Research Plan: Targeting Dihydroceramide Desaturase 1 (DES1) as a Method to Overcome Anoikis Resistance in Basal Breast Cancer

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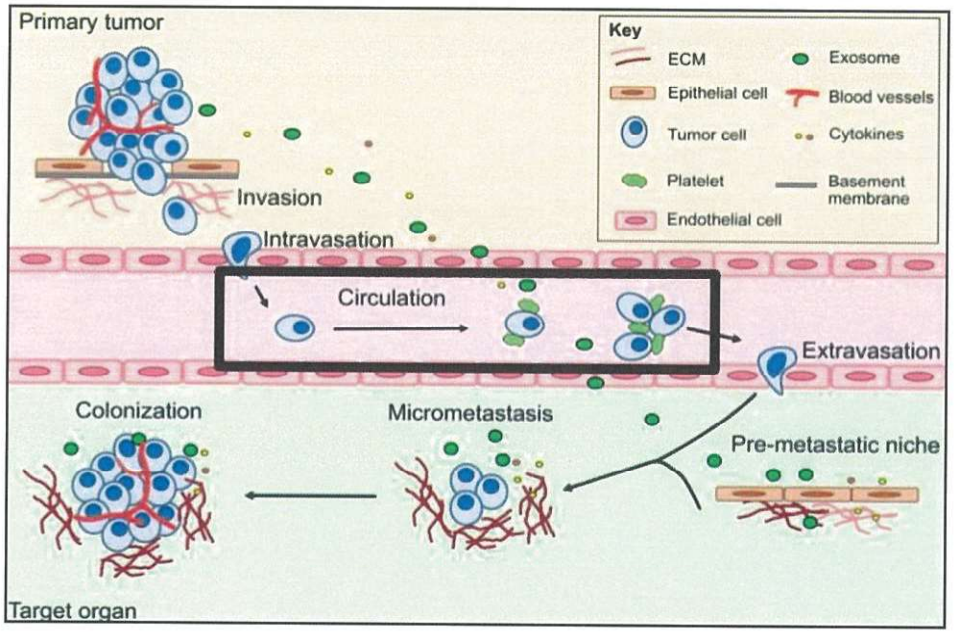
Rationale:

Cancer is a major public health problem across the globe. In the United States, it is the second leading cause of death for adults [1]. Breast cancer is the most commonly diagnosed cancer in American women (excluding cutaneous cancers) and, after lung cancer, is the second leading cause of cancer death among women [2]. It is estimated that in 2019, 268,600 U.S. women will be diagnosed with invasive breast cancer and another 48,100 will be diagnosed with in situ ductal carcinoma [2]. Overall, it is estimated that 41,760 women will die from the disease, with most of the deaths being attributed to stage IV breast cancer [2]. Localized breast cancer is fairly docile, with stage I and II breast cancers having 98 and 92 percent overall 5 year survival rates, respectively. Stage III survival rates drop to 75 percent. Metastatic stage IV breast cancer survival rates drop down to 27 percent [2]. It is estimated that about 30 percent of women diagnosed with early-stage (I and II) breast cancer will develop metastatic lesions [3]. Currently, treatment for patients with advanced metastatic breast cancer is centered on palliative care due to the lack of targeted therapies and innate resistance of metastatic tumors to conventional treatment [3].

Basal-like breast cancer (BLBC) is a subtype of breast cancer that is defined as lacking expression of estrogen (ER), progesterone, and human epidermal growth factor 2 (HER2) receptors but expressing various cytokeratins and/or epidermal growth factor receptors [5]. It often presents as large, advanced stage tumors at diagnosis. BLBC represents about 15-25% of cancer diagnoses and, on average, is diagnosed in younger patients and premenopausal women [6]. Due to its ER negativity, BLBC has relative sensitivity to cytotoxic chemotherapies but still has a poor prognosis and is at high risk of metastasis [6]. The majority of women with BLBC are at a high risk for early relapse in the first 2-5 years after treatment, leading to an overall lower 5 year survival rate [7]. In addition, the pattern of distant organ specific metastasis (lungs, liver, and central nervous system) unique to BLBC makes it an aggressive and difficult cancer to treat [7]. Current therapies for BLBC are limited to non-specific cytotoxic agents like doxorubicin [7]. The absence of targeted therapies for BLBC is representative of the limited research done in the area of BLBC and similar triple negative breast cancers.

Understanding the mechanisms and interactions leading to metastatic disease is critical for the development of effective targeted therapies. To metastasize, cancer cells must undergo a "metastatic cascade" involving multiple steps: (1) local invasion of tissues and bloodstream, (2) survival in circulation, (3) extravasation and initial survival in the distant organ, and (4) resuming uncontrolled growth in the distant organ [4].

Figure 1: anoikis resistance is crucial for survival in circulation [16]

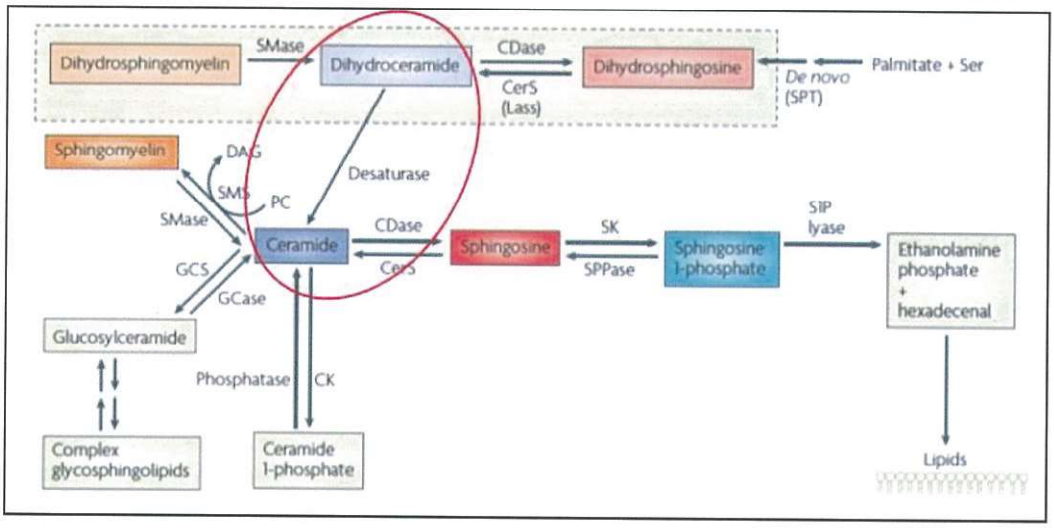


Resistance to anoikis — a unique form of apoptosis in the absence of attachment to the extracellular matrix (ECM) — plays a key role in the survival of cancer cells in the bloodstream [8]. Overall resistance to apoptosis (anoikis) is an important factor in the aggressiveness of BLBC [7]. Many molecular pathways are involved in the regulation (and subsequent dysregulation) of anoikis in breast cancer. Cancer cells can achieve resistance to anoikis through a switch in their integrins, undergoing epithelial-mesenchymal transition, deregulation of metabolism, constitutive pro-survival signaling, or dysregulation of cell death signaling as a result of internal and external factors [8]. Gaining a more in-depth understanding of these pathways has the potential to yield novel targets.

Sphingolipids (SLs), a family of bioactive lipids, are involved in almost all major cellular processes including cell survival, migration, inflammation, apoptosis, and senescence [9]. The deregulated metabolism and synthesis of sphingolipids has been well documented in cancer,

making them a potential target [10]. Currently, there is little research regarding how SLs connect to anoikis or anoikis resistance. Understanding the biochemical regulation of SLs by their enzymes is a key factor in the development of novel targeted therapies [11]. Among the many enzymes involved in SL regulation is dihydroceramide desaturase 1 (DES1), an enzyme in the last step of the *de novo* pathway leading to the production of ceramide (Cer). DES1 is responsible for inserting a double bond into dihydroceramide (dhCer), converting it to Cer [12]. Previously deemed inert, dhCer has more recently been found to have biological functions distinct from Cer and other SLs, playing a key role in apoptosis, cell proliferation, and oxidative stress [13].

Figure 2: schematic of the sphingolipid network;
note Cer in the central hub of the network and the role of DES in the *de novo* pathway [9]



Ongoing work in The Lipid Cancer Laboratory at Stony Brook University has identified DES1 as a target of interest in HER2+ breast cancer, suggesting that disruption of the *de novo* pathway as a result of targeting DES1 can lead to accumulation of dhCer, overcoming anoikis resistance in HER2+ breast cancer [15].

The goal of this study is to assess the importance of DES1 in making basal breast cancer cells resistant to anoikis. To test this, DES1 will be targeted through both a genetic and pharmacological approach. For the genetic approach, CRISPR will be used to knockout the DES1 gene, effectively eliminating all resultant gene products in the cell. For the

pharmacological approach, DES1 inhibitors fenretinide (4-HPR) and ABC294640 (ABC) will be used, interfering with DES1 function [17] [18].

Hypothesis:

The hypothesis was that targeting DES1 through pharmacological inhibitors and CRISPR mediated knockout can overcome anoikis resistance in basal-like breast cancer.

Methodology:

Cell culture - The 4T1 cell line (mouse basal/triple negative breast cancer, ATCC CRL-2539) will be cultured in RPMI 1640 (RPMI) media with 10% Fetal Bovine Serum (FBS) at 37 degrees Celsius in 5% carbon dioxide (CO2). Inhibitors will be added during plating at final concentrations of 5μM for 4-HPR and 20μM for ABC with dimethyl sulfoxide (DMSO) added as vehicle control.

Assaying DES1 activity - After removal of the DES1 gene using CRISPR technology, the cells will be plated in 60mm dishes with 300k cells per dish. After incubation for 24 hours, 1.5μL of C12-PyrdhCer will be added to each dish for one hour. Lipids will then be extracted and the levels of substrate plus product (C12-PyrCer) will be measured through mass spectrometry. DES1 activity will be calculated through the ratio of substrate to product.

Measuring cell death by Trypan Blue exclusion - After treatment, the attached cells will be trypsinized from plates and centrifuged for 5 minutes at 1200 RPM. After spinning, the cells will be resuspended in 2mL of RPMI with 10% FBS. The cells in suspension will be collected, centrifuged for 5 minutes at 1200 RPM, and then trypsinized for 30 seconds to 1 minute. The reaction will be then stopped with an equal volume of RPMI (+10% FBS) and the cells will be again centrifuged for 5 minutes at 1200 RPM. After spinning, the cells will be resuspended in 1mL of RPMI (+10% FBS). Both attached and suspended cells will be then stained with trypan blue dye (10μL cells + 10μL trypan blue) and counted using a hemocytometer. The data will be

expressed as cells trypan blue positive compared to total cells (dye trypan blue positive plus excluded).

Assaying in vitro tumorigenicity - 200k cells and 2X RPMI will be mixed with an equal volume of 0.8% agar (final 0.4% agar) and placed over a solidified layer of 0.8% agar with RPMI (+10% FBS). After solidifying, 150μL of RPMI (+10% FBS) will be added to the trays to prevent dehydration. The inhibitor (4-HPR) will be added to the upper layer of agar where needed. The colonies will be then stained with MTT dye, and incubated for 1-2 weeks at 37 degrees Celsius in 5% CO2. Then, pictures will be taken on an EVOS microscope with a 10x objective.

Risk and Safety:

Potentially Hazardous Biological Organisms - The 4T1 mouse basal breast cancer cell line was obtained from ATCC (catalog number CRL-2539). It is listed as a BSL-2 organism and has been determined to be as such through the risk assessment procedure. When handling the cells, proper personal protective equipment such as gloves, coats, masks, and goggles will be worn. In addition, all guidelines established by both the manufacturer (ATCC) and Stony Brook University safety protocols will be adhered to. The cells will be disposed of in a bleach solution and any related materials (trays, dishes, pipette tips, etc.) will be disposed of in clearly marked red biohazard disposal bins.

Hazardous Chemicals - The potentially hazardous chemicals used in the project are trypan blue dye and the inhibitors lapatinib (4-HPR) and ABC294640. Proper personal protective equipment (gloves, coats, masks, and goggles) will be worn when handling these chemicals. The qualified scientist will be present when performing protocols requiring these substances. These substances will be disposed of in red biohazard disposal bins.

Data Analysis:

The data collected will be analyzed using Prism 8 software and various tests (T tests, etc.) will be done to test for statistical significance. Other data will be analyzed through discussion with the supervisor/qualified scientist and prior experience will be used when making judgements.

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Addendum:

No changes were made from original research plan.