or $>14\%$).\textsuperscript{[44]}

**Breast Cancer Health Disparities**

In the United States, nearly 1 in 3 of all newly diagnosed female cancers are breast cancer each year.\textsuperscript{[45]} Globally 1 in 10 women are diagnosed with BC each year.\textsuperscript{[46]} However, the incidence and mortality rates vary among the different ethnic groups. Although women of African Ancestry (Black women) historically have had higher breast cancer incidence rates\textsuperscript{[47]}, recent data show that Black women have similar incidence rates compared to women of European ancestry (White women), however the incidence rate before age 45 is higher among Black women than White
women and significantly higher in White women than Black women between the ages of 60 and 84.\[^{48}\] The incidence and mortality rates for breast cancer are lower among women of other racial and ethnic groups compared to non-Hispanic White and Black women. Notably, Black women have the highest mortality rates from breast cancer at every age and Asian/Pacific Islander women have the lowest incidence and death rates compared to other racial and ethnic groups.\[^{47, 49}\]

Despite the racial and ethnic disparities in cancer survival, studies have identified theoretical causalities and solutions to reduce racial disparities. While TNBCs are categorically the most aggressive subtype of breast cancer, they occur more frequently in young women and Black women. In fact, Black women of diverse background have 3-fold more triple negative tumors than non-black women regardless of age and body mass index (BMI).\[^{50}\] Interestingly, studies have shown that while pregnancy and higher parity increase the risk of basal-like cancer, it reduces the risk of ER-positive/PR-positive breast cancer.\[^{51}\] Furthermore, the Carolina Breast Cancer Study discovered that breastfeeding was associated with a reduced risk of basal-like breast cancers.\[^{52}\]

Previous research on the disparity in the prevalence and death rates of Black and White breast cancer patients was attributed to various socioeconomic factors including access to health care and medical treatment.\[^{53}\] However emerging evidence indicates that these disparities may be due to the uniquely aggressive biology of the disease among African Americans.\[^{54}\] To date, numerous studies have identified possible differences in biological properties between Black and White women including: plasma levels of growth factors and hormones\[^{55}\], reproductive factors\[^{56, 57}\], susceptibly loci\[^{58, 59}\], primary tumor characteristics—including the presence and expression of steroid and
growth factor receptors\cite{57,60,61}, cell cycle proteins\cite{62,63}, tumor suppressor genes\cite{64,65}, and chromosomal abnormalities.\cite{66}

Hispanic women have an overall lower incidence of breast cancer compared to non-Hispanic White women, non-Hispanic Black Women, as well as American Indian/Alaska Native women.\cite{67} Asian-Americans who are recent immigrants have lower rates of breast cancer than those who have lived in the U.S. for many years. However, Asian-American women born in the U.S. have about the same risk as White women.\cite{48,67,68} Furthermore, while Chinese and Japanese women have the highest breast cancer survival rates, Black women have the lowest survival rate of any racial or ethnic group. Black women also have the highest breast cancer mortality rate compared to any other racial or ethnic group and this disparity continues to increase.\cite{48,67,68} Yedjou and colleges have eloquently reviewed the varying health and racial disparities in breast cancer by evaluating the socioeconomic and potential risk factors including: age and sex, family history and genetic mutations, lack of physical activity, and poor diet and obesity in further detail.\cite{48}

**Stages of Breast Cancer**

There are four stages of breast cancer: stages I-IV. However, breast cancer can involve additional characteristics. For example, in situ breast cancer can be classified as a Stage 0 breast cancer and is divided into two subcategories: lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). LCIS, in addition to being a risk factor, is an incidental microscopic finding of abnormal tissue growth in the lobules of the breast.\cite{69}
Although LCIS does not progress to breast cancer, it does increase the risk of subsequent invasive breast cancer of approximately 7 percent over 10 years.\textsuperscript{[69, 70]} DCIS is characterized by abnormal ductal epithelial cells that have yet to invade the myoepithelial cells that line the lower membrane of the ducts. Conversely, while DCIS is noninvasive, it can progress to invasive breast cancer.\textsuperscript{[9]}

Stage I breast cancer describes a cancer that has become invasive and is further divided into two subcategories. Stage IA are confirmed if the tumor measures up to 2 centimeters (cm) and if the tumor has not spread outside the breast. If a tumor does not exist in the breast but rather small groups of cancer cells are found in the lymph nodes, a patient is diagnosed with stage IB cancer. Alternatively, Stage IB breast cancer is diagnosed if a tumor less than or equal to 2 cm exists in addition to the presence of small groups of cancer cells within the lymph nodes.\textsuperscript{[71]}

Stage II breast cancer is also divided into two subcategories. Stage IIA breast cancer is diagnosed when a patient presents with three underlying conditions: the tumor is larger than 2 cm but doesn’t exceed 5 cm and small groups of breast cancer cells are found in the lymph nodes, the tumor is larger than 2 cm but no larger than 5 cm and the cancer has spread to 1 to 3 axillary lymph nodes or to lymph nodes near the breastbone, or if the tumor is larger than 5 cm but has not spread to the axillary lymph nodes.\textsuperscript{[71]} Stage IIB describes a tumor that is larger than 2cm but no larger than 5 cm and small groups of breast cancer cells are found in the lymph nodes. Stage IIB breast cancer can also describe a similarly sized tumor that has spread to 1 to 3 axillary lymph nodes or to lymph nodes near the breastbone. A tumor larger than 5cm that has not spread to the axillary lymph nodes can also be diagnosed as stage IIB breast cancer.\textsuperscript{[71]}

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Stage III breast cancer is divided into three subcategories: IIIA, IIIB, and IIIC. Stage IIIA can be diagnosed similarly with the exception of having tumors larger than 5 cm. Additionally, stage IIIB is diagnosed if cancer is found in 4 to 9 axillary lymph nodes or in the lymph nodes near the breastbone. Stage IIIB involves breast cancer that has invaded the chest wall and/or skin of the breast, resulting in swelling or an ulcer and has either spread to multiple axillary lymph nodes or the lymph nodes near the breastbone. In stage IIIC breast cancer, diagnosis can occur with a tumor of any size that has spread to the chest wall and/or the skin of the breast and the cancer has spread to 10 or more axillary lymph nodes. Alternatively, cancer that has spread to the lymph nodes above or below the collarbone or to axillary/breastbone lymph nodes is diagnosed as stage IIIC breast cancer.

Patients diagnosed with Stage IV breast cancer have “advanced” or “metastatic” cancer that has spread beyond the breast and nearby lymph nodes to other organs of the body. Cancer that has come back or “recurred” after initial treatment is called recurrent breast cancer. Recurrent breast cancer is a result of the growth of cancer cells that survived treatment.

**Therapeutic Strategies for Stages of Breast Cancer**

Each stage of breast cancer calls for a different method of treatment. Although DCIS can progress to invasive breast cancer, Stage 0 LCIS and DCIS are non-invasive breast cancers. As such, patients diagnosed with LCIS are often offered information about chemoprevention using selective estrogen receptor modulations (SERMs) such
as tamoxifen and aromatase inhibitors (AIs) such as anastrozole.[73] Patients with DCIS on the other hand, are usually recommended for breast-conserving surgery followed by radiation therapy.[74]

Treatment of stages I and II breast cancer can involve either surgery, radiation therapy, or adjuvant systemic therapies. Historically, mastectomies have been the standard of care for early-stage invasive breast cancers.[75] In recent years, breast-conserving surgeries are favored. Breast-conserving surgeries involve removing the tumor without removing excess healthy breast tissue. While breast-conserving surgeries boast the highest success rate in women with early-stage breast cancer, women at high risk of recurrence are not recommended to undergo this surgery.[76] This is because a higher risk of local relapse is associated with higher risk of distant disease and subsequent risk of dying of breast cancer.[77] Specifically, the status of axillary lymph nodes (ALNs) impacts the necessity for radiation therapy and adjuvant systemic therapy. Additionally, women with early-stage breast cancer may opt for mastectomy because of contraindications to radiation therapy or because of personal preference and vice versa.[69, 78] Factors such as presence of lymph nodes can affect treatment options. Specifically, the status of axillary lymph nodes (ALNs) impacts the necessity for radiation therapy and adjuvant systemic therapy.[69]

Radiation therapy (aka radiotherapy) is a form of cancer treatment that uses high doses of radiation to kill cancer cells, resulting in tumor regression.[79] Although studies have shown that radiation therapy in addition to surgery significantly reduces the five-year local recurrence rate,[80] radiation therapy is expensive and time-consuming. As a result, shorter and more affordable therapies are more appealing.[69]
Stage I-II breast cancer patients typically receive adjuvant systemic therapies. Chemotherapy, endocrine therapy, and targeted therapies are some of the most common forms of adjuvant systemic therapies. These therapies help increase the effectiveness of local therapies. Chemotherapy is an aggressive form of therapy meant to destroy rapidly growing cells in the body. Several factors are involved when introducing chemotherapy to a patient. Chronologic age and comorbidity in cancer patients are two of the major influences on the decision to use chemotherapy or radiation therapy. Endocrine therapies include SERMs, aromatase inhibitors, and gonadotropin-releasing hormone agonists. Aromatase inhibitors block the conversion of androgens to estrogen and are generally used as the first-line of defense in patients with hormone receptor-positive (HR+) breast cancer.

Stage III breast cancer is often known as locally advanced breast cancer (LABC) and is characterized by tumors larger than 5 cm, extensive regional lymph node involvement, direct involvement of underlying chest wall or skin, tumors considered inoperable but without distant metastases, and inflammatory breast cancer. Standard of care for patients presenting with these symptoms involves induction chemotherapy and adjuvant surgery and/or radiation therapy. Similar to stage I-II breast cancer, several therapies for stage III breast cancer patients are available. Induction systemic and local therapies are common forms of treatment.

In general, patients with LABC that have successful induction chemotherapy perform similarly to patients with early-stage breast cancer. Furthermore, 75% of patients undergoing induction chemotherapy have reported more than a 50% decrease in tumor size. In contrast, controversy surrounds preoperative chemotherapy in that, not...
only does it decrease tumor size and increases breast conservation rates, but the risk of local recurrence is also increased. Induction endocrine therapy includes tamoxifen alone or in combination with AIs and although it is less effective than chemotherapy, it stands as a “safer” alternative for older patients who are not willing to accept chemotherapy-related toxicity. Local therapy is dependent on how a patient/tumor responds to induction chemotherapy. Studies show that 50-90% of LABC patients are successfully treated with breast-conserving surgery after induction chemotherapy. Reports of residual pathologic tumors larger than 2 cm, multifocal residual disease, lymphovascular invasion, and extensive lymph node involvement following breast-conserving surgery after induction chemotherapy have been linked to increases of local recurrence.

Only about 23% of women with stage IV or metastatic breast cancer survive five years. LABC and early-stage breast cancer patients who relapse after treatment have progressed to metastatic breast cancer. For these cancers, endocrine therapy is generally better tolerated than chemotherapy. However, to ensure a timely response with women who progressive rapidly, chemotherapy is recommended. Trastuzumab alone or in combination is recommended for initial treatment of metastatic cancers overexpressing HER2. Studies show that 11% of patients treated with adjuvant therapies develop recurrence or relapse within 5 years and 20% develop recurrence or relapse within 10 years. If a patient relapses after breast-conserving surgery, mastectomy is recommended, followed by repeat axillary staging. The success of chemotherapy for breast cancer patients with recurrence is uncertain. However, clinical trials are ongoing to address this issue. Chemotherapy, although uncertain, is still
recommended for recurrent cancer. Endocrine therapy is recommended for hormone receptor-positive cancer and trastuzumab is recommended for HER2-overexpressing breast cancers.\[^{69}\]

**Therapeutic Strategies for Breast Cancer Subtypes**

Treatment regimens also vary depending on the different subtypes of breast cancer. A prevalent area of breast cancer research involves the development of novel targeted therapies for the treatment of breast cancer. Conventional therapies in the treatment of these diseases involve the use of synthetic small molecules or monoclonal antibodies.\[^{88}\] For example, Luminal BC (hormone receptor positive breast cancer) treatment mainly involves endocrine therapy that works to lower or block hormone levels or activity.\[^{89}\] These tumors represent 60%-80% of all BC cases in developed countries.\[^{90}\] Current available treatment options include aromatase inhibitors (AIs) such as anastrozole, estrogen uptake blockers such as tamoxifen, luteinizing hormone-releasing hormone analogs that suppress hormone production such as leuprolide, and ER degraders such as fulvestrant. Because endocrine drugs operate through different mechanisms, combination therapies are often used.\[^{89}\] Furthermore, metastatic HR\(^+\) can develop resistance to endocrine therapy.\[^{91}\] Therefore, novel therapies should involve reversing resistance to hormonal therapies.

Current therapies designed to reverse HR\(^+\) endocrine resistant disease include cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitors, phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (Akt)/Mammalian target of rapamycin (mTOR) pathway
inhibitors, and histone deacetylase (HDAC) inhibitors. CDK4/6 regulate cell cycle progression by their reversible interaction with cyclin D1 and are responsible for proliferation processes upon hormonal resistance.[92] The E2 factor (E2F) is a downstream effector of the retinoblastoma (RB) pathway that plays a role in cell division control.[93] CDK4/6 inhibitors work by blocking the phosphorylation of RB, downregulating the E2F-response genes to mediate G1-S cell cycle arrest.[89] These inhibitors include palbociclib, ribociclib, and abemaciclib. Hyperactivation of the PI3K/AKT/mTOR pathway is associated with drug resistance and cancer progression.[94] Inhibitors of this pathway therefore aim to reverse hyperactivation and regulate activity. Additionally, combination therapies are often used to target both HR and PI3K/Akt/mTOR pathways. For example, α-specific PI3K inhibitors (alpeisib and taselisib), are currently in phase III trials (NCT02437318 and NCT02340221) and have demonstrated promising efficacy, particularly in BC patients who have PIK3CA mutations.[95, 96] mTOR inhibitors such as everolimus is FDA approved for HR+ advanced BC in combination with exemestane after treatment failure with AIs, letrozole or anastrozole.[97] HDAC-mediated loss of ER expression in ER+ patients can lead to resistance. Histone deacetylases (HDAC) and transferases (HAT) are chromatin modifiers that lead to epigenetic changes in the cell and have been implicated in the development of drug resistance in breast and other forms of cancer.[98] Co-administering HDAC inhibitor vorinostat with anti-estrogen drug, tamoxifen, in advanced breast cancer patients reestablished sensitivity to anti-estrogens despite progression on multiple prior anti-estrogen therapies and chemotherapy.[99]
Current treatment regimens for HER2\(^+\) overexpressing BC include: anti-HER2 monoclonal antibodies, trastuzumab and pertuzumab, antibody-drug conjugates such as ado-trastuzumab emtansine, and tyrosine kinase inhibitors (TKIs) such as lapatinib.\(^{[89]}\) A combination of sequential chemotherapy and HER2-targeted therapy is the current standard of care followed by surgery, radiotherapy, 12-months of HER2-directed therapy, or endocrine adjuvant therapy if needed.\(^{[100]}\) While trastuzumab is a widely-used therapy for HER2\(^+\) overexpressing BC, acquired resistance to trastuzumab is a primary concern. Therefore, novel therapies and research for HER2\(^+\) overexpressing BC involve elucidating resistance mechanisms and new targeted agents and immunotherapies.\(^{[89]}\) Similar to HR\(^+\) BC, PI3k/Akt/mTOR inhibitors, such as buparlisib, have been shown to overcome trastuzumab resistance.\(^{[101]}\) Interestingly, the combination of nelipepimut-S, a vaccine used to prevent clinical recurrence in high-risk BC patients, and trastuzumab in HER2\(^+\) early BC is being investigated in a phase IIb trial (NCT02297698).\(^{[89]}\)

**Therapeutic Strategies for Triple Negative Breast Cancer**

Breast cancers that overexpress or lack certain genes/receptors such as estrogen, progesterone, or HER2 receptors are prone to poor prognosis. While targeted therapy options are available for patients with tumors expressing ER and/or HER2 amplification, breast cancer patients with tumors that do not express those genes do not benefit from such therapies. TNBC patients lack the expression of estrogen, progesterone, and HER2 receptors. As such, the scarcity of targeted therapy options
results in poor prognosis. Current treatment regimens for TNBC are limited to standard chemotherapy, radiation therapy, and/or breast-conserving surgery.\cite{102, 103} Interestingly, TNBC displays the most complete response to chemotherapy, however it is associated with higher rates of recurrence and metastasis than other BC subtypes.\cite{89, 104}

Novel research and therapeutic strategies for TNBC treatment are highly focused on developing effective targeted agents. These novel targeted agents and strategies are the subject and ultimate goal of this manuscript. Poly-ADP ribose polymerase (PARP) inhibitors, aryl hydrocarbon receptor ligands, and the reactivation of tumor suppressor genes are three novel strategies of particular interest. Poly-ADP ribose polymerase (PARP) is an enzyme involved in the repair of DNA damage.\cite{105-107} PARP1, in particular, is implicated in mediating various components of DNA metabolism including: single-strand break repair, double-strand break repair, nucleotide excision repair, stabilizing replication forks, and modulating chromatin structure.\cite{108} A more detailed discussion of PARP and various triumphs and challenges in exploiting synthetic lethality to combat TNBC will be addressed in Chapter 3 of this manuscript. TNBC patients that respond to PARP inhibitors, such as the FDA-approved olaparib, primarily possess tumors defective in DNA repair protein, breast cancer susceptibility gene 1 (BRCA1).\cite{109} Although olaparib demonstrates keen sensitivity in TNBC patients with defective DNA repair proteins BRCA1/BRCA2,\cite{110} TNBC patients with BRCA1-proficient tumors rarely benefit. Thus, a need exists to develop novel agents that can improve the efficacy of PARP inhibitors in TNBC patients with BRCA proficient tumors.

The aryl hydrocarbon receptor (AhR) mediates a myriad of cellular processes. Once AhR ligands bind to and activate the AhR, they behave like classical nuclear
receptors and is translocated into the nucleus where they form a heterodimer with the AhR nuclear translocator (ARNT). This complex then acts as a transcription factor and recognizes and binds to the xenobiotic response element (XRE) of target genes such as cytochrome P450 1A1 (CYP1A1) resulting in its transcriptional activation to promote the bioconversion of the ligand into its active form (Figure 1).\cite{111}

![Figure 1. Mechanism of the aryl hydrocarbon signaling pathway (Callero and Loaiza-Pérez 2011).](image)

Although prototypical AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) tend to display deleterious actions, we and others have shown that certain AhR ligands exhibit anticancer actions \cite{112-119} in breast cancer cells, including triple negative subtypes. For example, we have shown that AhR ligand 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) exhibits potent anticancer activity in TNBC cells.\cite{114, 120, 121} We and others have demonstrated the potential for AhR ligands to inhibit survival
signaling pathways, such as the PI3K/Akt signaling pathway.\textsuperscript{[122, 123]} Notably, PI3K/Akt inhibitors have been shown to synergize with PARP inhibitors.\textsuperscript{[124]} Emerging evidence suggest that pharmacological restoration of tumor suppressor genes (TSGs) represents a novel therapeutic approach to treat a variety of cancer types, including TNBC.\textsuperscript{[125]} Cytoglobin (CYGB) is a putative tumor suppressor gene that is often inactivated due to epigenetic silencing via hypermethylation of its promoter region.\textsuperscript{[126]} Studies also show that CYGB deficiency is linked to ER- or triple negative breast cancers and that its promoter region contains AhR binding sites.\textsuperscript{[114, 127]} Furthermore, we have previously shown the ability of 5F 203 to induce the expression of putative tumor suppressor CYGB in breast cancer cells.\textsuperscript{[114, 128]}

**Phytochemicals for Breast Cancer Treatment**

Despite the discoveries, evolutions, ingenuity, and success of recent novel therapies for BC, over the last 5 years, the estimated number of women diagnosed with BC has increased by approximately 12\%.\textsuperscript{[5]} In fact, current projections indicate that the number of new cancer cases is increasing at a fast rate and will increase from 14 million in 2012 to 22 million global cases by 2030.\textsuperscript{[129]} The causality of this increase deserves further investigation. However, several studies demonstrate that healthy diet and exercise can improve life expectancy as well as overall quality of life.\textsuperscript{[130-136]}

Older age (>65 years), genetic predispositions such as DNA mutations and BC family history, early menarche (<12 years), late menopause (>55 years), age at first pregnancy over 30 years, infertility and not having children, use of contraceptives,
hormonal treatment after menopause, and no history of breastfeeding have all been identified as risk factors for developing BC.\textsuperscript{[136-138]} While consistent and statistically significant data surrounding the relationship between consuming specific foods and BC development is lacking, it is proposed that diet may have a significant impact on BC outcomes. Specifically, studies show that adopting healthy dietary patterns based on high consumption of fruits, vegetables, whole grains, poultry and fish, and low consumption of red meat, refined foods, sweets and high-fat dairy products, may improve the overall prognosis and survival of women diagnosed with early-stage BC.\textsuperscript{[139]}

In fact, the American Cancer Society recommends in their guideline for breast cancer survivors that primary care clinicians counsel survivors to achieve a dietary pattern that is high in vegetables, fruits, whole grains, and legumes.\textsuperscript{[133]}

Globally, there is increased use of herbal medicines due to concerns associated with conventional pharmaceuticals. Often the prescribing physician is unaware of the herbal medicine use among patients which puts them at risk for toxicities and potentially reduced efficacy and toxicities due to drug-herbal interactions.\textsuperscript{[140]} Our understanding of the mechanisms by which these plant isolates confer their actions is often limited. However, studies investigating nutritional interventions during BC treatment have shown that nutritional counselling and supplementation with certain omega-3 fatty acids, may be useful in limiting drug-induced side effects, as well as in enhancing therapeutic efficacy.\textsuperscript{[136]} Therefore, understanding the role and mechanism of actions of plant isolates could help prevent some of the adverse effects that comes with herb-drug interactions as well as gain insight into their potential to interact synergistically with other agents.
Anticancer and Chemopreventive actions of plant isolate Glaucarubulone glucoside in MCF-7 breast cancer cells

We recently published the effects of a quassinoid isolated from the Simaroubaceae plant in breast cancer cells.\textsuperscript{[145]} Quassinoid isolates from members of the Simaroubaceae family possess a variety of biological actives including: antitumor, anti-viral, anti-inflammatory, antifeedant, antimalarial, and antiamebic effects.\textsuperscript{[141, 142]} Quassinoids have also been used in traditional folk medicine to treat a variety of medical conditions.\textsuperscript{[143]} While isolates from the \textit{Castella macrophylla} Urb. family member of Simaroubaceae has been reported to display anti-feedant activities against tobacco budworms, \textit{Plasmodium falciparum} and \textit{Plasmopara viticola}, we discovered that the \textit{Castella macrophylla} Urb. family member glaucarubulone glucoside exhibited chemopreventive and anticancer activity.\textsuperscript{[144, 145]}

Polycyclic aromatic hydrocarbons (PAHs) such as Benzo[a]pyrene (B[a]P) are ubiquitous chemicals produced by the incomplete pyrolysis of organic materials commonly released through the combustion of fossil fuels. PAHs are found in considerable quantities in vehicle exhaust, cigarette smoke, particulate matter in urban air, and charcoal-broiled food.\textsuperscript{[146]} Lipophilic PAHs easily diffuse into cells and bind with high affinity to the cytosolic aryl hydrocarbon receptor (AHR).\textsuperscript{[147, 148]} This PAH-activated transcription factor forms a nuclear complex with AHR nuclear translocator (ARNT), which transcriptionally activates genes containing the Ah locus, including the cytochrome P450 (CYP) 1 enzyme family members CYP1A1, CYP1A2 and CYP1B1.\textsuperscript{[149]} The dihydrodiol epoxide (DDE) pathway catalyzes B[a]P-metabolism via
CYPs and epoxide hydrolase (EH), to form highly mutagenic and reactive B[a]P metabolites.\textsuperscript{[150, 151]} CYP1 enzymes (particularly CYP1A1) carry out epoxidation at the 7,8 position, followed by hydrolysis to form B[a]P-trans-7,8-dihydrodiol. An additional CYP-catalyzed epoxidation produces the ultimate carcinogen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BDPE) (aka. (+)benzo[a]pyrene-7,8-dihydropiol-9,10-epoxide) (\textbf{Figure 2}).\textsuperscript{[150, 152]}

\textbf{Figure 2.} Metabolism of B[a]P by cytochrome P450s and other xenobiotic-metabolizing enzymes. 
(https://en.wikipedia.org/wiki/Benzo(a)pyrene#/media/File:Benzo(a)pyrene_metabolism.svg)
The first round of CYP-mediated B[a]P bioactivation leads to quinones that can undergo redox cycling to cause continuous release of prominent reactive oxygen species (ROS): superoxide anion radicals, hydroxyl radical and hydrogen peroxide via the Fenton reaction. The genotoxic actions of PAHs such as B[a]P occur either directly or through the formation of benzoquinones. Indeed, ROS are associated with DNA damage, cell damage and apoptosis.\textsuperscript{[153,154]} In addition, benzoquinones formed by B[a]P metabolism have been shown to increase cell proliferation, generate ROS and transactivate the epidermal growth factor receptor in MCF-10A breast epithelial cells and this provides a plausible mechanism for B[a]P-mediated tumour promotion.\textsuperscript{[155]}

Since quassinoids frequently display anticancer and chemopreventive actions, we hypothesized that the quassinoids isolated from the \textit{C. Macrophylla} plant exhibit cytotoxic actions in breast cancer cells by modulating oxidative stress and suppress B[a]P-mediated CYP1 induction. Specifically, glaucarubulone glucoside (Gg) and two related quassinoids, glaucarubulone and holacanthone, and a coumarin scopoletin were isolates that were investigated. We found that Gg reduced the viability of MCF-7 breast adenocarcinoma cells adenocarcinoma cells (IC\textsubscript{50} = 121 nM) (\textbf{Figure 3a}) to a greater extent than standard of care anticancer agents 5-fluorouracil, tamoxifen (IC\textsubscript{50} > 10 μM) and the tamoxifen metabolite 4-hydroxytamoxifen (IC\textsubscript{50} = 2.6 μM) (\textbf{Figure 3b}). Furthermore, we found Gg to display no cytotoxicity to non-tumourigenic MCF-10A breast epithelial cells (\textbf{Figure 3c}).
Furthermore, Gg blocked increases in reactive oxygen species in MCF-10A cells mediated by B[a]P metabolite, benzo[a]pyrene 1,6 quinone, yet down-regulated the expression of genes that promote antioxidant activity in MCF-7 cells (Figure 4). This implies that Gg exhibits anti-oxidant and cytoprotective actions in non-tumourigenic breast epithelial cells and pro-oxidant, cytotoxic actions in breast cancer cells.

Figure 3. Gg demonstrates greater anticancer activity than 5-Fluorouracil, tamoxifen or its metabolite 4OH-tamoxifen in MCF-7 breast cancer cells yet no cytotoxicity to non-tumorigenic MCF-10A epithelial cells. In A-C, cells were analysed for cell survival using the Alamar Blue™ assay following treatment. Statistical significance as indicated by * P < 0.05, **P < 0.01 or ***P < 0.01 versus vehicle control.
We found that Gg also induced MCF-7 breast cancer cell death (Figure 5a), inhibited CYP1A activity (Figure 5b) and attenuated the ability of B[a]P to induce CYP1A gene expression in MCF-7 cells (Figure 5c). These data indicate that Gg selectively suppresses MCF-7 breast cancer cell growth without impacting non-tumourigenic breast epithelial cells and blocks B[a]P-mediated CYP1A induction. Taken together, our data provide a rationale for further investigations of Gg and similar plant isolates as potential agents to treat and prevent breast cancer.
Figure 5. Gg induces apoptosis and inhibits CYP1 enzyme activities and suppresses B[a]P-induced CYP1A gene expression in non-invasive MCF-7 breast cancer cells. (A) MCF-7 cells were exposed to media containing Gg (0.01-1.0 μM) or 0.025% DMSO for 24 h before being analyzed for apoptosis using the Annexin-V-7AAD assay. (B) Human recombinant CYP1B1-catalysed 7-ethoxyresorufin activity (0.37μM), CYPs 1A1 catalyzed 7-ethoxy-3-cyano-coumarin deethylase activity (0.5μM), were determined in the presence of varying concentrations of Gg (0-20μM). Control enzyme activity (mean ± SEM) for CYPs 1B1 and 1A1 were 0.34 ± 0.08, 0.86 ± 0.01 μM/min/pmol of CYP respectively. (C) MCF-7 cells were exposed to B[a]P alone or in combination with Gg at indicated concentrations for 24 h. Cells were harvested, RNA extracted and quantitative real-time PCR analysis to evaluate CYP1A1 and CYP1A2 mRNA expression. Data represent the mean ± EM of three independent experiments. Statistical significance as indicated by ** P< 0.01 or *** P < 0.001 versus treatment with B[a]P only.
Specific Aims

Triple negative breast cancer (TNBC), characterized by tumors that lack estrogen receptor, progesterone receptor, and human epidermal growth receptor expression, represents one of the most aggressive subtypes of breast cancer.\[41\] TNBC carries a poor prognosis due, in part, to a lack of clinically available targeted therapy. Novel therapeutic strategies to combat TNBC primarily involve the use of synthetic small molecules.\[88\] However, the World Health Organization reports that there is increased use of natural remedies and traditional medicine to treat diseases.\[156, 157\] Our lab has investigated the chemotherapeutic and chemopreventive effects of a variety of plant isolates.\[145, 158\] As a result of these findings, our lab has sought to further investigate various plant isolates for the potential treatment of TNBC. This dissertation is a compilation of our findings on two separate plant isolates, dibenzyl trisulfide (DTS) and 3,3'-diindolylmethane (DIM) and their mechanisms of action in TNBC cell lines.

Dibenzyl trisulfide (benzyl trisulfide, DTS) is isolated from a perennial shrub called Petiveria alliacea L. (Guinea Hen weed or Anamu) found growing in tropical regions such as the Caribbean.\[159\] This plant is traditionally prepared for internal use as a tea, liquid, or paste and for treatment of a wide range of conditions such as arthritis, asthma, cancer, colds, headaches, and snakebites.\[160\] DTS is one of the major active phytochemicals isolated from this plant and has been shown to have potent anti-cancer actions in a variety of cancers including prostate, ovarian, and leukemia.\[160-162\] DTS also confers activity against in vitro and in vivo models of breast cancer.\[163, 164\] These studies, however, were performed in preclinical models derived from patients
of European ancestry. To date, such a study has not been undertaken using preclinical models derived from patients of West African ancestry. Importantly, a clinical trial (ClinicalTrials.gov Identifier: NCT04113096) is currently underway to investigate the effects of DTS in stage IV breast cancer patients in Jamaica where more than 90% of residents are of West African ancestry. While DTS has been shown to modulate mitogen activation protein kinase and ribosomal protein S6 kinase alpha 1 activity, the mechanism(s) by which DTS confers its anticancer actions remains ill-defined \cite{164}. We hypothesize that DTS induces potent chemotherapeutic and chemopreventive activity in TNBC.

The status quo in the use of targeted breast cancer therapy has primarily involved treating breast tumors that rely on ER or HER2 signaling for growth.\cite{165, 166} While these approaches have dramatically improved breast cancer outcomes for many women, those with TNBC do not benefit from such therapies. Furthermore, PARP inhibitor, olaparib demonstrates keen sensitivity in TNBC patients with defective DNA repair proteins BRCA1/BRCA2\cite{110} while TNBC patients with BRCA1-proficient tumors rarely benefit. An alternative and viable approach to the effective clinical management of TNBC is to use agents that sensitize olaparib to patients with BRCA1-proficient tumors. While clinical trials have been initiated to examine combination therapy of PI3K/Akt inhibitors and PARP inhibitors, none have investigated combination therapy with an AhR ligand derived from plant sources and a PARP inhibitor. We and others have demonstrated the potential for AhR ligands to inhibit survival signaling pathways, such as the PI3K/Akt signaling pathway.\cite{122, 123} Importantly, PI3K/Akt inhibitors have been shown to synergize with PARP inhibitors.\cite{124}
Emerging evidence suggest that pharmacological restoration of tumor suppressor genes (TSGs) represents a novel therapeutic approach to treat a variety of cancer types, including TNBC.\(^{125}\) We have previously shown the ability of 5F 203 to induce the expression of putative tumor suppressor CYGB in breast cancer cells.\(^{114, 128}\) A number of AhR ligands are plant-derived and the use of herbal medicine is growing in the US.\(^{160, 167}\) AhR ligand 3,3'-diindoylmethane (DIM), a metabolite of indole-3-carbinol and the active component of cruciferous vegetables, is a partial agonist that demonstrates anticancer activity.\(^{168, 169}\) DIM also enhances the anticancer actions of PARP inhibitor PJ34 and induces CYGB expression in BRCA-proficient TNBC cells. Therefore, we also hypothesize that plant-derived AhR ligand, DIM, and synthetic AhR ligand, 5F203 enhance PARP inhibitor efficacy in TNBC cells and upregulates CYGB.

**Aim 1:** Evaluate the mechanism of anticancer action for DTS in TNBC cells derived from patients of West African ancestry (Chapter 2). *We hypothesize that DTS induces apoptotic cell death and inhibits migration and proliferation in TNBC cells.*

**Aim 2:** Determine the mechanism by which CYGB mediates the anticancer actions of DIM (Chapter 4). *Our working hypothesis is that DIM transcriptionally upregulates CYGB leading to TNBC cell death and reduced TNBC migration.*

**Aim 3:** Evaluate the efficacy of DIM and 5F203 in combination with PARP inhibitor olaparib using in vitro models of TNBC (Chapter 4). *Our working
hypothesis is that AhR ligand, DIM and 5F203, sensitizes TNBC cells to PARP inhibitor, olaparib.

It is expected that this work would shed light on the benefits of plant isolates as agents to treat TNBC. Furthermore, healthy diets consisting of cruciferous vegetables may be clinically beneficial in sensitizing BRCA1-proficient TNBC tumors to olaparib, thereby providing patients a healthier lifestyle and improved long-term survival and quality of life.
References


42. NCBI, *The p53 tumor suppressor protein*. Genes and Diseases [Internet], 1998-.


CHAPTER TWO

DIBENZYLTRISULFIDE INDUCES CASPASE-INDEPENDENT DEATH AND LYSOSOMAL MEMBRANE PERMEABILIZATION OF TRIPLE-NEGATIVE BREAST CANCER CELLS

Abstract

The *Petiveria alliacea* L. (*P. alliacea*) plant is traditionally used in folklore medicine throughout topical regions of the world to treat arthritis, asthma, and cancer. Dibenzyl trisulfide (DTS) is one of the active ingredients within the *P. alliacea* plant. Triple-negative breast cancer (TNBC) is associated with a poor prognosis, particularly among women of West African ancestry, due in part to limited effective therapy. Though potent anticancer actions of DTS have been reported in a TNBC cell line, the mechanism of DTS-mediated cytotoxicity and cell death remains ill-defined. In the current study, we show that DTS exhibits cytotoxicity in a panel of triple-negative breast cancer (TNBC) cells derived from patients of European and West African ancestry. We found that DTS inhibits proliferation and migration of CRL-2335 cells derived from a patient of West African ancestry. DTS induces the expression of pro-apoptotic genes BAK1, GADD45a, and LTA in CRL2335 cells though it primarily promotes caspase-independent CRL-2335 cell death. DTS also promotes destabilization of the lysosomal membrane resulting in cathepsin B release in CRL-2335 cells. Finally, Kaplan-Meier survival curves reveal that higher expression of BAK1 and LTA in tumors from patients with TNBC is associated with longer relapse-free survival. Collectively, our data suggest that DTS confers promising antitumor efficacy in TNBC, in part, via lysosomal-
mediated, caspase-independent cell death to warrant furthering its development as an anticancer agent.

**Introduction**

Triple negative breast cancer (TNBC) is characterized by tumors that lack expression of the estrogen receptor (ER), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification. [1] Furthermore, women of West African ancestry are more than twice as likely as women of other ancestries to develop aggressive TNBC. [2] TNBC carries a poor prognosis due, in part, to limited targeted therapy options. While conventional anticancer therapies often entail the use of synthetic small molecules or monoclonal antibodies [3], studies by the World Health Organization and others have reported that the use of herbal medicines for various health challenges is rapidly expanding across the world. [4-6] This rapid global expansion is due, in part, to growing concerns regarding adverse effects associated with pharmaceuticals. Herbal remedies or medicines are often taken in tandem with prescribed pharmaceuticals, often unbeknownst to the prescribing physician. [7, 8] For example, 80% of Jamaican cancer patients have been reported to use medicinal plants along with prescription remedies, with limited physician awareness. [9] As a result, many of the adverse effects seen with pharmaceuticals could be due to alterations in metabolism as seen with drug-herbal interactions. [10] Our understanding of the mechanisms by which these plant isolates confer their actions is often limited. We therefore aimed to delineate the mechanism of anticancer action for dibenzyl trisulfide (DTS, Figure 1A).
DTS is isolated from a perennial shrub called Petiveria alliacea L. (P. alliacea) native to Tropical and Sub-tropical America, and found growing in the Amazon forest, Central America, Caribbean islands and Mexico, as well as specific regions of West Africa. [11] This plant is traditionally prepared for internal use as a tea, liquid, or paste and for treatment of a wide range of conditions such as arthritis, asthma, cancer, colds, headaches, and snakebites. [12] DTS is one of the active phytochemicals isolated from this plant with potent activity against a variety of malignancies such as prostate cancer, ovarian cancer, and leukemia. [12-14] DTS also confers activity against the MDA-MB-231 TNBC breast cancer cell line derived a patient of European ancestry. [15, 16] To date, such a study has not been undertaken using preclinical models derived from patients of West African ancestry. Importantly, a clinical trial (ClinicalTrials.gov Identifier: NCT04113096) is underway to investigate the effects of DTS in patients with stage IV malignancies including breast cancer in Jamaica where more than 90% of residents are of West African ancestry. While DTS has been shown to modulate mitogen activation protein kinase and ribosomal protein S6 kinase alpha 1 activity, the mechanism(s) by which DTS confers its anticancer actions remains ill-defined. [16]

In this study, we investigated the anticancer and chemopreventive potential of DTS, one of the active components of P. alliacea. DTS and other P. alliacea extracts were screened against a panel of TNBC cell lines derived from patients of West African and European ancestry. We then evaluated the anticancer and cell death mechanisms of DTS in a TNBC cell line derived from a patient of West African ancestry that displayed high sensitivity to DTS. To the best of our knowledge, this is the first study
investigating the potential mechanisms of DTS–mediated death in TNBC cells derived from patients of West African ancestry.

**Materials and Methods**

**Cell culture and reagents**

MDA-MB-468, MDA-MB-157, MDA-MB-436, MDA-MB-231 and CRL-2335 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). The cells were cultured as previously described.[17] MDA-MB-231 and MDA-MB-436 cells are derived from patients of European ancestry while the MDA-MB-468, MDA-MB-157 and CRL-2335 cells are derived from patients of West African ancestry. Purified and refined DTS was purchased from International Laboratories (San Francisco, CA, USA). Stock solutions of inhibitors and agents were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use. Cells were exposed to < 0.1% DMSO to avoid impacting cell behavior.

**Plant collection and preparation**

The *P. alliacea* plant was obtained from the botanical garden at the University of the West Indies (UWI) Mona campus, identified and deposited in the UWI Herbarium. Standardized aqueous and 65% ethanolic extracts were prepared as previously described[12] from the dried and fresh plants with voucher numbers # 35931 and # 36361. All extracts were prepared following ethnomedical practices.[18, 19] Briefly, the aqueous extracts were prepared by decocting the whole plant (leaf, root and stem) for 20 minutes in deionized water (1 g/100 mL). The resulting extract was filtered, freeze-
dried and stored at -20°C. The 65% ethanolic extracts were prepared by macerating the plant (leaf, root and stem) in 65% ethanol (1g/10mL) for 10 d and the resulting tincture was dried using a stream of nitrogen gas and stored at -20°C.

**Cell Viability Assay**

We evaluated DTS-mediated cytotoxicity using the Alamar Blue assay in a panel of TNBC cell lines as previously described. Briefly, cells were plated in 96-well plates at their appropriate densities in a total volume of 100 µL. After 24 h of incubation, cells were treated with medium containing DMSO (0.01%), *P. alliacea* extracts (100 pM-100 mM) or DTS for 48 h or 72 h. Alamar Blue dye (10%) mixed with fresh medium was then added to the wells followed by an additional 4–5 h incubation. Cytotoxicity was determined using an FLx800 microplate spectrofluorometer (BioTek Instruments, USA).

**Colony Formation and Wound Healing Assays**

For the colony formation assay, CRL-2335 human breast cancer cells were plated at a density of 2000 cells per well in a 6-well plate. The following day, cells were treated with either DMSO or DTS (100 nM-50 µM) for 48 h. The treatment was removed, and cells were allowed to grow for 2 weeks before the cells were fixed with 10% Formalin and stained with crystal violet solution. Colonies were imaged and counted using the open-source image processing software ImageJ (National Institutes of Health, Bethesda, MD). For the wound healing assay, CRL-2335 cells were plated in 24-well plates containing Ibidi culture inserts (to create the wound) at a density 3x10^5 cells/ml and allowed to recover overnight. The cells were then treated with DMSO, or 10
μM DTS, or 25 μM DTS for 48 h. Images were captured on an Olympus IX-71 microscope and quantified using SPOT software (Olympus Life Sciences Solutions, Waltham, MA).

**Cell Morphology and Apoptosis Determination**

The Annexin V/propidium iodide assay was used to detect apoptosis as previously described. [20] Briefly, CRL-2335 cells were treated with medium containing DTS (10 µM-50 µM) or DMSO for 48 h. In some studies, CRL-2335 cells were pretreated for 1 h with z-VAD-fmk (z-VAD, 100 µM) before treatment with DTS. Cell morphology was examined under relief contrast at different time points to assess the presence of apoptotic blebs before data acquisition using flow cytometry. Data were acquired using a MACSQuant Analyzer 10 (Miltenyi Biotec Inc, Auburn, CA) and analyzed using FlowJo (v9.7.5, TreeStar, Inc, Ashland, OR).

**Real-time quantitative RT-PCR (qPCR) analysis**

RNA was extracted from breast cancer cells after specified treatments, and cDNA synthesis was performed according to the manufacturer’s instructions and in accordance with a method previously described. [17] The primers for the GAPDH, BAK-1, GADD45A, and LTA, genes were obtained from Qiagen (Germantown, MD). Relative fold changes in gene expression were calculated using the 2^(-ΔΔCt) method.

**Immunoblotting**
CRL-2335 cells were seeded at 1-3 × 10⁶ per plate (100 mm). After 24 h, cells were treated with medium containing DMSO, 10 μM DTS, or 50 μM DTS for 48 h. After the treatment, cells were harvested, and Western blot analysis was performed as described. [17] Briefly, proteins were resolved on 4%-12% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked before an overnight incubation at 4°C in 5% milk–based buffer with mouse primary antibodies against LTA (1:1000), and rabbit primary antibodies against BAK-1 (1:1000), GADD45A (1:1000), PARP (1:1000) and caspase-3 (1:1000). Membranes were incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA or Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at the appropriate dilution before imaging.

**Lysosomal Membrane Permeability (LMP) Assay**

The Acridine Orange (AO, ImmunoChemistry Technologies, Bloomington, MN) method was used to analyze LMP as described previously. [21, 22] AO preferentially accumulates in the lysosomes due to photon trapping. At high concentrations (lysosomes), this dye emits red/orange fluorescence and green fluorescence at low concentrations (nucleus and cytoplasm) via blue light excitation. Briefly, CRL-2335 cells were seeded at 2 x 10⁵ in a 6-well culture plate and incubated overnight. The cells were then incubated in culture medium containing 0.1% DMSO or 10 μM DTS for 48 h. To ensure that the cells did not suffer any damage in the staining process, they were washed with cold PBS following treatment. PBS was replaced with cell culture medium.
containing 5 μM AO and counterstained with 5 μM Hoechst 33342 before incubating for 30 min at 37°C and examined with an EVOS cell imaging system (Invitrogen, USA).

**Cathepsin B Activation**

The fluorogenic substrate-based assay, Magic Red (Immunochemistry Technologies, Bloomington, MN), was used to detect Cathepsin B activity using the Magic Red MR-(RR)2 reagent as previously described. [21, 22] Briefly, CRL-2335 were treated with 0.1% DMSO or 10 μM DTS for 48 h. The cells were then exposed to the cathepsin B substrate MR-(RR)2 for 45 min, rinsed twice with cold PBS, and examined using the fluorescence microscope. Additionally, cells were counterstained with Hoechst 33342 to detect nuclear morphology.

**Statistical analysis**

Data are reported as mean ± SEM or mean ± SD. Statistical significance was assessed using the one-way analysis of variance (ANOVA) with Tukey’s test, the Dunnett’s test, or the Tukey-Kramer multiple comparison test when evaluating three or more groups. An unpaired Student’s t test with Welch’s correction was used to compare two groups. Statistical analysis was performed using GraphPad InStat 3.0. Differences were considered significant at p < 0.05. Kaplan-Meier plots were constructed using the KM plotter program and hazard ratio with 95% confidence intervals and log rank P values were calculated using gene expression of tumors from cohorts of TNBC patients in accordance with St. Gallen and PAM50 databases.
**Results**

*DTS but not aqueous- or ethanol-based extracts from P. alliacea reduce TNBC cell viability.*

To ascertain the cytotoxic response of TNBC cells to *P. alliacea*, we first exposed MDA-MB-468, MDA-MB-157, MDA-MB-436, MDA-MB-231, and CRL-2335 TNBC cells to aqueous- and ethanol-based *P. alliacea* extracts (100 pM to 100 μM). The percent of DTS per gram of crude extract was found to be at 0.2% and 0.06% in the ethanolic and aqueous extracts, respectively, of different parts of the *P. alliacea* plant and in undetectable levels in the fresh plant preparations using HPLC as well as LC-MS analyses.[12] We observed no significant cytotoxicity against any of the TNBC cells exposed to either of the extracts (Table 1; Supplementary figure 1). However, when these cells were exposed to DTS (100 pM-100 μM), a significant reduction in cell viability was observed following 48 h and 72 h of exposure (Table 1; Figure 6B-C). Notably, the CRL-2335 cell line is described as an acantholytic squamous cancer that is generally more aggressive and treatment-refractory compared to the other cell lines used in this study.[23, 24] Thus, we focused our investigation on the anticancer and death promoting actions of DTS in CRL-2335 cells.
Table 1. Relative cell viability IC$_{50}$ values (M) following exposure to *P. alliacea* extracts or DTS

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-157</th>
<th>MDA-MB-468</th>
<th>CRL-2335</th>
<th>MDA-MB-436</th>
<th>MDA-MB-231</th>
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<tr>
<td>GHW (AE) 48h</td>
<td>&gt; 1.00e-004</td>
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<td>GHW (EE) 48h</td>
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<tr>
<td>DTS 48h</td>
<td>~ 3.989e-006</td>
<td>~ 1.387e-005</td>
<td>5.022e-006</td>
<td>~ 8.583e-006</td>
<td>2.482e-006</td>
</tr>
<tr>
<td>GHW (AE) 72h</td>
<td>&gt; 1.00e-004</td>
<td>&gt; 1.00e-004</td>
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<tr>
<td>GHW (EE) 72h</td>
<td>&gt; 1.00e-004</td>
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<td>&gt; 1.00e-004</td>
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</tr>
<tr>
<td>DTS 72h</td>
<td>~ 1.108e-006</td>
<td>~ 8.927e-007</td>
<td>7.327e-007</td>
<td>3.487e-006</td>
<td>7.233e-006</td>
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Cells were exposed to extracts and isolates for 48 or 72 h at varying concentrations and IC$_{50}$ values determined in accordance with Materials and Methods. IC$_{50}$ is the concentration needed to reduce cell viability by 50%. Abbreviations: Guinea Hen Weed, GHW; aqueous extract, AE; ethanolic extract, EE.

Supplementary materials can be found at https://doi.org/10.1016/j.fitote.2022.105203
Figure 6. Impact of Dibenzyln Trisulphide (DTS) on the viability of triple-negative breast cancer cells. A) Structure of dibenzyl trisulfide (DTS), an active ingredient of *P. alliacea*. B-C) Cells were treated with DMSO or DTS (100 pM -100µM) for 48 h or 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. Data are reported as the mean of at least triplicate values. Bars, SEM
**DTS inhibits proliferation and migration in TNBC cells**

We used a colony forming assay to determine whether DTS decreases proliferation of CRL-2335 cells. DTS exposure resulted in significantly fewer colonies compared to the control at concentrations below 500 nM (Figure 7). We performed a wound healing assay involving IBIDI inserts to determine whether DTS decreases CRL-2335 cell migration (Figure 8). Our data reveal that DTS suppresses cancer cell migration at 10 µM and completely blocks it at 25 µM. These data suggest DTS not only thwarts proliferation and migration but also TNBC cell invasion and metastases.
Figure 7. Colony Forming and Wound Healing Assay Analysis. CRL-2335 Cells were treated and allowed to grow for 2 weeks before staining with crystal violet. Colonies were counted using Image J and quantified using GraphPad Prism version 9. Data are reported as the mean of triplicates. Bars, SD. Not significant (ns), *** P <0.001, **** P < 0.0001 as compared to DMSO treatment group.
Figure 8. Wound Healing Assay of CRL-2335 Breast Cancer Cells. Cells were plated in IBIDI culture inserts and allowed to grow to confluency before being treated with DTS (10 µM and 25 µM). A) Images were captured using an Olympus IX-71 microscope at 0h, 24h, and 48h. B) Quantification of cell migration following given treatments was performed using GraphPad Prism version 9. Bars, SD ** P < 0.01, *** P < 0.001, **** P < 0.0001, relative to the DMSO-treated group.
**DTS promotes caspase-independent TNBC cell death.**

We next sought to determine whether DTS induces TNBC cell death using the Annexin V-FITC assay and relief contrast microscopy. We show that DTS promotes early apoptotic cell death of CRL-2335 as evidenced by Annexin V positive cells (Figure 9A-C). Morphological imaging also revealed apoptotic bodies after exposure to DTS in a dose- and time-dependent manner in MDA-MB-468 and CRL-2335 TNBC cells (Figure 9C; Supplementary Figure 2). To further elucidate the mechanism of DTS-mediated cell death, we investigated the role of caspase activation in cells treated with DTS. Pretreatment with a pan-caspase inhibitor (Z-VAD-FMK) minimally rescued cells from DTS-mediated TNBC cell death (Figure 9A-C). Taken together, these data suggest DTS promotes cell death primarily via caspase-independent mechanisms.
Figure 9. DTS induces caspase-independent CRL-2335 cell death. A) Flow cytometry data of cells treated with either 0.01% DMSO, 50 µM DTS, 10 µM Staurosporine (STS, positive control), or DTS in combination with caspase inhibitor Z-VAD-FMK. (Z-VAD) B) Quantification of cells in early apoptosis in cells exposed to the treatments as described in A, ** P < 0.01 as compared to 0.01% DMSO, Bars, SD. Numbers enlarged in the lower right quadrant indicate the average percentage of cells in early apoptosis. C) Morphology of CRL-2335 breast cancer cells after DTS treatment. CRL-2335 cells were exposed to DMSO, DTS (1-50 µM) and STS (10 µM) for the indicated times. Images were captured using the Olympus IX71 microscope (X40 magnification).
To gain further insight into the mechanism of DTS-mediated cell death, we treated CRL-2335 cells with 0.01% DMSO, 10 μM DTS, or 50 μM DTS and analyzed the expression of three proapoptotic genes: BAK-1, GADD45a, and LTA. BAK-1 promotes apoptosis and counteracts protection from apoptosis provided by Bcl-2. GADD45a plays a role in p38-mediated p53 activation. LTA induces tumor necrosis factor receptor 1-dependent apoptosis and necroptosis. Our studies revealed that DTS significantly induced BAK1, GADD45a, and LTA mRNA expression at 50 μM (Figure 10). We then sought to evaluate the relationship between BAK1, GADD45a, and LTA tumor expression from patients with TNBC and survival. Figure 11 shows that higher BAK1 and LTA expression levels in tumors from patients with TNBC are associated with increased relapse-free survival according to PAM50 and St. Gallen databases. We did not observe this trend with GADD45a (data not shown). Though a similar trend in overall survival for these genes was also observed, it did not achieve statistical significance. The downstream cleavage of various cytoplasmic or nuclear substrates, including PARP, by effector caspases such as caspase 3 mark many of the morphological features of apoptotic cell death. Thus, we sought to determine whether DTS impacted the cleavage of PARP and caspase 3 in CRL2335 cells. We also sought to evaluate the impact of DTS on BAK1, GADD45a, and LTA protein expression in CRL2335 cells. We found that DTS induced PARP cleavage, but only minimal and non-significant caspase 3 cleavage in CRL-2335 cells (Figure 12). Importantly, DTS significantly induced the expression of proapoptotic proteins BAK-1 and LTA but not GADD45a (Figure 12A-B) in CRL-2335 cells. These data suggest that DTS not only exhibits anticancer
activity in TNBC but diminishes the potential for recurrence among patients by inducing caspase-independent cell death and inducing the expression of certain pro-apoptotic genes associated with increased relapse-free survival.
Figure 10. Proapoptotic gene expression in CRL-2335 cells following treatment with DTS. Cells were exposed to DMSO or DTS for 48h before they were harvested, RNA extracted, and quantitative PCR analysis. *P < 0.05, ** P < 0.01 Bars, SD.
Figure 11. Kaplan-Meier survival curves correlating BAK1 and LTA gene tumor expression relapse-free survival predicted among patients with triple negative breast cancer (TNBC). (A-B) Relapse free survival (RFS) predicted among TNBC patients with tumors that express BAK1 as significantly higher than that among those with lower expression in accordance with the PAM50 and St. Gallen databases respectively. (C-D) RFS predicted among patients with tumors expressing LTA as significantly higher than that among those with lower expression in accordance with the PAM50 and St. Gallen databases respectively.
Figure 12. Apoptosis analysis of CRL-2335 cells. A) Immunoblot determination of pro-apoptotic protein expression in CRL-2335 cells. B) Quantification of relative protein expression. *P < 0.05, ** P < 0.01, *** P <0.001, **** P < 0.0001. Bars, SEM.
DTS promotes lysosomal membrane permeabilization and cathepsin B release in CRL2335 cells

Because our data suggest DTS mediates cell death via mechanisms that don’t rely on caspases, we sought to determine whether DTS-mediated cell death was related to lysosomal membrane permeabilization. Lysosomes are organelles that mediate the degradation of intracellular macromolecules. Various stressors can induce lysosomal membrane permeabilization (LMP) which releases lysosomal contents such as cathepsins into the cytoplasm.\textsuperscript{[30]} Furthermore, LMP-induced cathepsin release activates classical caspase dependent and independent apoptosis\textsuperscript{[31]}

To delineate the role of DTS in mediating LMP and cathepsin activity, we analyzed lysosome and cathepsin activity in cells exposed to acridine orange (AO) and Magic Red (MR) respectively. We found that DTS caused LMP in CRL-2335 cells, as evidenced by a reduction in intact, punctate red fluorescent lysosomes (Figure 13A). Similarly, DTS promoted cathepsin B release, as evidenced by diffuse red fluorescent dye staining throughout the cell (Figure 13B).
Figure 13. DTS induces lysosomal membrane permeabilization and cathepsin B release in CRL-2335 cells. CRL-2335 cells after treatment with DMSO (CTL) or DTS (10 μM) for 48 h followed by analysis using acridine orange (A) or the Magic Red (B) assays to measure LMP and cathepsin B release, respectively (X40 magnification).
Discussion

TNBC carries a poor prognosis due, in part, to the scarcity of efficacious, targeted therapy. Therefore, it is crucial to develop agents with the ability to effectively treat TNBC. While aqueous- and ethanol-based preparations of *P. alliacea* have been used to treat a variety of medical conditions including breast cancer, our data suggest that such preparations may be less efficacious due to the low content of DTS in the ethanolic and aqueous extracts of 0.2% and 0.06% respectively. The low concentration levels of DTS may explain the low bioactivity observed in the extracts. As DTS is insoluble in water, it is no surprise that the fresh plant extract, which has a higher water content than that of the dried plant, would possess even lower concentrations of DTS. Although DTS content is higher in the ethanolic extract, it is still in low yield to incite cytotoxicity within the tested parameters. Additionally, like all natural extracts, ethanolic extracts of *P. alliacea*, likely contain multiple secondary metabolites, accounting for possible competitive binding in the cell viability assays. Our study does suggest that DTS itself possesses promising anticancer actions due in part to its ability to confer caspase-independent death of TNBC cells.

The effectiveness of DTS as an anticancer agent has been thoroughly documented in human pancreatic, ovarian, prostate, lung, and breast cancer cell lines.\[15\] In particular, the anticancer activity of DTS has been screened in MCF7 and MDA-MB-231 breast cancer cell lines with the MDA-MB-231 cell line showing more sensitivity to DTS.\[32, 33\] MCF-7 cells are luminal since they express hormone receptors, and MDA-MB-231 breast cancer cells are classified as TNBC; both are derived from patients of European ancestry.\[34\] Importantly, Phase I clinical trials (ClinicalTrials.gov Identifier:
NCT04113096) are underway to investigate the effects of DTS in patients with stage IV cancer, including those with breast cancer. While the P. alliacea plant extracts exhibited minimal efficacy in TNBC cells, the isolated phytochemical DTS showed significant activity in these cells irrespective of the ancestry of the patients from where they were derived (Figure 6). The CRL-2335 cells demonstrated promising antiproliferative and antimigration actions (Figures 7-8) to suggest it has potential to thwart metastases in TNBC. Furthermore, while some plant isolates require concentrations of at least 100-200 μM to display activity, DTS treatment resulted in IC\textsubscript{50} values less than 10 μM in most TNBC cells irrespective of ancestry (Table 1). Previously, DTS was shown to exhibit potent cytotoxicity in MDA-MB-231 cells and our data agree with these findings \cite{16}. Notably, studies have shown that extracts of P. alliacea as well as DTS display no cytotoxic activity against non-tumorigenic, human hepatocellular carcinoma cell line, HB 8065 (Hep G2) \cite{35, 36}, and this suggests that DTS is selectively cytotoxic to tumorigenic cells. It is important to note that cancer cells lack the complex cytochrome P450 systems found in preclinical mouse models and in humans crucial to converting the aqueous and ethanolic extracts to active metabolites. As a result, we cannot conclude that such extracts are ineffective in humans just because we were unable to detect activity in the TNBC cell lines used in the current study.

We recently found that DTS suppresses aryl hydrocarbon receptor (AhR)-independent cytochrome P450 1 (CYP1) activity \textit{in vitro} and \textit{in vivo} and binds to the CYP1A1 active site.\cite{37} CYP1A1 has been shown to promote the bioconversion of procarcinogens such as benzo-A-pyrene into carcinogenic metabolites which promote
the progression of breast cancer after activating the AhR signaling pathway. Thus, DTS has potential use as a chemopreventive agent. Our data show that DTS suppresses CYP1A2 expression in MDA-MB-468 and CRL2335 cells (Supplementary figure 3). We also found DTS suppressed TNBC proliferation (Figure 7) and cell migration (Figure 8). These findings suggest DTS confers chemopreventive actions in addition to its anticancer actions in TNBC.

Apoptosis can occur via extrinsic or intrinsic pathways. Extrinsic apoptosis occurs through the activation of death receptors such as CD95 or TRAIL, while intrinsic apoptosis is initiated via a diverse array of non-receptor-mediated stimuli. Diverse forms of cellular stress can activate the intrinsic-apoptotic mitochondrial pathway by inducing the formation of pores in the outer mitochondrial membrane by proapoptotic members of the Bcl-2 family of proteins. BAK1 and the protein it encodes belong to the Bcl-2 family members that promote mitochondrial outer membrane permeabilization (MOMP). MOMP releases pro-apoptotic components such as cytochrome c into the cytosol, initiating the caspase cascade to mediate apoptosis. GADD45a is a DNA damage-inducible protein that plays a role in the cell’s response to DNA damage and in the maintenance of its genomic integrity. Induced Gadd45a enables the dissociation of Bcl-2 family member Bim from microtubule-associated components which are then translocated to the mitochondria to promote MOMP, release cytochrome c, and activate the caspase cascade. We found that 50 μM DTS induced BAK1, GADD45a, and LTA gene and protein expression though DTS-mediated induction of GADD45a protein expression did not reach statistical significance (Figures 10 and 12). While the St. Gallen database showed that higher GADD45a tumor expression correlated with
increased relapse-free survival, this association was not significantly observed when we used the PAM50 database (data not shown). LTA or tumor necrosis factor-beta (TNF-β) is a pleiotropic cytokine that mediates a myriad of antiviral, inflammatory, and immunostimulatory responses.[45-48] LTA induces apoptosis extrinsically through the death receptor p55TNFR, resulting in the subsequent activation of caspases.[49] Furthermore, increased survival was found among patients with tumors that possessed increased expression of BAK and LTA (Figure 11). This suggests that these pro-apoptotic genes are important components to improving patient outcomes and DTS-mediated induction of these genes represents an important component to its mode of anticancer action.

From our results and due to the role of BAK1 and LTA genes in mediating caspase cascade activation, we initially theorized that the anticancer actions of DTS were dependent on the activation of caspases. However, when caspase activity was inhibited via the pan caspase inhibitor Z-VAD, DTS-mediated cell death was insignificantly inhibited (Figure 9). In contrast, Z-VAD pretreatment of cells significantly suppressed staurosporine-mediated cell death (data not shown). Staurosporine has been shown to induce caspase-dependent or caspase-independent apoptosis depending on cell context.[50-52]

Other cellular components are involved in the cell death pathway. Poly(ADP-ribose) polymerases (PARPs) play pivotal roles in counteracting genotoxic stress and other forms of DNA damage.[53] PARP-1, the most abundantly expressed, is one of several known cellular substrates of caspases. Cleavage of PARP-1 via caspase 3, is a hallmark of apoptosis.[54-56] Our data show that DTS induces PARP in a dose-dependent
manner (Figure 12) and cleaved PARP is expressed more robustly than cleaved caspase 3. However, studies show that cathepsins and TGF-β are involved in caspase-independent PARP-1 cleavage. Therefore, the lack of caspase 3 cleavage may correlate with the DTS-mediated cathepsin B release resulting in cleaved PARP-1 fragments. Additionally, we observed DTS-induced expression of proapoptotic proteins (Figure 12A-B). Notably, BAK exists as an inactive monomer. Upon apoptotic stimuli, activated BAK undergoes oligomerization by forming Bcl-2 homology 3 (BH3): groove homodimers that represent the basic stable oligomeric unit. For this reason, the ~50 kDa band often observed in our Western blots correlates with the dimerized form of Bak (25 kDa, anti-bak; Cell Signaling Technology, Inc.) resulting from apoptotic stimuli.

Apoptosis is a specific and specialized form of cell death characterized by distinct morphological characteristics such as cell shrinkage, pyknosis, blebbing, and formation of apoptotic bodies. We observed cellular bodies closely resembling advanced apoptosis at high concentrations of DTS (Figure 9, supplementary figure 2). Furthermore, we show DTS induces LMP (Figure 13A) and cathepsin B release (Figure 13B). Interestingly, LMP is a hallmark of not only apoptosis and necrosis, but necroptotic and autophagic cell death. Taken together, these data suggest that DTS induces TNBC cell death primarily via caspase-independent mechanisms. Caspase-independent cell death carries two advantages over caspase-dependent cell death. Firstly, caspase-independent death prevents the caspase-dependent oncogenic effects of apoptosis. Secondly, caspase-independent death can activate anti-tumor
immunity, suggesting that research focused on the signals that accompany a cell during its death rather than the execution of cell death may be prove to be beneficial.[64]

In conclusion, DTS represents a promising agent with potential to treat refractory forms of breast cancer including TNBC which disproportionately impacts women of West African ancestry. With the continued growth in popularity, the potential for interactions between medicinal plant products and pharmaceutical drugs is a growing health concern for many governments and regulatory bodies worldwide. Though most of our results were obtained after 48 h or 72h of treatment, it is recommended that patients use *P. alliacea* twice daily for an extended period[65], to allow sufficient levels to accumulate in the plasma. A thorough understanding of the pharmacokinetics of DTS, including any activated metabolites, would therefore be beneficial. It is possible that factors such as drug metabolizing enzymes (e.g., CYP1A) influence the efficacy of this phytochemical over time. It is crucial to further elucidate the mode of DTS-induced cell death and efficacy using *in vivo* models of TNBC as such preclinical studies are needed to validate the results of our study. These preclinical studies will be essential to provide a basis for further clinical evaluation of not only DTS but *P. alliacea* extracts with the goal of enhancing clinical outcomes for patients with refractory forms of cancer.
References


CHAPTER THREE

POLY(ADP-RIBOSE) POLYMERASE INHIBITION: TRIUMPHS AND CHALLENGES IN EXPLOITING SYNTHETIC LETHALITY TO COMBAT TRIPLE NEGATIVE BREAST CANCER

Abstract

When healthy cells encounter DNA damage, they initiate responses including intricate repair mechanisms to preserve the genome. When these DNA damage repair mechanisms go awry, disorders such as cancer can occur. Emerging evidence suggest that poly(ADP-ribose) polymerase 1 (PARP1) regulates a myriad of DNA repair mechanisms to maintain genomic integrity. PARP inhibitors (PARPi) represent the first targeted therapy approach to exploit synthetic lethality in the clinics to treat malignancies with a deficiency in the homologous recombination (HR) pathway. Malignancies such as triple negative breast cancer (TNBC) characterized by tumors that lack expression of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, are typically unresponsive to most targeted therapy. The recent approval of PARPi has resulted in promising efficacy in a significant subset of these patients harboring mutations in BRCA1 and BRCA2, critical tumor suppressors and mediators of HR. However, a number of patients who initially respond to PARPi acquire resistance while others show de novo resistance to PARPi. Thus, it is crucial that we enhance our understanding of how PARP coordinates DNA repair to enable us to develop more effective therapeutic strategies to counteract PARPi resistance. In this
review, we discuss the roles of PARP in mediating DNA repair processes in breast epithelial cells, mechanisms of PARPi resistance in TNBC and recent advances in the development of agents designed to overcome PARPi resistance in TNBC.

Introduction

Cells encounter various forms of DNA damage. Fortunately, intricate processes exist within cells that are designed to detect and respond to DNA damage. DNA damage triggers DNA repair mechanisms crucial to maintaining genomic stability. Such mechanisms represent critical responses to DNA damage as they aid in the prevention of cancer development.

Several mechanisms are implemented to repair DNA damage in epithelial cells. For instance, scaffold protein XRCC1 enables crucial interactions between enzymes such as AP endonuclease, DNA ligase III, DNA polymerase β, and poly(ADP-ribose) polymerase 1 (PARP1) during base excision repair (BER). PARP1 binds to damaged DNA, triggering the activation of the catalytic portion of the C-terminal domain and nicotinamide adenine dinucleotide (NAD+) hydrolysis \[^{1-3}\]. Poly(ADP-ribose) polymerase 2 (PARP2) represents another key contributor to DNA repair, providing up to 10% of all the PARP activity \[^{4}\]. PARP1 enables the resealing of single strand breaks that occur in BER \[^{3}\] as well as in the repair of topoisomerase I cleavage complexes \[^{5}\] and DNA double strand breaks (DSBs) \[^{6-8}\]. Both PARP1 and PARP2 undergo extensive autoPARylation which enables them to dissociate from DNA to ultimately complete DNA repair \[^{9}\].
Homologous recombination (HR) DNA repair is triggered when single strand breaks (SSBs) encounter replication forks to form DSBs \[^{10-12}\]. In sporadic breast cancer cells, HR DNA repair is particularly elevated\[^{13}\]. Indeed, mutations in HR DNA repair increase susceptibility to the cytotoxicity of certain genotoxic anticancer agents such as PARP inhibitors (PARPi). A detailed discussion of HR DNA repair has been previously reviewed \[^{14}\] and is outside of the scope of this manuscript.

Individuals with mutations in breast cancer susceptibility genes (BRCA1 and BRCA2), exhibit a defect in HR DNA repair that heightens their reliance on enzymes such as PARP \[^{15}\]. BRCA1 and BRCA2 genes function as tumor suppressors which initiate DNA repair in cooperation with other proteins to maintain genomic stability \[^{16, 17}\]. Mutations in BRCA1 and BRCA2 are known to substantially increase one’s likelihood of developing breast and ovarian malignancies. Certain patients with these mutations including those with triple negative breast cancer (TNBC), respond favorably to recently approved PARP inhibitors olaparib and talazoparib \[^{18, 19}\]. PARPi monotherapy confers exceptional efficacy in certain patients. Mutations in HR DNA repair in certain breast cancer cells render them more susceptible to the cytotoxic actions of certain genotoxic agents and also to PARPi. Indeed, One key benefit of PARP inhibition is its keen ability to enhance the DNA damaging actions of chemotherapeutic agents in TNBC cells.

The efficacy of PARPi is based on the premise of synthetic lethality such that tumors from patients with germline mutations in BRCA (gBRCAm) also have deficiency in HR, which results in their increased sensitivity to PARPi. Patients with gBRCA mutations differ from patients with somatic mutations in BRCA in that gBRCA mutations are inherited while somatic mutations are acquired from damage to genes in an
individual cell during a person’s life.\textsuperscript{[20]} One limitation in the use of PARPi to treat TNBC is that not all of these patients possess gBRCAm \textsuperscript{[21]}. In addition, among those who respond to PARPi initially, a substantial number acquire resistance \textsuperscript{[22]}. Intriguingly, a subset of patients with gBRCAm display intrinsic resistance to PARPi via mechanisms that are not entirely understood \textsuperscript{[23, 24]}. In contrast, some reports suggest that some patients with TNBC expressing wildtype BRCA1 and BRCA2 have the potential to respond to certain PARPi \textsuperscript{[25]}. Though DNA damage repair mechanisms are essential to maintaining genomic stability, they are also culprits to mediating chemoresistance in TNBC. In this review, we will primarily focus on DNA damage repair mechanisms that involve PARP in TNBC. We will also discuss the clinical development of PARPi and therapeutic strategies to overcome PARPi resistance which frequently entails the implementation of combination therapy. Understanding the context of PARPi sensitivity is indeed crucial to appropriately stratifying care among patients with TNBC.

\textbf{Roles of PARP in mediating DNA damage repair in breast epithelial tissues}

The integrity and stability of DNA in breast epithelial tissues are key factors in breast homeostasis. DNA, however, can be damaged or compromised. Defects in DNA repair due to mutations substantially increases the likelihood a person will develop breast cancer, including aggressive TNBC. Fortunately, among individuals with intact DNA damage repair mechanisms, breast cancer development is far less likely since DNA damage is readily repaired, regardless of whether it is caused by environment factors (exogenous) or by errors in replication (endogenous).
Poly(ADP-ribose) polymerases (PARPs) are a family of proteins that are loosely based on structural similarity and function and are comprised of two ribose moieties and two phosphates per unit polymer. [26]. These polymerases play pivotal roles in counteracting genotoxic stress and other forms of DNA damage [27]. Although PARP1 is the most abundantly expressed and characterized isoform, PARP2 also plays important and overlapping roles in DNA repair pathways and in maintaining genomic stability [27, 28]. More than 90% of ADP-ribosylation in cells is carried out by PARP1 mechanisms while PARP2 is responsible for up to 15% of the cell’s poly(ADP-ribose) production [29].

The major forms of endogenous DNA damage include DNA mutations, such as deletions, insertions or inappropriate repetitions, and DNA chromosomal abnormalities [30]. DSB, SSB, and chromosome fusion represent common DNA chromosomal abnormalities. PARP1 detects these DNA strand interruptions and promotes the synthesis of poly(ADP-ribose) (PAR) using the coenzyme, NAD+ as a substrate [31] (Figure 14). PAR is a post-translational modification that regulates a myriad of DNA damage responses including methylation, ubiquitination, acetylation, and phosphorylation [32]. Since NAD+ provides the ADP-ribose units that PARP attaches to proteins, PAR cannot occur without NAD+ [33]. Generally, the roles of PARP1 in response to DNA damage involve the PAR-mediated recruitment of repair factors and the regulation of chromatin structure and composition [34].

While PARP1 involvement in the BER pathway to promote SSB repair is fairly well-known, PARP1 is also implicated in the regulation of classical non-homologous end-joining (C-NHEJ), alternative non-homologous end-joining (A-NHEJ), nucleotide excision repair (NER), DNA mismatch repair (MMR), homologous recombination (HR),
microhomology-mediated end-joining (MMEJ), and maintenance of replication fork stability \cite{34}. Detailed reports elucidating the roles of PARP1 in different pathways have been discussed in several recent reviews; however, damage detection, recruitment, and activation of DNA ligase III, polymerase β, and several scaffolding proteins are common components in the DNA repair process \cite{34-36}.

**PARP1 mediates excision repair in breast epithelial cells**

SSB repair can occur via BER, NER or MMR. BER is considered the most important mechanism for handling SSB repair and PARP contributes to this repair mechanism. When nitrogenous bases are damaged, either by radiation or alkylating agents, BER is important for removing these damaged bases by a DNA glycosylase that recognizes specific types of base damage and removes them from the DNA backbone, forming an abasic or apurinic/apyrimidinic (AP) site \cite{29, 37}. AP sites are the central intermediate in DNA base excision repair (BER) and must be processed by 5' AP endonucleases. Apurinic endonuclease 1 (APE1) is a critical enzyme in the repair of oxidized bases that detects, recognizes, and cleaves the DNA phosphodiester backbone 5' of AP sites to create a free 3'-OH end for DNA polymerase repair synthesis \cite{38, 39}. Reports suggest that PARP1 is a protein in the BER pathway that physically interacts with APE1 \cite{40, 41}. Studies have shown a co-dependency in expression between the PARP1 and APE1 proteins—where downregulating PARP1 yielded APE1 deficiency—as well as the synthetic lethality of inhibiting both PARP and APE1 in TNBC \cite{38, 42, 43}. Chen and colleagues confirmed that the expression of APE1
helps maintain the expression of PARP1 and that the deletion of APE1 expression may contribute to the resistance of PARPi olaparib \[44\]. Interestingly, one report demonstrates that PARP1 is not absolutely required for the completion of BER and rather promotes the trapping of a single cell intermediate upon treatment with PARP inhibitors \[45\]. Evidence of the importance of PARP trapping will be discussed later in this review.

PARP promotes NER by stabilizing the DNA damage binding protein 2 (DDB2) and recruiting ALC1 \[46\]. PARP also activates the NER pathway to aid in the removal of UV-induced DNA lesions \[47\]. However, more recent studies demonstrate that TNBC cells with a deficiency in ERCC5, an NER gene, were not particularly responsive to PARP inhibition and that other TNBC cells did not exhibit NER deficiency \[48\]. This suggests that PARP-mediated regulation of NER does not readily occur in TNBC.

\textit{PARP plays a pivotal role in variety of NHEJ pathways in breast epithelial cells}

HR and C-NHEJ, are the two major mechanisms of DSB repair. While HR is the more accurate recombination due to the use of a sister chromatid as a template to maintain genomic stability, C-NHEJ involves the direct ligation of DNA ends, generating programmed deletions during the diversification of antigen receptor genes \[49\]. However, inappropriate activation of C-NHEJ can also promote genomic rearrangements and translocations associated with the onset of cancer. Therefore, genomic stability depends on the activation of appropriate pathways for DSB repair \[50\]. Once DSBs occur, tumor suppressor p53-binding protein 1 (53BP1) is rapidly recruited to the site of
DNA damage where it interacts with modified nucleosomes to inhibit nucleolytic DNA end resection. This process ultimately favors DNA joining by C-NHEJ. However, in BRCA1-deficient cells, 53BP1 inhibits HR and promotes toxic C-NHEJ that leads to genomic instability [51]. Becker and colleagues demonstrated that the protein dimerization hub, DYNLL1, stimulates 53BP1 oligomerization to promote its recruitment to DSB-associated chromatin. Deletion of DYNLL1 or its transcriptional regulator ASCIZ occurs in BRCA1-deficient tumor cells and results in PARPi resistance. The DYNLL1-53BP1 complex is therefore speculated to provide stability to 53BP1 oligomers within chromatin-binding domains to provide appropriate chromatin interactions for successful resection inhibition of C-NHEJ [52].

E3 ubiquitin ligase ring finger protein 168 (RNF168)-mediated ubiquitination of proteins on damaged chromatin is also critical for appropriate DSB repair. BRCA1 and 53BP1 are among the recruited proteins under normal physiological conditions [53]. However, under cancer-associated ubiquitin starvation, their recruitment is inhibited. Chroma and colleagues reported that in TNBC cells with overexpressed RNF168 and altered recruitment of 53BP1, the DSB repair mechanism shifts from HR to C-NHEJ upon exposure to PARPi [54]. Furthermore, the deregulated RNF168/53BP1 pathway could promote tumorigenesis by selecting for a more appropriate cancer cell phenotype. REV7 is a DNA damage response (DDR) factor that is recruited to DSBs by 53BP1 and blocks the resection of HR to promote C-NHEJ. Xu and colleagues reported that cells resistant to olaparib treatment displayed diminished REV7 expression levels and had high levels of colony formation after PARPi treatment [55].
A-NHEJ pathways utilize microhomologies to connect the broken ends of DNA and are used as backup pathways for NHEJ. MMEJ plays a role in chromosomal rearrangement and genomic instability and is upregulated in HR-deficient tumors [56]. Studies on PARP1-null cells showed a deficiency in synapsis and joining of linear DNA fragments by MMEJ and that purified PARP1 tethered DNA fragments. Sfeir and Symington recently discussed that cells treated with PARPi show reduced frequencies of translocations and have a reduced amount of microhomologies at translocation junctions [57]. Furthermore, DNA ligase 3 (LIG3), an enzyme responsible for catalytic functions in mitochondria that is crucial for cell survival, and PARP1 display higher steady state levels in therapy-resistant breast cancer cells and may be genetically predisposed towards MMEJ [57].

**PARP promotes HR-mediated DNA repair in breast epithelial cells**

DSBs caused from either exogenous agents such as UV radiation or endogenous processes, such as errors from SSB repair are corrected by HR and NHEJ repair mechanisms [29]. Breast cancer susceptibility genes BRCA1 and BRCA2 are both required for HR, although BRCA1 is the most characterized and extensively studied accessory to DNA damage responses (DDRs) [58, 59]. BRCA1 is among the various proteins recruited by PARP1 to the site of DNA damage where it is poly-ADP-ribosylated, decreasing its DNA binding affinity and fine-tuning its function in HR repair [60].
PARP regulates BRCA1 to enable it to drive HR-mediated DNA repair \[^{60}\] (Figure 14). BRCA1 mediates the repair of DNA DSBs via HR. BRCA1 and BRCA2 protect the stalled replication forks through the prevention of their degradation \[^{61}\]. During replication stress, PARP1 protects the integrity of replication forks \[^{62}\]. The majority of TNBCs possess HR DNA repair that would render them sensitive to PARPi and a mutation signature determined through whole genome sequencing has the potential to provide predictive biomarkers for PARPi responsiveness \[^{63, 64}\]. PARP inhibition exhibits synthetic lethality for HR-mediated DNA repair even in TNBC \[^{65}\] (Figure 15). A pivotal study revealed that a shieldin complex mediates the restoration of HR as a means of conferring PARPi resistance \[^{66}\].
Figure 14. PARP coordinates base excision repair and homologous recombination pathways to repair single strand breaks and double strand breaks respectively. When DNA receives a single strand break, PARP is recruited to the site along with XRCC to detect the lesion. NAD+ serves as a substrate for PAR formation and is recovered in cells from nicotinamide via ATP. This enables PARP to catalyze the polymerization of ADP-ribose also known as PARylation which can be reversed by enzymes such as PARG. DNA ligase III and pol β work in conjunction with XRCC to promote ligation and fill the gap. Following DDB, PARP is also recruited and the MRE 11 is recruited along with ATM. Later BRCA1 partnered with BARD1 and RAP80 are recruited to the site with RAP80 serving to stabilize BRCA1 following PARylation. The cohesion complex composed of core subunits: SMC1, SMC3, Rad21 and SA encircle the DNA strands and mediate sister chromatid cohesion during mitosis to promote DSB repair. Later WAPL and PDS5 act to release cohesion (not shown). RPA is recruited and later exchanged for RAD51 filaments placed there by BRCA2 (not shown). Strands undergo invasion and then resolution occurs. SSB: single strand breaks; XRCC: X-ray repair cross-complementing protein; NAD+: nicotinamide adenine dinucleotide +; PAR: poly(ADP-ribose); PARP: poly(ADP-ribose) polymerase; PARG: poly(ADP-ribose) glycohydrolase; DSB: double strand breaks; MRE 11: meiotic recombination 11; pol β: DNA polymerase β; structural maintenance of chromosome: SMC; ATM: ataxia telangiectasia mutated; BRCA1: breast cancer gene 1; BARD1: BRCA1-associated RING domain protein 1; RPA: RNA polymerase A.
Recent advances in the use of PARPi to treat triple negative breast cancer

Preclinical development of PARPi to treat triple negative breast cancer

The success of PARPi in breast cancer patients are well documented \[^{67-70}\]. The discovery and development of PARPi began nearly fifty years ago with the actual nicotinamide \[^{71}\]. Later, PARPi were designed containing nicotinamide and benzamide functional groups to enable them to bind to the catalytic portion of PARPs \[^{72}\]. Interestingly, an initial candidate agent (iniparib) evaluated as a PARPi for TNBC failed clinical trials and a definitive in vitro study revealed that the agent did not appreciably inhibit PARP \[^{75}\].

More recent discovery efforts for PARPi led to olaparib and talazoparib which demonstrate utility in gBRCAm-associated breast tumors and this enabled their approval in 2018 \[^{76, 77}\]. In a recent study, different PARPi were evaluated in 12 breast cancer cell lines with and without gBRCAm; 5 of the 8 TNBC cell lines were susceptible to PARPi regardless of their BRCA-status \[^{78}\]. Talazoparib, niraparib, olaparib, and rucaparib also demonstrated effective inhibitory potency in both advanced TNBC and ER-/HER2+ cells, irrespective of gBRCAm status. This suggests that factors other than gBRCAm play a role in determining a patient’s responsiveness to PARPi. These data also indicate that PARPi may be useful in treating gBRCAm-associated breast tumors that display BRCAness characteristics or contain aberrant DNA-damage response gene expression \[^{78}\]. BRCAness is a state in which tumors display similar sensitivity to PARPi due to defects in HR repair yet don’t possess gBRCAm.
Improvements in the efficacy of PARPi derivatives are ongoing. A recent study revealed the successful development of four conjugated complexes of PARPi derivatives coordinated to the ruthenium(II) ion\textsuperscript{[79]}. These drugs were designed to more effectively inhibit PARP enzymatic activity. In a panel of breast cancer cell lines (including BRCA-null), these Ru(II)-arene complexes displayed promising efficacy. The Ru(II)-arene complex C2 \([(\eta^6-p\text{-cymene})\text{Ru}(L1)\text{Cl}]\text{PF}_6\) displayed the highest efficiency of PARP1 enzymatic activity inhibition followed by C4 \([(\eta^6-p\text{-cymene})\text{Ru}(L2)\text{Cl}_2]\), C1 \([(\eta^6-toluene)\text{Ru}(L1)\text{Cl}]\text{PF}_6\), and then C3 \([(\eta^6-toluene)\text{Ru}(L2)\text{Cl}_2]\). The C1 complex displayed the highest antiproliferative activity as well as the highest intracellular accumulation and distribution, entering BRCA1-deficient CRL-2336 TNBC cells within 24h. The C1 complex also displayed the highest DNA binding activity, resulting in S phase cell cycle arrest\textsuperscript{[79]}. Overall, these data suggest a promising new field of study for increasing PARPi efficacy and treatment of breast cancer regardless of BRCA mutation status.

It is important to note that the initial PARPi were designed to attack the catalytic portion of the enzyme and synthetic lethality of PARPi in gBRCAm cells was later discovered\textsuperscript{[80-82]}. These pioneers attributed most of the cytotoxic action of PARPi due to SSB accumulation following PARP inhibition. More recent studies suggest that the ability of PARPi to trap PARP along with their enzyme inhibitory actions dictates cell responsiveness\textsuperscript{[83]}. Different forms of PARPi have variable abilities to trap PARP-1. While some PARPi drive PARP-1 allostery to promote release from a DNA break, others drive allostery to retain PARP-1 on a DNA break. Recently, Zandarashvili and colleagues generated a new PARPi compound, converting an allosteric pro-release compound to a pro-retention compound and increasing its ability to kill cancer cells\textsuperscript{[84]}. 

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How crucial PARP trapping is in comparison to PARP enzyme inhibition depends, in part, on which agents PARPi are used in combination with [85]. Interestingly, more recent evidence reveals that the cytotoxicity of PARPi correlates more with autoPARylation of PARP1 on DNA as opposed to PARP-DNA trapping [86].

**Clinical development and application of PARPi**

Currently, PARPi olaparib, talazoparib, niraparib, rucaparib, veliparib, and pamiparib are in various phases of clinical development to treat cancer. In 2018, the FDA approved the use of Olaparib in patients with gBRCAm, HER2- metastatic breast cancer (MBC) previously treated with chemotherapy in the neoadjuvant and adjuvant settings. That same year, the FDA also approved talazoparib for patients with deleterious or suspected deleterious gBRCAm, HER2- locally advanced or MBC based upon an FDA-approved companion diagnostic. Niraparib and Rucaparib have been FDA-approved for patients suffering from cancers other than breast cancer, while Pamiparib and Veliparib are not yet FDA approved for treatment of any cancer patients but are in clinical trials.

Niraparib is under investigation as a neoadjuvant therapy in HER2-, gBRCAm TNBC in a small pilot study with results pending (NCT03329937) [Table 2]. Researchers are also investigating Niraparib in larger cohort studies of patients with TNBC and advanced stage solid tumors in combination with radiation or the immunotherapy agent pembrolizumab (NCT03945721, NCT02657889) [Table 2]. Vinayak found that among 47 of 55 patients enrolled, niraparib in combination with pembrolizumab achieved an objective response rate (ORR) of 21% and a disease control rate of 49%, which suggests that niraparib in combination with pembrolizumab offers promising antitumor
activity in patients with advanced or metastatic TNBC \cite{87}. Therefore, this combination should be investigated further.

<table>
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<tr>
<th>NCT #</th>
<th>Phase</th>
<th>Study Population</th>
<th>Setting</th>
<th>Stage</th>
<th>Experimental Arm</th>
<th>Control Arm</th>
<th>Primary Endpoint</th>
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<tr>
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<td>I</td>
<td>TNBC</td>
<td>Neoadjuvant</td>
<td>NA</td>
<td>niraparib + radiation therapy</td>
<td>Niraparib</td>
<td>MTD</td>
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<td>Ib/II</td>
<td>Solid Tumors including TNBC, MBC Advanced BC</td>
<td>Adjuvant/ Neoadjuvant</td>
<td>I-IV</td>
<td>1. niraparib + pembrolizumab (dose escalation) 2. niraparib (recommended 1. dose) + pembrolizumab 200mg</td>
<td>Placebo</td>
<td>DLTs, AEs, ORR</td>
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<tr>
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<td>HER2-, BRCAmut BC</td>
<td>Neoadjuvant</td>
<td>I/II</td>
<td>Niraparib</td>
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<tr>
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<td>Solid tumors including TNBC</td>
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<td>III/IV</td>
<td>1. rucaparib + lucitanib 2. rucaparib + sacituzumab govitecan</td>
<td>N/A</td>
<td>safety, TLDs, PKs, ORR</td>
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<td>NCT03542175</td>
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<td>Rucaparib</td>
<td>N/A</td>
<td>MTD</td>
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<tr>
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<td>Solid tumors including HR deficient BRCAmut TNBC</td>
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<td>1. Pamiparib and TMZ (dose escalation) 2. Pamiparib and TMZ (dose expansion)</td>
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<td>TNBC, ovarian</td>
<td>Adjuvant/ Neoadjuvant</td>
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<td>ORR, AEs, safety</td>
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<td>Neoadjuvant</td>
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<td>TNBC</td>
<td>Adjuvant/Neoadjuvant</td>
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<td>MBC (ER-, PR-, HER2-) BRCAm Advanced/d/metastatic TNBC</td>
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<td>IV</td>
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<td>capecitabine , eribulin, gemcitabine, or vinorelbine</td>
<td>PFS, ORR</td>
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<td>I–III</td>
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Rucaparib is under investigation in combination with other agents such as immunotherapy, vascular epidermal growth factor receptor (VEGFR)-inhibitors or radiotherapy to treat solid tumors including TNBC (NCT03992131, NCT03542175) [Table 2]. The study assessing the efficacy of radiation in combination with Rucaparib was completed in 2020, though results are not yet published and studies evaluating rucaparib in combination with immunotherapy or VEGFR inhibitors are slated to reach completion in 2023. Interestingly, Chopra and colleagues recently published data from their 2015-2019 RIO window clinical trial (EudraCT 2014-003319-12) in which patients newly diagnosed with TNBC were treated with the PARPi rucaparib prior to surgery. After rucaparib treatment, there was no association between change in aggressiveness (as measured by the proliferation marker Ki67) and HR-deficiency. In contrast, it was found that rucaparib was beneficial in suppressing amounts of circulating tumor DNA in blood samples from patients with mutation-signatures that include BRCA and HR-deficient TNBC. Furthermore, rucaparib induced expression of interferon response genes in HR-deficient cancers. Taken together, the research group believes that since
the majority of TNBCs have a defect in DNA repair, identifiable by mutational signature analysis, the use of PARPi offers a promising treatment option as a single agent prior to resection or chemotherapy \cite{63}.

Pamiparib (BGB-290), a new PARPi undergoing clinical evaluation in patients with ovarian cancer and TNBC, has the ability to inhibit PARP1 and PARP2 (NCT03333915) [Table 2]. It is also being investigated for efficacy in solid tumors including TNBC as a monotherapy and in combination with the chemotherapy agent temozolomide (TMZ) (NCT03150810). Veliparib is also being investigated in combination with radiation to treat patients with advanced TNBC, and thus far the results are pending (NCT01618357). A few years ago, veliparib was investigated in combination with carboplatin in gBRCAm tumors (NCT01149083). Somlo and colleagues discovered that a number of patients experienced dose-limiting toxicities (DLTs) including nausea, dehydration, and thrombocytopenia. The ORR was 56% and 3 patients achieved complete response (CR) for more than 3 years. Progression free survival (PFS) and overall survival (OS) were 8.7 and 18.8 months respectively. In a phase II trial, the PFS and OS were 5.2 and 14.5 months in 44 patients respectively, with a 14% ORR in patients with BRCA1 mutations (n = 22) and 36% in patients with BRCA2 mutations (n = 22). These findings suggest that safety and efficacy are encouraging with veliparib alone and when used in combination with carboplatin to treat BRCA-mutant associated MBC \cite{88}.

Talazoparib, is being evaluated for use to treat patients with early stage gBRCAmutant HER2- breast cancer (NCT03499353). It was previously investigated as a monotherapy in phase II and III trials (NCT04039230, NCT01945775) [Table 2]. Results
are pending for the phase II trial but in the phase III trial, Litton and colleagues found that PFS was significantly longer in the talazoparib group compared to the standard-therapy group (8.6 months vs. 5.6 months). The ORR was also significantly higher in the talazoparib group compared to the standard-therapy group (62.6% vs. 27.2%). About 55% of the patients who received talazoparib experienced hematologic adverse events compared to 38% of the patients on standard therapy. It is important to note that fewer patients on talazoparib therapy experienced nonhematologic adverse events as compared to those on standard therapy (32% vs 38%) [89].

**Oncogenes and DNA damage repair mechanisms confer PARPi resistance in TNBC**

While DNA damage repair mechanisms are crucial for maintaining genomic stability, these same mechanisms triggered in cancer cells have the ability to promote the onset of chemoresistance. While TNBC tends to be highly aggressive, one great challenge associated with treating TNBC is the extensive heterogeneity that exists within this subtype. Mechanisms of PARPi resistance in gBRCAm cells including TNBC cells include decreased PARP trapping and restoration of HR as detailed in a previous review [90]. HR becomes restored in gBRCAm carriers via additional BRCA mutations that restore the open reading frame and ultimately re-express BRCA1 [91-93]. Loss of BRCA promoter methylation has also been shown to promote PARPi resistance [94, 95]. HR restoration via de novo rearrangements in the BRCA1 locus in patient derived xenografts with methylated BRCA1 resulted in BRCA1 re-expression leading to PARPi
resistance [96]. Additional mechanisms of PARPi resistance have been identified more recently. We will focus on select oncogenes and DNA repair processes in relationship to BRCA1.

The oncogenic transmembrane protein mucin 1 (MUC1) C-terminal subunit (MUC1-C) is overexpressed in TNBC and is implicated in the downregulation of BRCA1 expression [97]. MUC1-C, appears to regulate PARP1 to promote resistance to PARPi and chemotherapeutic agents [98]. During the DNA damage response, polycomb group proteins BMI1 and EZH2 form complexes with PARP1 at the site of DNA damage. BMI1 regulates the DDR by ubiquitinating histone H2A and γH2AX, which mediates the repair of DSBs via HR and NHEJ activation. On the other hand, EZH2 is a direct target of PARP1 that induces H3K27 trimethylation (H3K27me3) under normal physiological conditions and inhibits EZH2 histone methyltransferase activity after DNA damage [33]. MUC1-C was found to regulate BMI1, a stemness promoting gene and regulate the nuclear localization of EZH2 and its function in catalyzing H3K27me3—a necessary component for DSB repair. Additionally, targeting MUC1-C downregulated BMI1-induced H2A ubiquitylation, EZH2-mediated formation of H3K27me3, as well as PARP1 activity, and sensitized mutant and wild-type BRCA TNBC cells to olaparib [98]. Notably, studies also suggest the potential for MUC1-C to drive dedifferentiation of TNBC cells [97].

The induction of functional HR marker RAD51 is implicated in PARPi resistance [99, 100]. Amplification in the MYC gene correlates with elevations in the HR gene RAD51, with both being associated with poor prognosis as well as PARPi resistance [101]. These investigators found that down-regulation of MYC in conjunction with PARPi resulted in
potent synthetic lethality in TNBC cells. Others found that RAD51 promotes resistance in TNBC stem cells \cite{102} and this held true for those derived from both BRCA mutant and BRCA wild type TNBCs. Furthermore, DDB2 protects cells from PARPi by stabilizing RAD51 in TNBC cells and depleting DDB2 aids in sensitizing these cells to PARPi \cite{103}.

When DSBs occur, kinases ATM, ATR and CHK2 are activated, phosphorylating BRCA1. Simultaneously, a complex of Fanconi anemia proteins ubiquitinates the D2 protein, leading to its interaction with BRCA1 and subsequent formation of the Fanconi anemia of complementation group D2 (FANCD2) complex \cite{29}. FANCD2 also complexes with BARD1 for stability and plays a role in the ubiquitination and degradation of RNA polymerase 2 (RPA), inhibiting transcription and RNA processing to remove prematurely terminated transcripts and clearing the damaged DNA region for recombination enzymes (Figure 15). RAD51 is a recombination enzyme that serves as a functional marker for HR DNA repair and complexes with BRCA2 for subsequent strand invasion and Holliday junction formation at the site of DNA damage \cite{104}. Studies have shown that induction of RAD51 nuclear foci after neoadjuvant chemotherapy was a common feature in Patient derived xenograft (PDX) models and patient samples with primary or acquired PARPi resistance and its expression can be used to predict treatment response \cite{99,100}. Furthermore, in the absence of BRCA1 and BRCA2, studies have shown that tumor cells can become resistant to PARPi via the restoration of HR DNA repair and/or by stabilizing their replication forks.
Figure 15. Synthetic Lethality from PARPi during homologous recombination (HR) in HR proficient versus HR-deficient cells. PARPi suppress PARP activity and trap the proteins in the DNA following single strand breaks. Repair is thus inhibited. The replication forks then become stalled and often DNA double strand breaks form. In HR-deficient cells, the fork collapse following stalled replication leads to cell death. On the other hand, in HR-proficient cells, the HR DNA repair process gets activated leading to normal cell replication and resumption of the cell cycle. PARPi: PARP inhibitors; SSB: single strand breaks; DSB: double strand breaks; HR: homologous recombination.
Therapeutic strategies to overcome PARP inhibitor resistance in TNBC

TNBC cells frequently encounter genotoxic stress following exposure to chemotherapy. We previously demonstrated that small molecules that activate aryl hydrocarbon receptor (AhR) signaling induce SSB and oxidative DNA damage in TNBC cells to confer anticancer activity \textsuperscript{[106, 107]}. We and others have also demonstrated that AhR ligands induce other forms of DNA damage including DSB in different cancer cells including TNBC cells\textsuperscript{[108-111]}. We and others exhibit this damage in conjunction with PARP cleavage \textsuperscript{[107, 112, 113]}. Historically, AhR agonists have been shown to confer toxic actions. For instance, AhR agonist tetrachloro-dibenzo-dioxin (TCDD) induces oxidative DNA damage and activates PARP particularly in TNBC cells though it is known to promote cancer \textsuperscript{[114]}. It is important to note that the tumor promoting actions precludes development of TCDD as an anticancer agent, but at the correct dose and during brief exposures, this agent can exhibit anticancer actions. Unlike TCDD, AhR agonists such as 5F 203 and NAP-6 have been shown to induce DNA damage in TNBC cells and display potent anticancer actions \textsuperscript{[111, 115-118]}. While the above-mentioned anticancer AhR agonists have not been used in combination with PARPi, their DNA damaging actions resemble those of genotoxic chemotherapeutic agents. It is therefore appropriate to consider the use of AhR agonists in combination with PARPi to treat TNBC. Extensive work however is necessary to fine-tune the safety profile of anticancer AhR agonists first.

PARPi exploit the deficiency in a cell’s DNA repair mechanisms most notably by HR as seen in BRCA-deficient tumors. This forces the cell to depend almost completely
on SSB repair mechanisms. Therefore, by eliminating PARP so crucial for the cells’ survival, the cell is forced to initiate cell death mechanisms. Although PARPi olaparib demonstrates keen sensitivity in patients with BRCA1 or BRCA2 mutant breast cancer, those with BRCA1-proficient tumors typically do not benefit. Indeed, the phenomenon of PARPi resistance in breast cancer has been well documented [70, 90, 105, 119, 120]. Fortunately, several recent studies have reported potential novel therapies to counteract this resistance.

The de novo resistance of PARPi is potentially mediated by factors such as tumor heterozygosity or isoforms of BRCA1 with reduced function [121]. Acquired resistance to PARPi often occurs via the restoration of the HR repair pathway [100, 122]. Ataxia telangiectasia and RAD3-related protein (ATR) is a crucial element for the survival of PARPi resistant BRCA-deficient cancer cells [123]. Studies have shown that inhibiting ATR reverses PARPi resistance in BRCA1-deficient models with acquired resistance to PARPi by resolving stalled replication forks [124].

PARPi resistance is mediated by several mechanisms [105] which include increases in oncogene actions. For instance, increased EZH2 oncogene activity has the potential to promote PARPi resistance [125]. EZH2 inhibitors such as GSK343 sensitize breast cancer cells to olaparib in preclinical BRCA-deficient models. [126] Bromodomain and extra-terminal (BET) proteins are epigenetic readers that regulate DNA repair genes including BRCA1 and RAD51 [127, 128]. Data from Yang and colleagues demonstrate that bromodomain inhibitor, JQ1, synergizes with olaparib to decrease the expression of BRCA1 and RAD51 reduces HR activity to subsequently enhance PARPi-induced DNA damage in TNBC cells [129].
Another mechanism of PARPi resistance involves secondary BRCA mutations in TNBC tumors during or post-treatment. Pulliam and colleagues investigated the response of BRCA-wildtype and mutant breast cancer to combinations of PARPi talazoparib and DNA methyltransferase inhibitor (DNMT inhibitor) guadecitabine\textsuperscript{[130]}. Importantly, guadecitabine synergizes with talazoparib, irrespective of gBRCAm status both \textit{in vitro} and \textit{in vivo}. Furthermore, this combination increases reactive oxygen species accumulation, promotes PARP activation and further sensitizes, in a cAMP/PKA-dependent manner, breast cancer cells to PARPi. The combination also decreased xenograft tumor growth and increased overall survival (OS) in BRCA-proficient high-grade serous TNBC models. Taken together, the data provide a rationale to develop DNMT inhibitor-PARPi combination therapy for patients with either intrinsic or acquired PARPi resistance\textsuperscript{[130]}. Interestingly, the authors did not discuss the likelihood that the DNMT inhibitors reversed the silencing of BRCA1 to counteract PARPi resistance.

Several studies have reported that receptor tyrosine kinases (RTKs), such as the hepatocyte growth factor receptor c-MET, are involved in resistance to various anti-cancer agents, including PARPi. However, the mechanism by which c-MET contributes to acquired resistance to PARPi in TNBC is not yet fully understood. Chu and colleagues showed that hyperactivated c-MET is detected in TNBC cells that have acquired PARPi resistance, and the combination of talazoparib synergizes with crizotinib (a multi-kinase inhibitor that inhibits c-MET) and this ultimately inhibits the proliferation of PARPi-resistant cells. However, depleting c-MET expression had little effect on talazoparib sensitivity in PARPi-resistant cells. Additionally, Chu found
epidermal growth factor receptor (EGFR) hyperactivation and increased interaction of EGFR/c-Met in these cells [131]. Of note, combining EGFR inhibition and PARPi resulted in greater inhibition of c-MET-depleted TNBC cells. Also, combining c-MET inhibition and EGFR inhibition increased sensitivity to talazoparib in PARPi-resistant TNBC cells. Collectively, these data suggest that combined c-MET and EGFR inhibition could potentially re-sensitize TNBC that is resistant to PARPi.

Promising efficacy has been demonstrated using PARPi in combination with PDL1 inhibitors [63]. The efficacy of olaparib in combination with various immunotherapy agents is being evaluated in multiple clinical trials in patients with solid tumors and metastatic TNBC including those without gBRCA mutations (NCT03167619, NCT02734004, and NCT03801369 & NCT03544125) [Table 2]. The rationale for employing this combination is that PARPi upregulate PD-L1 within tumors of patients with TNBC [132, 133]. Results from a published clinical trial reveal that PDL1 inhibitor, durvalumab, in combination with olaparib and VEGFR 1-3 inhibitor, cediranib was well tolerated among patients in a dose-escalation study. [134]. It is expected that further studies involving PD-L1 inhibitors and PARPi will take place provided this combination offers increased efficacy with minimal adverse effects.

**Perspectives and Conclusions**

TNBC is highly aggressive and defined by heterogeneity and poorer overall survival as compared to the other breast cancer subtypes. Until recently, this condition
largely lacked targeted therapy agents. However, with the FDA approval of olaparib and talazoparib in 2018, those with gBRCAm TNBC are now being offered targeted therapy. Approximately 15% of patients with TNBC have gBRCAm that make them candidates for the PARPi. While clinical trials demonstrate promising activity for those with gBRCAm TNBC, emerging evidence suggest that in some instances, patients with TNBC can derive benefit without gBRCAm in part due to their deficiencies in other forms of HR DNA repair. Agents used in combination with PARPi appear to demonstrate the capacity to phenocopy gBRCAm in such patients by inducing BRCAness. It is quite plausible however, that mechanisms yet to be discovered are at play to enable TNBC patients without gBRCAm to derive benefit from PARPi.

A key challenge that persists is how to devise strategies to overcome PARPi resistance that is predicted to occur in a substantial number of patients who show encouraging responsiveness to PARPi initially. An even greater challenge is deciphering the mechanisms of intrinsic PARPi resistance among patients with gBRCAm. Delineating mechanisms of PARPi resistance is crucial to rendering appropriate care to patients with TNBC. It will be necessary to use a myriad of molecular approaches to clarify determinants of PARPi responsiveness which would will likely entail the use of gene editing approaches such as CRISPR-cas9 or the use of selective pharmacological inhibitors. Results from these studies should promote the development of predictive biomarkers for existing PARPi and for the design and development of novel PARPi. This should enable us to ultimately provide effective treatment strategies for TNBC and other more refractory forms of breast cancer.
References


88. Somlo, G., et al., *Efficacy of the PARP Inhibitor Veliparib with Carboplatin or as a Single Agent in Patients with Germline BRCA1- or BRCA2-Associated Metastatic


111. Callero, M.A. and A.I. Loaiza-Perez, *The role of aryl hydrocarbon receptor and crosstalk with estrogen receptor in response of breast cancer cells to the novel


CHAPTER FOUR
SELECTED UNPUBLISHED DATA

ARYL HYDROCARBON RECEPTOR LIGAND 3,3'-DIINDOLYLMETHANE AS A POTENTIAL AGENT TO TREAT TRIPLE NEGATIVE BREAST CANCER

Abstract

Triple negative breast cancer (TNBC), characterized by tumors that lack expression of the estrogen receptor (ER), progesterone receptor (PR), and human growth factor receptor 2 (HER2) carries a poor prognosis due, in part, to the scarcity of targeted therapy options for TNBC. Furthermore, a subset of TNBC possesses mutant BRCA, making it more likely to respond to PARP inhibitor therapy. This discovery has led to the recent approval of PARP inhibitors such as Olaparib. We present data in this chapter to indicate that aryl hydrocarbon receptor ligands have potential as novel agents to treat TNBC because they not only exhibit anticancer actions but have potential to synergize with PARPi and others are able to restore the expression of putative tumor suppressors. We investigated the ability of AhR ligand and plant isolate DIM to confer anticancer activity, enhance PARP inhibitor efficacy, and induce the expression of tumor suppressor CYGB in TNBC cells. We observed that the anticancer actions of DIM were impaired in TNBC cells with stable AhR knockdown. Chromatin immunoprecipitation-sequencing data revealed that DIM and synthetic AhR ligand, 5F203 promoted the recruitment of AhR to the CYGB promoter in breast cancer cells. Interestingly, this recruitment was observed in MDA-MB-231 cells that are largely
refractory to 5F203 treatment. Furthermore, DIM induced CYGB gene expression and inhibited migration and proliferation in TNBC cells. Finally, using Alamar Blue and colony formation assays, we observed that 5F203 and DIM both enhanced Olaparib anticancer efficacy. Taken together, our data suggest that DIM and 5F203 upregulate CYGB and sensitize BRCA-proficient TNBC cells to olaparib through an AhR-dependent manner, providing a rationale for incorporating the use of AhR ligands that are natural products as a strategy to enhance PARP inhibitor efficacy in patients with TNBC that have BRCA1-proficient tumors.

**Introduction**

TNBC is characterized by tumors that lack expression of estrogen (ER), progesterone (PR), and human epidermal growth factor 2 (HER2) receptors. While targeted therapy options are available for patients with tumors expressing ER and/or HER2 amplification, patients with TNBC do not benefit from such therapies.

Poly-ADP ribose polymerase (PARP) repairs DNA damage.[1-3] TNBC patients that respond to PARP inhibitors such as the FDA-approved olaparib primarily possess tumors defective in DNA repair protein, breast cancer susceptibility gene 1 (BRCA1).[4] Thus, a need exists to develop novel agents that can improve the efficacy of PARP inhibitors in TNBC patients with BRCA proficient tumors.

The aryl hydrocarbon receptor (AhR) mediates a myriad of cellular processes. Although prototypical AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) tend to display deleterious actions, we and others have shown that certain AhR ligands
exhibit anticancer actions\textsuperscript{[5-12]} in breast cancer cells, including triple negative subtypes. For example, we have shown that AhR ligand 5F 203 exhibits potent anticancer activity in TNBC cells.\textsuperscript{[7, 13, 14]} We and others have also demonstrated the potential for AhR ligands to inhibit survival signaling pathways, such as the PI3K/Akt signaling pathway.\textsuperscript{[15, 16]} Notably, PI3K/Akt inhibitors have been shown to synergize with PARP inhibitors.\textsuperscript{[17]}

Emerging evidence suggest that pharmacological restoration of tumor suppressor genes (TSGs) represents a novel therapeutic approach to treat a variety of cancer types, including TNBC.\textsuperscript{[18]} We have previously shown the ability of 5F 203 to induce the expression of putative tumor suppressor CYGB in breast cancer cells.\textsuperscript{[7, 19]} A number of AhR ligands are plant-derived and the use of herbal medicine is growing in the US.\textsuperscript{[20, 21]} AhR ligand DIM, a metabolite of indole-3-carbinol (I3C) and the active component of cruciferous vegetables, is a partial AhR agonist that demonstrates anticancer activity.\textsuperscript{[22, 23]} Our rationale is that data derived from the current study will help determine whether clinical trials should be implemented to evaluate whether a diet consisting of plant-derived AhR ligands have the potential to reactivate tumor suppressor genes and sensitize PARP inhibitors to confer anticancer actions in TNBC.
Synthetic and Plant-derived AhR Ligands inhibit TNBC cell viability

For decades, the activation of the AhR has received negative connotations as a therapeutic approach due to the potential toxic effects of AhR ligands that induce the drug metabolizing cytochrome P450 enzymes.[24] However, following years of research, it is now understood that the AhR is a regulator of environmental toxins and serves as a key immunomodulator where ligands induce a variety of cellular and epigenetic mechanisms to attenuate inflammation and anticancer actions.[24] Consequently, the AhR has become an emerging therapeutic target for a variety of diseases and malignancies. While many AhR ligands have been formulated and synthesized in labs, many AhR ligands are found in a variety of foods we eat. DIM (Figure 16A), a metabolite of I3C, found in cruciferous vegetables such as broccoli, cabbage and Brussel sprouts, is a partial AhR agonist that demonstrates anticancer activity.[22, 23] These cruciferous vegetables contain bioactive precursor compounds known as glucosinolates. Glucobrassicin is the most abundant glucosinolate in these vegetables. Enzymatic breakdown of glucobrassicin by myrosinase during plant storage, preparation, and/or chewing yields various indoles including I3C. Finally, an acid-catalyzed condensation rapidly converts I3C to oligomers that include DIM, the major indole bioactive compound.

The first step in understanding the role a new compound has in TNBC cells is to establish relative levels of sensitivity. As such, our first aim was to ascertain the relative cytotoxicities of DIM by treating a panel of TNBC cell lines with DIM and assess cell survival using the Alamar Blue assay. We previously reported that synthetically
formulated AhR ligand, 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) (Figure 16B) inhibits viability of TNBC cells[7] and was subsequently used as a comparison. Interestingly, TNBC cells treated with varying concentrations of DIM displayed no appreciable cytotoxicity except for BRCA-deficient MDA-MB-436 cells at 24h, 48h, or 72h (Figure 17A, B, C). In comparison, 5F203 significantly inhibited the viability of BRCA-proficient MDA-MB-468 cells at 48h and 72h (Figure 18A, B, C). The results of this preliminary investigation suggest that monotherapy treatment of TNBC cells with AhR ligands vary between specific cell types.
Figure 16. Structure of A) 3,3'-Diindolylmethane (DIM), a major metabolite of cruciferous vegetables and B) 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203), a derivative of the lysylamide prodrug Phortress.
Figure 17. Impact of 3,3’ Diindolylmethane (DIM) on the viability of triple-negative breast cancer cells. Cells were treated with DMSO or DIM (100 pM -100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. Data are reported as the mean of at least triplicate values. Bars, SEM.
Figure 18. Impact of 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) on the viability of triple-negative breast cancer cells. Cells were treated with DMSO or 5F203 (100 pM - 100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. Data are reported as the mean of at least triplicate values. Bars, SEM
**DIM Inhibits TNBC Cell Proliferation and Migration**

Our lab has extensively investigated the effects of 5F203 in TNBC cells.\(^{[7, 19]}\) Therefore, for the majority of this project, we focused on evaluating the mechanisms of anticancer action of DIM in TNBC cells. Based on the results of our Alamar Blue, we were prompted to examine how DIM affects TNBC cell proliferation for further confirmation of its sensitivity. The clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. While the colony formation assay is used to determine cell proliferation, it can also be used to determine the effectiveness of other cytotoxic agents.\(^{[25]}\) As such, we employed the colony formation assay to ascertain the effectiveness and sensitivity of DIM. We found that DIM exposure resulted in significantly fewer colonies in MDA-MB-468 TNBC cells compared to the control and concentrations below 25 µM (Figure 19). Furthermore, we performed a wound healing assay involving IBIDI inserts to determine whether DIM decreases TNBC cell migration (Figure 20). Our data reveal that DIM completely suppresses cancer cell migration at 10 µM and 25 µM. These data suggest DIM not only thwarts proliferation and migration but also TNBC cell invasion and metastases. Results from this experiment are preliminary and will be repeated in the future in order to report statistical significance in terms of fold change.
Figure 19. Colony Forming Assay Analysis. MDA-MB-468 cells were treated and allowed to grow for 2 weeks before staining with crystal violet. Colonies were counted using Image J and quantified using GraphPad Prism version 9. Data are reported as the mean of triplicates. Bars, SD. Not significant (ns), **** P < 0.0001 as compared to DMSO treatment group.
Figure 20. Wound Healing Assay of MDA-MB-468 Triple Negative Breast Cancer Cells. Cells were plated in IBIDI culture inserts and allowed to grow to confluency before being treated with DIM (10 µM and 25 µM). A) Images were captured using an Olympus IX-71 microscope at 0h, 24h, and 48h. B) Quantification of cell migration following given treatments was performed using GraphPad Prism version 9. Bars, SD ** P < 0.01, *** P < 0.001, **** P < 0.0001, relative to the DMSO-treated group.
DIM Induces Tumor Suppressor CYGB Gene Expression

The reactivation of inactive or suppressed tumor suppressor genes represents a novel therapeutic approach to treat a variety of cancer types, including TNBC.\cite{18} Cytoglobin (CYGB) is a putative tumor suppressor gene that is often inactivated due to epigenetic silencing via hypermethylation of the CYGB promoter region.\cite{26} In a previous study where we treated breast cancer cells with a synthetic AhR ligand, 5F 203, and used an oxidative stress PCR array to profile genes that regulated oxidative stress, MDA-MB-468 cells—derived from an African American patient—exhibited the most sensitivity to 5F 203 and demonstrated the greatest induction of CYGB following treatment.\cite{7} In that same study, we found that MDA-MB-468 cells were deficient in CYGB expression, consistent with the findings of others for TNBC cells. Furthermore, their relative expression is substantially less than non-tumorigenic MCF-10A cells. In this current project, we investigated whether the plant-derived AhR ligand induces the expression of CYGB. Our investigations revealed that CYGB expression in TNBC cell lines MDA-MB-468 and CRL-2335 is significantly induced (Figure 21).
Figure 21. Quantitative PCR (qPCR) analysis of CYGB expression in DIM-exposed TNBC cells. (A) MDA-MB-468 and (B) CRL-2335 cells were treated with either a control (0.1% DMSO) or 15 µM DIM and CYGB expression measured using qPCR. Bars, SD. Statistically significant in comparison with control or where indicated as designated *P < 0.05, or ****P < 0.0001. CYGB, cytoglobin; control is designated as CTL which are dimethyl sulfoxide (DMSO) exposed cells.
**DIM and 5F203 Induces AhR Recruitment to promoter region of CYGB**

Based on our observation that DIM induces CYGB expression, we aimed to delineate a potential mechanism of CYGB upregulation. In a previous study, we pretreated cells with an AhR antagonist and then with 5F 203 before measuring CYGB mRNA expression using qPCR.[7] Similarly, in order to obtain mechanistic information, we aimed to modulate the expression of AhR, and then evaluate the ability of DIM and 5F203 to induce CYGB expression in the modified cells. ChIP-quantitative PCR (ChIP-qPCR) was subsequently performed to establish more definitive mechanistic conclusions. Through this assay, we confirmed that 5F203 promotes the recruitment of AhR to the promoter region of the CYGB gene (Figure 22). DIM also promotes the recruitment of AhR to the CYGB promoter, although statistical significance was not achieved (Figure 22). Results from this experiment are preliminary and will be repeated in the future in order to report statistical significance in terms of fold change. Furthermore, for this assay, we used MDA-MB-231 cells which demonstrate resistance to AhR ligands such as 5F 203 and DIM. As such, it is interesting that there is any appreciable recruitment of AhR to the CYGB promoter with 5F 203 and DIM. Future studies will therefore evaluate the potential for 5F203 and DIM to promote AhR recruitment to the CYGB promoter in MDA-MB-468 cells that are highly sensitive to these AhR ligands.
Figure 22. Chromatin immunoprecipitation (ChIP) analysis of the CYGB promoter region following treatment of MDA-MB-231 TNBC cells with AhR ligands. MDA-MB-231 TNBC cells were treated with 0.1% DMSO, 10 µM DIM or 1 µM 5F203 1h before ChIP analysis followed by NextGen sequencing to assess the recruitment of AhR to the promoter of the CYGB Gene. *P<0.05 compared with DMSO AHR.
Our observations of the role of AhR in CYGB expression led us to further investigate if AhR plays a direct role in the ability of DIM and 5F203 to elicit cytotoxicity. To determine this, Alamar Blue Assays were performed in MDA-MB-468 cells transfected to eliminate AhR expression (AhRKO) and control MDA-MB-468 cells transfected with empty vectors (EMPTY). The MDA-MB-231 AhRKO cells were generated as previously described.[27] Briefly, validated CompoZr knockout ZFN plasmids targeting AHR were transfected into MDA-MB-231 cells followed by several serial dilutions and clone selection. Clones were screened for the presence of indels at the ZFN recognition site in exon 1 of AHR by DNA sequencing, and AhR knockout was confirmed by assessing downstream targets if AhR (CYP1A1) with qPCR. Our data revealed that 5F203 displayed no appreciable activity in AhRKO cells in either 24 h, 48 h, or 72 h treatments compared to the control cells (Figure 23). This suggests that 5F203 is dependent on AhR activation in order to elicit cytotoxicity. These findings were anticipated since we previously showed an attenuation of 5F203 to exhibit toxicity in the presence of an AhR antagonist.[7, 19] Interestingly, we observed that DIM displayed similar inactivity from either the AhRKO or control cells after 24 h, 48 h, or 72 h treatments (Figure 24). However, these data are comparable to the data observed in the non-transfected MDA-MB-468 cells (Figure 17).
Figure 23. Impact of 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) on the viability of AhRKO cells. AhRKO and EMPTY vector TNBC cells were treated with DMSO or 5F203 (100 pM -100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. Data are reported as the mean of at least triplicate values. Bars, SEM.
Figure 24. Impact of 3,3' Diindolylmethane (DIM) on the viability of AhRKO cells. AhRKO and EMPTY vector TNBC cells were treated with DMSO or DIM (100 pM -100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. Data are reported as the mean of at least triplicate values. Bars, SEM.
AhR Knockout Cells Inhibit Cell Proliferation

Our data observed in the Alamar Blue studies involving DIM and its cytotoxicity in AhRKo cells was insufficient to generate a general understanding of the role of AhR in its mechanism of action. Therefore, we performed colony formation assays in these cells in order to better ascertain the relationship between DIM and the AhR. In these studies, we indeed observed a significant difference in the number of colonies generated in the AhRKo cells compared to the control cells. While the control cells behaved similar to the non-transfected cells (Figure 19) with significant decreases in colony formation compared to the control and concentrations below 10 µM (Figure 25), there were no observable significant decreases in colony formation in AhRKo cells until high concentrations of DIM (≥ 75 µM) were reached (Figure 26). These data suggest that AhR plays a role in the ability of DIM to inhibit the formation of colonies and subsequent proliferation of TNBC cells. Results from this experiment are preliminary and will be repeated in the future in order to confirm statistical significance.
Figure 25. EMPTY Colony Forming Assay Analysis. EMPTY vector MDA-MB-468 TNBC cells were treated with DIM and allowed to grow for 2 weeks before staining with crystal violet. Colonies were counted using Image J and quantified using GraphPad Prism version 9. Data are reported as the mean of triplicates. Bars, SD. Not significant (ns), **** P < 0.0001 as compared to DMSO treatment group.
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![Image showing colony formation assay](image)

**Figure 26. AhRKo Colony Forming Assay Analysis.** AhRKo MDA-MB-468 TNBC cells were treated with DIM and allowed to grow for 2 weeks before staining with crystal violet. Colonies were counted using Image J and quantified using GraphPad Prism version 9. Data are reported as the mean of triplicates. Bars, SD. Not significant (ns), * P < 0.05, ** P < 0.01 as compared to DMSO treatment group.
AhR Knockout Suppresses CYGB Gene Expression

Based on our previous investigations of the ability of DIM to induce the expression of CYGB, we further examined the relationship between DIM and the AhR on the reactivation of CYGB. To accomplish this, we treated AhRKO cells with DIM and assessed the relative levels of CYGB expressing using reverse-transcriptase quantitative PCR (RT-qPCR). We observed that CYGB expression was considerably reduced in comparison to the control cells (Figure 27). Taken together, our data suggest that DIM relies on the AhR to induce CYGB expression and cytotoxicity in TNBC cells. Results from this experiment are preliminary and will be repeated in the future in order to report statistical significance in terms of fold change.
Figure 27. Quantitative PCR (qPCR) analysis of CYGB expression in DIM-exposed AhRKo TNBC cells. AhRKo and EMPTY vector TNBC cells were treated with either a control (0.1% DMSO) or 10 μM DIM and CYGB expression measured using qPCR. CYGB, cytoglobin; control is designated as CTL which are dimethyl sulfoxide (DMSO) exposed cells.
AhR Ligands Sensitize BRCA Proficient TNBC to Olaparib

Few targeted therapy options exist for patients with TNBC since they have tumors lacking ER, PR, and HER2 expression. While the PARP inhibitor, olaparib has been recently approved by the FDA to treat TNBC patients, the effectiveness of this inhibitor is limited to patients with BRCA mutations.\(^4\) Therefore, a need exists to develop novel agents that can improve the efficacy of PARP inhibitors in TNBC patients with BRCA proficient tumors. We previously demonstrated the ability of synthetic AhR ligands to inhibit Akt signaling in breast cancer cells.\(^15\) Notably, PI3K/Akt inhibitors have been shown to synergize with PARP inhibitors.\(^17\) Our final aim for this project was to therefore investigate the potential of synthetic and plant-derived AhR ligands to sensitize BRCA-proficient TNBC cells to FDA approved PARP inhibitor, olaparib.

We began our investigation with establishing relative cytotoxicity’s of olaparib to various TNBC cell lines using the Alamar Blue assay. As expected with BRCA-proficient cells, we observed no significant decrease in cell viability in cells treated with olaparib at 24 h, 48 h, or 72 h (Figure 28A, B, C). MDA-MB-231 cells were the only cell line to reach acceptable cytotoxic levels with an IC\(_{50}\) value of 10 \(\mu\)M after 72 h of treatment.

We then performed another Alamar Blue assay after pretreating the same cell lines with either 5F203 or DIM for 48 h and then added varying concentrations of olaparib for an additional 24 h or 48 h. We observed that the AhR ligands in combination with olaparib significantly reduced the viability of TNBC cells compared to our prior investigations (Figure 28D, E, F, G, H, I). Notably, 50 \(\mu\)M DIM monotherapy displayed no cytotoxicity in our TNBC cell lines (Figure 17). However, when combined
with olaparib, significant toxicity was observed in both MDA-MB-468 and MDA-MB-231 BRCA-proficient TNBC cells, even at picomolar concentrations of olaparib (Figure 28l). These data suggest that AhR ligands indeed sensitize BRCA-proficient TNBC cells to PARP inhibitor olaparib.
Figure 28. Impact of 3,3' Diindolylmethane (DIM) and 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) in combination with PARP Inhibitor Olaparib on the viability of triple-negative breast cancer cells. Cells were treated with DMSO or olaparib (100 pM -100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. TNBC cells were pretreated with either 100 nM 5F203, 10 µM DIM, or 50 µM DIM for 48 h before treatment with DMSO or olaparib (100 pM -100 µM) for D-E) 24 h or F-I) 48 h. Data are reported as the mean of at least triplicate values. Bars, SEM
**AhR Knockout Combination Therapy Efficacy**

Our observations with the combination therapy led us to further investigate the mechanism of action of AhR ligands and olaparib. As such, we performed additional Alamar Blue assays using AhRKo cells treated with the drug combinations. Similar to our investigations with non-transfected cells, we first evaluated the cytotoxic potential of olaparib in AhRKo cells. We found no appreciable decrease in cell viability in these cells after treatment with olaparib (Figure 29A, B, C).

We then performed another Alamar Blue assay in the AhRKo cells using the same combination conditions as the non-transfected cells. We observed that 5F203 was ineffective at sensitizing TNBC cells to olaparib as evidenced by treatments not reaching IC$_{50}$ compared to the control cells (Figure 29D, G,). Interestingly however, we observed that pretreating cells with 50 µM DIM sensitized cells to olaparib regardless of AhR status. Taken together, our data suggest an AhR mechanism for the ability of 5F203 to increase TNBC cell sensitivity to olaparib and that DIM may enhance PARP sensitivity via AhR-independent mechanisms. Results from these studies are preliminary and will be repeated in the future in order to confirm statistical significance in terms of % cell viability.
Figure 29. Impact of 3,3’ Diindolylmethane (DIM) and 2-(4 amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) in combination with PARP Inhibitor Olaparib on the viability of AhRKo triple-negative breast cancer cells. Cells were treated with DMSO or olaparib (100 pM -100 µM) for A) 24 h, B) 48 h or C) 72 h respectively and the viability of the cells was examined using the Alamar Blue assay. TNBC cells were pretreated with either 100 nM 5F203, 10 µM DIM, or 50 µM DIM for 48 h before treatment with DMSO or olaparib (100 pM -100 µM) for D-E) 24 h or F-I) 48 h. Data are reported as the mean of at least triplicate values. Bars, SEM.
References


CHAPTER FIVE
GENERAL DISCUSSION

Triple negative breast cancer (TNBC), characterized by tumors that lack estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth receptor (HER2) expression is one of the most aggressive subtypes of breast cancer. TNBC carries a poor prognosis due, in part, to a lack of clinically available targeted therapy. As such, it is imperative that novel therapeutic targets be investigated. Due to growing concerns with pharmaceuticals, there has been a general shift in patients choosing alternate, more natural solutions to treat diseases.[1, 2]

The AhR was initially identified as the receptor that bound the environmental toxicant TCDD and other structurally related compounds and by-products. Animal models further confirmed that this receptor was necessary to mediate the effects of toxic substances such as TCDD.[3] As a result, the AhR has dealt with much controversy over the years as to its benefits as an anticancer agent. Over the past two decades, it has been well established that various compounds including endogenous AhR ligands, phytochemicals, and AhR-active pharmaceuticals bind the AhR.[4-8] In recent years, studies have identified AhR ligands that can act as agonists or antagonists to block many of the hallmarks of cancer.[3] As a result, the AhR has become a widely accepted novel target for cancer research. However, the mechanism of action of an AhR ligand to either cause angiogenesis or anticancer effects presents an interesting area of research. It is plausible that the actions of an AhR ligand to elicit various effects may depend on the way the individual ligand binds to and fits within the pocket of the
receptor. Furthermore, the resulting conformational rearrangement between the receptor and its xenobiotic response element may also contribute to how an AhR ligand is defined as a “good” agent or a “bad” agent. Molecular modeling techniques present a promising area of research to elucidate those mechanisms.

Furthermore, numerous compounds, including natural products, have been shown to be ligands of the AhR and/or substrates of the drug metabolizing cytochrome P450 (CYP) enzymes.\cite{9,10} In our attempt to address the need of more efficacious targeted therapy for TNBC and the increased demand for more natural solutions for diseases, we investigated two AhR ligands derived from plant sources and their potential use for breast cancer treatment.

While aqueous- and ethanol-based preparations of \textit{P. alliacea} have been used to treat a variety of medical conditions including breast cancer\cite{11}, our observations revealed that these extracts are not efficacious in TNBC cells. As discussed in Chapter 2 of this manuscript, the content of DTS even in the ethanol extract was notably low (0.2%), which may not be sufficient to incite observable cytotoxicity over the 48 and 72-hour space. Therefore, additional experiments involving longer incubation times may be necessary to achieve significant \textit{in vivo} activity.

Looking at these results of the extracts pharmacologically, \textit{P. alliacea} is comprised of numerous secondary metabolites. This was inferred in our previous studies also where despite the low content of DTS, aqueous and ethanolic extracts were capable of binding to and inhibiting the activity of human cytochromes P450 enzymes.\cite{11} It is therefore plausible that one of the more abundant ligands found in the mix of chemicals inside the extracts has a binding affinity with a dissociation constant
(K_D) value much lower compared to DTS, and therefore has a greater binding affinity to the tested cancerous cells. This may be why there is less activity as compared to isolated DTS from within the extracts in the cell viability assays. Furthermore, investigations of various other ethanolic extracts have also reported inactivity in cell viability assays.\textsuperscript{[12, 13]} This may also be due to competitive binding interferences. Additionally, \textit{in vitro} models may not necessarily translate what is observed with the more complex biological systems found in \textit{in vivo} models. While the extracts may display inactivity within \textit{in vitro} models, the extracts may display more significant activity in \textit{in vivo} mouse models in part because \textit{in vitro} models may not account for interactions between cells and biochemical processes that occur during turnover and metabolism. In previous studies, we observed \textit{in vivo} efficacy (and pharmacological relevance) of DTS in a Zebrafish model on the activity of CYP1 enzymes. Future directions should therefore involve assessing aqueous and ethanolic extracts of \textit{P. alliacea} in preclinical breast cancer animal models to establish more definitive efficacies for breast cancer therapy.

DTS is one of the major compounds isolated from \textit{P. alliacea} and was recently identified as a putative AhR ligand based on its ability to inhibit the downstream CYP enzymes of the AhR signaling pathway.\textsuperscript{[10]} Furthermore, it should be noted that, from a chemical point of view, the structure of DTS does not carry any significant anticancer potential. It is more likely that the anticancer effects observed with DTS come from the formation of free radicals after metabolic/ enzymatic breakage of the disulfide bond \textsuperscript{[14]}. Our observations of this natural product revealed significant potency in TNBC cell lines, leading us to further investigate its mechanisms of action.
The AhR is historically known to mediate the actions of carcinogens such as TCDD, a side product of burning organic materials and pro-carcinogens such as B[a]P found in tobacco smoke. Emerging evidence reveal that certain AhR agonists inhibit cancer cell growth, invasion, and metastasis.\[^{15}\] Furthermore, selective aryl hydrocarbon receptor modulators (SAhRMs) may block the binding of more potent pro-cancer agonists such as BAP.\[^{16}\] Interestingly, benzoquinones formed by B[a]P metabolism have been shown to increase cell proliferation, generate reactive oxygen species (ROS) and transactivate the epidermal growth factor receptor in MCF-10A breast epithelial cells, providing a plausible mechanism for B[a]P-mediated tumor promotion.\[^{17, 18}\] We therefore performed a colony formation assay with B[a]P-pretreated MCF-10A breast epithelial cells and investigated the resulting activity of DTS. We observed that although proliferation increased in wells pretreated with B[a]P compared to those without B[a]P treatment, DTS still significantly inhibited the number of colonies formed after 48 h additional treatment (data not shown). Therefore, DTS may also behave as a chemopreventive agent as well as an anticancer agent.

Apoptosis is a very specific and specialized form of cell death characterized by distinct morphological characteristics such as cell shrinkage, pyknosis, blebbing, and formation of apoptotic bodies.\[^{19}\] We observed cellular bodies closely resembling advanced apoptosis at high concentrations of DTS. Furthermore, we show DTS induces cathepsin B release and LMP, a hallmark of both apoptosis and necrosis, as well as necroptosis and autophagic cell death.\[^{20-22}\] Collectively, these data suggest that DTS may induce cell death via caspase-independent mechanisms. However, at the conclusion of this dissertation, it may be premature to suggest that DTS does not induce
apoptosis because apoptosis can occur through caspase-independent mechanisms, mainly through the activation of the BNIP-3 protein.\[23\]

Specifically, two aspects may be involved when discussing the potential caspase-independent mechanism of DTS we observed in our studies. First, it is well established that caspases are important mediators of apoptosis. Caspase-3 in particular is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins.\[24\] In fact, cleavage of PARP-1 by effector caspases, such as caspase 3, is a well-established hallmark of apoptosis.\[25-27\] In our studies, we observed that DTS did not induce cleavage of caspase 3 and surmised that caspase-independent mechanisms were at play. However, effector caspases also include caspases 6 and 7. Therefore, while caspase 3 may display inactivity when cells are treated with DTS, caspases 6 and 7 may still play a role. Second, studies have shown that cathepsins and TGF-β are involved in caspase-independent PARP-1 cleavage.\[28\] Consequently, the lack of caspase 3 cleavage may correlate with the DTS-mediated cathepsin B release that was also observed in our studies, resulting in cleaved PARP-1 fragments.\[29\] Nonetheless, further experiments should investigate the activity of the other effector caspases 6 and 7 to more accurately confirm caspase-dependent or caspase-independent mechanisms. Fortunately, a variety of assays are commercially available to elucidate that mechanism such as fluorometric and colorimetric assays for caspases 3/7 and 6, and immunoprecipitation assays specific to caspase 7.

Additionally, our studies revealed that DTS potently induced pro-apoptotic genes in sensitive breast cancer cells. Interestingly, caspase-independent cell death mechanisms can also occur via apoptosis-independent mechanisms. Tait and Green
reported a number of different modes and mechanisms of caspase-independent cell death\textsuperscript{[30]} that likely contribute to the mechanism(s) of anticancer activity for DTS that are worth pursuing in future investigations. However, based on our results, it is unlikely that DTS induces necrosis due to the processed PARP band observed in the western blot (89 KDa). PARP is also processed during necrosis but at a major fragment of 50 kDa. To confirm or rule out other modes of cell death, necroptosis studies can be performed by commercially available Nec-1 and MLKL inhibitors. Furthermore, while LMP is a hallmark of necroptosis, LMP can also occur without necroptosis. Cathepsin inhibitors can be used to demonstrate this.

Our investigations in elucidating mechanisms of DTS anticancer activity also revealed that caspase inhibitor, ZVAD was ineffective at inhibiting apoptosis in cells treated with DTS. However, exposure time to the caspase inhibitor may be a factor. Additional morphological studies revealed that at 12h DTS treatment, apoptotic body formation was not visible in cells pretreated with ZVAD via microscopic imaging (data not shown). Therefore, ZVAD may prove to be more impactful during shorter DTS treatments. Nonetheless, future directions will involve further investigations assessing caspase inhibition in cells treated with DTS for shorter time periods.

Our data suggest that DTS involves caspase-independent mechanisms. Giampazolias and Tait reported two potential benefits of caspase-independent cell death as an anti-cancer treatment: 1) it prevents caspase dependent oncogenic effects of apoptosis 2) it can activate anti-tumor immunity.\textsuperscript{[31]} DTS’ ability to induce caspase-independent non-apoptotic cell death mechanisms increases the likelihood it will bypass
chemoresistance mediated by caspase-dependent apoptosis since cancer cells survive primarily via apoptosis dysregulation.\textsuperscript{[32, 33]}

Recent trends in drug development involve the identification of drug combinations or multi-target agents that effectively modify multiple modes of disease-associated networks. These polypharmacological effects may reduce the risk of emerging drug resistance by means of attacking the disease networks through synergistic and synthetic lethal interactions.\textsuperscript{[34]} Furthermore, by inhibiting or blocking multiple pathways in cancer cell progression, growth, and metastasis, we can more effectively ensure the elimination of cancer cell survival and recurrence.

Most BRCA1-mutations in breast cancers are triple negative and basal-like.\textsuperscript{[35]} BRCA1 is a key protein involved in homologous recombination repair of damaged DNA.\textsuperscript{[36]} Poly-ADP ribose polymerase (PARP) is a key enzyme in repairing single strand DNA damage breaks via base excision repair mechanisms. As discussed previously, an emerging field of research involves the use of PARP inhibitors to treat TNBC. Among the different variety of PARP inhibitors, olaparib is of particular importance because of its recent approval by the FDA to treat TNBC patients. However, olaparib is primarily used for patients deficient in BRCA1. By understanding these mechanisms of DNA repair, we can understand why olaparib is so successful in BRCA-deficient tumors. Olaparib exploits the weakness in the cell’s DNA repair mechanisms. Due to the absence of BRCA1 in BRCA-deficient patients, successful homologous recombination of DNA double strand breaks is crippled, making the cell almost completely dependent on single strand break repair mechanisms. As such, by eliminating or inhibiting PARP,
another pathway for the cancer cells’ survival, the cell is forced to initiate cell death mechanisms.

Pharmacological restoration of tumor suppressor genes is another emerging approach for treating TNBC. We have previously shown that synthetic AhR ligand 5F203 exhibits potent anticancer activity in TNBC cells and induces the expression of putative tumor suppressor CYGB in breast cancer cells.\[^{37, 38}\] Additionally, several natural products and plant-derived compounds behave as AhR ligands. Notably PARP inhibitors have been shown to synergize with inhibitors of the PI3K/Akt signaling pathway.\[^{39}\] In addition to evaluating the novel plant-derived compound, DTS, as potential therapy for refractory breast cancer, this manuscript attempts to combine everything we know about natural products, AhR ligands, PARP inhibitors, and tumor suppressors into a novel multi-pathway treatment for treating TNBC. We therefore hypothesized that the plant-derived AhR ligand, DIM, enhances PARP inhibitor efficacy in BRCA-proficient TNBC cells and upregulates CYGB.

3,3’-diindoylmethane (DIM), a metabolite of indole-3-carbinol and the active component of cruciferous vegetables such as broccoli, cabbage, and cauliflower, is a partial AhR agonist that demonstrates anticancer activity.\[^{40, 41}\] Cruciferous vegetables are the primary source of DIM in the human diet.\[^{42}\] Interestingly, the concentration of DIM increases during cooking due, in part, to the thermal activation of myrosinase—the enzyme responsible for breaking down Glucobrassicin.\[^{43}\] This increase is evidenced by a 6-fold increase in DIM concentrations in boiled cabbage compared with uncooked cabbage.\[^{43}\] Furthermore, studies point to an overall protective role regarding the consumption of cruciferous vegetables and breast cancer risk and recurrence.\[^{42}\]
Interestingly, a meta-analysis of 13 case–control and prospective cohort studies and 18,673 individual cases suggested that overall high intake of cruciferous vegetables was significantly associated with a 15% lower risk of breast cancer.\textsuperscript{[42, 44]} A diet high in cruciferous vegetables corresponds more or less to one portion, or about 250 grams per day.\textsuperscript{[45]} For visual representation, this correlates to the size of about two and a half medium tomatoes per day. On average, 100 g of cruciferous vegetables contains up to 30 mg of glucobrassicin, which is estimated to convert to approximately 2 mg of DIM.\textsuperscript{[42]} Taken together, data suggests that 4.5 mg of DIM per day is associated with a 15% lower risk of breast cancer.

Our investigations revealed that DIM is ineffective at reducing breast cancer cell viability at concentrations below 100 µM. However, given that diets high in cruciferous vegetables convert to higher concentrations of DIM, future studies should involve additional cell viability assays using higher concentrations of DIM that better correlate with average and high diets of cruciferous vegetables. However, we observed that DIM at low concentrations (10 µM - 25 µM) potently inhibits TNBC proliferation and migration and induces the expression of tumor suppressor CYGB in an AhR-dependent manner. However, the role of CYGB in mediating the anticancer actions of DIM remain to be understood. Previous studies demonstrated that silencing CYGB reduced 5F203-mediated apoptosis, caspase-3/7 activation, LMP, and cathepsin B release, indicating a definitive role in the role reactivating CYGB plays in the ability of 5F203 to promote TNBC cell death.\textsuperscript{[38]} Future studies should therefore investigate the ability of DIM to elucidate chemotherapeutic actions in CYGB knockout TNBC cells in order to elucidate if CYGB mediates DIM anticancer activity. Interestingly, DIM was found to inhibit chemical
carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in Sprague-Dawley rats, suggesting a chemopreventive role. Future studies should therefore include studies to confirm mechanisms of chemoprevention.

While clinical trials have been initiated to examine combination therapy of PI3K/AKt inhibitors and PARP inhibitors, none have investigated combination therapy with an AhR ligand derived from plant sources and a PARP inhibitor. Clinical trials have also examined the combination of olaparib with other chemotherapy agents. However, enhanced toxicity has been problematic in these studies. For example, the addition of gemcitabine and cisplatin to olaparib was intolerable to patients due to excessive myelosuppression. To the best of our knowledge, this is the first study of its kind to propose the implementation of natural products in combination with PARP inhibitors such as olaparib. In our studies, we observed that 5F203, significantly sensitized TNBC cells to olaparib in an AhR-dependent manner and that DIM significantly sensitized TNBC cells to olaparib in a mechanism that may not completely rely on AhR activation. However, our combination studies performed using the Alamar Blue assay may not truly reveal if there is an enhancement. Further combination studies will therefore involve colony formation assays as well as using the CalcuSyn program to determine whether true synergy is achieved. Interestingly, 5F203 displayed no cytotoxicity to MCF-10A breast epithelial cells, even at high concentrations to reveal its ability to selectively target breast cancer cells.

In our in vitro studies, each cell line represents a patient with distinct ethnicities and genome ancestry. However, it was recently revealed upon a deeper investigation and analysis of genetic ancestry, that there has been a misclassification of commonly
used cancer cell lines.[48] For example, while the MDA-MB-468 breast cancer cell line used in our studies has been classified by The American Type Culture Collection (ATCC) as a 51 year old black female patient, studies revealed that this cell line carries 23% Native American ancestry, suggesting possible Afro-Hispanic/Latina ancestry.[48] Furthermore, Ramamoorthy and colleagues reported that race and ethnicity can contribute to interindividual differences in drug exposure and/or response, which may alter risk–benefit in certain populations.[49] In fact, within the past 6 years, approximately 1/5th of newly approved drugs have demonstrated differences in exposure and/or response across different racial/ethnic groups. In some cases, these differences translate to population-specific prescribing recommendations. The mechanisms involved in these differences remains to be elucidated. However, differences in the genetic makeup of different races and ethnicities may contribute to the differences observed in various health disparities. Emerging data show that differences in gene expression between African American and Caucasian TNBC patients that may account for the disparate outcomes in survival.[50, 51] These varying expressions of genes may contribute to the varying levels of efficacy observed in our mono and combinatorial drug treatment experiments when comparing our African American and European American patient-derived TNBC cells.

It is important to note that while cancer investigational studies often involve two-dimensional (2D) cell culture methods, they are not without their limitations. These limitations include the disturbance of interactions between the cellular and extracellular environments, changes in cell morphology, polarity, and method of division.[52] Additionally, one of the key challenges of developing novel therapies is the translation of
scientific knowledge from bench to bedside. Because many cancer models inadequately represent the patient’s tumor, many drugs that may perform well in preclinical cancer models ultimately fail in clinical trials.\cite{53} Animal models are a generally more accepted representation of how a novel drug therapy may perform in the clinic. However, their generation is time consuming, expensive, and it is argued that these models often do not faithfully recapitulate pathogenic processes in patients.\cite{53, 54} Fortunately, the recent advances in *in vitro* 3D culture technologies, such as organoids, present new possibilities for the development of novel, more physiological human cancer models.\cite{53}

Over the past few years, patient-derived cancer organoids have become more prominent in pre-clinical and translational research and have been generated for most common solid tumors. Studies have shown that cancer organoids retain key genetic and phenotypic characteristics of their tissue of origin, tumor subtype and maintain intratumoral heterogeneity.\cite{55} As a result, cancer organoids have the potential to be used as predictors for individualized treatment response. In particular, studies have shown that for some small-molecular compounds, organoids expressed drug transporters, efflux transport activity, and the activation of the drug-metabolizing enzyme cytochrome P450. Taken together, further studies should employ patient-derived TNBC organoids for pharmacologic and pharmacokinetic assessment of how DTS, DIM, and 5F203 impacts the CYP enzymes, as well as investigate more accurately how a combinational approach might translate to better TNBC patient outcomes clinically.

In conclusion, DTS, DIM, and 5F203 represent promising agents with potential to treat refractory forms of breast cancer including TNBC BRCA-proficient tumors. With the
continued growth in popularity, the potential for interactions between medicinal plant products and pharmaceutical drugs is a growing health concern for many governments and regulatory bodies worldwide. Our investigations of DTS demonstrated potent anticancer and chemopreventive activity in TNBC cells. Finally, our investigations of DIM suggest that TNBC patients with BRCA-proficient tumors may benefit from olaparib treatment by incorporating more cruciferous vegetables in their diet. Taken together, AhR ligands, particularly those derived from plant sources, should be further investigated for potential use clinically to treat refractory forms of breast cancer.
References


