Pre-clinical trials with precision-medicine based therapeutics in basal-like patient-derived xenografts

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Pre-clinical trials with precision-medicine based therapeutics in basal-like patient-derived xenografts

A thesis/dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

David Charlie Boyd

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Abstract

Breast cancer treatments have improved over time, but the diseases seeing the most benefit from these improvements have the estrogen receptor, progesterone receptor, or are positive for HER2. Basal-like breast cancer tends to not have these biomarkers, which necessitates their treatment to be traditional, untargeted therapeutics which are less effective and tend to have harsh adverse effect profiles – this is an important unmet need. These studies utilize a variety of techniques, including tissue culture, viability assays, high-throughput screening, in vivo drug treatments and imaging, pathway analyses, molecular techniques such as Western blot, antibody arrays, RNA sequencing, sc RNA sequencing, and many others. This project is divided into 4 studies, each with important findings. First, a characterization of the Glowing Head mouse model showed that the model is suitable for most metastatic settings, though the endogenous signal of the luciferase produced by the mouse will confound studies which study bone or brain metastasis. Then, a study in EGFR inhibitor resistance identified LCN2 as an upregulated marker of resistance which could reduce sensitivity to erlotinib by aiding in the recycling of EGFR. Another study discovered compounds that doxorubicin treated cells will become resistant to and which show a trend of reducing senescent cells’ viability. Finally, the core project identified three compounds when combined with BYL-719, a PIK3CA inhibitor, have synergistic activity in the reduction of tumor sizes of basal-like PI3K aberrant PDXs. It is my hope that these studies may be used as preliminary data for further study, both preclinical and clinical.
Chapter 1: Introduction

Cancer incidence overall

Cancer, which is likely named after the crab-like tendrils spreading from a central mass[1], is overall commonly diagnosed as almost two million cancer cases are projected to be diagnosed in the US this year[2]. Cancer is caused by a number of different possible pathological events, mostly environmental in nature[3]. When DNA is damaged, repair mechanisms or apoptotic signals activate, when there is a problem in these processes, cancer can arise[4]. There is certainly a genetic component to cancer, as some genes regulate genetic stability such as TP53[5], for example, people with germline pathogenic TP53, a rare condition known as Li-Fraumeni Syndrome, have a massively increased chance of developing cancer in their lifetimes[6]. When TP53 is mutated somatically it results in especially aggressive cancers, such as triple-negative breast cancer (TNBC)[7].

Breast cancer statistics and subtypes

Breast cancer is one of the most diagnosed cancers. This year, just over 300,000 cases of newly diagnosed breast cancers are projected[2]. Breast cancer comprises almost a third of female cancers and 1 out of every 8 women in the United States will get cancer at some point in their lives[8] and 2,800 men are predicted to get breast cancer this year[9]. Invasive ductal carcinoma is 9 times more common relative to generally less aggressive invasive lobular carcinoma, the difference pathologically being that the former forms when cells from milk ducts become cancerous and the latter resulting from cells from milk lobules, where milk is produced, become cancerous[10].
Figure 1. Percentage share of each PAM50 subtype among breast cancer patients. Percentages come from FFPE samples from breast cancer patients.

**PAM50 subtypes**

Prediction Analysis of Microarray 50 (PAM50) is a gene signature that is used to sort cancers into intrinsic genomic subtypes. Originally, this tool was used to sort breast cancers into luminal A, luminal B HER2 enriched, and basal-like breast cancers[11]. Luminal A is by far the most common of the PAM50 subtypes (Figure 1)[12]. Luminal A and B tend to be ER and/or PR positive, with luminal B comprising a more aggressive, higher HER2 and/or Ki-67 expression disease. HER2 disease is faster in growth rate and historically has had a worse prognosis than luminal A/B disease. Basal-like breast cancers have high heterogeneity, higher mutational burden, and are aggressive with limited treatment options, and is characterized by the lack of ER, PR, or HER2 expression. All breast cancer instances carry a risk of metastasis and/or recurrence to metastasize to brain, liver, and lung. Luminal B disease has an increased risk of bone metastasis [13]. Later, the claudin-low subtype was identified, which is a type of triple negative breast cancer that is more stem-like and has high epithelial to mesenchymal (EMT) gene expression. Another subtype added to the PAM50 is normal-like breast cancer, which is likely samples
with high percentage of noncancerous cells from the patient[14] or can also be thought of as non-basal-like triple negative breast cancer[15].

The Vanderbilt TNBC subtypes

Another valuable perspective in classifying TNBC is the Vanderbilt subtypes. Most of these would generally fall under the basal-like PAM50 subtype except for LAR which tends to cluster with other luminal BRCR. These are divided into basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor. Basal-likes 1/2 are high in replication genes, proliferation, and DNA damage response. Basal-like 2 differs from basal-like 1 in that it is more enriched in genes of growth factor signaling, such as EGFR. The immunomodulatory subtype is enriched for multiple immune cell processes and has the best prognosis of the Vanderbilt types. The mesenchymal subtype is enriched for EMT genes as well as growth factor signaling and other pathways such as WNT and ERK. The mesenchymal stem-like differs from the mesenchymal subtype in that it has reduced proliferation rates and higher mesenchymal stem like markers. Luminal androgen receptor subtype is the most different among the TNBCs, as it is luminal and hormone receptor positive, though still TNBC, because the hormone receptor on which it relies is the androgen receptor[16].
Figure 2. Molecular chart of PIK3CA in the PI3K/mTOR/AKT pathway. In purple is the influence of the activity on downstream effects of PI3K pathway activity.

**Importance of PIK3CA in breast cancer**

PIK3CA one of the most commonly pathogenically mutated genes breast cancer[17]. As proteins, class 1 PI3Ks phosphorylate the lipid PIP2 to produce PIP3, which drives proliferative and anti-apoptotic downstream effects[18] (Figure 2). The p110α subunit can bind with its regulatory subunit, p85α, or one of its splice variants, p55α or p50α[19]. Knock-in of activating mutations of PIK3CA have been shown to have oncogenic effects[20]. PIK3CA expression is found in all tissue and cancer types and has low specificity between tissue and cancer types[21]. PTEN is diametrically opposed to PIK3CA and serves as intrinsic brakes to PI3K pathway, so another way PI3K activity is increased is loss of PTEN, which is common in basal-like cancer[22]. PI3K inhibitors have been tested in clinical trials, but only 1 has been approved for breast cancer. Because PI3K pathway is so important in cell processes and is found in every cell type, inhibiting it comes with many possible adverse events. PI3K inhibition also has been shown to have numerous possible resistance mechanisms. Buparlisib, pictilisib, copanlisib, taselisib, idelalisib are each PI3K inhibitors which have been shown to have extensive toxicities in clinical trials, while alpelisib still showed some toxicity, it was more favorable than more toxic PI3K inhibitors like taselisib[23].
Because of these challenges, additional compounds in use with PI3K inhibitors to possibly reduce overall side effects by improving activity with lower doses are highly desirable.

**PI3K targeting and Combinatorial Agents with PI3KCA Inhibitors**

Most of the PI3K inhibitors that have been produced are ATP competitive[24]. There were over forty drugs targeting one or more PI3K isoforms being used in ongoing clinical trials as single agents or with standard of care established drugs and 44 clinical trials using PI3Ki for patients with breast cancer that in various stages as of 2019[25]. Despite the attention of researchers on PIK3CA, alpelisib is the only p110α inhibitor that is FDA indicated for use in breast cancer and only in conjunction with fulvestrant, an estrogen receptor antagonist[26]. This combination is only approved for patients with ER+ HER2- breast cancer that are male or post-menopausal female with PI3KCA mutant after disease progression after or while being treated with endocrine-based treatment[27]. PI3K inhibition can induce significant toxicity[28], but combining treatments can help utilize a usually toxic compound, because it allows for reduced concentrations of drugs, which can reduce toxicity[29]. Additional promising combinations with PIK3CA inhibitors have been investigated since 2012 when PI3Ki were tested in combination with PARP inhibitors[30] and CDK4/6 inhibitors[31]. PI3Ki have also been investigated as possible agents of overcoming drug resistance of cancer cells to other treatments[27], [32]–[35]. Antidiabetic drugs can possibly help to prevent PI3Ki resistance, as PI3K signaling can be induced with insulin[36], [37]. PI3KCA inhibitors have been shown to increase the survival of patients across many types of cancer, but toxicity leading to adverse events limit maximum doses, cause treatment interruptions, discontinuation, and nonadherence in some patients[38], [39]. Alpelisib and taselisib both had the highest fold change efficacy of drugs tested on PIK3CA mutated cell lines relative to wild type PIK3CA containing cell lines[40] and alpelisib was shown to have limited drug-drug interactions, making it ideal for combination therapy[41].
**Breast cancer treatment and resistance**

There are many treatment options for breast cancer, including surgery, radiotherapy, and pharmaceuticals. For ER+ disease, drugs that either inhibit the estrogen receptor’s ability to bind, degrade the estrogen receptor, or deplete estrogen by inhibiting aromatase are highly effective in many cases, and are called endocrine therapy[42]. HER2 enriched disease has seen a marked increase in patient outcome due to trastuzumab and its ADC derivatives and these treatments have even been applied to a subsection of triple negative disease now being referred to as “HER2 low” to favorable effect[43]. For basal-like disease, which is triple negative for the aforementioned actionable drug targets, drugs that preferentially target cells with higher proliferation rates like cisplatin and doxorubicin are standard of care, but these drugs carry hefty side effect profiles[44]. All current drugs have multiple resistance mechanisms which can either be endogenous or arise, called intrinsic or acquired resistance. These mechanisms are myriad, but include mutations that reduce binding to the drug target, efflux of drugs out of cells, senescence as a means of escaping, changes in target regulation or compensatory pathway and many others[45].

**Methods in cancer research**

A wide array of methods has been used to discover and refine treatments in cancer. Paclitaxel, the first discovered taxane[46], refined from the tree, the pacific yew, was discovered through high through-put screening of 30,000 plant and animal compounds[47]. Mouse models have been created for cancer research, such as NOD-SCID mice which are missing an adaptive immune system, or more recently, the glowing head mouse model, which is immune competent while harboring endogenous GFP and luciferase in order to utilize them with GFP or luciferase labeled cells without inducing an immune response to those otherwise-foreign proteins[48]. Cell lines have been developed, cancer cells from a human patient or other animal, which have been invaluable for their ability to reasonably approximate
patient cancer in an in vitro or in vivo environment. Patient-derived xenografts are cell lines, with the
distinction that their cells are only passaged in mice, which more closely approximates a human
environment, and more closely approximates patient cancer than traditional cell lines[49].

Approach

In these studies, we sought out to address areas of knowledge in need of exploration. First, the glowing
head model was characterized for the metastatic setting. Then, mechanisms of intrinsic and acquired
resistance to erlotinib were compared, which allowed us to discover genes shared among both models.
Next, senescent cell production was optimized, and drugs were tested and compared relative to their
effect on senescent or proliferative cells. Finally, high-throughput screening was used to discover
combinations of drugs that when applied with BYL-719, a PIK3CA inhibitor, showed synergistic
cytotoxicity relative to the single drugs alone in the in vivo and in vitro settings.

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Chapter 2: The utility of the “Glowing Head” mouse for breast cancer metastasis research

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DCB, MAZ, JCH: experimental design; MAZ: portal vein and tail vein injections; DCB, JCH: manuscript writing; DCB, MAZ: IVIS imaging and mouse breeding.

Abstract

The expression of cellular reporters to label cancer cells, such as green fluorescent protein (GFP) and luciferase, can stimulate immune responses and effect tumor growth. Recently, a mouse model that expresses GFP and luciferase in the anterior pituitary gland was generated to tolerize mice to these proteins; the “Glowing Head” mouse. Mice were obtained from a commercial vendor, bred, and then used for tumor growth and metastasis studies. The transgene expression of luciferase was assessed within tumor-naïve mice as well as mice with mammary tumors or metastases. Tumor-free mice with white fur, compared to black fur, allowed for stronger luciferase transgene expression to be observed in the pituitary, sternum, and femur. Growth of four different luciferase-expressing mouse cancer cell lines readily occurred in the mammary gland. Though sternum expression of the luciferase transgene occurred in cancer-free mice, growth or death of luciferase positive cancer cells in the lung could be observed. Liver metastases seeded by portal vein injections of luciferase positive cancer cell lines were completely distinct from luciferase transgene expression. Though lung and brain metastasis studies have limitations, the Glowing Head mouse can be useful to inhibit immune system rejection of luciferase or GFP expressing cancer cells. This mouse model is most beneficial for studies of mammary tumors and liver metastases.
Background

Mouse models have been used in medical research since Dr. William Castle in 1902 [1]. One of the pioneers of metastatic research, Dr. Isaiah Fidler, began prolifically publishing studies utilizing injections of cultivated melanoma cells into mice in the 1970s [2, 3]; these models of metastasis were ideal given that the dark color of the cells facilitated observations of metastatic growth. Over the past three decades, the majority of metastasis studies have incorporated the use of foreign proteins to label and track cancer cell dissemination and growth. This includes the use of beta-d-galactosidase [4], green fluorescent proteins (GFP) [5–7], coral reef fluorescent proteins [8–11] and luciferase [12]. These technologies have greatly advanced metastasis research by providing the ability to non-invasively measure growth or death of metastatic cells.

The majority of cancer cell metastasis tracking studies have been performed within immune-compromised mice; either athymic nude mice, which were discovered and characterized in the 1960s [13], or more recently, NOD SCID (NSG) mice [14]. NSG mice have been used in many patient derived xenograft (PDX) and cell line studies [15–18]. Since immunotherapy is showing significant promise in certain cancer types, ideally xenograft studies with human cells would be performed in parallel with immune-competent murine carcinoma models. A problem with GFP or Luc expressing mouse-derived cell lines is that the immune systems of the mice attack the cells expressing the Luc or GFP antigen [19–21], which can significantly confound results. One approach to overcome this GFP-antigen problem was by using eGFP induced bone marrow transplants, which was found to prevent rejection of GFP + skin grafts [22]. Along these lines, a novel advancement, the “Glowing Head” mouse, was recently developed [23]. These mice were generated with a transgenic construct containing the Luc-GFP fusion gene under the control of the growth hormone promoter injected into blastocysts. These mice were shown to limit
adverse immune responses to GFP-Luc labeled Lewis Lung Carcinoma or Mvt1 mouse mammary cancer cells. Three versions of the Glowing Head mouse are available from The Jackson Laboratory; FVB/N, BALB/c, and C57BL/6.

Different versions of the polyomavirus middle T-antigen (PyMT) murine mammary carcinoma cell line are often utilized for in vivo studies within the C57Bl/6 background. Two of them, pB2 and pB3, were characterized recently [24]. The pB2 cells exhibited more epithelial and more differentiated characteristics while pB3 tumors were more mesenchymal. The Py230 is another PyMT derived “luminal” line [25]. The E0771 is a mouse carcinoma cell line that was developed nearly 7 decades ago and has been extensively studied [26].

The goal of this study was to qualitatively assess the Glowing Head Mouse model for use in breast cancer metastasis research. We found that tumor-naïve mice express luciferase in the pituitary, as well as the sternum and epiphysis of the femur. Though the sternum and femur expression may potentially confound luciferase based quantification of lung and bone metastases, studies of mammary tumors and liver metastases were ideal. Ultimately, these studies support expanded metastasis research with this model.

**Methods**

**Glowing head mouse**

All studies were approved by the VCU Institutional Animal Care and Use Committee; protocol AD10001247. C57Bl/6-Tyrc–Brd Tg(Gnrhr-luc/EGFP)D8Mrln/J (stock 027662) male and female mice were purchased through the Jackson Laboratory. Mice were maintained in a breeding colony for use within in vivo studies. Mice injected with cell lines were monitored during the study and euthanized once reaching the humane endpoint of tumor size.
**IVIS**

IVIS imaging was conducted on anesthetized mice using the IVIS Spectrum In Vivo Imaging System hardware and Living Image software.

**Cell lines**

The pB2 and pB3 cell lines were acquired as a gift as part of a Material Transfer Agreement with Dr. Robert Weinberg from Massachusetts Institute of Technology. E0771 cells were purchased from CH3 Biosystems and Py230 cells were purchased from American Type Culture Collection (ATCC). These cell lines were transduced with a lentivirus encoding for firefly luciferase (gentarget; LVP323) and cells expressing the construct were selected with blasticidin. Luminescence was used as an indicator for cell number, which has been an established metric in previous studies [18].

**Establishment of tumors and metastases**

Tumors and metastases were induced using injections into the mammary fat pads for primary tumors, tail veins for lung metastases, and portal veins for liver metastases of mice, as described in previous PDX studies [15, 16]. Mammary tumors were measured twice a week until they reached 100 mm², at which point the mice were euthanized. Matrigel was used in the injection to create an artificial matrix in which the cells could grow in some of the mammary fat pad injections. Cells injected in the tail vein were inoculated with luciferin immediately prior to the injection and the mice were IVIS imaged immediately after implantation to confirm that cells had colonized the lungs. Mice with tail vein and portal vein established metastases were monitored for two weeks after cell injection.

**Results**

**Phenotype of the breeding colony**
The mice that were originally received from the vendor all had black fur. IVIS imaging identified that four of seven of the cancer-free mice had a detectable luminescence signal in the pituitary gland. Two of seven of the mice also had a strong signal in the tail, thoracic cavity, and leg (Fig. 1a). The most luminescent male and female mice were selected for breeding. Of the first generation produced in the mouse colony, 62% (5 of 8) expressed the transgene in the pituitary and were luciferase positive. Three of these mice were albino (Fig. 1b), which corresponds with how they were originally generated. The albino mice with the highest luminescence were selected for breeding and all of the second and later generations were albino. Of the first generation of all albino mice, 75% had strong signals in the pituitary gland, the thoracic cavity, the femur bones, and the tail (Fig. 1c). Continuous breeding resulted in ~75–100% of offspring expressing the luciferase transgene. Mice that were 4 or 8 weeks of age were imaged and had similar expression of luciferase in their pituitary glands, legs, and tails; some mice, age-independent, had stronger signals in the spine and thoracic cavity (Fig. 2a).
Fig. 1
IVIS imaging of Glowing Head mice obtained from The Jackson Laboratory. a IVIS imaging of the stock Glowing Head mice. b Ventral and dorsal imaging of the first generation of offspring. c The offspring of the second generation of breeding Glowing Head mice with white fur.

Fig. 2
IVIS imaging of Glowing Head mice implanted with or without cancer cells. a IVIS imaging of Glowing Head mice at 4 and 8 weeks age. b Luciferase induced pB2 and pB3 cells: 2500; 5000; 7500; 10,000 cells. c Glowing head mice with pB2, pB3, E0771, and Py230 cell lines injected into mammary fat pads with Matrigel on their left and without Matrigel on their right. d pB2 and pB3 cells induced with luciferin and imaged, injected into a mammary gland (MGT), or injected into the tail vein (TV) and imaged at days 0, 4, and 11. e pB2 and pB3 liver metastases, established through portal vein (PV) injection and imaged at days 3, 7, and 13. Scale bar indicates low to high luciferase expression.

Initiating studies with cancer cells
Four different mouse mammary carcinoma cell lines (PyMT: pB2, pB3, Py230; E0771) were transduced with a lentivirus that encodes for firefly luciferase and GFP. Stable cell lines were then imaged in the IVIS to confirm luciferase expression. Higher numbers of cells per well yielded increased radiance, or photons emitted per second, demonstrating that luminescence could be a metric for estimating relative cell count (Fig. 2b).

**Imaging of mammary tumors**

Each cell line was intraductally injected into the abdominal mammary gland to generate tumors either with or without Matrigel. Imaging of mice two weeks later showed that Matrigel supported the growth of each cell line (Fig. 2c). Growth of the tumors often led to the inability to visualize pituitary or sternum transgene expression in some of the mice, however, transgene expression in other mice remained abundant. In all cases, if the primary tumor signal was blocked, transgene expression was observed.

**Observing metastases**

Cells from the pB2 and pB3 lines were next stimulated with luciferin and then injected into the mammary gland or tail-vein to seed lung metastases (Fig. 2d). Over an 11 day period, the pB2 metastases’ luciferase intensity diminished in the lung, denoting loss of cell viability; subsequently, pituitary transgene expression was again visible. In contrast, the pB3 lung metastases intensified, indicating metastatic growth. The lung metastases within the lobes of the lung appeared slightly lower and slightly leftward or rightward compared to the location of the sternum expression of the luciferase transgene (Figs. 1, 2a, d).

To generate liver-specific metastases, portal vein injections were performed with pB2 or pB3 cells. Immediately after injection, a luciferase signal was present in the liver, which was distally located away
from any transgene expression. The signals increased on day 7 and further on day 13. Ex vivo imaging of the liver at the end of the study confirmed extensive metastatic outgrowth (Fig. 2e).

Discussion

These studies expand on data presented by Day et al. [23], which highlighted the utility of the Glowing Head model to study murine lung (Lewis Lung Carcinoma (LLC)), mammary (MVT1 cells), and melanoma (HGF/CDK4R24C cells) tumors and metastasis. We found that white fur, as compared to black fur, facilitated increased luciferase-based photon capture, which expands the pituitary and testis-specific transgene expression to also include the femur, sternum, and spine. Transgene expression was variable from mouse-to-mouse, among individual tumor-naïve Glowing Head mice. The luminescence of mammary tumors and metastases during IVIS imaging often caused the pituitary signal of the transgene to no longer be recorded by the camera. It follows then, that if the pituitary signal of the mouse has a brighter luminescence level than a small metastasis, the metastasis may go undetected during luciferase imaging. Other researchers have discovered that small metastases located near large primary tumors are unable to be imaged unless the primary tumor was removed [19] or covered with opaque black paper. Longitudinal studies of brain and lung metastases therefore may lose accuracy due to tissue transgene expression close to or within these organs. End-point assessments of metastatic distribution or measuring the effects of drug treatments on metastases in these organs are possible, if brains (with pituitary excluded) or lungs are excised, and then imaged ex vivo.

Previous studies found that the Glowing Head mouse immune environment promoted the anti-cancer properties of Crizotinib for melanoma metastases and Gemcitabine for LLC metastases [23]; liver metastases were not formally assessed. Since our studies show that liver metastases are distally located away from endogenous luciferase expression, the treatment of hepatic lesions are ideal for this model. Other groups have utilized the Glowing Head mouse to discern immune contributions to targeting the
perivascular niche in order to sensitize disseminated tumor cells to chemotherapy [27]. Given the importance of incorporating the immune system into cancer treatments, the data presented in Day et al. [23], and our own studies herein, we encourage other researchers to utilize the Glowing Head mouse for metastasis research.

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Footnotes

Conflict of Interest The authors declare that they have no conflict of interest.

References


Chapter 3: Transcriptomic changes underlying EGFR inhibitor resistance in human and mouse models of basal-like breast cancer

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Abstract

The goals of this study were to identify transcriptomic changes that arise in basal-like breast cancer cells during the development of resistance to epidermal growth factor receptor inhibitors (EGFRi) and to identify drugs that are cytotoxic once EGFRi resistance occurs. Human patient-derived xenografts (PDXs) were grown in immunodeficient mice and treated with a set of EGFRi; the EGFRi erlotinib was selected for more expansive in vivo studies. Single-cell RNA sequencing was performed on mammary tumors from the basal-like PDX WHIM2 that was treated with vehicle or erlotinib for 9 weeks. The PDX was then subjected to long-term erlotinib treatment in vivo. Through serial passaging, an erlotinib-resistant subline of WHIM2 was generated. Bulk RNA-sequencing was performed on parental and erlotinib-resistant tumors. In vitro high-throughput drug screening with > 500 clinically used compounds was performed on parental and erlotinib-resistant cells. Previously published bulk gene expression microarray data from MMTV-Wnt1 tumors were contrasted with the WHIM2 PDX data. Erlotinib effectively inhibited WHIM2 tumor growth for approximately 4 weeks. Compared to untreated cells, single-cell RNA sequencing revealed that a greater proportion of erlotinib-treated cells were in the G1 phase of the cell cycle. Comparison of WHIM2 and MMTV-Wnt1 gene expression data revealed a set of 38 overlapping genes that were differentially expressed in the erlotinib-resistant WHIM2 and MMTV-Wnt1 tumors. Comparison of all three data types revealed five genes that were upregulated across all erlotinib-resistant samples: IL19, KLK7, LCN2, SAA1, and SAA2. Of these five genes, LCN2 was most
abundantly expressed in triple-negative breast cancers, and its knockdown restored erlotinib sensitivity in vitro. Despite transcriptomic differences, parental and erlotinib-resistant WHIM2 displayed similar responses to the majority of drugs assessed for cytotoxicity in vitro. This study identified transcriptomic changes arising in erlotinib-resistant basal-like breast cancer. These data could be used to identify a biomarker or develop a gene signature predictive of patient response to EGFRi. Future studies should explore the predictive capacity of these gene signatures as well as how LCN2 contributes to the development of EGFRi resistance.

**Subject terms:** Breast cancer, Breast cancer, Cancer genomics, Cancer therapy, Cancer therapeutic resistance, Cancer

**Introduction**

Triple negative breast cancer (TNBC) is an aggressive, highly metastatic breast cancer subtype that is characterized by a lack of hormone receptors and human epidermal growth factor receptor 2 (HER2)[1,2]. Thus, TNBC patients are not candidates for endocrine therapies or targeted therapy with anti-HER2 agents. TNBC patients face limited therapeutic options; chemotherapy is standard of care. TNBCs are a heterogeneous class and can be categorized into distinct subtypes: basal-like (1 and 2), claudin-low, immunomodulatory, mesenchymal-like, mesenchymal stem-like, and luminal androgen receptor positive[3,4]. These subtypes are transcriptionally distinct and display unique biology, immune composition, and sensitivity to chemotherapy[5]. Basal-like TNBCs are associated with the worst prognoses of all TNBC subtypes, indicating a need to identify efficacious treatment strategies for basal-like TNBC[6]. Drug resistance is a major clinical problem in basal-like TNBCs; we were interested in identifying strategies to overcome this clinical deficit by using targeted drugs, particularly EGFR inhibitors (EGFRi).
Basal-like breast cancers express relatively high levels of epidermal growth factor receptor (EGFR) compared to other breast cancer subtypes[7]. The EGFR family is composed of four categories of transmembrane tyrosine kinase receptors (ERBB1-4)[8]. Upon ligand binding, the inactive EGFR monomers dimerize to form active heterodimers. Dimerization is necessary for phosphorylation of the intracellular receptor kinase domain and activation of downstream pathways[8]. Once phosphorylated, EGFR can activate the PI3K/AKT and RAS signaling pathways. EGFRs are overexpressed and/or mutated in many cancers, including breast cancer[9]. EGFR overexpression or mutation can lead to aberrant signaling and promotion of uncontrolled cell growth and proliferation. EGFR overactivation in cancers is associated with poorer prognoses[9]. Currently, EGFRi are standard of care for patients with EGFR mutation-positive non-small cell lung cancer (NSCLC)[10]. However, the majority of patients treated with EGFRi for EGFR mutation-positive NSCLC will develop resistance to EGFRi[10]. Secondary mutations to the EGFR ligand binding domain, activation of compensatory pathways, and impairment of EGFR-EGFRi mediated apoptotic pathways are all mechanisms of resistance[11–13]. HGF overexpression, low BIM expression, PIK3CA mutations, and PTEN deletions have been associated with primary resistance to EGFRi[14]. In TNBC clinical trials, EGFRi have exhibited a modest response in combination with platinum compounds in a subset of patients, highlighting the need for predictors of therapeutic selection[15,16].

PDXs have been shown to largely maintain the properties of the patient tumors from which they were derived[17]. Previous studies have found that basal-like PDXs have transcriptional profiles and metastasis patterns similar to patient samples within The Cancer Genome Atlas (TCGA) breast cancer cohort dataset[18]. In addition, several of these models have been found to be insensitive to chemotherapeutics[19,20]. In this study, we sought to identify a basal-like patient-derived xenograft (PDX) that was sensitive to EGFRi treatment, develop an EGFRi-resistant subline, and then, identify transcriptomic alterations underlying acquired resistance through bulk and single-cell RNA sequencing. Parallel analyses of transcriptomic data from isogenic transgenic mouse models of basal-like disease
were incorporated to identify shared transcriptomic characters underlying EGFRi resistance. We also sought to identify drugs that demonstrate high levels of cytotoxicity in erlotinib-resistant basal-like PDXs via high-throughput drug screening. We hypothesize that these insights could be beneficial for (1) stratification of patients that could be responsive to EGFRi and (2) identification of effective therapies for patients with EGFRi-resistant disease.

**Methods**

**In vivo growth of breast cancer PDX models**

The following basal-like triple-negative breast cancer PDX models were used in this study: (HCI-001, UCD52, WHIM2). HCI-001 was obtained from the Huntsman Cancer Institute, University of Utah; WHIM2 was obtained from Washington University, St. Louis; UCD52 was obtained from the University of Colorado. All studies involving mice were approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (IACUC) (Protocol # AD10001247), and all experiments were performed in accordance with IACUC guidelines and regulations, as well as the ARRIVE guidelines 2.0. Tumor fragments were grown in the mammary fat pads of female non-obese diabetic severe combined immunodeficient gamma (NSG) mice (The Jackson Laboratory, strain #005557). When tumors reached approximately 10 mm × 10 mm, the mice were euthanized with isoflurane anesthesia, cervical dislocation and thoracotomy, and the tumors were excised. Tumors were prepared into single-cell suspensions using a previously described protocol [21]. Single-cell suspensions were used for serial passaging by suspending tumor cells 1:1 in Matrigel (Corning) or Cultrex (Bio-Tech) and injecting tumor cells (500,000 cells per injection) into the mammary fat pads of mice. Single-cell suspensions were also used for in vitro drug screens.

**In vivo drug treatments**
For the study in Supplementary Fig. 1, all drugs were dissolved in a solution of 1% methylcellulose + 0.1% Tween-80 and administered via oral gavage. Drugs were administered daily for 10 days: CO-1686 [100 mg/kg], erlotinib [100 mg/kg], gefitinib [200 mg/kg], dacomitinib [10 mg/kg], lapatinib [100 mg/kg], and afatinib [50 mg/kg]. For longer-term erlotinib treatment, mice received 367 ppm erlotinib-incorporated mouse chow (Envigo) ad libitum until a resistant phenotype arose.

**High-throughput drug screens**

Single-cell suspensions of PDX cells were plated in 96-well plates at 16,000 cells per well in M87 medium and treated with 516 drugs (ApexBio DiscoveryProbe FDA-approved Drug Library) at 10 µM[22]. After 72 h, the CellTiter-Glo Luminescent Viability Assay (Promega) was used according to the manufacturer’s protocol. Cell viability was quantified by normalizing treated wells to vehicle (0.1% DMSO) wells to produce a percent of vehicle value. Drug cytotoxicity was compared between the parental WHIM2 and erlotinib-resistant WHIM2 PDXs. Three separate tumors were tested in duplicate, and replicates were then averaged for each PDX.

**Single-cell RNA sequencing**

Single-cell transcriptomes were obtained from 1,314 parental tumor cells and 844 erlotinib-resistant tumor cells with a 10× Genomics Chromium single-cell controller utilizing a 10× Genomics Single Cell 3’Reagent kit standard protocol. Libraries were then sequenced on an Illumina Nextseq500/550 with 42-bp paired end reads, or a HiSeq2500 v4 with 125-bp paired end reads. The 10X Genomics CellRanger v6 software suite of tools was used to align samples and calculate gene expression. An in-house R script utilizing the Seurat v3.1.5 package was used to remove poor quality or dead cells. Additional filtering and realignment were performed to remove mouse cells. A final merged dataset containing only human cells was created using CellRanger. 10X Loupe Cell Browser v6.0.0 was used to visualize cell clusters and perform differential gene expression analyses across clusters[23]. Chi-squared test was performed using
GraphPad Prism v.9.2.0 to identify if differences in the proportion of cells in each phase of the cell cycle were statistically significant between parental and erlotinib-treated tumors.

**Bulk RNA-sequencing**

Parental WHIM2 (n = 4) and erlotinib-resistant WHIM2 (n = 3) mammary tumors tissues were excised and flash frozen. RNA was prepared with the Qiagen RNeasy mini kit. Sequencing libraries were prepared with NEBNext Ultra II RNA Library Prep Kit for Illumina using manufacturer’s instructions (New England Biolabs). The sequencing libraries were multiplexed and clustered onto a flowcell. After clustering, the flowcell was loaded onto the Illumina HiSeq instrument according to manufacturer’s instructions. The samples were sequenced using a 2 × 150 bp Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification. Approximately 30 M reads were obtained per sample. Reads were aligned to a hg38 human reference genome. DEseq2 was utilized to identify fold change in gene expression and genes significantly differentially expressed in erlotinib-resistant WHIM2 tumors; P < 0.05 was considered statistically significant[24]. Gene Set Enrichment Analysis (GSEA) was performed to identify upregulated and downregulated genetic programs[25,26].

**Ingenuity pathway analysis**

Qiagen Ingenuity Pathway Analysis (IPA) was used to perform network analyses[27]. False discovery rate adjusted p-values (q-values) were calculated for bulk RNA-sequencing data to identify significantly differentially expressed genes in parental and erlotinib-resistant WHIM2 samples. IPA was performed on 228 genes meeting the following parameters: log experimental fold change > 2.0 and q-value < 0.05.
Pathway analyses revealed upregulated and downregulated gene expression programs between samples.

**Immunohistochemistry**

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded WHIM2 tumors. Heat-induced antigen retrieval was performed in pH 9 Tris–EDTA using a Dakocytomatin Pascal Pressure Chamber. EGFR (Cell Signaling Technology, 2232S) antibody was diluted 1:50 in SignalStain Antibody Diluent (Cell Signaling Technology) and was applied to sections from parental and erlotinib-resistant WHIM2 tumors. Detection was performed using the Rabbit Dako EnVision System (Agilent K406511–2). Slides were imaged using Zeiss Axio Observer with Zen software. Quantification of immunohistochemical staining was performed using ImageJ plugins IHC Toolbox and IHC Profiler[28]. T-tests were performed on GraphPad Prism v.9.2.0 to determine if differences in staining were statistically significant between groups.

**Wnt1-Early vs Wnt1-Late gene expression analysis**

Gene expression microarray data from 37 untreated, FVB MMTV-Wnt1 tumors were downloaded from the UNC Microarray Database as log2 Cy5/Cy3 ratios, filtering for probes with Lowess normalized intensity values greater than ten in both channels and for probes with data on greater than 70% of the microarrays[29,30]. After median centering the expression of each probe and imputing via the ten-nearest neighbor gene values, the dataset was collapsed by averaging the expression of probes corresponding to the same gene symbol. A two-class (Wnt1-Early vs Wnt1-Late) Significance Analysis of Microarrays (SAM) analysis was performed to identify differentially expressed genes[31].

**Identification of 38 overlapping differentially expressed genes in basal-like erlotinib-resistant samples**
DEseq2 identified 1641 significantly differentially expressed genes in erlotinib-resistant WHIM2 compared to parental WHIM2 (Padj < 0.05). Of these genes, 521 had a fold change greater than or equal to 1.5. SAM analysis identified 9424 significantly differentially expressed genes in Wnt-Late compared to Wnt-Early (SAM q-value < 5%). Of these genes, 2833 genes had a fold change greater than or equal to 1.5. Significantly differentially expressed genes with a fold change ≥ 1.5 in erlotinib-resistant WHIM2 and Wnt-Late were compared. There were 92 overlapping genes. Of these genes, 38 were upregulated or downregulated in the same direction in erlotinib-resistant WHIM2 and Wnt-late samples. Heatmaps depicting differential gene expression were created using Morpheus (https://software.broadinstitute.org/morpheus/). Data were hierarchically clustered by both samples (erlotinib-sensitive vs resistant) and genes using the one minus Pearson correlation metric and average linkage method. Boxplots of TCGA data were generated with http://ualcan.path.uab.edu/index.html[32,33].

Transient knock-down of LCN2 using siRNA lipofection

WHIM2 erlotinib-resistant cells were harvested and transfected per the manufacturer’s protocol. For the experimental group, siGENOME Human LCN2 siRNA SMARTPool (Dharmacon) was utilized, which contained four targeting siRNAs for the LCN2 transcript. For the control group, a non-targeting siGENOME siRNA control pool (Dharmacon) of four siRNAs were used. Both siRNAs and DharmaFECT 1, a lipo-based transfection reagent, were diluted into serum-free, antibiotic-free media separately and shortly incubated. Then, for each group, the siRNA and DharmaFECT media were plated together in 100 mm dishes for another incubation period. Cells were suspended in antibiotic-free media containing serum, plated on top of the siRNA and DharmaFECT media solution, and gently mixed. Optimization led to a transfection period of 1 day. Transfection was ended by replacing transfection media with M87 media. Knock down was confirmed via western blot analysis.
Ethical approval and consent to participate

All animal and cell line work were done according to VCU Institutional Animal Care and Use Committee protocols.

Results

Select EGFR inhibitors reduced the growth of the WHIM2 PDX

In previous studies, the WHIM2 basal-like PDX was not responsive to the chemotherapeutic carboplatin[19,20]; however, several different EGFRi demonstrated cytotoxic activity in vitro21. To identify EGFRi that were effective in vivo, NSG mice bearing palpable WHIM2 mammary tumors were treated daily with one of six different EGFRi: CO-1686, erlotinib, gefitinib, dacomitinib, lapatinib, or afatinib. At the concentrations tested, erlotinib, gefitinib, dacomitinib, and afatinib each prevented tumor growth over a 10-day period (Supplementary Fig. 1). Since erlotinib is FDA-approved for clinical use and has a well-characterized side effect profile, it was chosen as the EGFRi to be used for the remainder of the study. A separate cohort of WHIM2 tumor-bearing mice was then treated with erlotinib-incorporated mouse chow, which also successfully inhibited tumor growth (Supplementary Fig. 1).

Development of an acquired erlotinib-resistant PDX model

The tumor growth inhibitory activity of erlotinib was next assessed across two additional basal-like PDXs: UCD52 and HCI-001; however, no significant effect on tumor growth was observed in these models (Fig. 1A,B). Longer-term treatment of the WHIM2 PDX found that erlotinib effectively prevented tumor growth for at least 4 weeks before tumor growth resumed (Fig. 1C). Drug-resistant tumors were serially passaged into new recipient mice (2nd passage) which were then treated with erlotinib chow.
This process was repeated a second time (3rd passage), and the tumor growth rate was similar to the parental WHIM2 (Fig. 1D). The erlotinib-resistant PDX was termed WHIM2/ErlR.
Figure 1
Development of acquired erlotinib-resistance in an erlotinib-sensitive PDX. (A) UCD52; (B) HCl-001; and (C) WHIM2 PDXs were treated with erlotinib-incorporated mouse chow ad libitum once tumors were palpable; (D) WHIM2 tumors were treated with erlotinib chow until resistance arose (1st passage) (n = 2). The first cohort of resistant tumors were passaged into a second cohort of mice (2nd passage) (n = 4), and mice were treated with erlotinib chow once tumors were palpable. The second cohort’s tumors were then passaged into a third cohort of mice and treated with erlotinib chow. The third cohort’s tumors were considered erlotinib-resistant (n = 10).
Identification of compounds that were cytotoxic to WHIM2/ErlR cells

When primary or secondary drug resistance occurs in the clinical setting, new therapeutic approaches are needed. Therefore, 516 clinically-utilized compounds were individually tested for cytotoxic activity on single-cell suspensions obtained from WHIM2 or WHIM2/ErlR cells. Interestingly, the majority of compounds displayed similar efficacy on both the parental and WHIM2/ErlR cells (Fig. 2). Notable exceptions that demonstrated greater efficacy on WHIM2/ErlR cells included elvitegravir (GS-9137), an integrase inhibitor used to treat human immunodeficiency virus infection, and atovaquone, a quinone antimicrobial. Conversely, several drugs demonstrated reduced efficacy on the WHIM2/ErlR cells, including birinapant, aprepitant, and imatinib. Importantly, most of the highly cytotoxic drugs resulted in cell death to both parental and WHIM2/ErlR cells. Examples include topoisomerase inhibitors (doxorubicin, idarubicin, epirubicin), proteasome inhibitors (MLN2238/Ixazomib, CEP-18770, carfilzomib), HDAC inhibitors (belinostat, PCI-24781), and other EGFR inhibitors (neratinib, dacomitinib, afatinib), among others.
Assessment of 516 FDA-approved and clinically used drugs on parental and erlotinib-resistant WHIM2 PDXs. Tumors were excised from mice, prepped into single cell suspensions, plated in 96-well dishes, and treated with 10 μM of drug. Cell viability was assessed with CellTiter-Glo after 72 h of treatment and normalized to DMSO vehicle control. Parental (n = 3) and erlotinib-resistant (n = 3) tumors were treated in triplicate.

**Single-cell transcriptional responses to erlotinib treatment**

Single-cell RNA-seq was used to identify transcriptional changes that occurred at the single-cell level upon the development of erlotinib resistance in the WHIM2 PDX. Mice with palpable WHIM2 PDX tumors were treated with erlotinib until drug-resistance occurred (9 weeks). Treated and control tumors were prepared into single-cell suspensions, and scRNA-seq was performed. In total, 1,314 control cells and 844 treated cells were analyzed. Uniform Manifold Approximation and Projection (UMAP) based images of all cells were developed based on transcriptomic data from all significantly differentially expressed genes (Fig. 3A). Cell cycle phase specific gene expression signatures were assessed for each cell to determine if there were differences in the proportion of cells in each phase of the cell cycle due to treatment (Fig. 3B). Chi-squared test revealed a significant association between
erlotinib treatment status and proportion of cells in each phase of the cell cycle ($X^2 = 208.6$, df = 2, $P < 0.0001$). In the erlotinib-treated sample, there was an increase in the proportion of cells that were identified in the G1 cell state compared to the untreated sample (Fig. 3C). Conversely, there was a decrease in the proportion of cells that were identified in the S and G2M cell state in the erlotinib-treated sample compared to the untreated sample. Next, we identified differentially expressed genes which could have mediated erlotinib-resistance. Gene expression was quantified as percentage of cells in the sample expressing the gene transcript. There were 713 unique gene transcripts that were more abundantly expressed in the WHIM2 erlotinib-treated cells than in the WHIM2 parental cells; 390 other transcripts were more abundantly expressed in the parental cells than the erlotinib-treated cells (Fig. 3D). The 25 most differentially upregulated genes in the erlotinib-treated or control cells are shown (Fig. 3E). Examples of genes with more expression per cell in the erlotinib-treated group are PLAAT3, LCN2, CEBPD, and SAA1; conversely, LDHB is shown as an example for transcripts more abundant in control tumor cells. MKI67 is shown as a marker of the proliferating-G2/M population (Fig. 3F).
Figure 3

Single-Cell RNA sequencing identified subpopulations that mediate erlotinib resistance. Uniform Manifold Approximation and Projection (UMAP) plots of scRNA-seq data from vehicle or erlotinib-treated WHIM2 tumors. UMAP plots are two-dimensional plots depicting clusters of specific cell types (e.g., vehicle vs erlotinib-treated). UMAP plots are colored based on (A) treatment status or; (B) cell cycle status as assessed through cell cycle phase-specific gene signatures; (C) The proportion of cells from the vehicle tumor and erlotinib-treated tumor in each phase of the cell cycle, as shown in plot B; (D) Plotted are 1,103 transcripts identified as significantly differentially expressed between treatment conditions. Each axis shows the percentage of cells in each treatment condition that expressed the RNA transcript; (E) Plot depicting the 25 most differentially upregulated genes each treatment condition, as determined by the percentage of cells in the sample expressing each transcript; (F) UMAP plots depicting examples of genes that were differentially expressed following erlotinib treatment; MKI67 is shown to label G2/M cells.
Bulk RNA-seq analysis of erlotinib-resistant WHIM2 tumor cells

After the WHIM2/ErlR subline was generated via serial passaging of erlotinib-treated tumors, immunohistochemical staining for EGFR was performed on parental WHIM2 and WHIM2 Erl/R tumors. Both were found to heterogeneously express EGFR (Fig. 4A). This suggests that selection for EGFR-negative cells was not the mechanism of resistance, nor was the gross upregulation of EGFR to compensate for its inhibition. Bulk RNA-sequencing data was then generated from the parental and WHIM2/ErlR PDXs. There were 521 transcripts identified as differentially expressed in the WHIM2/ErlR compared to WHIM2 parental (P < 0.05) (Fig. 4B). GSEA found that hallmark genetic programs of hypoxia, TNF-α signaling via NFκB, and epithelial mesenchymal transition were activated (Fig. 4C). Ingenuity Pathway Analysis was used to perform network analyses. Activation at p-value < 0.001 and z-score > 2.0 was predicted in 40 functions related to cellular movement in the WHIM2/ErlR (Fig. 4D). This suggests that cellular movement gene expression programs were upregulated in the development of erlotinib resistance in WHIM2.
Figure 4

Assessment of erlotinib-resistance mechanisms in the WHIM2 PDX. (A) Immunohistochemical staining for EGFR on formalin-fixed, paraffin-embedded parental and erlotinib-resistant WHIM2 tumors and comparison of mean staining intensity of parental and erlotinib-resistant WHIM2 tumors (P = 0.41); (B) Each point represents a single gene. Plot depicts fold change in gene expression in erlotinib-resistant tumors relative to vehicle tumors. Blue points represent genes that were significantly differentially expressed in the erlotinib-resistant WHIM2 tumor (P < 0.05); (C) Examples of gene signatures upregulated in erlotinib-resistant tumors as determined by gene set enrichment analysis. Hallmark gene signatures for hypoxia, TNF-α via NFκB, and epithelial to mesenchymal transition are depicted; (D) Ingenuity Pathway Analysis identified significantly up- and down-regulated cellular gene expression programs in the WHIM2 erlotinib resistant cells.

Comparison of the WHIM2 and MMTV-Wnt1 transgenic models

The MMTV-Wnt1 transgenic basal-like mouse model spontaneously generates mammary tumors in a bimodal distribution, either early (6.5 weeks) or late (22.5 weeks). Interestingly, the Wnt1-Early and Wnt1-Late tumors are transcriptionally distinct and respond differently to erlotinib[29]. Genomic differences between these isogenic tumors were contrasted with the WHIM2 parental and WHIM2/ErlR tumors. In total, 38 shared genes were found to be upregulated or downregulated during the development of erlotinib-resistance in the WHIM2 PDX; each of these genes was also increased or decreased in the same direction in the Wnt1-Late (erlotinib-resistant) and WHIM2/ErlR models (Fig. 5A,B). Five of these genes were also found to be significantly differentially expressed in the same direction in the scRNA-seq data (Fig. 5C). Analysis of TCGA breast cancer cohort dataset found that three of the genes were expressed in triple-negative breast cancers[33] (Fig. 5D–F); LCN2 showed high transcriptional expression level in some tumors, similar to the above transcriptomic analyses.
Figure 5

An overlapping set of 38 differentially expressed genes correlated with erlotinib response was identified within the Wnt1 transgenic model and the WHIM2 PDX model. Heat maps depicting the set of 38 overlapping genes within the (A) Wnt1 tumors and; (B) WHIM2 PDX tumors; (C) Five genes that were upregulated in all 3 types of data analyzed; (D–F) Plots depicting expression of the five genes found to be upregulated in all 3 data types were created using The Cancer Genome Atlas and UALCAN. Plots depict gene expression by major breast cancer subtype; plots depicting the three genes that were expressed in TNBC are shown.

Differential LCN2 protein expression between parental and erlotinib-resistant tumors

Since LCN2 was found to be highly expressed in a subset of TNBCs, immunohistochemical staining for LCN2 was performed on parental (erlotinib-sensitive) and Erl/R WHIM2 tumors to assess differential LCN2 protein expression. Compared to staining for EGFR, LCN2 expression appeared bimodal and less diffuse, in that each cell either expressed or did not express LCN2 protein. Consistent with the transcriptomic data, immunohistochemical staining of parental and Erl/R WHIM2 tumors found a significantly greater proportion of LCN2 positive cells in Erl/R tumors compared to parental tumors (P < 0.05) (Fig. 6A–H). To investigate LCN2’s role in erlotinib resistance, LCN2 siRNA transfection was used to knock down LCN2 expression in WHIM Erl/R PDX cells using non-targeting siRNAs for a control. Relative LCN2 protein was visualized via western blot (Fig. 6I). Transiently transfected cells were treated with 1, 3, 5, 7, and 9 µM of erlotinib in vitro. At 5, 7, and 9 µM, erlotinib demonstrated significantly greater cytotoxicity towards LCN2 siRNA transfected cells compared to non-targeting siRNA transfected cells (Fig. 6J).
Figure 6

(A–F) Representative images of immunohistochemical staining for LCN2 on formalin-fixed, paraffin-embedded WHIM2 parental and WHIM2 Erl/R mammary gland tumors; (G) Quantification of LCN2-negative cells and LCN2-positive cells in each image; (H) Percentage of total cells in the image that were LCN2-positive (P = 0.021). (I) Western blot of lysates from LCN2 siRNA and non-targeting siRNA transfected WHIM2 erlotinib-resistant PDX cells. (J) Erlotinib treatment on WHIM2 erlotinib-resistant PDX cells with LCN2 siRNA or non-targeting siRNA transfection (P < 0.05 at 5 μM, P < 0.001 at 7 μM and 9 μM).
Discussion

In these studies, we sought to identify transcriptomic changes that accompany the development of EGFRi resistance in basal-like TNBC PDXs in order to identify biomarkers and gene signatures predictive of EGFRi response. In pilot studies, we identified an EGFRi that demonstrated in vivo antitumor activity in the WHIM2 basal-like TNBC PDX. Of these EGFRi, erlotinib was chosen for further study. We derived an erlotinib-resistant WHIM2 PDX from the parental PDX. ScRNA-seq and bulk-RNA sequencing were performed to identify transcriptomic changes underlying the development of erlotinib resistance. GSEA and IPA utilizing bulk-RNA sequencing data identified increased epithelial-to-mesenchymal transition (EMT) and cell movement, respectively, in WHIM2 Erl/R compared to parental WHIM2. This suggests that EGFRi resistance may be associated with genomic programs that are also increased during metastasis. Furthermore, drugs targeting EMT and cell movement may be effective in the restoring EGFRi sensitivity.

Further analysis of bulk-RNA sequencing data from WHIM2 and MMTV-Wnt1 transgenic mouse models identified 38 genes that were differentially expressed in erlotinib-resistant strains. Of the upregulated genes, five genes were also significantly upregulated in scRNA-seq. These genes included: SAA1, SAA2, KLK7, LCN2, and IL19. These genes could potentially serve as predictive biomarkers of erlotinib response. Interestingly, López-Ayllón et al. (2015) identified sixteen genes that were upregulated in erlotinib-resistant NSCLC tumors compared to erlotinib-sensitive tumors[34]. These genes included LCN2, MET, PIGR, and SAA1, all of which were found to be upregulated in WHIM2/ErlR and Wnt-late tumors compared to WHIM2 parental and Wnt-early tumors. Interestingly, many of the activated genes found within the model system presented herein and in these previous studies encode for proteins within inflammatory and natural immunity processes, suggesting that these biological processes are
contributing to drug resistance. Krysan et al. also found that LCN2 overexpression was associated with erlotinib-resistance in NSCLC[35].

Of the five genes found to be upregulated in all three data types analyzed, LCN2 was the only gene found to be overexpressed in TNBC. Previous studies have found a correlation between LCN2 expression and disease aggression. For example, LCN2 has been positively associated with tumorigenesis, invasiveness, migration, metastasis[36–39]. Interestingly, LCN2 was included in several IPA cell movement gene signatures found to be upregulated in the EGFRi resistant setting. This suggests that LCN2 may contribute to observed increases in EMT, cell movement, and metastasis observed in the EGFRi resistant setting.

The mechanism by which LCN2 may contribute to acquired EGFRi resistance requires further study, but it may be related to EGFR recycling. Yammine et al. demonstrated that LCN2 increases EGFR abundance on the cell membrane[40]. They also found that LCN2 is involved in intracellular trafficking of EGFR and promotes recycling of EGFR to the plasma membrane. In this way, LCN2 upregulation may serve to salvage EGFR signaling in the presence of an EGFRi by returning EGFRs to the plasma membrane for further stimulation and activation of downstream pathways (Fig. 7). Previous analysis of MMTV-Wnt tumors revealed EGFR pathway amplification in Wnt-late tumors compared to Wnt-early tumors29. LCN2 upregulation may allow for EGFR pathway amplification via recycling of EGFRs and thereby promote resistance to EGFRi. When LCN2 was knocked down in Erl/R WHIM2 PDX cells in vitro, cells were more responsive to EGFRi. This suggests that LCN2 causally contributes to acquired EGFRi resistance, at least in the WHIM2 model of basal-like disease.
Hypothetical model depicting how LCN2 upregulation in erlotinib-resistant tumors may contribute to EGFR recycling back to the plasma membrane and allow for continued oncogenic EGFR pathway activity despite EGFR inhibition by erlotinib. Schematic of EGFR recycling via LCN2 was adapted from Yammine et al.40. Created with BioRender.com.

Despite transcriptomic differences, however, the WHIM2 parental and WHIM2/ErlR demonstrated similar responses to a panel of drugs including topoisomerase inhibitors, proteasome inhibitors, HDAC inhibitors, and other EGFR inhibitors. Notable exceptions were elvitegravir and atovaquone, both of which demonstrated greater cytotoxicity in WHIM2/ErlR than WHIM2 parental. Interestingly, several drugs demonstrated decreased efficacy in the WHIM2/ErlR line, including birinapant (SMAC inhibitor) and imatinib (ABL inhibitor). These data suggest that, in general, drugs that demonstrate high cytotoxicity in erlotinib-sensitive models are also cytotoxic towards erlotinib-resistant models.
Although basal-like tumors exhibit high levels of EGFR expression, EGFRis have only demonstrated modest antitumor activity in a subset of TNBC patients. Through these studies, we found a set of genes that could potentially serve as predictive biomarkers of erlotinib response, and more generally, EGFRi response in basal-like TNBCs. This gene set could be used to identify patients that would best benefit from EGFRi. Future studies should evaluate the predictive capability of the described gene signatures, as well as how these genes contribute to the development of EGFRi resistance. Future studies should also focus on the identification of drugs that demonstrate increased cytotoxicity towards EGFRi-resistant models, as well as drugs that may re-sensitize EGFRi-resistant cells to EGFRi.

Supplementary Information

Supplementary Figure 1. (page 103)

Author contributions


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**Data availability**

Single-cell RNA-sequencing: GSE189324. WHIM2 bulk RNA-sequencing: GSE189257. SuperSeries (links to both of the above): GSE189325. Wnt1 gene expression microarray data: GEO GPL11383. Other data are available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare no competing interests.

**Footnotes**

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**Supplementary Information**

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**References**


Chapter 4: Investigating potential senolytic agents and shared induced chemotherapeutic resistance

The following study is unpublished at this time.

Abstract

Breast cancer treatments have seen improvements in recent times, but basal-like breast cancers, which lack ER, PR, and HER2, targetable biomarkers, have the most limited treatment options relative to its other intrinsic counterparts. Doxorubicin, a chemotherapeutic prescribed for basal-like breast cancer patients, can induce a senescent phenotype. These cells acquire resistance to doxorubicin, but also other chemotherapeutics. Because it is thought that these cells are the kind that can cause recurrence and metastasis in patients, we utilized high-throughput screening to help identify drugs which may be more effective on senescent cells. We also identified 35 drugs which are effective on cells that are proliferative, untreated with doxorubicin, but not on their senescent counterparts, elucidating drugs that should be potentially avoided as follow-up treatment after doxorubicin.

Introduction

Breast cancer comprises 30% of annual female cancer incidence[1]. Triple negative breast cancers lack ER, PR, and HER2, which are targets for therapeutics that have been more successful for patients whose cancer has those features and though while treatment options have improved, basal-like breast cancer treatment is still an unmet need[2]. Doxorubicin, one of the most commonly used treatments for basal-like breast cancer, because it can achieve a pathologic complete response in basal-like disease of up to 17%[3]. Unfortunately, the adverse event profile of doxorubicin is notoriously rough, with side effects ranging from fatigue, nausea, vomiting, and many others[4], but also increased risk of other cancers such as acute myelogenous leukemia and myelodysplastic syndrome[5].
Doxorubicin, like all cancer chemotherapeutics, will have attenuating effectiveness for some patients due to resistance. There are many avenues for resistance, such as efflux of the drug. Reduced doubling rate has been identified as one potential mechanism[6]. After exposure to doxorubicin, some models will slow down so much that they exhibit a senescent phenotype[7]. Senescence has been identified as a potential mechanism for recurrence and metastasis[8].

Being that doxorubicin is currently used as a standard of care in the breast cancer setting[9], and patients commonly are moved to another drug when resistance arises, it was important to characterize additional compounds which doxorubicin-treated senescent cells become resistant to. Furthermore, drugs which could be used as a lead for treatment development focusing on clearing senescent cells produced by doxorubicin is an unmet need. This study sought to accomplish those goals by utilizing high-throughput screening.

**Methods and Materials**

**Cell culture, drug screens, and inducing senescence**

Cells were cultured and screened using a 516 drug library of FDA approved drugs as described previously[10]. MDA-MB-231 (MDA-231) cells were acquired from ATCC. Doxorubicin was used to induce senescence at 200nM for a one-hour pulse before changing the media, inducing a senescent phenotype that emerged by day 3, when media was changed again before viability assays.

**Microscope and software**

The Keyence BZ-X800 microscope was used to take high-quality images the cells, BZ-H4A software was used to stitch images together and export those images.

**Beta-galactosidase staining**
The “Senescence β-Galactosidase Activity Assay Kit” (23833S) from Cell Signaling was used to stain for beta-galactosidase (b-gal), fixed cells were pHed, rinsed with PBS, and incubated at 0% CO2 and 37°C overnight as per manufacturer instructions. 70% glycerol was used for imaging media.

Results

![Workflow of producing isogenic senescent and proliferative cells with comparable timepoints for viability assays.](image)

**Figure 1.** Workflow of producing isogenic senescent and proliferative cells with comparable timepoints for viability assays.

**Senescent phenotype was induced in MDA-231 cells**

MDA-231 Cells induced with senescent with doxorubicin as described in Figure 1 were imaged, far fewer cells remained after 3 days, those that remained were much larger in size than the untreated cells, and their proliferation rate was drastically reduced. Doses of doxorubicin from 125nM to 750nM at pulses of 1 or 2 hours of treatment before media change were tested, ultimately settling on 200nM for a 1-hour pulse because it produced cells with a strong senescent phenotype with bold staining. Formalin-fixed MDA-231 cells that had been treated with doxorubicin or vehicle for 1 hour 3 days before fixing were then stained with b-gal staining, yielding more stain per cell for doxorubicin pulsed cells and the blue rings around their nuclei (hematoxylin counterstained) that are emblematic of senescent cells (Figure 2).
Figure 2. Senescent cells were produced and stained. Doxorubicin pulsed (A) and vehicle treated (B) cells. Each were stained with b-gal yielding a blue stain where b-galactosidase is present. A hematoxylin counterstain was used to visualize nuclei.

Drugs which preferentially clear senescent or proliferative cells

Senescent and proliferative cells were used to conduct high-throughput screening using 516 FDA approved compounds at two screens each with two technical replicates (Figure 3A). Two-way ANOVA using Holm-Šídák's multiple comparisons test to correct for false discovery was conducted, 35 drugs with significantly different effect on proliferative cells relative to senescent cells were identified (Figure 3B). Senescent cells showed an acquired resistance to both doxorubicin and doxorubicin HCl. No drugs were identified as statistically significantly reducing senescent cells’ viability relative to proliferative (Figure 3C), this is likely due to the high variability of senescent cell assays and the non-normal distribution of the viability data (Figure 3D). Instead, what is shown in Figure 3C is 77 drugs which reduce the viability of senescent cells by 25 percentage points of viability on average, one of which is the positive control, the known senolytic agent navitoclax, which had the fourth highest percentage point difference.
Figure 3. Senescent cells and proliferative cells were screened using 516 FDA approved compounds. Viability of in vitro cell cultures (A), drugs that are significantly more effective on proliferative cells (B), drugs that are 25 percentage points more effective on senescent cells (C), and (D) QQ plot showing departure from normality.

Discussion

When treated with doxorubicin, MDA-231 cells became senescent and resistant to doxorubicin. Other topoisomerase inhibitors that those cells were resistant to included idarubicin HCl, daunorubicin HCl, irinotecan HCl trihydrate, nadifloxacin, sitafloxacin hydrate, gatifloxacin, epirubicin HCl. Other drugs targeting cell cycle and/or proliferative cells that senescent cells were resistant to were microtubule inhibitors, PI3K/AKT/MTOR pathway inhibitors, and STAT inhibitors. Treatment with doxorubicin is likely to induce resistance to topoisomerase inhibitors in general, which should be avoided as follow-up treatment after doxorubicin.
The drug with the largest average viability reduction in senescent cells relative to proliferative cells was sodium ascorbate, which is a salt of vitamin C, which has been shown to delay senescent onset[11]. Sodium butyrate[12] was also a high difference-of-viability drug in killing senescent cells and has been used to induce senescence in previous studies. A diverse background of drugs were identified in this 77 drug cohort and further testing is merited to confirm their senolytic effects and untangle the mechanism of action of that activity.

Further studies utilizing this data as preliminary are warranted, in particular, higher n screening of fewer drugs of interest would help to achieve statistical significance to weed out which ones might be better at clearing senescent cells, then drugs identified should be tested in living systems.

Acknowledgements:

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DCB: In vitro assays, manuscript writing

References:


Chapter 5: Discovering Synergistic Compounds with BYL-719 in PI3K Overactivated Basal-like PDXs

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A note referencing challenges from transferring from in vivo to in vitro dose ratios is included in the appendix, page 102.

Simple Summary

Basal-like breast cancers comprise the majority of triple-negative breast cancers (TNBC) and lack effective treatment options that have a sustained response. Part of the reason that they are hard to eliminate is that they exhibit high levels of genomic instability and cellular diversity. Most basal-like tumors have high levels of Phosphoinositide 3-Kinase (PI3K) pathway activity. A key driver of this pathway is PIK3CA. Many compounds have been made to target PIK3CA and have become standard-of-care in some estrogen-dependent patients; however, in TNBC patients, PI3K inhibitors (PI3Ki) as single agents thus far have shown limited duration at tolerable doses. The goal of this study was to identify and/or repurpose drugs that, when combined with PI3Ki, yield a significant inhibition of tumor growth. When treated in conjunction with the PI3Ki BYL-719, which is clinically prescribed as alpelisib, 20 potent drug combinations were identified and formed a basis toward clinical studies with these molecules.

Abstract

Basal-like triple-negative breast cancer (TNBC) tumor cells are difficult to eliminate due to resistance mechanisms that promote survival. While this breast cancer subtype has low PIK3CA mutation rates when compared to estrogen receptor-positive (ER+) breast cancers, most basal-like TNBCs have an overactive PI3K pathway due to gene amplification or high gene expression. BYL-719 is a PIK3CA inhibitor
that has been found to have low drug-drug interactions, which increases the likelihood that it could be useful for combinatorial therapy. Alpelisib (BYL-719) with fulvestrant was recently approved for treating ER+ breast cancer patients whose cancer had developed resistance to ER-targeting therapy. In these studies, a set of basal-like patient-derived xenograft (PDX) models was transcriptionally defined with bulk and single-cell RNA-sequencing and clinically actionable mutation profiles defined with Oncomine mutational profiling. This information was overlaid onto therapeutic drug screening results. BYL-719-based, synergistic two-drug combinations were identified with 20 different compounds, including everolimus, afatinib, and dronedarone, which were also found to be effective at minimizing tumor growth. These data support the use of these drug combinations towards cancers with activating PIK3CA mutations/gene amplifications or PTEN deficient/PI3K overactive pathways.

**Keywords:**

patient-derived xenograft; precision medicine; basal-like breast cancer; triple-negative breast cancer; synergism; BYL-719 (alpelisib); everolimus; dronedarone; afatinib

**1. Introduction**

**1.1. Etiology of Triple Negative Breast Cancer in the US**

In total, 300,590 new breast cancer diagnoses and 43,700 breast cancer-related deaths are predicted for 2023 [1], an increase from the year before [2]. Basal-like tumors have the most limited treatment options of the breast cancer intrinsic subtypes because they lack ER, PR, and HER2, which are susceptible to inhibition with anti-estrogens or HER2-targeted agents. They also have high rates of DNA mutations and amplifications, which increases the heterogeneity of cancer cells and can lead to more genetically diverse subpopulations [3]. Basal-like breast cancer patients have a low 5-year survival rate, partially because of their cancers’ tendency to metastasize to lung, bone, brain, and other organs [4]. Because of
these challenges, the basal-like disease has a worse prognosis than breast cancer overall and is most in need of new effective therapeutic options.

1.2. Importance of PIK3CA in Breast Cancer

PIK3CA is the second most commonly aberrant gene in breast cancer, after TP53 [5]. PIK3CA codes for the catalytic subunit p110α that converts the lipid PIP2 into PIP3. This acts as a substrate for AKT and its activating kinases, which have oncogenic downstream effects [6]. PIK3CA often has an activating mutation that drives oncogenic transformation [7,8]. Overexpression of PIK3CA is also pathogenic: a gain of 1 or 2 copies is enough to see a significant increase in PI3K expression [5]. PIK3CA expression is often associated with resistance to therapeutics, such as EGFR inhibitors [9]. The loss of a regulatory protein of p110α, PTEN, often happens concurrently with PIK3CA mutations [10] but can increase PI3K pathway activity without PIK3CA alteration. PTEN can have normal CNV on the DNA level but be lost on the RNA level through gene silencing via methylation of the promoter [11]. Activating mutations of PIK3CA occur in basal-like tumors at a rate of approximately 10%, yet nearly all exhibit robust upregulation of the PI3K pathway. This central network is also activated through mutations or loss of PTEN or INPP4B or changes in RTKs [12] and AKT3. Collectively, PI3K is most highly expressed in basal-like breast cancers compared to other subtypes [13]. Despite the attention of researchers on PIK3CA, alpelisib (BYL-719) is the only p110α inhibitor that is FDA-indicated for use in breast cancer and only in conjunction with fulvestrant, an estrogen receptor antagonist [14]. This combination is only approved for patients with ER+ HER2- breast cancer that are male or post-menopausal female with PIK3CA mutant after disease progression after or while being treated with endocrine-based treatment [15]. A novel combination therapy approach with BYL-719 may provide pharmacokinetic synergism and improve meaningful clinical efficacy, such as disease-free survival, all while lessening the likelihood of severe adverse events through reduced drug exposure.
1.3. Patient-Derived Xenografts

PDXs (patient-derived xenografts) are similar to cancer cell lines but differ in that they are maintained in a physiological setting as soon as they are isolated from the patient and for subsequent passages. These models are valuable for preclinical trials because PDX models have been shown to closely match their patient counterparts [16,17], both in genomic profile and response to treatment [18]. In comparison, some cell lines have been shown to diverge from human patient tumors and lose intratumor heterogeneity and have alterations in protein levels revealed using histopathology [19]. PDX models have unique difficulties that cell lines do not have in establishing; one among them is establishment rates as low as 4% [20], as well as requiring mouse implantation instead of media-based tissue culture. Among other institutions, cohorts of human breast cancer PDXs have been created at Huntsman Cancer Institute [17] (HCI), Baylor College of Medicine [21] (BCM), University of Colorado, Denver [22] (UCD), and Washington University, St. Louis which developed the Washington Human in Mouse (WHIM) [23] PDXs.

1.4. Approach

BYL-719 is approved as part of a multi-drug treatment strategy for ER+ disease, but it is not curative as a single agent due to the development of drug insensitivity. To overcome resistance, an additional agent would be required. In this study, high throughput screening was utilized to identify synergistic candidates. Everolimus, an mTOR inhibitor, afatinib, an Epidermal Growth Factor Receptor (EGFR) inhibitor, and dronedarone, a multi-ion channel inhibitor, were selected and tested in vivo and in vitro in PI3K aberrant and PI3K overactive PDXs. All three combinations proved promising in PIK3CA aberrant basal-like breast cancer in the mouse models.

2. Materials and Methods

2.1. Cell Culture and Cell Lines
Cells were passaged in tissue culture-treated filter flasks in a 37 °C, 5% CO2 environment. To passage and in assays, the cells were grown in DMEM media with 10% FBS and 2% Pen/Strep for MDA-MB-453. Isogenic MCF10A wild type, E545K, and H1047R PIK3CA, kind gifts from Ben Ho Park [24] were utilized in a similar media that contained horse serum instead of FBS.

2.2. Targeted Mutation Profiling

To identify clinically actionable mutations targeted next-generation sequencing (NGS) was performed by VCU Pathology Molecular Diagnostics laboratory with clinically validated methodology used for diagnostic tumor profiling. PDX tumors were excised from mice, flash frozen, prepared into frozen sections using optimal cutting temperature (OTC) compound, and total nucleic acids extracted. NGS using the Oncomine Comprehensive Assay v3 (ThermoFisher, Waltham, MA, USA) was performed as previously described [25] to identify DNA mutations, DNA copy number variations, and RNA fusions across cancer-related genes.

2.3. PDXs and Passaging

The UCD52, WHIM30, HCI-001, HCI-010, and HCI-013 PDXs were utilized. PDXs were passaged in vivo by injecting single cell suspensions in a 1:1 ratio of HF (Hanks’ Balanced Salt Solution + 2% FBS) and Matrigel into the abdominal mammary fat pad of female NSG mice. At the end of a passage, the tumor-bearing mouse was euthanized, and its primary tumor or tumors were immediately excised and placed in PBS. The tumors were minced with a sterile razor blade and placed in tumor digestion solution (DMEM/F12 with 5% FBS, 0.0533 mg/mL hyaluronidase, and 2.4 mg/mL collagenase) on a thermoregulated tube cycler at 37 °C for 1 h. Solutions were centrifuged, and their supernatants were discarded. Pellets were resuspended in red blood cell lysis buffer, centrifuged, and again supernatants discarded into aspiration bleach traps. Pellets were resuspended in trypsin. Cells were resuspended at 500,000 cells per 100 μL.
HF; this was mixed 1:1 with Matrigel or Cultrex before injecting into the recipient mouse. Tumor area was calculated using volume = length times width.

2.4. Cell Culture Viability Assays

Cells were plated as described above for secondary cell lines or PDX single cell suspension in 96 or 384 well plates by hand or by automated robotic micropipette (Integra, Hudson, NH, USA, Assist Plus). PDX cells were plated in M87 media [26]. Drugs or control vehicles were pipetted into the wells immediately after plating for PDX single cell suspensions or after allowing cells to adhere, 2 h minimum for adherent cell lines. After the desired timepoint, a Cell Titer Glo luminescence assay was performed using a plate reader (BMG LABTECH, Ortenberg, Germany, POLARstar OPTIMA), and percent viability was calculated relative to vehicle control.

2.5. In Vivo Drug Trials and Mouse Observations

All in vivo studies were approved by VCU IACUC protocol approval through Animal Care and Use Program, an AAALAC-accredited program. BYL-719 (50 mg/kg) and afatinib (25 mg/kg) were administered via oral gavage (OG) in 100 μL of 1% methylcellulose 6 times a week. Everolimus (10 mg/kg) was administered via OG 3 times a week. Dronedarone (50 mg/kg) was administered via intraperitoneal (IP) injection in 100 μL of 10% DMSO, 40% PEG300, 5% Tween-80, and 45% Saline solution 6 times a week. Tumors were measured 3 times a week for health checks. Mouse weights were recorded once or twice a week. Mice that reduced in weight by 10% or reached maximum tumor burden by protocol guidelines were euthanized.

2.6. Bulk RNA Sequencing

Dry ice flash frozen tumors were processed using Qiagen Rneasy Kit in conjunction with QIAshredder tubes and RNA Zap. The quality of the sample was tested using Nanodrop. To construct the library, the
RNA sample was first quantified with the Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and checked for its integrity with TapeStation (Agilent Technologies, Santa Clara, CA, USA). Then, NEBNext Ultra II RNA Library Prep Kit for Illumina was used to prepare the RNA sequencing library according to manufacturer’s instructions (New England Biolabs, Ipswich, MA, USA). Afterward, the mRNAs were briefly enriched with Oligod(T) beads and then fragmented for 15 min at 94 °C. Subsequently, the first- and second-strand cDNA fragments were synthesized, after which their 3’ ends were end-repaired and adenylated. Following this, universal adapters were ligated to the cDNA fragments, and then index addition and library enrichment via PCR were performed with limited cycles. Finally, to validate the finished sequencing library, Agilent TapeStation (Agilent Technologies) was used, and the library was quantified with the Qubit 2.0 Fluorometer (ThermoFisher Scientific) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA).

Sequencing was performed by Azenta. Sequencing libraries were first multiplexed and clustered onto a flow cell, after which the flow cell was inserted into the Illumina HiSeq 4000 instrument according to manufacturer’s instructions. A 2 × 150 bp paired-end (PE) configuration was selected to sequence the samples. The HiSeq Control Software (HCS) was utilized to conduct image analysis and base calling. Once the raw BCL data were generated, it was converted into FASTQ format and, lastly, demultiplexed with the Illumina BCL2FASTQ 2.17 program. For index sequence identification, one mismatch was allowed.

2.7. Bulk RNA-Seq Quality Control and Pre-Processing

Bulk RNA-seq data were preprocessed as previously described in Alzubi et.al. [27] Briefly, FastQC v0.11.8 [28] was used to assess sequencing quality and adapters and low-quality base pairs were removed using CutAdapt v1.15 [29]. High-quality reads were aligned to a merged human/mouse genome using STAR v2.5.2b [30] (see Alzubi et al. for merged genome construction) with the following command line options: “--outSAMtype BAM Unsorted --outSAMorder Paired --outReadsUnmapped Fastx --quantMode
TranscriptomeSAM --outFilterMultimapNmax 1. The Salmon v0.8.2” [31] “quant” algorithm was used to obtain read counts from the aligned BAM files using the “IU” library type. Read counts were loaded into R to calculate Log2 TPM (transcript per million) values used for gene signature computations (methods below) and PAM50 subtyping using the genefu v2.11.2 R package [32]. New and previously generated data were analyzed (Supplementary Table S1).

2.8. Gene Signatures and Clustering

A set of 13 previously published gene signatures [33,34,35,36,37,38,39,40] were scored by averaging bulk RNA-seq Log2 TPM expression values over all genes in each signature to create a gene signature profile for each PDX. Morpheus was used to cluster these gene signature PDX profiles, with hierarchical clustering using the one minus Pearson’s correlation coefficient as the distance metric and average linkage method to cluster both rows and columns. PDX gene signatures were then compared using Pearson’s correlation coefficient to generate a similarity matrix.

2.9. Single-Cell RNA Sequencing, Quality Control, and Preprocessing

Single-cell RNA-Seq (scRNA-Seq) was performed on four TNBC cell lines (HCC1143, HCC1187, MDA-MB-468, and SUM149) and 13 PDX samples using the Chromium single Cell Gene Expression Kit (10x Genomics) per the manufacturer’s protocol and sequenced in the VCU Genomics core. The TNBC cell line samples were aligned to the GRCh38 version of the human genome and gene expression was calculated using the 10x Genomics CellRanger v6.0 software suite of tools. Dead and poor-quality cell removal was performed using an in-house R v4.1.3 script with the Seurat v4.3.0 package [41]. Briefly, cutoffs for number of genes detected (nFeature), number of molecules detected (nCount), and percent mitochondrial expression (percent.mt) were calculated for each sample individually using 3 median absolute deviations (MADs) above the median value for that sample (the median for the percent.mt attribute was calculated using only cells with ≤50% mitochondrial expression). Cells were deemed poor
quality if their nFeature or nCount value was greater than 3 MADs from the median in either direction, and if the percent.mt was above the 3 MAD cutoff, or a hard cutoff of 25%, whichever was lower. PDX samples followed a similar pipeline, however, they were first aligned to the 10x Genomics merged human/mouse genome (human genome version GRCh38 and mouse genome version mm10) to identify and remove mouse cells. Once mouse cells were removed the remaining human cells were re-aligned to the GRCh38 human genome, followed by the dead and poor-quality cell filtering described above. All PDX samples were then normalized using log normalization and merged using Seurat’s merge() function. A principal component analysis (PCA) was performed using a 100-gene PI3K activity signature from Gatza et al. [33], instead of the default most variable features in the Seurat pipeline. This modified PCA matrix was utilized for the generation of UMAP and tSNE visualizations, as well as Seurat’s graph-based clustering. An in-house script was then used to export the Seurat object to a 10x Genomics formatted file, and the CellRanger “reanalyze” function was run to reformat the data so that it could be imported into the 10x Loupe Cell Browser v6. Data analysis scripts are available on GitHub at https://github.com/AmyOlex/Boyd_SynCompounds_PI3K (accessed on 31 January 2023). Note that for this study the raw data were re-processed with a newer version of the 10x CellRanger software than previously reported.

2.10. Western Blot

Lysates were made using homogenized tumors in RIPA with protease and phosphatase inhibitors, sonicated, and cold centrifuged at 4 °C for 20 min. Lysates were quantified using Bradford reagent using spectrophotometer (ThermoScientific BioMate 160) to quantify absorption. Electrophoresis samples were made from those lysates, Laemmli buffer, and beta-mercaptoethanol, heated to 95 °C. Electrophoresis was run using (BIO-RAD, Hercules, CA, USA, mini protein TGX 4–15% gradient) in using (BIO-RAD PowerPac Basic). Gels were transferred using semi-dry blotter (BIO-RAD Tran-Blot Turbo) onto
methanol-activated nitrocellulose membranes using WypAll sheets soaked in transfer buffer. Cell
Signaling anti-Vinculin, RPS6 p-RPS6 rabbit primaries were used. LI-COR 680 florescent anti-rabbit
donkey were used as secondary antibodies, which were detected using (LI-COR, Lincoln, Nebraska,
Odyssey FC) using ImageStudio. Drying the membrane and reimagining were utilized to reduce
background. P-RPS6 levels were used as a known metric for PI3K pathway inhibition [42].

2.11. Ray Biotech Array C55

Ray Biotech’s C55 arrays were incubated with lysates that were produced from MDA453 and UCD52
cells, which had been treated either with DMSO control or 5 μM BYL-719, which were previously
sonicated and cold centrifuged. The membranes were imaged using ImageStudio. Relative development
was quantified using ImageStudioLite, each probe was normalized relative to background, those values
were normalized relative to positive control probes normalized to their own background.

2.12. Immunohistochemistry (IHC)

Using the DAKO envision system HRP kit for rabbit primaries, formalin-fixed, paraffin-embedded tissues
were cut and sections placed on slides. These sections were melted at 60 °C and rehydrated using
stepwise xylene to ethanol to water baths. Antigen retrieval was performed using 9pH EDTA, TRIS
Antigen retrieval buffer in a decloaking chamber (DakoCytomation, Glostrup, Denmark, Pascal). Slides
were washed in TBST. A solution of 0.3% hydrogen peroxide was used as a peroxidase block. Then, slides
were washed in TBST. Anti-PTEN, Anti-RPS6, and Anti-p-RPS6 rabbit primary Cell Signaling antibodies
were used for overnight incubation at 4 °C. Then they were washed again in TBST. HRP-conjugated anti-
rabbit secondaries were used to incubate the sections, then they were washed again in TBST before DAB
incubation. Finally, they were washed in TBST one more time before hematoxylin counterstaining for 1
min, which was rinsed repeatedly with tap water. Dehydrating was performed using stepwise water to
ethanol to xylene baths. Permount was used as mounting media. Slides were imaged using ZenBlue software.

2.13. Upstream Regulator Pathway Analysis

Normalized values from protein array data were uploaded into Ingenuity Pathway Analysis (IPA) and analyzed to produce Z scores that denote relative activity of known clusters of molecules contributing to pathway activity. IPA was also utilized to predict upstream regulators, which are molecules that it determined were likely to cause the state of uploaded expression values.

3. Results

3.1. Mutation Profiling Revealed PIK3CA Aberrations Are Common

To assess clinically actionable targets of these models, NGS was utilized to characterize pathogenic genetic profiles of 14 cell lines and 20 PDX samples (Table 1), which revealed pathogenic PIK3CA aberrations in 37% of models tested; 5 cell lines and 7 PDXs. PIK3CA was the second most commonly identified pathogenic driver among these models after TP53 confirmed previous observations [43]. The most common PIK3CA aberrations found in patient tumors were identified in this cohort; this included amplification through copy number gain and two of the activating mutations, E542K and H1047R (Figure 1A).
Figure 1. High PIK3CA gene aberration and pathway enrichment in many PIK3CAwt PDX models. (A) Percent oncogenic aberrations. (B) IHC for PTEN for 6 basal-like breast cancers. (C) Hierarchical clustering of PDX models by previously published PIK3CA-related gene signatures and (D) Similarity matrix of those signatures.
Table 1. Oncomine V3 revealed clinically actionable pathogenic calls. The 34 BCRR models probed with Oncomine sequencing of 229 targeted oncogenic aberrations, returning the status of known mutations, DNA CNVs, and RNA fusion drivers. Models with genes with * were sequenced twice, those marked appeared both times. Pathogenic aberrations in PIK3CA are highlighted in red and in PTEN are highlighted in orange. “CR” denotes treatment with carboplatin.

<table>
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<th>Name</th>
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<th>Oncomine Amplification</th>
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3.2. Binary Single Gene Status Is Not Enough to Assess PI3K Pathway Activity

Human-specific bulk RNA sequencing data of 82 samples (45 previously unpublished) from 21 PDXs were used to stratify PDX models based on their proliferation rate (11-gene proliferation signature) [44] and PI3K pathway activity defined through 13 previously published PI3K gene signatures [33,34,35,36,37,38,39,40]. Most basal-like PDXs had low PTEN expression when assessed by IHC, but BCM3887CR (a model that has become carboplatin resistant, CR) was an example of high PTEN expression (Figure 1B). Figure 1C shows the hierarchical clustering of these signatures and PIK3CA and PTEN as individual gene values overlayed for reference. UCD52 overexpresses PIK3CA and scored highly on the signatures despite high PTEN expression, which usually inhibits PI3K activity. The reason UCD52 scored highly may have been that the PTEN it expresses has a deactivating mutation (Table 1). WHIM30 and HCI-010 scored highly despite modest PIK3CA single gene expression and no pathogenic mutation, but these PDXs are PTEN deficient. WHIM2 is an example of a basal-like breast cancer that is not PI3K overactive, having moderate PTEN expression and low PIK3CA expression and scoring relatively lowly on PI3K activity signatures. WHIM2 exhibiting relatively low PI3K gene expression levels was similarly observed in luminal models (BCM15034, BCM5097, HCI-011, HCI-013, HCI-009), clustering more closely to them than to other basal-like models. Two of the ER+, HCI-011 and HCI-013, scored relatively lowly on most of these gene signatures, despite containing an activating mutation of PIK3CA. UCD52 appears to have reduced PI3K activity after treatment with carboplatin (UCD52CR), but this may be because of a reduction in average RNA expression (from 2.17 to 2.01 log2 TPM), whereas HCI-001 and WHIM30 did not have as large of a change (from 2.15 to 2.14 and from 2.16 to 2.20 log2 TPM, respectively). An all-by-all pairwise comparison of the PDX gene signature enrichment scores using Pearson’s correlation coefficient (Figure 1D) revealed these signatures were, overall, highly similar to one another. Only 4 out of 91 signature pairs obtained a negative correlation, with the lowest correlation being ~0.12.
(REACTOME PI3K CASCADE to Lung 545k DEG Viglietto) and the highest correlation being 0.91 (Scorr PTEN Absent PNAS.2007 to YALE PIK3CA Pathway Ann.Oncol.2017) on a scale of from −1 to 1.

3.3. Single-Cell RNA-Seq Identifies Cell Subpopulations with High PI3K Activity

Because PI3K inhibitors have not been found to be effective as single agents, we sought to determine if subpopulations of cells existed within PDXs that would be more or less therapeutically targetable for inhibitors of PI3K. Single-cell RNA-seq data containing 37,851 total cells from 18 samples (6 published previously) across 12 models (4 cell lines and 8 PDX) were utilized to compare cells and subpopulations. A 100-gene PI3K activity signature from Gatza et al. [33] was used to map these models, some of which, such as UCD52 and WHIM30, formed distinct subpopulations. (Figure 2A) These populations contained different proportions of the PIK3CA single gene (Figure 2B) and AKT1, AKT2, and AKT3 average expression (Figure 2C). Individual re-cluster analyses of UCD52 or WHIM30 cells identified two or three distinct subpopulations within each PDX (Figure 2D–G). Each model in the cluster was assessed for PIK3CA and AKT1-3 percent cell positivity (>0) (Figure 2H,I).
Figure 2. Subpopulations less reliant on the PIK3CA pathway were identified among 2 basal-like PDX models. (A) UMAP using the Gatza et al. The 2017 PIK3CA signature of scRNA sequencing data of basal-like models. Cells positive for (B) PIK3CA expression marked in purple and (C) by the level of AKT1-3 average expression. Unsupervised reclustering of (D) PIK3CA positive cells in UCD52 and (E) AKT 1-3 expression. Unsupervised reclustering (F) WHIM30 PIK3CA positive and (G) AKT1-3 expression. Percent positive (>0) (H) PIK3CA and (I) AKT1-3 cells per model.

3.4. Alpha-Specific PIK3CA Inhibitors Were More Effective on PIK3CA Aberrant Cells

To determine if the PIK3CA aberrations found within the models tested were targetable and resulted in the loss of cell viability, two clinically tested PI3K inhibitors (PI3Ki) were utilized; BYL-719 and GDC-0032 (marketed as taserlisib). HCI-013, an ER+ PIK3CAH1047R mutant PDX, was the most responsive to both
drugs at higher doses (Figure 3A and Supplemental Figure S1A). When grouped by pathogenic PIK3CA status, those with mutations were significantly (p > 0.01) more responsive on average to the PI3Ki in vitro at 5 μM and 10 μM using two-way ANOVA with Šidák’s correction for multiple comparisons (Figure 3B and Supplemental Figure S1B).

Figure 3. Pathogenic PIK3CA-containing models were more responsive to BYL-719. (A) Dose to viability by model in vitro. (B) Dose to viability by, grouped by PIK3CA oncogenic status.

3.5. Inhibition of PIK3CA Yielded Related Predicted Upstream Regulators in Pathway Analysis

We next sought to contrast the downstream effects of PI3K inhibition on TNBC cells from a PDX as compared to a cell line often utilized for PI3K inhibition studies, MDA-MB-453. Protein lysates from two basal-like models containing pathogenic PIK3CA aberrations, UCD52 and MDA-MB-453, were prepared from in vitro cultures, which were treated with BYL-719 or vehicle and applied to antibody arrays for orpheus-proteins relating to the PI3K/mTOR/AKT pathway (Supplemental Figure S2). Phospho-protein levels were quantified and analyzed using IPA, and fold changes (Figure 4A,B) were used to predict upstream regulators. The effects of BYL-719 were predicted by IPA to be seven upstream regulators to be downregulated and three upstream regulators to be upregulated, including PTEN, of the top 15 z-scores (Figure 4C,D). PTEN downstream effects are the same as BYL-719, to reduce PIP3 levels in the plasma.
membrane, as PI3K activity is to convert PIP2 into PIP3, so a reduction in PIK3CA activity from BYL-719 would be predicted to have a similar or potentially identical downstream effect as an increase in PTEN activity.

Figure 4. Confirmation of BYL-719’s PIK3CA inhibitory effects. Fold changes of phosphoprotein levels for (A) UCD52 and (B) MDA-MB-453 from phosphoprotein antibody arrays. (C) Highest z-score predicted upstream molecules in Ingenuity Pathway Analysis. (D) Model of how the highest predicted upstream regulators would share downstream effects with PI3K pathway suppression.
3.6. Identification of More Effective Single Compounds Relative to PIK3CA Mutational Status

Since the sensitivity or resistance to drugs that were not intended for targeted treatment can arise from a mutation in another gene, it was important to test the effect of PIK3CA activating mutations in controlled models. MCF10A wild type, E545K, and H1047R PIK3CA variant containing cell lines, when screened, responded to a similar number of drugs at 40% viability or lower (Figure 5A). The two mutant variants responded similarly (R = 0.97) to each drug as one another (Figure 5B), but when the average value of each of those was compared to the wild type containing cell line, viability was more dissimilar (R = 0.76) (Figure 5C); generally, each oncogenic mutant version caused cells to react the same to most drugs, but having one of these mutations changes the response of the cell to far more drugs relative to wild type containing cells. Different viability in parental versus mutation-containing cells was observed in 45 drugs, significant at 0.01 or lower p-value using a two-way ANOVA correcting for multiple comparisons using Tukey’s post-hoc test (Figure 5D). No drugs were significant with the same statistical comparison when comparing the effects of those drugs relative to mutation.
Figure 5. High throughput viability screening of 516 drugs on MCF10AWT, MCF10AE545K, and MCF10AH1047R revealed drugs that were more differentially effective relative to PIK3CA mutation status. (A) Drugs on each variant are ranked by model by viability. (B) Each drug’s effect on the viability of both mutant-containing models was compared. (C) The average of both mutant-containing models’ viability when treated with each drug compared to parental viability for that drug. (D) Significant (p < 0.01) drugs when comparing MCF10AWT viability to that of the average MCF10A mutant viability. (** denotes p < 0.01, *** denotes p < 0.001, **** denotes p < 0.0001).
3.7. Identification of Synergistic Compounds with BYL-719

Because BYL-719 as a single agent only had modest effects in vivo (Supplemental Figure S3A) while still inhibiting PI3K activity (Supplemental Figure S3B), it was of interest to discover agents that potentiated BYL-719's effect on these models. High-throughput screening (HTS) with 516 single drugs alone and those agents with BYL-719 was performed on MDA453, UCD52, and HCI-013 (Figure 6A–C). Of drugs that were found to be synergistic using the coefficient of drug interaction (CDI), there were 20 for the two basal-like PIK3CA oncogenic aberration containing model, and half of those were also found to be synergistic with BYL-719 in HCI-013, an ER+ model which also has oncogenic PIK3CA. In total, 20 drugs were identified as synergistic in both basal-like PIK3CA aberrant-containing models, half of which were also synergistic in the ER+ PIK3CAH1047R (Figure 6D). Some drugs were highly effective as single agents, such as YM-155 and Digoxin, so significant synergy was not observed in combination with BYL-719. The PIK3CAWT basal-like PDX HCI-001 had fewer drugs with which BYL-719 was synergistic, and none of those found in the aberrant models were found to be synergistic in HCI-001 (Figure 6E).
Figure 6. High throughput synergism screening revealed drug combinations of interest. A library of 516 drugs with and without BYL-719 with the viability of each drug (left x-axis) and CDI (right x-axis) for each drug combination in (A–C) PIK3CA pathogenic models and (D) Venn diagrams of drugs under 0.7 CDI for oncogenic PIK3CA containing models. (E) Viability of single and combinatorial agents and CDI in the PIK3CAWT containing HCI-001.
3.8. CompuSyn Was Utilized to Confirm Synergistic Effects of Candidate Therapies

To investigate these drugs at a higher level of rigor, afatinib, dronedarone, and everolimus, three of the drugs which were discovered in HTS, were assessed with CompuSyn using 5 dose ratios per drug and 5 or more dose escalations per ratio in MDA453 (Supplemental Figure S4). Each of these combinations was predicted to have at least one dose ratio that was synergistic below one combination index (CI) and at a high fraction affected (FA), denoting that each combination had at least one dose ratio that performed better than the expected effects of both drugs alone combined. Each combination was predicted to have dose reduction index (DRI) greater than one at high FA with at least one dose ratio (Figure 7A–C), denoting that each combination could have one or the other of the drug’s concentrations reduced and be predicted to see the same effects. Highlighted in yellow is the range of interest, FA of 0.75 or higher, and CI below one or DRI above one.
Figure 7. Compusyn was utilized to confirm synergism with higher rigor and to determine effective dose ratios in vitro on MDA453 cells. (A) Projected combination indexes per fractions affected for BYL-719 and afatinib and projected dose-reduction indexes per fractions affected for each drug at the dose ratio, BYL-719 to afatinib, listed. The same is shown for the combinations of BYL-719 with (B) dronedarone and (C) everolimus.
3.9. In Vitro Synergism Testing of Select Dose Ratios of Synergistic BYL-719 Combinations

Those three drug combinations were then tested at two dose ratios each, each at a lower and higher escalation of doses, for four conditions total per drug combination. In total, 11 models were tested in vitro. Two dose ratios showed synergistic to significantly synergistic response in all models tested, the BYL-719 10 μM with afatinib 3.125 μM combination and the same dose of BYL-719 with 6.25 μM dronedarone. No dose ratio with everolimus showed synergism across each model, but the 10 μM BYL-719 with 7.5 μM everolimus had synergistic to significantly synergistic proliferation reduction in HCI-013, UCD52, WHIM30, and WHIM30CR. The only models that these dose ratios that were not synergistic were the ER+ HCI-011, which does contain pathogenic PIK3CA, and the PIK3CAWT, PTENWT basal-like PDX, BCM2147 (Figure 8).

Figure 8. In vitro synergism screening revealed synergistic dose ratios across multiple PDX models. CDI for each drug combination at 4 dose ratios each, green denotes significant synergy, blue denotes synergism, and red denotes antagonistic effects, while white is additive.
3.10. In Vivo BYL-719 Synergism Trial on PI3K Overactive Basal-like PDXs

Finally, the drugs needed to be tested in living systems. BYL-719 alone, afatinib alone, the combination, and vehicle control were each administered to mice bearing tumors of the PIK3CA overactive UCD52 basal-like PDX. The combinatorial group showed significant (p < 0.0001) and significantly synergistic activity when comparing the final tumor area (CDI = 0.32) (Figure 9A,B). The combination of dronedarone and BYL-719 was significantly more effective than either agent alone (p < 0.0001) (Figure 9C). Everolimus with BYL-719 also showed significant efficacy compared to its single-agent components (p < 0.0001) (Figure 9E). Each of these combinations yielded significant synergism (CDI = 0.69 and 0.18, respectively) (Figure 9D,F). PI3K pathway activity was assessed using IHC and Western blot of p-RPS6 relative to total RPS6. Each group treated with BYL-719 alone does not show the same reduction in p-RPS6 that shorter in vivo trials show, which also occurred after BYL-719 single-agent resistance began. The afatinib + BYL-719 group had reduced total RPS6 (Supplemental Figure S5), as did each group treated with dronedarone on IHC (Supplemental Figure S6). Treatment with everolimus in UCD52 reduced p-RPS6 even at longer time points. (Supplemental Figure S7).
Figure 9. The combinations of everolimus, afatinib, or dronedarone with BYL-719 were significant and significantly synergistic in UCD52. BYL-719 tested in vivo in UCD52, comparing to alternative single agents, (A,B) afatinib, (C,D) dronedarone, and (E,F) everolimus and the combination of each with BYL-719. **** denotes p < 0.0001 significance, ANOVA after Tukey correction for multiple comparisons, comparing final tumor area. * on the days since seeded axis denotes the day that the treatment was started.

These three combinations were tested in the same way on mice with xenografts of WHIM30, a PTEN deficient PIK3CA wild type basal-like PDX, with the everolimus and BYL-719 combination showing a reduction in tumor growth for each treatment group relative to the vehicle and synergistic effects (p <
0.05 and CDI 0.77) (Figure 10A,B) and everolimus treatment also reduced p-RPS6 in HCI-010 (Supplemental Figure S8). The combinations of dronedarone and afatinib with BYL-719 were tested, and while showing a trend, the effectiveness of BYL-719 alone drove the phenotype statistically (Supplemental Figure S9). The BYL-719 and everolimus combination was tested in HCI-010, another PTEN deficient PIK3CA wild type basal-like PDX, yielding significant difference (p < 0.01) and significantly synergistic effects (0.66) (Figure 10C,D). Once again, each everolimus-treated group had a reduction of p-RPS6 (Supplemental Figure S10).

Figure 10. The combination of everolimus with BYL-719 performed better than the expected additive effects of both drugs in the PTEN-deficient WHIM30 and HCI-010. BYL-719 tested in vivo in WHIM30, compared to alternative single agents everolimus and the combination with BYL-719 in (A,B) WHIM30 and (C,D) HCI-010. * denotes p < 0.05, ** denotes p < 0.001 significance, ANOVA after Tukey correction for multiple comparisons, comparing final tumor area. * on the days since seeded axis denotes the day that the treatment was started.
4. Discussion

BYL-719 is currently approved for some cases of PIK3CA mutated ER+ breast cancer, though such as with HCI-011, targeting PIK3CA in ER+ disease, even with pathogenic PIK3CA, does not always yield an effective treatment.

In clinical trials not related to breast cancer, the most common side effects of dronedarone were nausea at about 5% and diarrhea at about 10%, a very manageable adverse event profile, at least as a single agent [45]. Dronedarone, a multi-ion channel inhibitor, and afatinib, an EGFR inhibitor, have been studied in breast cancer previously [46,47] but not in combination with BYL-719. Afatinib has been approved as a single agent in some lung cancers [48], but despite clinical trials so far in the breast cancer setting, it has not been shown to be effective as a single agent in breast cancer [49]. These combinations did show synergistic effects in the PI3K overactive basal-like breast cancers tested. The combination of BYL-719 plus everolimus, an mTOR inhibitor, has been studied in PIK3CA mutant breast cancer cell lines previously [50] and was reconfirmed through this study. Toxicity has been tested in a phase 1b clinical trial for HR+ HER2- breast cancer to test the safety of the drugs, resulting in a manageable safety profile with no observed interactions between the two drugs [51], which is a promising outcome since both drugs have serious, but manageable side effects alone. The efficacy of the drugs was not able to be assessed in that trial because of the sample size. The results of previous research of the BYL-719 and everolimus combination point towards utilizing it on PIK3CA mutant cancers only, but they showed to be synergistic and effective in the PTEN deficient PIK3CA WT PDXs WHIM30 and HCI-010, suggesting that the precision medicine potential of BYL-719 plus everolimus should be considered in the treatment for PTEN lacking basal-like cancers, which are three or more times or more common than PIK3CA mutation containing basal-like breast cancer.

5. Conclusions
Further research is merited, but based on these results, clinical trials could be considered using BYL-719 in combination with everolimus for basal-like tumors with PI3K pathway overactivity, either through PTEN loss or pathogenic PIK3CA and the combinations of either dronedarone or afatinib with BYL-719 should be considered for testing for patients with basal-like breast cancer with pathogenic PIK3CA.

**Supplementary Materials** (pages 104 – 116)

The following supporting information can be downloaded at:

https://www.mdpi.com/article/10.3390/cancers15051582/s1, Supplemental Table S1. Bulk and scRNA samples used. Supplemental Figure S1. Pathogenic PIK3CA-containing models were more responsive to GDC-0032. (A) Dose to viability by the model in vitro. Dose to viability by model, grouped by PIK3CA oncogenic status. Supplemental Figure S2. Phospho-protein array membranes of (As) untreated and treated (Bs) from lysates made with UCD52 and MDA453 treated with BYL-719. Each number denotes the numbered membrane from the RayBiotech C55 phosphoprotein array. Supplemental Figure S3. Preliminary single-agent trials of BYL-719 showed some efficacy in WHIM30 and UCD52, but resistance arose. In HCI-001 and BCM2147CR6, the drug performed as well as the vehicle. Supplemental Figure S4. Full output from CompuSyn shows multiple drug ratios that were effective in MDA453 for combination index (CI) and for dose reduction index (DRI) and high fraction affected (FA). Supplemental Figure S5. Treatment did not show a reduction in p-RPS6. (A) H + E and IHC of RPS6 and p-RPS6 of each treatment group in the BYL-719 with Afatinib trial in UCD52. (B) Western blots of Vinculin loading control and RPS6 and p-RPS6. Supplemental Figure S6. Total RPS6 was reduced with dronedarone treatment in UCD52 in IHC but not in western. (A) H + E and IHC of RPS6 and p-RPS6 of each treatment group in the BYL-719 with Dronedarone trial in UCD52. (B) Western blots of Vinculin loading control and RPS6 and p-RPS6. Supplemental Figure S7. The levels of p-RPS6 were reduced with treatment of either BYL-719 or Everolimus and even further with combination therapy in UCD52. (A) H + E and IHC of RPS6 and p-RPS6
of each treatment group in the BYL-719 with Everolimus trial in UCD52. (B) Western blots of Vinculin loading control and RPS6 and p-RPS6. Supplemental Figure S8. The levels of p-RPS6 were reduced with treatment of either BYL-719 or Everolimus and even further with combination therapy in WHIM30. (A) H + E and IHC of RPS6 and p-RPS6 of each treatment group in the BYL-719 with Everolimus trial in WHIM30. (B) Western blots of Vinculin loading control and RPS6 and p-RPS6. Supplemental Figure S9. Further combination therapies showed a trend of an effect in WHIM30. The final tumor area of the combinations of (A + B) Afatinib and (C + D) Dronedarone with BYL-719 was reduced relative to either treatment alone, but the effect of the combination was not significant relative to the effect of BYL-719 alone. Supplemental Figure S10. The levels of p-RPS6 were reduced with the treatment of Everolimus in HCl-010. (A) H + E and IHC of RPS6 and p-RPS6 of each treatment group in the BYL-719 with Everolimus trial in HCl-010. (B) Western blots of Vinculin loading control and RPS6 and p-RPS6. Supplemental Figure S11. Complete Western blots of in vivo trials from tumors of the UCD52, WHIM30, and HCl-010 PDXs that were treated with vehicle, single agents, or combinations. Supplemental Figure S12. Complete Western blots of in vivo trials from tumors of the UCD52, WHIM30, and HCl-010 PDXs that were treated with vehicle, single agents, or combinations values indicate densitometry.

Author Contributions


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Institutional Review Board Statement

This study was approved by Virginia Commonwealth University IACUC, protocol number AD10001247.

Informed Consent Statement

Not applicable as study does not involve human subjects.

Data Availability Statement

The scRNA seq data presented in this manuscript is available online. Full Western blots are included in the supplemental figures. All other data used, including genes which comprise each gene signature, was submitted as supplemental uploads to the journal.

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Conflicts of Interest

The authors declare no conflict of interest.

References


Chapter 6: Conclusions

Each of these studies provide important insights into breast cancer research and/or treatment.

The characterization of pros and cons of the Glowing Head Mouse Model provides useful insights to researchers. Given that immune competent mouse models are necessary when studying the effects of the immune system on cancer in response to or in tandem with treatments or other conditions. The confoundment of the immune system targeting GFP or luciferase expressing cell line xenograft studies is an important concern. Metastatic studies are paramount in breast cancer research because the majority of breast cancer deaths are from metastatic disease. The findings of that project is that the Glowing Head Mouse Model is useful for studying some types of metastatic disease, but that glowing head mice have an endogenous luciferase signal in the brain and bones, which cannot be disentangled from the potential signal of metastasized cancer cells.

WHIM2 cells that had become resistant to Erlotinib, an EGFR inhibitor, and Wnt1-Late tumors, which are intrinsically resistant to erlotinib, both showed significant increase relative to their erlotinib sensitive counterparts of LCN2 and 4 other genes. LCN2 may play a role in erlotinib resistance by recycling EGFR and the transient knock-down of LCN2 restored sensitivity to erlotinib.

A workflow for developing inducing senescence in MDA-231 cells was developed and optimized. High-throughput screening revealed drugs to which these cells were also resistant. Drugs which senescent cells had a trend of being more sensitive to were also identified, providing interesting avenues of study for future projects.

BYL-719, having been approved for ER+ patients as a third-line therapy, is an interesting drug in the basal-like setting, since the majority of basal-like breast cancers are PI3K pathway overactive. Still, it has to date not been approved by the FDA in that setting, likely due to its ineffective nature as a single
agent. This study identified three drugs that, in combination with BYL-719, have significantly synergistic activity in vitro and in vivo. These drug combinations have the potential to be tested in future clinical trials for patients with PI3K overactive basal-like disease.

The projects were of a manageable scope for a dissertation project, and they came to full fruition, but any project could be better. If resources were unlimited, for the project which characterized the glowing head mouse model in the metastatic setting, a characterization of endogenous GFP would have been pursued. Recently, a method for imaging endogenous GFP in mice has been developed [1], and while not used in glowing head mice, could be used to determine which tissues express GFP endogenously that would interfere with signals from cancer cells. Importantly, naïve mice would be compared to mice with metastasis. This would be incredibly time intensive, as the entire mouse morphology would have to be characterized, requiring sections from all tissue types to be analyzed.

The project assessing transcriptomic changes in erlotinib resistant cells, if given more time and resources, would seek to assess if the same pathways are acquired in additional PDXs when treated with erlotinib and again with other EGFR inhibitors like afatinib could have been tested to see if they produce similar resistance mechanisms. Then, those afatinib or other EFGR inhibitor resistant cells could be high-throughput screened to determine if the same compounds were effective on those cells as the ones that were effective on erlotinib resistant cells. These cells would then have been used for multi-omic analyses and pathway analysis, to determine if key molecules in erolinib resistance would also be responsible for afatinib or other EGFR resistance.

The first improvement that could be made in the senolytic agents project given more time and resources would be to utilize more models to show that the ability of these drugs to clear senescence is transferable to cancer cells of a diverse background. Another improvement would be further testing of
compounds discovered at a higher level of rigor, with fewer drugs focused on a higher number of replicates and more doses of drug.

Given more time and resources, the way the core project of the dissertation, which identified three synergistic drug combinations to be used with the PIK3CA inhibitor, BYL-719, could have been further refined in many ways. More drugs could have been tested, which could have broadened the net to include more possible synergistic companion drugs. Then, during the shift from high throughput to the time-intensive CompuSyn, three drugs of 20 that were synergistic in both basal-like models were tested, but with more time and resources, each of these drugs could have been tested. Other drugs of similar classes to those identified could have been tested. Then, before taking the drugs to the in vivo setting, different synergism tools could have been utilized, to confirm the synergistic effects of the combinations. More models could have been tested to further characterize the suggest use cases of these drugs, they could have been tested at a higher number of replicates to further empower the statistical tests, and they could have been tested for longer in order to further test the durability of the responses to the combinations. The most successful drugs could have been used in lead development as a dual-inhibitor to refine response, and iterations of those drugs could have been tested among multiple models to determine which is best for primary tumors and again tested in the metastatic setting.

A key decision in the BYL-719 synergy project was to choose from the 20 drugs which were synergistic in both basal-like PIK3CA mutant containing models to study in vivo. Three drugs were ultimately chosen. Dronedarone was chosen because of the novel combination of targets. Afatinib was chosen because many of the drugs of that set of 20 were RTK inhibitors and it was chosen to represent that class as it has a favorable side effect profile. Everolimus was chosen because dual MTOR/PIK3CA inhibitors have been developed and tested, giving legitimacy to the co-targeting of those proteins.
Each of these possible improvements to the projects are also avenues for follow-up projects to expand upon these findings. Given that each of these projects uncover important findings, there of course are new directions that they make possible to future research. The key findings from the glowing head project allow for investigation of liver and lung metastasis research and possibly even the refinement of the model itself by future researchers. A possible future direction for the erlotinib resistance study could be the development of LCN2 inhibitors to reproduce the re-sensitizing to erlotinib that was seen in the LCN2 knockdown experiment. If those were successful, then a dual LCN2 + EGFR inhibitor would be produced and tested in a preclinical setting, to provide an option for patients whose cancer shows an increase in LCN2 histologically and a reduction in efficacy for EGFR inhibitors. Another direction after this study would be to design PROTAC drugs that degrade EGFR, which may result in a treatment for EGFR which circumvents the EGFR recycling seen after erlotinib treatment. To further study senescent based on the findings of that study, mice with a basal-like PDX which responds to doxorubicin would be treated to clear their cancer as best as possible, this would likely cause senescent cells, but when recurrence or metastasis occurred, the compounds discovered from screening, to see if their senolytic effects were maintained in the in vivo setting. Another way of furthering research based on this study would be to seed senescent cells only into mice and test with those drugs to determine if they could clear cells while they are still senescent, to determine if the drugs would be valuable as a prophylactic against metastasis or recurrence after doxorubicin treatment. Lastly, to follow up the core project, the BYL-719 synergism study, the biggest payoff for those efforts would be a clinical trial. As for other preclinical research, an investigation as to the mechanism of action of the drug combinations would be warranted. For the afatinib or everolimus with BYL-719 combinations, there is some intuitive logic as to how they work: cells are reliant on a pathway and find a way to upregulate that pathway, but when an RTK or mTOR are also inhibited, as they are upstream and downstream of PIK3CA respectively.
The mechanism behind synergism of dronedarone and BYL-719 is much less intuitive and would benefit from investigations into why these drugs work well together.

These studies help advance knowledge related to basal-like treatment and research. Our hope is that further studies, both preclinical and clinical, are conducted based on the findings of this project.

Reference

Appendix

Chapter 5 supplemental note

Challenges of transitioning from in vitro to in vivo preclinical drug trials

In Chapter 5, synergistic drug combinations were tested in vitro to assess models that would be explored in vivo. While this data was valuable in the decision-making process for the next step, a challenge for making this transition is that the concentration of the drug in media will not be the same as that of the drug in a mouse’s serum. Furthermore, the concentration of the drug in the cells is not the same as that of the media or serum for in vitro or in vivo studies respectively. Each drug will have different pharmacokinetics, resulting in an unknown drug ratio in the cells for either study type.

To address this, a researcher could conduct an assay, such as mass spectrometry, to identify the concentration of each drug in cells in vitro, then dose mice, harvest tumors, and continuously adjust dosing regimens in order to achieve the same dose ratios in vivo. That would be a time consuming and costly process. Instead, another approach could be to treat media and serum as somewhat analogous and adjust dosing of the mice until the blood concentrations of the drugs match the dose ratio of the in vitro conditions. This approach would be much more manageable but would not account for distribution of the drug at the intratumor level but would be useful in providing a starting point for dose ratio optimization.
Supplementary Figure 1. Assessment of EGFR inhibitors on growth of the basal-like patient-derived xenograft WHIM2. Effect of daily oral gavage treatment with various EGFR inhibitors (n = 1 per drug): CO-1686 [100mg/kg], erlotinib [100mg/kg], gefitinib [200mg/kg], dacomitinib [10mg/kg], lapatinib [100mg/kg], afatinib [50mg/kg]. A: Tumor size; B: Excised tumor mass; C: Picture of excised tumors; D: A second cohort of mice was treated with erlotinib-incorporated mouse chow (367ppm) ad libitum (n = 1 control, n = 4 erlotinib treated). Tumor size over the course of treatment is depicted.
### Chapter 5 Supplemental Figures

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