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

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Abstract:

Despite advances in early stage breast cancer treatment, metastatic breast cancer still has low survival rates and limited therapies. Certain subtypes such as basal breast cancer (BLBC) are more aggressive and difficult to treat, often lacking any targeted therapies. Resistance to anoikis — a form of cell death — has emerged as an important biology allowing aggressive cancers like BLBC to metastasize. While sphingolipids (SLs) have been indicated as key mediators of cell death and are known to be altered in cancer, the relationship between SLs and anoikis resistance has not been definitively studied. Because of its broader role in anoikis resistance, the enzyme dihydroceramide desaturase (DES1) has been indicated as a potential target in metastatic disease.

The hypothesis considered whether targeting DES1 through both a genetic and pharmacological approach could overcome anoikis resistance in BLBC. By using CRISPR to knockout DES1 in 4T1 cancer cells and the inhibitors fenretinide and ABC294640 to inhibit DES1 function, it was shown that the loss of DES1 did overcome anoikis resistance by decreasing cell survival in anchorage independent conditions and reducing colony growth and tumorigenicity. These results indicate DES1 as a potential target for the future treatment of BLBC.

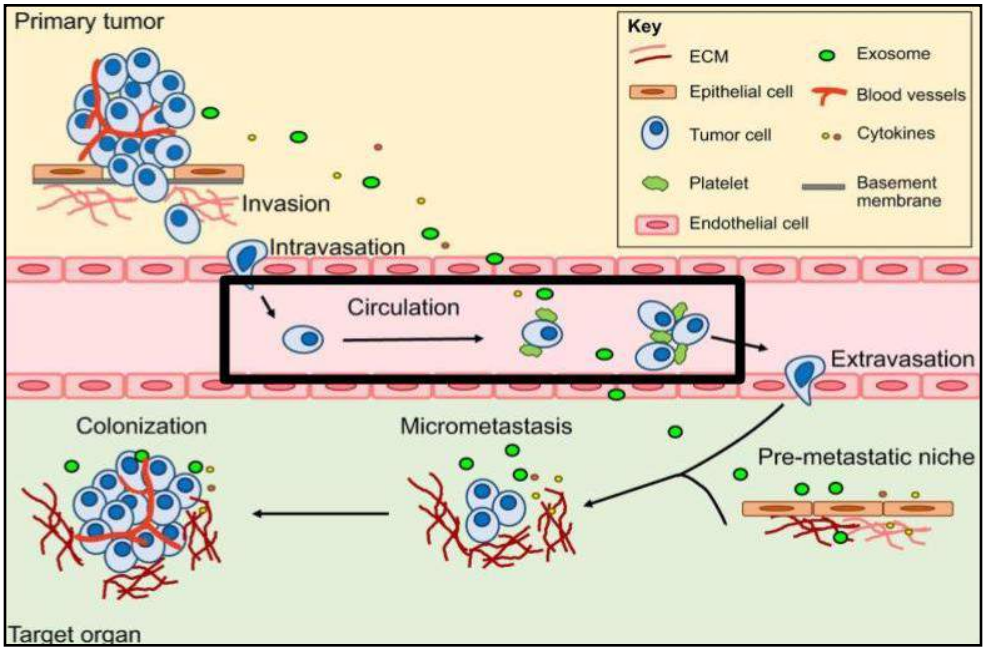
Introduction:

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]

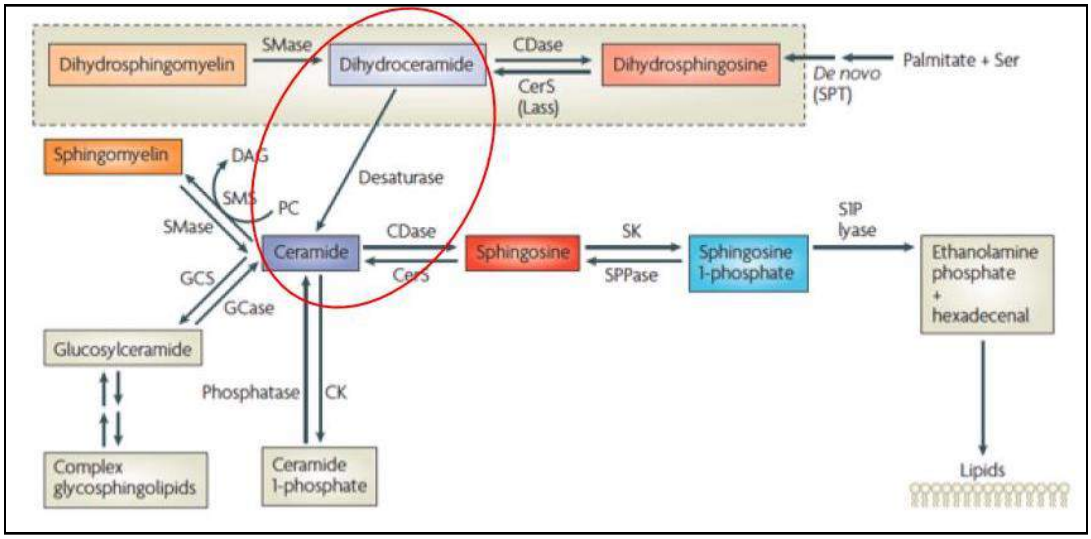


Resistance to anoikis — a unique form of apoptosis in the absence of attachment to the extracellular matrix (ECM) — plays a key role in the survival of cancer cells in the bloodstream [8]. Overall resistance to apoptosis (anoikis) is an important factor in the aggressiveness of BLBC [7]. Many molecular 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 was to assess the importance of DES1 in making basal breast cancer cells resistant to anoikis. To test this, DES1 was targeted through both a genetic and pharmacological approach. For the genetic approach, CRISPR was 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) were used, interfering

with DES1 function [17] [18]. 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) was cultured in RPMI 1640 (RPMI) media with 10% Fetal Bovine Serum (FBS) at 37 degrees Celsius in 5% carbon dioxide (CO2). Inhibitors were 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 were plated in 60mm dishes with 300k cells per dish. After incubation for 24 hours, 1.5μL of C12-PyrdhCer was added to each dish for one hour. Lipids were then extracted and the levels of substrate plus product (C12-PyrCer) were measured through mass spectrometry. DES1 activity was calculated through the ratio of substrate to product.

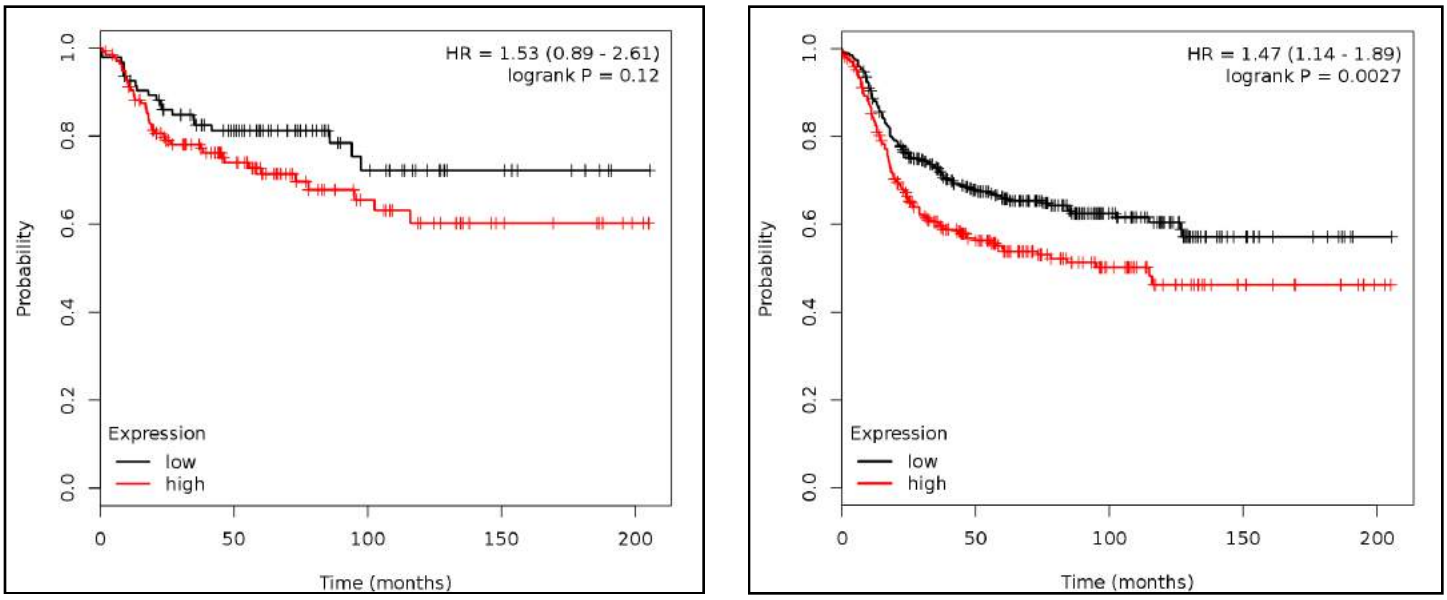
Measuring cell death by Trypan Blue exclusion - After treatment, the attached cells were trypsinized from plates and centrifuged for 5 minutes at 1200 RPM. After spinning, the cells were resuspended in 2mL of RPMI with 10% FBS. The cells in suspension were collected, centrifuged for 5 minutes at 1200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

dehydration. The inhibitor (4-HPR) was added to the upper layer of agar where needed. The colonies were then stained with MTT dye, and incubated for 1-2 weeks at 37 degrees Celsius in 5% CO2. Then, pictures were taken on an EVOS microscope with a 10x objective.

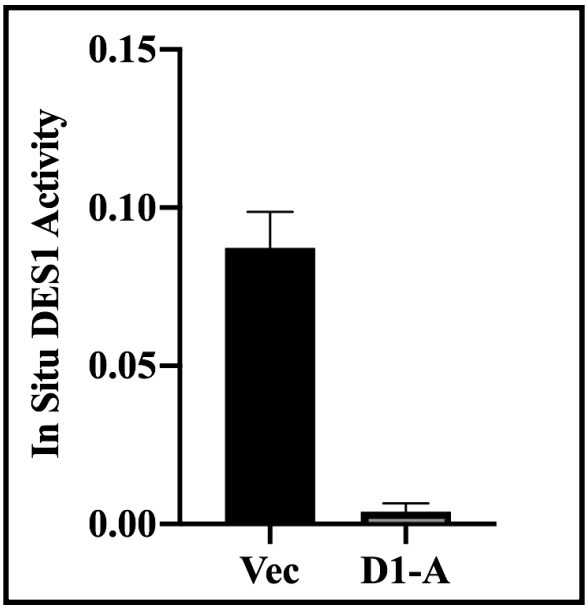
Results:

Figure 3: Kaplan Meier Plots for DMFS (left) and RFS (right)



Kaplan Meier (KM) analysis was done on patients diagnosed with BLBC to look for a clinical correlation between DES expression and both relapse free survival (RFS) and distant metastasis free survival (DMFS). Figure 3 shows the two KM plots generated from publicly available data with auto selected cutoffs. The results indicated a statistically significant correlation between low DES1 expression and higher probabilities of RFS ($p<0.01$, HR = 1.47, n=618). The correlation for DMFS (n=232) was not significant (p=0.12), but the hazard ratio (HR) of 1.53 does suggest a relationship.

Figure 4: Confirming loss of DES1 activity in D1 knockout cells



The substrate:product ratios were calculated after mass spectrometry. The difference in in situ DES1 activity between the control (MT-Vec) and knockout (D1-A) 4T1 cells was statistically significant ($p<0.05$). The mean activity in the unaltered cells was 0.087. The mean activity in the knockout cells was .004. The difference in means was 0.083± 0.012.

Figure 5: Percentage of Cell Death in D1 knockout cells compared to MT-Vec control in Attached (left) and Suspended (right) Conditions

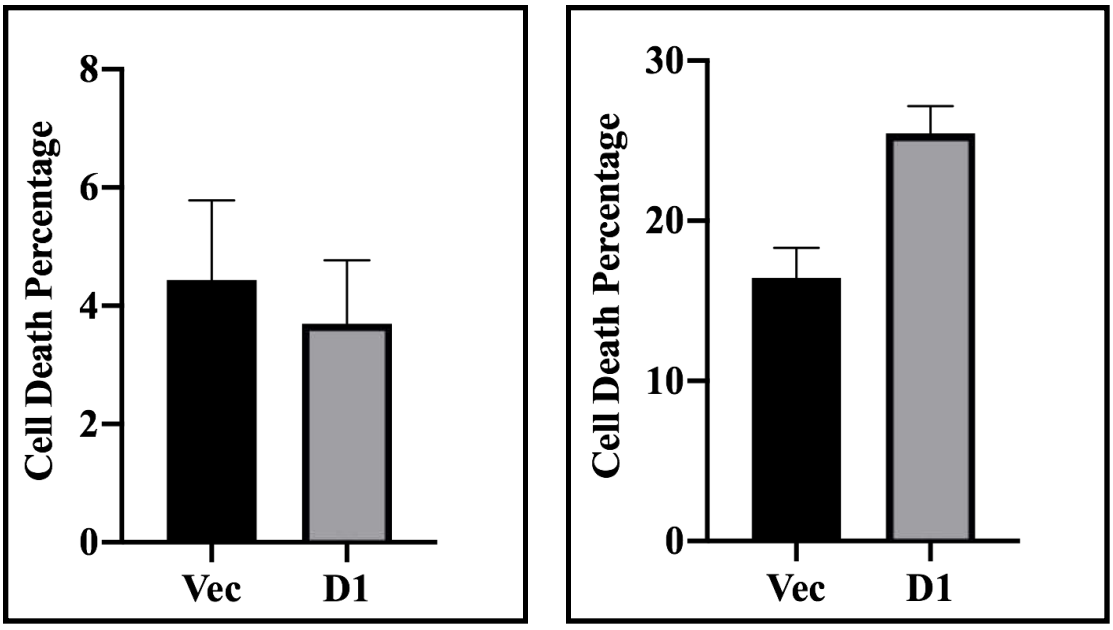
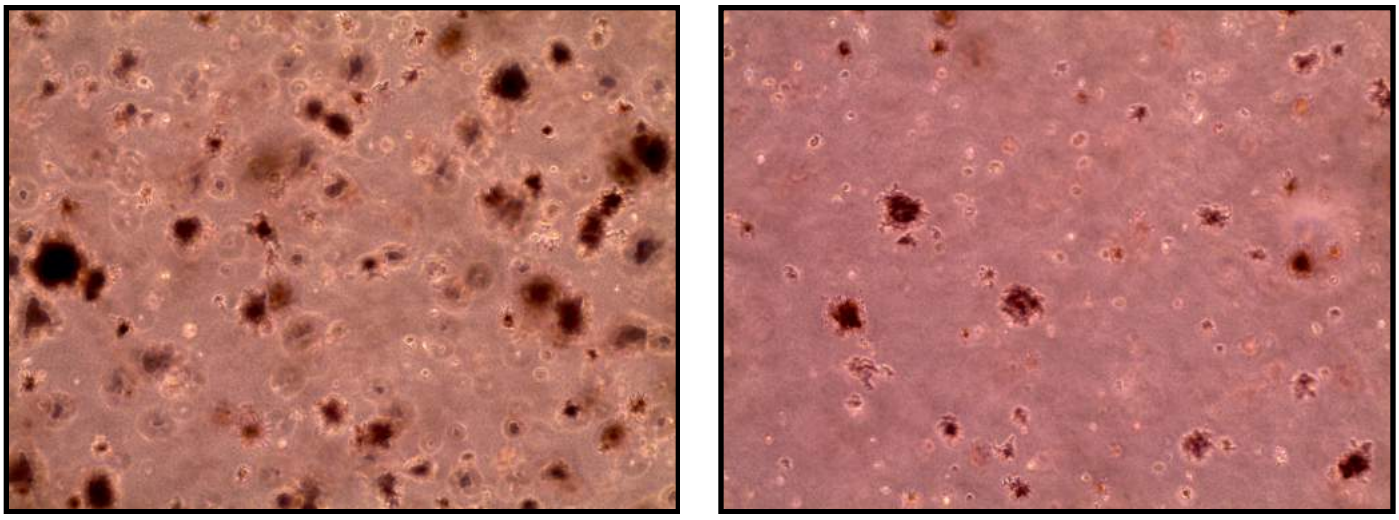


Figure 6: Colony Growth in Vec Control (left) and D1 Knockout (right) 4T1 Cells



The cell death percentages were calculated using trypan blue positive (dead) cells as a percentage of total cells (trypan blue positive plus excluded (live) cells). In attached conditions, loss of DES1 did not result in a statistically significant difference in cell survival ($p=0.69$). The mean cell death percentage in the attached MT-Vec control cells was 4.4% and 3.7% in D1 knockout cells. The difference between the means was 0.7 ± 1.7% (Figure 5). In suspension, there was a statistically significant difference ($p<0.01$) between MT-Vec control and D1 knockout cells. The mean cell death percentage in the MT-Vec control cells was 16.4% compared to 25.5% in the D1 knockout cells with a difference of 9.0 ± 2.5% (Figure 5). In addition, loss of DES1 had a significant effect on colony formation, with D1 knockout cells growing smaller and more sporadic colonies compared to the larger and denser colonies of the MT-Vec control cells (Figure 6).

Figure 7: Effect of DES1 inhibitors in suspension compared to Vehicle Control

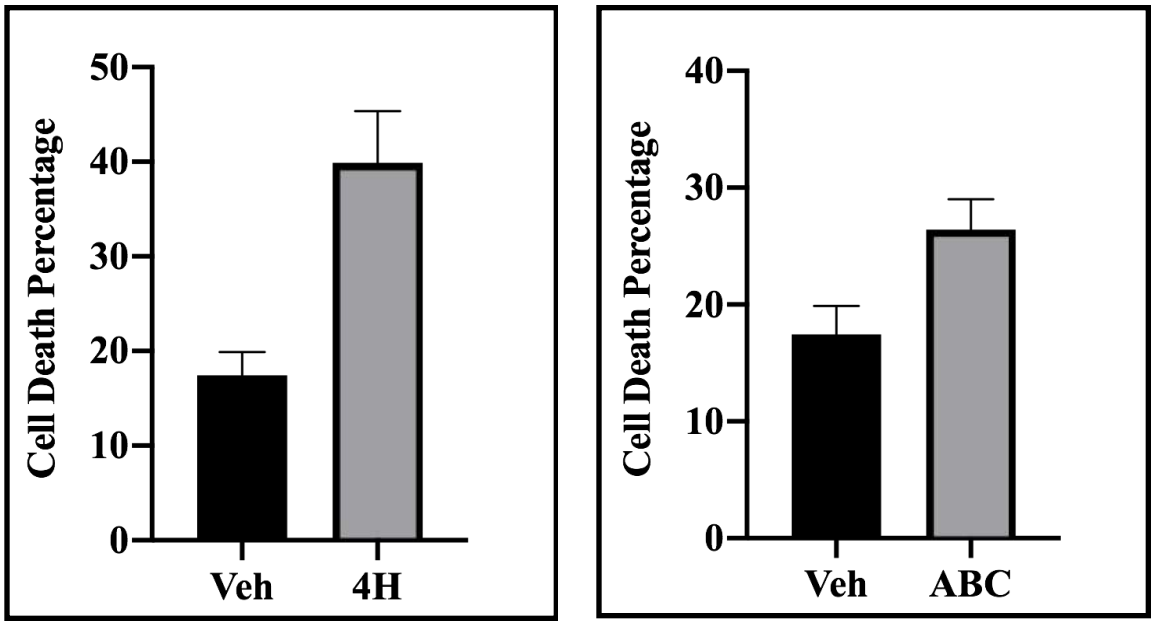
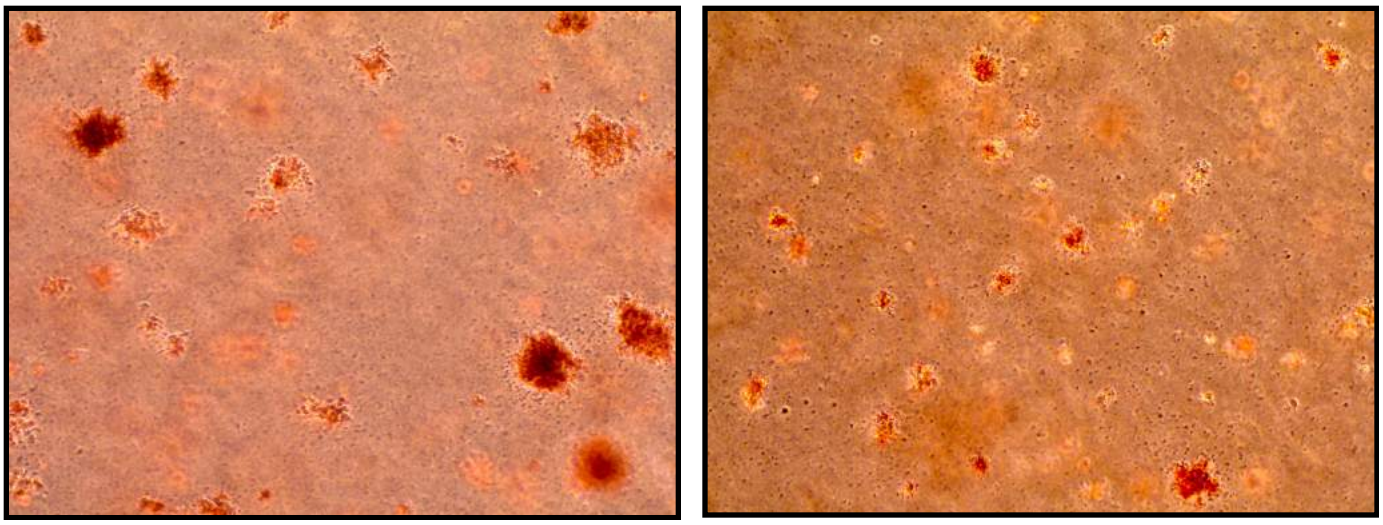


Figure 8: Colony Growth in 4T1 Cells Treated with 4-HPR (right) compared to Vehicle Control (left)



The inhibition of DES1 using fenretinide (4H) did result in a statistically significant difference in cell death in cells growing in suspension ($p<0.05$) compared to the control (Veh). The mean cell death in the control was 17.5%, compared to 40.0% in the fenretinide group. The mean difference was 22.5 ± 6.0% (figure 7). Inhibiting DES1 using ABC had a modest effect but did not result in a statistically significant difference ($p=0.06$) in cell death percentage between treated and untreated cells growing in suspension. The mean for the control was again 17.5%,

compared to 26.4% in the ABC group, with the difference being 9.0 ± 3.6% (Figure 7). Again, inhibiting DES1 with fenretinide did produce a noticeable effect in colony growth, with treated cells growing smaller and more sporadic colonies compared to the higher numbers of larger colonies in the vehicle control (Figure 8).

Discussion:

Anchorage independent growth allowing for survival in circulation (Figure 1) is a key part of the metastatic cascade leading to cancer cell metastasis. Anoikis resistance has emerged as a recent biology of interest due to its role in allowing cells to grow in anchorage independent conditions. Despite this, there are no current targeted therapies to overcome anoikis resistance in highly aggressive metastatic cancers like BLBC.

The major finding of this project was the necessity of DES1 for anoikis resistance in BLBC, and that subsequent targeting of DES1 can overcome anoikis resistance in BLBC. This finding is consistent with prior data from The Lipid Cancer Lab showing DES1 as a target of interest in HER2+ breast cancer [15] as well as Kaplan Meier analysis of publicly available data showing a correlation between high DES1 expression and worse survival in patients with BLBC (Figure 3). This project took two different approaches to targeting DES1 in BLBC. The genetic approach (D1 knockout) confirmed that, in suspension, the loss of DES1 decreases cell survival (Figure 5) and can overcome anoikis resistance in BLBC. Consistent with this, colony formation data (Figure 6) showed that DES1 knockout resulted in decreased tumorigenicity. This is consistent with prior studies linking aggressive cancers to anoikis resistance [8]. An interesting finding was that targeting DES1 was broadly more effective in suspension than in monolayer adherent cells (Figure 5) suggesting that the role of DES1 in regulating cell death may be specific to this context. The DES1 activity assays (Figure 4) serve to confirm the knockout, showing that DES1 function is effectively lost in knockout (D1-A) cells.

The knockout experiments were able to show that DES1 was necessary for anoikis resistance in BLBC. The inhibitor treatments are a logical next step as in the long term they can facilitate translation into clinical treatment. The results showed a significant increase in cell death percentage when treated with fenretinide (Figure 7) but not following treatment with ABC.

The modest effect of ABC is most likely due to its decreased effectiveness at inhibiting DES1 when compared to 4-HPR. This is also the reason for the difference in concentrations (5μM for 4-HPR, 20 μM for ABC) used for treatment. The colony growth assays mirror the above results, with cells treated with 4-HPR growing smaller and more sporadic colonies compared to the control, indicating that targeted inhibition of DES1 also decreases aggressiveness and tumorigenicity in BLBC. The advantage of the inhibitor treatments is the potential for translation into clinical trials and in vivo studies. There are current ongoing clinical trials for 4-HPR [19] as well as ABC [20] that indicate varying degrees of success. If these trials are successful, there is potential for these drugs to gain FDA approval and be available for treatment. Such an outcome would help promote the rapid translation of these findings into the clinical setting.

Most major SL research is focused on targeting Cer through up regulation in order to induce cell death. The unique aspect of this project is that instead of targeting Cer, it focused on dhCer and DES1 as inducers of apoptosis, showing that the accumulation of dhCer (and dissipation of Cer) as a result of DES1 inactivity still leads to cell death. This has many distinct advantages. As shown in Figure 2, there are five separate pathways that lead to the production of Cer at the central hub of the sphingolipid network. This means that if one pathway was targeted, the others could potentially compensate and restore Cer balance in the cell. DhCer is only present in one pathway (de novo), meaning that targeting dhCer balance through a decrease in DES1 activity is more likely to have its intended effect as there are minimal avenues for compensation. In addition, there are only two DES enzymes in the human body, DES1 and DES2. DES2 expression is naturally very low, making DES1 the only effective target of interest. In context, it is more feasible to target DES1 and dhCer instead of the more commonly researched Cer pathways. Finally, the analysis of publicly available clinical data (Figure 3) suggests that there is a subset of BLBC tumors with high levels of DES1 expression that have worse clinical outcomes. The data here suggests that this could be a consequence of increased anoikis resistance making them more aggressive. Indeed, the resultant reduction in colony formation as shown in both D1 knockout and inhibitor data is representative of decreased tumorigenicity. An important next step to test this hypothesis would be to determine if increasing DES1 expression is involved

in giving BLBC its characteristic aggressiveness and tumorigenicity. This also suggests that DES1 levels may be a marker of metastasis-prone BLBC

It is important to note the various limitations of this project. The experimentation was performed in vitro, meaning that the results might not accurately reflect what happens during metastasis. Further in vivo experimentation and, assuming positive results, an entry into clinical trials could provide a more complete picture. In addition, only a single cell line was used, which might not be representative of all BLBC. Further studies involving more cell lines would address this issue. While the pharmacological approach yielded positive results, there is potential for some effects of the inhibitors to be a result of off target effects. Using two distinct inhibitors was an attempt at resolving this issue, but the development of more potent and selective DES1 inhibitors would be of more use as potential therapies and limit any potential off target effects. Lastly, this project showed that DES1 was necessary for anoikis resistance, but further experimentation including the over-expression of DES1 would solidify its role as an oncogenic driver of anoikis resistance and indicate it as a major target, yielding new therapies for aggressive, metastatic cancers like BLBC.

Conclusion:

Overall, targeting DES1 was effective at overcoming anoikis resistance in basal breast cancer. This was confirmed by two distinct approaches — genetic and pharmacological — with both giving positive results including decreased cell survival in suspension and less anchorage independent growth resulting from decreased tumorigenicity. Despite the limitations discussed above, the findings establish DES1 as a major potential target in the treatment of aggressive, potentially metastatic tumors like BLBC.

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References:

1. Siegel, R. L., Miller, K. D. and Jemal, A. (2019), Cancer statistics, 2019. CA A Cancer J Clin, 69: 7-34. doi:10.3322/caac.21551

2. DeSantis, C. E., Ma, J. , Gaudet, M. M., Newman, L. A., Miller, K. D., Goding Sauer, A. , Jemal, A. and Siegel, R. L. (2019), Breast cancer statistics, 2019. CA A Cancer J Clin, 69: 438-451. doi:10.3322/caac.21583

3. Redig, A. J., & McAllister, S. S. (2013). Breast cancer as a systemic disease: a view of metastasis. Journal of internal medicine, 274(2), 113–126. doi:10.1111/joim.12084

4. Jin, X., & Mu, P. (2015). Targeting Breast Cancer Metastasis. Breast cancer : basic and clinical research, 9(Suppl 1), 23–34. doi:10.4137/BCBCR.S25460

5. Milioli, H.H., Tishchenko, I., Riveros, C. et al. Basal-like breast cancer: molecular profiles, clinical features and survival outcomes. BMC Med Genomics 10, 19 (2017) doi:10.1186/s12920-017-0250-9

6. Bertucci, F., Finetti, P., & Birnbaum, D. (2012). Basal breast cancer: a complex and deadly molecular subtype. Current molecular medicine, 12(1), 96–110. doi: 10.2174/156652412798376134

7. Toft, D. J., & Cryns, V. L. (2011). Minireview: Basal-like breast cancer: from molecular profiles to targeted therapies. Molecular endocrinology (Baltimore, Md.), 25(2), 199–211. doi: 10.1210/me.2010-0164

8. Paolo Paoli, Elisa Giannoni, Paola Chiarugi, Anoikis molecular pathways and its role in cancer progression, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, Volume 1833, Issue 12, 2013, Pages 3481-3498, ISSN 0167-4889, https://doi.org/10.1016/j.bbamcr.2013.06.026.

9. Hannun, Y., Obeid, L. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 9, 139–150 (2008) doi:10.1038/nrm2329

10. Giussani, P., Tringali, C., Riboni, L., Viani, P., & Venerando, B. (2014). Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. International journal of molecular sciences, 15(3), 4356–4392. doi:10.3390/ijms15034356

11. Hannun, Y.A., Obeid, L.M. Author Correction: Sphingolipids and their metabolism in physiology and disease. Nat Rev Mol Cell Biol 19, 673 (2018) doi:10.1038/s41580-018-0046-6
12. Mireia Casasampere, Yadira F. Ordoñez, Ana Pou, Josefina Casas, Inhibitors of dihydroceramide desaturase 1: Therapeutic agents and pharmacological tools to decipher the role of dihydroceramides in cell biology, Chemistry and Physics of Lipids, Volume 197, 2016, Pages 33-44, ISSN 0009-3084, https://doi.org/10.1016/j.chemphyslip.2015.07.025.
13. Siddique, Md Monowarul & Li, Ying & Chaurasia, Bhagirath & Kaddai, Vincent & Summers, Scott. (2015). Dihdyroceramides: From Bit Players to Lead Actors. The Journal of biological chemistry. 290. 10.1074/jbc.R115.653204.
14. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, Breast Cancer Res Treatment, 2010 Oct;123(3):725-31.
15. R.W. Linzer, G. Khaliffe, J.M. Snider, J. Aminov, B. Buyukbayraktar, P. Wang, C. Pan, J. Ren, C.J. Clarke. Targeting Dihydroceramide Desaturase (DEGS1) to Overcome Anoikis Resistance in HER2 positive Breast Cancer, Federation of European Biochemical Societies Special Meeting in Sphingolipid Biology, 2019
16. Laura Gómez-Cuadrado, Natasha Tracey, Ruoyu Ma, Binzhi Qian, Valerie G. Brunton. Mouse models of metastasis: progress and prospects, Disease Models & Mechanisms 2017 10: 1061-1074; doi: 10.1242/dmm.030403
17. Venant H, Rahmaniyan M, Jones EE, et al. The Sphingosine Kinase 2 Inhibitor ABC294640 Reduces the Growth of Prostate Cancer Cells and Results in Accumulation of Dihydroceramides In Vitro and In Vivo. Mol Cancer Ther. 2015;14(12):2744–2752. doi: 10.1158/1535-7163.MCT-15-0279
18. Mehrdad Rahmaniyan, Robert W. Curley, Jr, Lina M. Obeid, Yusuf A. Hannun, Jacqueline M. Kraveka. Identification of Dihydroceramide Desaturase as a Direct in Vitro Target for Fenretinide, J. Biol. Chem. 2011 286: 24754-. doi:10.1074/jbc.M111.250779
19. Mohrbacher AM, Yang AS, Groshen S, Kummar S, Gutierrez ME, Kang MH, Tsao-Wei D, Reynolds CP, Newman EM, Maurer BJ. Phase I Study of Fenretinide Delivered Intravenously in Patients with Relapsed or Refractory Hematologic Malignancies: A California Cancer

Consortium Trial. Clin Cancer Res. 2017 Aug 15;23(16):4550-4555. doi: 10.1158/1078-0432.CCR-17-0234. Epub 2017 Apr 18.

20. Britten CD, Garrett-Mayer E, Chin SH, Shirai K, Ogretmen B, Bentz TA, Brisendine A, Anderton K, Cusack SL, Maines LW, Zhuang Y, Smith CD, Thomas MB. A Phase I Study of ABC294640, a First-in-Class Sphingosine Kinase-2 Inhibitor, in Patients with Advanced Solid Tumors. Clin Cancer Res. 2017 Aug 15;23(16):4642-4650. doi: 10.1158/1078-0432.CCR-16-2363. Epub 2017 Apr 18.