Investigating Biomarkers of Response to CDK4/6 Inhibition in Combination with Anti-Estrogen Therapy in Breast Cancer Cell Lines

by

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Abstract

Breast cancer is the most common cancer diagnosed in women and remains the second leading cause of cancer related deaths. For decades, anti-estrogen therapy (AET) has been used to treat estrogen receptor positive (ER+) breast cancer. The development of novel targeted therapies, like cyclin dependent kinase 4/6 inhibitors (CDK4/6i), provide additional treatment options for women with late-stage disease. CDK4/6i in combination with AET has delayed cancer progression and is approved for use in metastatic ER+, human epidermal growth factor receptor 2 negative breast cancer. However, it is estimated that almost half of these patients don't derive a meaningful benefit from this treatment combination, resulting in unnecessary drug toxicities. Furthermore, unnecessary cancer treatment is a burden to the healthcare system, particularly with expensive drugs like CDK4/6i which cost ~$8000 per month of treatment. The identification of predictive biomarkers represents a top cancer research priority. Biomarker testing improves treatment selection so that patients and our health care system derive maximal benefit from these effective but expensive drugs. To date, ER positivity remains the only molecular biomarker for CDK4/6i treatment selection.

For this research, I performed a comprehensive literature review of CDK4/6i biomarkers and analyzed publicly available molecular/clinical databases to identify potential biomarkers. I then conducted *in vitro* tests of palbociclib (CDK4/6i) and tamoxifen (AET) on a comprehensive panel of breast cancer cell lines. From this, I optimized the *in vitro* experimental assays to ensure they measured the cytostatic activity of palbociclib to accurately rank drug efficacy amongst the cell lines. Next, using reverse phase protein array (RPPA), I performed a discovery-based proteomic analysis to identify biomarkers of resistance to palbociclib +/- tamoxifen.

My analysis of public databases failed to identify any statistically significant biomarkers, further highlighting the need for biomarker research. Western blot analysis identified p16 overexpression and Rb loss as markers of resistance to palbociclib. RPPA analyses identified an additional thirteen differentially expressed proteins associated with palbociclib resistance. I also identified twelve proteomic biomarkers of resistance to the combination therapy. This research identifies predictive biomarker candidates for validation and highlights the importance of experimental assay optimization for translatable *in vitro* testing.

Lay Summary

Breast cancer is the most common cancer in Canadian women. New molecular drugs are now improving outcomes for these patients, but they are very expensive. The purpose of my research is to study the molecular make-up of breast cancer cell lines treated with a new drug called palbociclib. The goal is to identify proteins (biomarkers) that predict which patients will not benefit from palbociclib. Breast cancer cell lines were treated with palbociclib alone and in combination with tamoxifen, a commonly used breast cancer drug. Fifteen different proteins were identified that might predict resistance to this drug. Using biomarker testing, patients can be selected for treatment so that those taking the drug will benefit. Also, by predicting when the drug won't work, patients can be spared unnecessary treatment, toxicity, and side effects. This research can help inform patient selection which can save health care dollars to spend on more effective treatments.

Preface

Under the supervision of Dr. Mark Carey and Dr. Marta Llaurado Fernandez, I participated in the design of the project and the experiments.

The research conducted in this study was approved by the institutional human ethics review board at the University of British Columbia (H22-00544).

I was responsible for all experimental work for the project in the thesis below, including:

-performing a literature review and identifying datasets for bioinformatic interrogation. The bioinformatic work was done with the help of Nemat Haroon, a co-op student in Dr. Carey's laboratory.

-obtaining breast cancer cell lines from collaborators

-maintaining all breast cancer lines for experiments

-Western blots

-*in vitro* cell proliferation experiments

-cell counting experiments

-database processing of proteomic data, and data analysis of proteomic data using SPSS

-processing all *in vitro* proliferation, cell counting and drug synergism data

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List of Abbreviations

AACR GENIE	American Association for Cancer Research Genomics Evidence Neoplasia Information Exchange
AET	Anti-Estrogen Therapy
AI	Aromatase Inhibitor
AKT1	AKT Serine/Threonine Kinase 1
AMP	Amplification
AR	Androgen Receptor
BRAF	Serine/threonine-protein Kinase B-Raf
CBR	Clinical Benefit Rate
CCLE	Cancer Cell Line Encyclopedia
CCND1	Cyclin D1
CCNE1	Cyclin E1
CDK4/6	Cyclin Dependent Kinase 4/6
CDK4/6i	Cyclin Dependent Kinase 4/6 Inhibitor
CDKN2A	Cyclin Dependant Kinase Inhibitor 2A
CI	Combination Index
CNA	Copy Number Alteration
ER	Estrogen Receptor
ERBB3	Erb-B2 Receptor Tyrosine Kinase 3
FDA	Food and Drug Administration
FFPE	Formalin-fixed Paraffin-embedded
FGFR2	Fibroblast Growth Factor Receptor 2
FISH	Fluorescence *In Situ* Hybridization
GAB2	GRB2 Associated Binding Protein 2
GATA3	GATA Binding Protein 3
GR	Growth Rate
HER2	Human Epidermal Growth Factor Receptor 2
HOMDEL	Homozygous Deletion
HR	Hormone Receptor
IC50	Half Maximal Inhibitory Concentration

IHC	Immunohistochemistry
MCLP	MD Anderson Cell Line Project
mRNA	Messenger RNA
OS	Overall Survival
PD-1	Programmed Cell Death Protein 1
PFS	Progression Free Survival
PKCα	Protein Kinase C Alpha Subunit
PLB	Palbociclib
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog deleted on Chromosome 10
Pts	Patients
Rb	Retinoblastoma Protein
RPPA	Reverse Phase Protein Array
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
STR	Short Tandem Repeat
TMX	Tamoxifen
TNBC	Triple-Negative Breast Cancer

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I would like to thank Dr. Mark Carey for his continued support and mentorship throughout my time as a student in his laboratory. I would also like to thank Dr. Marta Llaurado-Fernandez for her daily support and feedback on this project. I am also grateful for the technical support with bench top experiments provided by Hannah Kim, and the scientific discussion and support from Dr. Nelson Wong. In addition, a special thanks to the technicians and co-op students that have helped with this project during their time in the Carey laboratory, specifically Nemat Haroon, for helping with the bioinformatic analysis of the GENIE database.

I would like to thank my supervisory committee, Dr. Michael Anglesio, Dr. Christian Klausen, and Dr. Paul Yong, as well as my external examiner Dr. Martin Koebel for their feedback on this project. I would also like to thank Dr. Deborah Anderson for sharing a collection of her breast cancer cell lines with our laboratory. I would also like to acknowledge Dr. Gordon Mills, Kangjin Jeong and Soonyoung Park at the MD Anderson Cancer Center for providing me with the reverse phase protein array dataset.

Finally, I would like to thank family and my partner for their unwavering support during the last two years.

Chapter 1- Introduction

1.1 Project Overview

Breast cancer is the most commonly diagnosed cancer in women, with 1 in 8 Canadian women being diagnosed during their lifetime [1]. This cancer remains the second leading cause of cancer-related deaths in Canadian women, and approximately 5,500 women will succumb to the disease each year [1]. The stage of disease at time of diagnosis is a substantial prognostic factor. The average 5-year survival rate across all stages of breast cancer is 89%, decreasing to 22% for patients with stage 4 disease (Figure 1.1.1) [1]. Treatment options for breast cancer include surgery, radiation, chemotherapy or targeted drug treatments depending on the molecular subtype of the tumor [1].

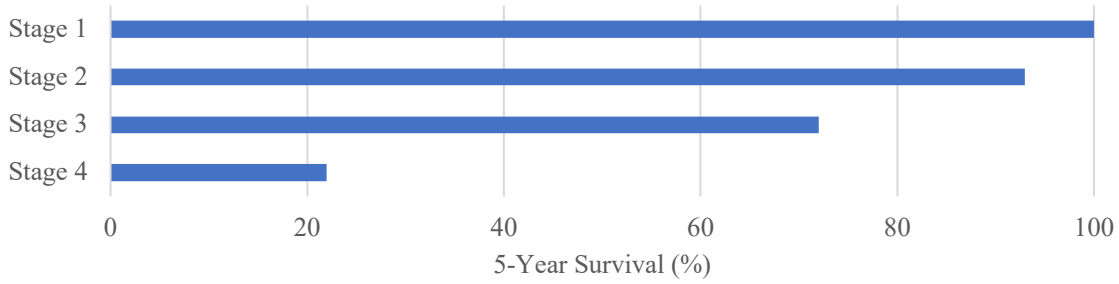

Figure 1.1.1 Breast cancer 5-year survival by stage [1].

A unique feature characterizing breast cancer is the presence of hormone receptors. Estrogen and progesterone receptors (ER and PR) regulate malignant growth and progression in this cancer type. With the identification of human epidermal growth factor receptor 2 (HER2), breast cancers are now characterized using these biomarkers [2]. Breast cancer treatment represents a leading example of precision medicine with the design of therapeutics directed at these molecular targets (ER and HER2), [3]. More recently, it has been recognized that breast

cancer tumours show significant alterations in cell cycle regulation, specifically loss of the tumour suppressor p16, which has led to the development of cell cycle inhibitors including cyclin dependent kinase 4/6 inhibitors (CDK4/6i) [4]. CDK4/6i are now used routinely for the treatment of ER-positive (ER+) advanced/recurrent breast cancers that are also negative for HER2 expression. CDK4/6i are almost always used in combination with anti-estrogen therapies (AETs - fulvestrant and letrozole).

Several pivotal clinical trials (PALOMA-1, -2 and -3) led to the approval of the CDK4/6i, palbociclib (PLB), for the treatment of advanced/recurrent breast cancer. These trials demonstrated a significant improvement in progression free survival (PFS) with the addition of PLB compared to AET alone. However, two of these randomized trials failed to show an overall survival benefit [5–7]. The only statistically significant overall survival benefit was seen in the PALOMA-3 trial where the addition of PLB to fulvestrant (AET) resulted in a 6 month improvement [8]. Based on the literature, it is estimated that up to 40% of patients who are treated with CDK4/6i + AET combinations do not obtain a substantial benefit from the CDK4/6i [9–13]. Rising drug costs relating to the use of novel targeted therapies represents a significant funding challenge facing our health care system today [14]. There is growing concern about the financial toxicity associated with using novel targeted therapies and the resulting impacts to both patients and the health care system [14].

Advanced breast cancers have been routinely treated with CDK4/6i for the last 7 years and there has been considerable research on the efficacy of CDK4/6i. Yet, it is remarkable that there are still no clinically validated, specific biomarkers to guide patient treatment [15]. As mentioned, most patients diagnosed with ER+/HER2- advanced/recurrent breast cancer are considered appropriate for treatment. There is a pressing need to ensure patients are treated with

new drugs that work, ensuring they have access to the most effective treatment options and avoid toxicities from ineffective treatments. Our laboratory has focused on predictive biomarker discovery for targeted therapies as a research priority [16,17]. Therefore, the purpose of this study was to address this research gap by evaluating CDK4/6i (PLB) response in breast cancer cell lines to identify biomarkers that can help to predict drug efficacy with and without AET (tamoxifen- TMX) treatment.

1.1.1 Project Aims and Hypothesis

The main aim of this project was to identify proteomic biomarkers of drug efficacy in a panel of breast cancer cell lines treated with the novel CDK4/6i PLB. In addition to PLB treatment alone, I also sought to evaluate predictive biomarkers of combination AET treatment (TMX). Based on the existing knowledge of cell cycle pathway regulation and cyclin dependent kinase 4/6 (CDK4/6) as a drug target, I hypothesized that I would identify predictive biomarkers of CDK4/6i efficacy using a discovery based proteomic approach. My specific objectives for identifying predictive biomarkers were highlighted as follows:

OBJECTIVE 1: To conduct an extensive literature review to search for predictive biomarkers of PLB efficacy and assess the current literature relating to the validity of these biomarkers (Chapter 2).

OBJECTIVE 2: To identify biomarkers of CDK4/6i efficacy using publicly available molecular/clinical breast cancer databases. These databases will be analyzed to determine if there are any molecular alterations that may be predictive of CDK4/6i efficacy (Chapter 2)

OBJECTIVE 3: To identify predictive proteomic biomarkers of PLB alone or in combination with TMX in breast cancer cell lines. To accomplish this, I established the following sub-objectives for this work:

3a) To comprehensively evaluate the experimental conditions and cell proliferation assays to accurately measure PLB drug efficacy (Chapter 3).

3b) To utilize the optimized experimental conditions to evaluate PLB and TMX efficacy and synergy in the panel of breast cancer cell lines (Chapter 4).

3c) To screen the categorized cell lines for differentially expressed proteins using reverse phase protein array to identify predictive biomarkers of resistance to PLB alone and in combination with TMX (Chapter 5).

1.2 Background on Breast Cancer

1.2.1 Breast cancer subtypes and contemporary treatment

With the use of molecular subtyping, precision medicine approaches are now being used to guide breast cancer treatment [2]. These molecular subtypes have distinct gene expression profiles that determine prognosis and help guide treatment decisions for targeted therapies (Table 1.2.1.1) [2]. Breast cancer is categorized into four main subtypes based on the expression level of three molecular markers: ER, PR, and HER2, which are summarized below.

Luminal Subtypes

Luminal breast cancer is characterized by the expression of ER. This subtype is further classified into two distinct groups, luminal A and luminal B. Luminal A breast cancer is characterized by high expression of ER and/or PR, and negative or low expression of HER2

(Table 1.2.1.1). High ER positivity is defined as >10% of tumour nuclei stained positive by immunohistochemistry (IHC) [18]. Thus, the range of ER positivity is wide and goes from >10 to 100%. Within the luminal B subtype, most tumours (~85%) express low ER levels (low ER is defined as 1-10% of tumor nuclei stained by IHC) and no HER2 expression; the remaining ~15% of luminal B tumours express both ER and HER2 (Table 1.2.1.1) [18,19]. Luminal B breast cancer has an increased proliferation rate and worse prognosis as compared to the luminal A counterpart. These ER-dependent tumours exhibit activation of the estrogen signaling pathway that drives cancer growth through the transcription of genes involved in DNA replication, apoptosis, and the cell cycle [20]. Because of this estrogen dependency, the estrogen receptor has become an obvious target for cancer therapeutics. Multiple AETs have been developed since the 1970s, and they remain the standard-of-care for treating pre- and post-menopausal hormone receptor positive (HR+) (ER+ and PR+/-) subtypes [21]. Three types of AETs have been approved for use: 1) selective estrogen receptor modulators (SERMs), 2) selective estrogen receptor degraders (SERDs), and 3) aromatase inhibitors (AIs) (Table 1.2.1.1).

Table 1.2.1.1 Breast cancer subtype characteristics, treatment options and 5-year survival rate for metastatic disease.

Subtype	Receptor Status	Incidence Rate [19,22]	Characteristics [19]	Treatment Options	Response Rates For Metastatic Disease [9,10,23–27]	5-year Survival Rate for Metastatic Disease [22]
Luminal A	HR+/HER2-	~50%	-Low proliferation rate -Good outcome	AET (SERD, SERM, AI)	30-40% tamoxifen 40-50% letrozole ~10% fulvestrant	32%
Luminal B	HR+/HER2- (low ER levels) **Or** HR+/HER2+	~20%	-High proliferation rate -Aggressive subtype	HER2 targeted agents, AET or chemotherapy	~40% HER2+AI ~30% AI	46%
HER2-enriched	HR-/HER2+	10-15%	-High proliferation rate -Poor outcome	HER2 targeted agents	30-60%	39%
Triple Negative	HR-/HER2-	15-20%	-High proliferation rate -Poor outcome -Aggressive subtype	Chemotherapy	20-70%	12%

Although AETs have increased luminal breast cancer patient survival outcomes in the last few decades, response rates for metastatic disease vary from 10%-50% depending on the type of AET received (Table 1.2.1.1). In patients who derive benefit from AET, the development of acquired resistance to these targeted therapies remains a clinical challenge [28]. Mechanisms of AET resistance have been extensively studied to better understand how to overcome, reverse or delay this resistance. Activation of both the PI3K/mTOR and CDK4/6/Rb pathways have been described as mechanisms of resistance [29]. Within the cell cycle pathway, upregulation of cyclin D1 has been linked to AET resistance in ER+ breast cancer [20,30]. Accordingly, co-targeting activated pathways is currently under evaluation in both the pre-clinical and clinical setting as a way to delay or prevent AET resistance [31].

Human Epidermal Growth Factor Receptor 2-Enriched Subtype

The HER2-enriched breast cancer subtype accounts for 10-15% of new cases [22]. These tumours are negative for ER and PR and have >10% of the tumour nuclei strongly staining for HER2 by IHC (Table 1.2.1.1) [32]. HER2 overexpression activates downstream signaling pathways, leading to tumorigenesis and increased cell proliferation [33]. Similar to exploiting ER for drug development, HER2 overexpression has become the target for novel therapies including Trastuzumab (monoclonal antibody) and lapatinib (tyrosine kinase inhibitor) [33]. Unfortunately, 70% of HER2+ patients experience *de novo* or acquired resistance to single agent Trastuzumab [34]. Mechanisms of resistance to HER2-targeted therapies include activation of PI3K, mTOR and MEK pathways [33]. Pre-clinical work by Goel *et al*., found that targeting the cyclin D1/CDK4/CDK6 axis can re-sensitize tumours to HER2 targeted agents [35]. To overcome resistance, multiple combination therapies have been explored and have demonstrated increased efficacy for treating HER2+ patients [33]. The development of antibody drug conjugates (T-MD1, T-DXd) have greatly increased response rates for HER2+ metastatic breast cancers through targeted drug delivery of chemotherapy in combination with Trastuzumab HER2 blockade [23].

Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is an aggressive subtype with the worst clinical outcome, and accounts for ~15% of breast cancer diagnoses (Table 1.2.1.1) [36]. These tumours have low or absent expression of ER, PR, and HER2, and therefore are not responsive to ER or HER2-targeted therapies [36]. The majority of TNBCs (~75%) can be further classified into the basal-like subtype, with the remaining 25% of TNBCs being comprised of luminal androgen

receptor and mesenchymal subtypes [26]. Chemotherapy remains the standard-of-care for TNBC, even though ~50% of TNBCs develop resistance to this treatment [36,37]. There are currently no approved targeted therapies for TNBC, highlighting the need for novel therapeutics to provide these patients with better treatment options [38].

In summary, the development of targeted agents has increased overall patient survival in HR+ and HER2+ patients; however, resistance to these drugs remains a pressing clinical challenge [31,39]. Additionally, 5-year survival rates for metastatic disease remain low, with a 12-46% survival rate across all breast cancer subtypes (Table 1.2.1.1) [22]. Novel treatment options are needed to increase patient response rates and to overcome inevitable treatment resistance.

1.2.2 The cell cycle

The cell cycle is regulated by the ER and HER2 pathways, making it a promising drug target following the development of resistance to ER and HER2-targeted therapies. In normal cells, progression of the cell cycle through its distinct phases is tightly regulated by a series of checkpoints. The cyclin D1/CDK4/6 complex is a key regulator of cell cycle progression through the G1/S phase. The tumor suppressor, p16 - a natural CDK4/6i - regulates the formation of this complex by binding to CDK4 and inhibiting kinase activity. As shown in Figure 1.2.2.1, in the absence of p16, the cyclin D1/CDK4/6 complex mono-phosphorylates the downstream retinoblastoma (Rb) tumour suppressor, leading to the release of initial E2F transcription factors. Initial E2F transcription factors induce the expression of cyclin E1, allowing for the formation of the cyclin E1/CDK2 complex that hyper-phosphorylates Rb. The Rb tumour suppressor becomes

inactive and fully releases E2F transcription factors, ultimately stimulating progression into the S phase of the cell cycle (Figure 1.2.2.1).

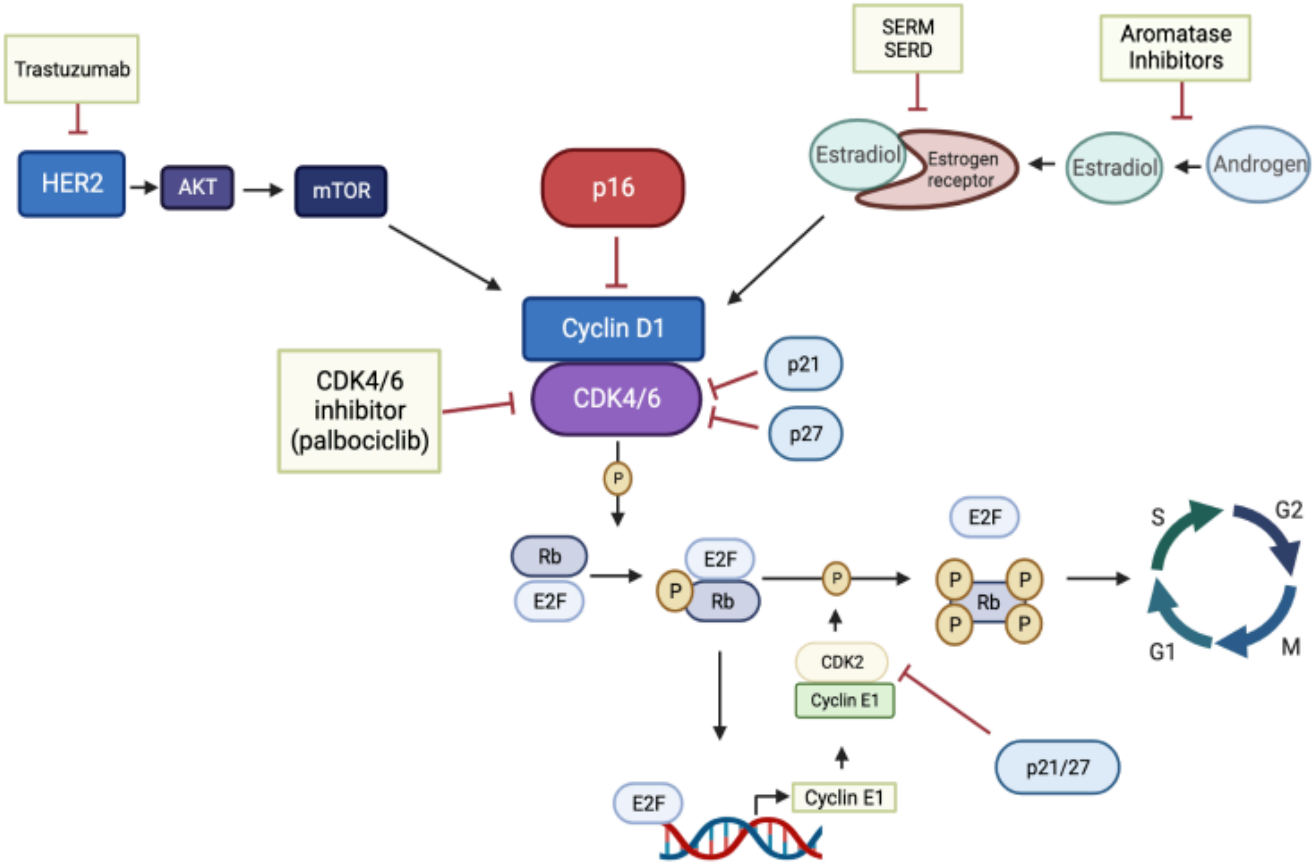

Figure 1.2.2.1 Cyclin D1/CDK4/6 pathway in breast cancer, and interactions of common signaling pathways on cyclin D1 expression. Pathway schematic adapted from Sobhani *et al.* (2019) [40] and created using BioRender.com [41]. SERM: Selective estrogen receptor modulator, SERD: selective estrogen receptor degrader.

Alterations within the cell cycle pathway at the G1/S phase are common in all breast cancer subtypes, as highlighted in Table 1.2.2.1. Negative p16 expression occurs more frequently in luminal and HER2-enriched subtypes, and increased cyclin D1 expression is seen in 60-70% of luminal breast cancer subtypes, as determined by IHC [42]. The Rb tumour suppressor is often retained in HER2-enriched and luminal subtypes in comparison to 20% of TNBC, which lack

functional Rb [43]. Elevated cyclin D1 has also been described as a marker of AET resistance [20]. These alterations which perpetuate an unchecked cell cycle present a targetable vulnerability in breast cancer, and have led to the evaluation of cell cycle inhibitors has a novel therapeutic option for breast cancer patients [30,44].

Table 1.2.2.1 Common cell cycle alterations in breast cancer subtypes [42,43,45].

Subtype	CDK4 gene amplification [43]	Cyclin D1 expression >50% (IHC) [42]	Negative Rb (IHC) [45]	Negative p16 (IHC) [45]
Luminal A	14%	60%	8%	23%
Luminal B	25%	71%	15%	30%
HER2+	24%	31%	13%	26%
TNBC	-	11%	19%	6%

1.2.3 Development of CDK4/6 inhibitors as a targeted therapy for breast cancer

The cell cycle is a promising target for cancer drugs to regulate unchecked proliferation. As a key cell cycle regulator, the frequent loss of p16 prompted the evaluation of CDK4/6i and showed that these inhibitors could reverse the effects of p16 loss on cell cycle progression at the G1/S phase (Figure 1.2.2.1) [4]. Currently, three CDK4/6i are approved for use in combination with AET to treat both pre- and post-menopausal advanced or metastatic ER+ breast cancer : PLB, ribociclib, and abemaciclib [46].

PLB was the first CDK4/6i approved by the FDA in 2015 and is extensively used to treat metastatic breast cancer [5]. Thus, PLB was chosen as the CDK4/6i to evaluate for this project. PLB is a highly selective reversible cell cycle inhibitor with an half maximal inhibitory concentration (IC50) of 11nM and 15nM for CDK4 and CDK6, respectively [47]. This CDK4/6i

elicits cytostatic effects by preventing the progression of cells from the G1 phase into the S phase. Finn *et al.* (2009) evaluated PLB in the pre-clinical setting, where they found luminal breast cancer cell lines exhibited increased sensitivity to PLB. Furthermore, synergistic effects were seen when PLB was combined with TMX [44]. TMX is a SERM that competitively binds to ER, eliciting a cytostatic effect due to the downstream stalling of cell proliferation within the G0 and G1 phases of the cell cycle [48]. In this project, I treated breast cancer cell lines with PLB and TMX to identify predictive proteomic biomarkers of efficacy at the pre-clinical level. TMX was selected as it is one of the most commonly used AET for adjuvant treatment in both pre- and post-menopausal women and the drug has been used extensively for *in vitro* testing [49].

1.2.4. Clinical trials evaluating palbociclib

In the pre-clinical research reported by Finn *et al.* (2009), PLB efficacy was evident, particularly in luminal and HER2+ breast cancer subtypes [44]. Confirmatory results subsequently led to the development of multiple clinical trials to study PLB in breast cancer patients. Multiple clinical trials using PLB to treat breast cancer patients are currently ongoing in all breast cancer subtypes as highlighted below (Table 1.2.4.1 and Appendix B-B1).

HR+/HER2- clinical trials

PLB was evaluated both as a monotherapy and in combination with AET (AI or SERD) in the TREnd trial [50]. A clinical benefit was observed with single PLB treatment, though the duration of benefit favoured the combination arm when compared to PLB alone (PFS of 11.5 versus 6 months, respectively) (Table 1.2.4.1) [50]. The combination of PLB plus AET was

further evaluated in the PALOMA trials [51]. The phase I/II PALOMA-1 trial evaluated PLB in combination with letrozole as a first-line treatment for post-menopausal recurrent ER+/HER2- breast cancer. This study showed a 10-month improvement in PFS compared to letrozole alone (Table 1.2.4.1), these results led to the accelerated approval of PLB by U.S. Food and Drug Administration (FDA) [5,51]. The PALOMA-1 findings were further validated in a larger phase 3 trial, PALOMA-2, where the combination resulted in a 13.1 month increase in PFS compared to letrozole alone [52]. PLB was well tolerated, with the most common adverse reaction being neutropenia. From these results, PLB received regular approval by the FDA for use in combination with letrozole as a first line treatment for recurrent/metastatic breast cancer [5]. Despite the remarkable improvements in PFS, upon long term follow up no statistically significant overall survival benefit was seen with the addition of PLB to letrozole treatment in either the PALOMA-1 or PALOMA-2 trials [6,7].

PLB was further evaluated in the PALOMA-3 trial, these results led to the expanded approval of PLB to include treating pre- and post-menopausal HR+/HER2- metastatic breast cancer in combination with fulvestrant following disease progression on AET [53]. The combination of PLB + fulvestrant resulted in a 6.6 month increase in PFS compared to fulvestrant alone [54]. The PALOMA-3 trial was the only PALOMA trial to demonstrate statistically significant improvement in overall survival (34.9 months with PLB + fulvestrant combination vs 28 months with fulvestrant alone) [54]. Additional trials are ongoing to further evaluate PLB efficacy in the ER+/HER2- subtype as outlined in a review by Chen *et al.* (2019) [55], including three trials evaluating PLB + TMX combination. Results from the single arm phase 2 trial, NCT0266866, found that PLB + TMX as a first line treatment for metastatic breast cancer elicited a 30% response rate in the 47 patients evaluated, and the combination was well

tolerated [56]. Results from the other PLB + TMX trials are pending (Table 1.2.4.1).

HER2-enriched and TNBC subtype clinical trials

In the HER2+ subtype, pre-clinical studies found that targeting the cell cycle may re-sensitize cells to HER2-targeted agents after the development of resistance [23]. Multiple clinical studies are underway to further evaluate PLB efficacy in the HER2+ breast cancer subtype. Treatment with single PLB and PLB in combination with HER2-targeted agents, chemotherapy or AET (for luminal B HER2+ patients only) are being evaluated in HER2-enriched patients (Appendix B-B1). The NA-PHER2 trial evaluated neoadjuvant dual HER2 therapy + fulvestrant + PLB, in non-metastatic ER+/HER2+ breast cancer patients. During an interim analysis, results on the primary outcome measure, a change in Ki67 levels, were reported [57]. The authors reported that the four drug combination decreased the expression of the Ki67 proliferation marker when compared to the pre-treatment baseline [57]. This treatment combination was well tolerated, neutropenia was the most common adverse effect (seen in 29% of patients), and warrants further evaluation [57]. The results from the other ongoing trials are pending (Appendix B-B1).

Although TNBC patients are thought to be poor candidates for CDK4/6i, due to their increased frequency of *RB1* mutations or loss of Rb, a subset of TNBCs that express the androgen receptor may derive benefit for CDK4/6i as they commonly retain Rb function [39]. A clinical trial (NCT02605486) is currently evaluating PLB in combination with androgen receptor-targeted agents for this subset of patients. Additional trials are exploring various combinations with PLB, including chemotherapy and MEK inhibitors in TNBC patients, these are ongoing and summarized in Appendix B-B1.

Table 1.2.4.1 Clinical trials evaluating palbociclib in combination with anti-estrogen therapy (fulvestrant, letrozole or tamoxifen) in HR+/HER2- breast cancer. AI: Aromatase Inhibitor; AET: Anti-estrogen therapy; CBR: Clinical benefit rate; ER+: Estrogen receptor positive; HER2-: Human epidermal growth factor receptor 2 negative; HR+: Hormone receptor positive; OS: Overall survival; PFS: Progression free survival; PLB: Palbociclib; pts: Patients. Clinical benefit rate is defined by the percentage sum of complete responses, partial responses, and stable disease for at least 24 weeks according to RECIST 1.1 criteria.

Clinical Trial Name	Trial phase	Trial duration	Drug combination	Patient population		Treatment line (prior treatment allowed)	Sample size	Primary outcome (combo vs control)	Secondary outcome (combo vs control)	Overall Survival Difference	Objective Response Rate for measurable disease	References
				Menopausal status	Disease characteristics		#pts in the analysis (#pts recruited)					
PALOMA-1/TRIO (NCT00721409)	Phase I/II	2008-2017	Palbociclib + Letrozole vs Letrozole + Placebo	Post-menopausal	Cohort 1: ER+/HER2- recurrent breast cancer. Cohort 2: ER+/HER2- with Cyclin D1 amplification and/or p16 loss	First Line (no prior therapy for advanced disease)	165 (400)	PFS 20.2 vs 10.2 months (Hazard Ratio 0.49, p<0.001)	OS 37.5 vs 34.5 months, not statistically significant	**3 months**	55.4% vs 39.4%	[6,10,51]
PALOMA-2 (NCT01740427)	Phase III	2012-present	Palbociclib + Letrozole vs Letrozole + Placebo	Post-menopausal	ER+/HER2- recurrent or metastatic breast cancer	First Line (no prior therapy for advanced disease)	666 (875)	PFS 27.6 vs 14.5 months (Hazard Ratio 0.56, p<0.001)	OS 53.9 vs 51.2 (Hazard Ratio 0.956) months, not statistically significant	**2.7 months**	60.7% vs 49.1%	[9,12,52]
PALOMA-3 (NCT01942135)	Phase III	2013-present	Palbociclib + Fulvestrant vs Fulvestrant + Placebo	Pre- and post-menopausal	HR+/HER2- metastatic breast cancer with progression	Second line (any line of AET treatment, ≤1 chemotherapy allowed)	521 (711)	PFS 11.2 vs 4.6 months (Hazard Ratio 0.50, p<0.001)	OS 34.9 vs 28.0 months (Hazard Ratio 0.814, p=0.09)	**6.9 months**	24.6% vs 10.9%	[11,13,54,58]
TREnd (NCT02549430)	Phase II	2015-2017	Palbociclib + AET (AI or Fulvestrant) vs Palbociclib	Post-menopausal	ER+/HER2- metastatic breast cancer	Second line (1-2 lines previous AET, ≤1 chemotherapy line allowed)	110 (115)	CBR 54% vs 60% (p=0.52) (combo vs PLB)	PFS 10.8 vs 6.5 months (Hazard Ratio 0.69, p=0.13); duration of benefit 11.5 vs 6 months	N/A	11% vs 7% (combo vs PLB)	[50,59]
NCT02668666	Phase II	2016-2021	Palbociclib + Tamoxifen	Pre- and post-menopausal	HR+/HER2+ de novo metastatic or recurrent breast cancer	First Line (no prior therapy for advanced disease)	47 (49)	PFS 14.6 months for de-novo MBC, 6 months for recurrent	Adverse effects 51% pts developed grade 3 neutropenia	N/A	30%	[56]
NCT03423199 (PATHWAY)	Phase III	2018-present	Palbociclib + Tamoxifen vs TMX alone	Pre- and post-menopausal	Asian HR+ HER2- advanced or MBC	First or second line (progressed on AI)	180	PFS	OS	N/A	N/A	[60]
NCT02384239	Phase II	2015-present	Palbociclib + Fulvestrant or Palbociclib + Tamoxifen	Pre- and post-menopausal	HR+ locally advanced or metastatic breast cancer	Prior treatment with PI3Ki	70	Percentage of patients with grade 3/4 neutropenia	PFS	N/A	N/A	[25]

1.2.5 Precision medicine and achieving best outcomes using CDK4/6 inhibition

Novel targeted therapies provide substantial benefit to patients with metastatic breast cancer whose tumours develop *de novo* or acquired resistance to currently available drugs. CDK4/6i are now routinely used in combination with AET for the treatment of advanced or metastatic ER+ breast cancer. In the 7 years since its approval, PLB has been prescribed to over 450,000 patients across 100 different countries [61]. Recently, real-world data from a large retrospective database analysis found the addition of PLB to AI treatment increased overall survival by 5.9 months and PFS by 5.4 months when compared to AI treatment alone [61]. It is important to note that the response rates from the PALOMA trials, which lead to the approval of PLB, were limited to 25-60% [9–11]. CDK4/6i are generally well tolerated and toxicities can be managed with dose reduction; however, some patients still experience neutropenia, leukopenia and fatigue [46].

Novel targeted therapies are very expensive, the costs associated with these treatments have become a financial burden to the health care system and some question the sustainability of using targeted therapies without biomarkers to guide treatments [14,62]. Treatment with PLB for one month costs ~$8,000, resulting in an annual cost of ~$96,000 for one patient [63]. Thus, predictive biomarkers of PLB efficacy are of high importance to address the increasing financial burden of cancer care, specifically to ensure that drug expenditure is optimized. There is also a pressing need for biomarker research that is designed in such a way where biomarkers can be validated for use in the clinical setting. This research aims to identify predictive biomarkers that will lead to better patient selection for treatment, reduce toxicities, and ensure efficient use of our health care resources.

Chapter 2 – Analysis of existing biomarkers from literature and online databases

2.1 Background and Rationale

In recent years, the knowledge of molecular mechanisms for drug efficacy has expanded exponentially, resulting in a multitude of predictive biomarker publications [64]. With advancing -omic technologies, biomarker research is expanding into all aspects of cancer cell regulation, including genomics, transcriptomics, proteomics and epigenetics [65]. Despite this, it has been challenging to translate biomarkers from preclinical discovery into clinical application. In fact, there are a limited number of clinically validated biomarkers currently used to predict drug response [64]. As part of my thesis, I undertook two strategies to obtain information on existing biomarkers for PLB efficacy in breast cancer. Firstly, I performed a literature review on PLB predictive biomarkers to establish the background for this chapter (Objective 1). Second, I analyzed existing clinical databases with both outcomes for patients treated with CDK4/6i and molecular data from a translational component (Objective 2).

Current state of knowledge relating to PLB biomarkers: Existing pre-clinical data

For my literature review (Objective 1), I examined existing publications to identify PLB predictive biomarkers. Preclinical work by Finn *et al.* (2009) found three transcripts directly related to the CDK4/6 pathway to be predictive of increased PLB response in breast cancer cell lines [44]. Lower levels of *CDKN2A* and higher levels of *CCND1* and *RB1* mRNA were correlated with PLB sensitivity [44]. The predictive value of these cell cycle markers was further explored in the PALOMA trials. The PALOMA-1 trial included a cohort of patients with cyclin D1 (*CCND1)* gene amplification and p16 loss (CDKN2A), as determined by fluorescence *in situ*

hybridization (FISH); however, this cohort analysis was terminated early as no increased benefit to PLB + letrozole combination was observed [51]. The PALOMA-2 trial evaluated common cell cycle molecules at both protein (cyclin D1, Rb, and p16) and mRNA (*CCND1, CCNE1/2, CDK2/4/6, RB1*, and *CDKN2A*) levels in formalin-fixed paraffin-embedded (FFPE) breast cancer tissues; however, no correlation with response to PLB + letrozole was seen [66,67]. Instead, this trial found that lower mRNA levels of *PD-1* and increased mRNA levels of *FGFR2* and *ERBB3* were associated with PLB + letrozole sensitivity [67]. Biomarker evaluation from the PALOMA-3 trial found lower levels of *CCNE1* (mRNA from FFPE tissue) were associated with PLB + fulvestrant sensitivity [68]. The potential biomarkers (lower mRNA levels of *PD-1* and *CCNE1*, and increased mRNA levels of *FGFR2* and *ERBB3*) identified in these large-scale correlative trials have yet to be further validated [67,68]. Multiple clinical trials have evaluated the predictive value of several biomarkers with a lack of success [12,13,51]. Technical limitations, such as the use of analytical techniques based on measuring mRNA expression in FFPE tumor tissues, could be one explanation for failed efforts to discover useful biomarkers [69].

Although low p16 levels were not predictive of PLB response in the PALOMA clinical trials, additional preclinical work has found overexpression of p16 to be correlated with CDK4/6i resistance. CDK4/6i resistance was seen in ER+ breast cancer cell lines and both ER+ and ER- patient derived xenografts that overexpressed p16 [70,71]. P16 overexpression is a rare occurrence in ER+/HER2- breast cancer but is more frequently seen in ER- breast cancer, therefore this biomarker may hold more predictive value in ER- breast cancer [15].

Rb expression was not found to be predictive of PLB response in the PALOMA trials, however Rb loss has been described as a mechanism of intrinsic and acquired resistance to CDK4/6i [43,72]. In the absence of Rb, cell cycle regulation bypasses the CDK4/6 axis resulting

in constitutive activation of E2F transcription factors (Figure 1.2.2.1). The PALOMA-3 trial evaluated circulating tumor DNA from pre- and post- treatment plasma samples, it was found that 5% of patients acquired an *RB1* mutation after PLB + fulvestrant treatment [73]. Recently, Palafox *et al.* (2022) identified heterozygous loss of *RB1* as a marker of acquired resistance to CDK4/6i (ribociclib) in patient derived xenografts [71]. Another proposed mechanism of resistance is the activation of cyclin E1/CDK2 axis as this complex phosphorylates Rb leading to the release of E2F factors, which could bypass CDK4/6 inhibition (Figure 1.2.2.1). Amplification of *CCNE1* has been described as a marker of resistance to PLB in multiple *in vitro* studies [74,75]. This finding was strengthened in the PALOMA-3 trial where they found that lower levels of *CCNE1* were associated with PLB sensitivity [68].

Other pre-clinically identified biomarkers of PLB response include T172 phosphorylation of CDK4 [70] and low levels of Y88 phosphorylated p27 [76], each of which have been correlated with PLB sensitivity. Loss of function *FAT1* mutations have been described as a marker of resistance [72]. A retrospective analysis by Lee *et al.* (2022) using next-generation sequencing data from patients who had previously received a CDK4/6i, identified several genomic alterations that were predictive of CDK4/6i resistance [77]. These alterations included *PTEN* and *CDKN2A* loss, *FGFR1, CDK4, MDM2* and *FRS2* amplification and *BRCA1* and *ERBB2* mutations [77].

While multiple biomarkers have been identified in the pre-clinical setting, further evaluation and validation are required for them to be used in a clinical setting. As the literature review did not reveal any validated biomarkers for clinical use, I undertook an analysis of existing databases containing molecular and clinical data from PLB-treated breast cancer patients to search for predictive biomarkers of CDK4/6i efficacy (Objective 2).

2.2 Methods

Three publicly available databases were identified containing treatment data on metastatic breast cancer patients:

1) Count Me In: The Metastatic Breast Cancer Project;
2) The Cancer Genome Atlas within the National Cancer Institute Genomic Data Commons Data Portal;
3) American Association for Cancer Research Genomics Evidence Neoplasia Information Exchange (AACR GENIE).

Each database was searched to identify clinical and molecular data on patient samples previously treated with a CDK4/6i. The Count Me In: The Metastatic Breast Cancer Project database did not contain information on CDK4/6 treatment, and the Cancer Genome Atlas within the National Cancer Institute Genomic Data Commons Data Portal was limited to radiation treatment only. The AACR Project GENIE AKT1 cohort was the only database that contained CDK4/6i data [78]. Accordingly, the AACR GENIE database alone was mined to 1) identify novel biomarkers and 2) interrogate previously evaluated biomarkers that were identified in the clinical trials highlighted above (PALOMA-1, -2, -3).

In the AACR GENIE AKT1 cohort, samples from patients who underwent CDK4/6i treatment were collected as FFPE tissues from five different sites, including MD Anderson Cancer, Princess Margaret Cancer Centre, Memorial Sloan Kettering, Dana-Farber Cancer Institute, and Vanderbilt-Ingram Cancer Center. This database contained gene mutation and copy number alteration (CNA) data generated by each center using different gene sequencing panels. Therefore, the number of genes analyzed varied depending on the collection site of the sample.

The analysis of the AACR GENIE database was conducted using the cBioPortal platform with R for statistical analysis. Since data on PFS was not available, I used CDK4/6i treatment duration (long versus short) as a surrogate measure of PFS. Non-parametric Wilcoxon rank-sum tests were used to compare clinical attributes (including age, race, tumour stage, previous treatment) between long and short CDK4/6i treatment duration groups (p-value < 0.05). All figures and tables presented in this chapter were generated using cBioPortal, and raw data is available on that platform (cBioPortal.org) [79].

2.3 Results

The AACR GENIE AKT1 cohort contained clinical and molecular information on 428 breast cancer patients, of which 126 patients were treated with a CDK4/6i. Multiple treatment types were reported, including chemotherapy, fulvestrant, and AIs. This database reported duration of CDK4/6i treatment (rather than PFS) and did not specify which CDK4/6i or AET the individual patients were treated with.

To identify potential genomic biomarkers of CDK4/6i response or resistance, the CDK4/6i treatment duration data from 126 patients was divided into quartiles. The lower quartile - shortest duration (0-2.3 months) - and upper quartiles - longest duration of CDK4/6i treatment (10.3-29.0 months) - were selected for further analysis and comparison. All patients included in the analysis were female and the patient characteristics were similar between the two groups (Table 2.3.1). The short CDK4/6i treatment duration group had an OS of 52.34 months and the long CDK4/6i treatment duration group had an OS of 95.72 months (Figure 2.3.1). When comparing the clinical characteristics of the two groups, the shorter CDK4/6i treatment group

had statistically significant shorter first and second line treatment durations and an increased number of chemotherapy lines when compared to the long treatment group (Table 2.3.2).

Table 2.3.1 Baseline characteristics of breast cancer patients with short versus long CDK4/6i treatment duration.

	Short duration of CDK4/6i treatment (0-2.3 months) n=32	**Long duration of CDK4/6i treatment (10.3-29.0 months) n=31**
Age at primary diagnosis	52 (26-69)	50 (28-65)
Metastatic	13 (41%)	17 (55%)
Primary	15 (47%)	10 (32%)
ER+/PR+ expression	24 (75%)	25 (81%)
ER+/PR- expression	5 (16%)	3 (10%)
ER- expression	2 (6%)	1 (3%)
HER2- expression	28 (88%)	27 (87%)
Previous endocrine sensitivity	23 (72%)	31 (100%)
Average therapies in metastatic setting	4 (1-13)	2 (1-9)
Received CDK4/6i in first line	14 (43%)	7 (23%)
Received CDK4/6i in second line	8 (25%)	6 (19%)
Received CDK4/6i in unknown line	10 (32%)	18 (58%)

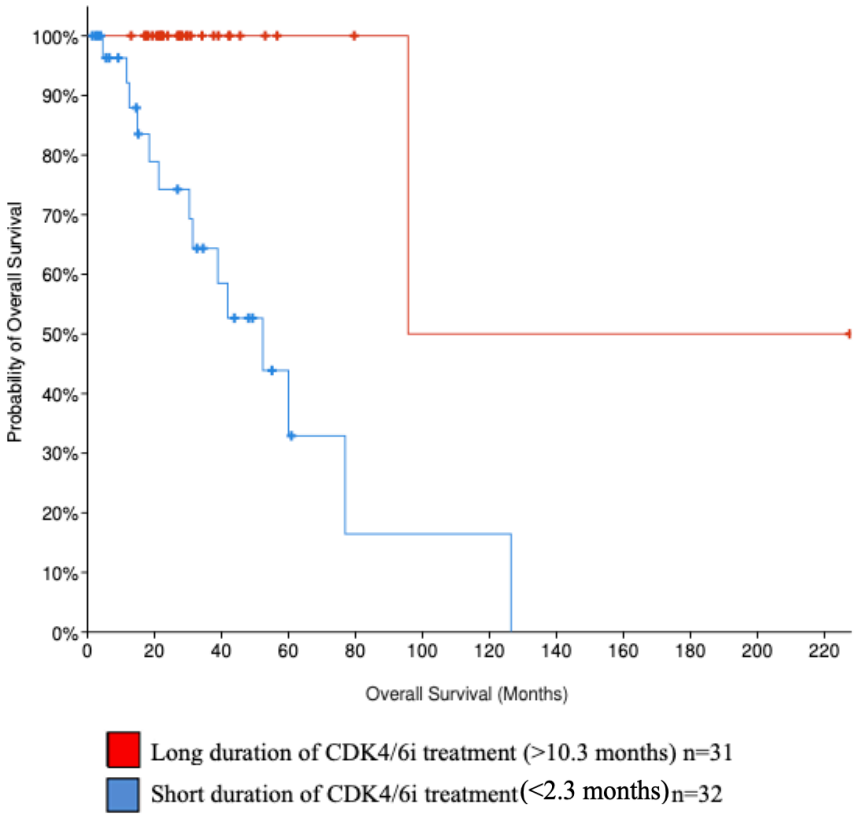

Figure 2.3.1 Kaplan Meier survival curve for breast cancer patients with long (>10.3 months) and short (<2.3 months) duration of CDK4/6i treatment. Overall survival was determined using the time from metastatic diagnosis to death or date of last follow-up. The graph was generated using cBioPortal.org [79].

Table 2.3.2 Statistically significant clinical attributes of breast cancer patients with short duration of CDK4/6i treatment (<2.3 months) compared to patients with a long duration of CDK4/6i treatment (>10.3 months). The table generated from cBioPortal.org [79].

Clinical Attribute	Statistical Test	p-Value
Duration of CDK4/6 treatment (any line) (Months)	Wilcoxon Test	≤0.05
Chemotherapy lines received in Metastatic Treatment	Wilcoxon Test	≤0.05
Duration of first-line treatment (Months)	Wilcoxon Test	≤0.05
Duration of second-line treatment (Months)	Wilcoxon Test	≤0.05
Total number of therapies received in metastatic disease treatment	Wilcoxon Test	≤0.05

Copy Number Alterations

Some differences in CNA were observed between the shortest (<2.3 months) and the longest (>10.3 months) CDK4/6i treatment duration groups but were not statistically significant. Of interest, *RIT1* amplification and *RB1* homozygous deletion were more commonly seen in the

short treatment duration group, whereas *MYC, NBN* and *RAD21* amplification were most common in the long treatment duration group (Table 2.3.3). Despite this, CNA frequency was not higher than 16.1%. Similar to the inconclusive results of the PALOMA trials, alterations within the cell cycle pathway were common in both short and long responders [51,67]. Although the long duration of treatment group had a higher percentage of alterations in *CCND1* and *CDKN2A*, these findings were not statistically significant (Table 2.3.3).

From the PALOMA-2 and -3 trials, increased *FGFR2* mRNA and low levels of *CCNE1* mRNA were found to correlate with PLB + AET response [67,68]. Although this data base did not contain mRNA expression data, CNA of *FGFR2* and *CCNE1* were evaluated. Amplification of *CCNE1* was observed in 3.2% and 0% of samples in the long and short CDK4/6i treatment duration groups, respectively. *FGFR2* amplification was only seen in one sample in both the long and short CDK4/6i treatment groups. Due to the low frequency of these alterations seen in the explored database, further validation of *CCNE*1 and *FGFR2* mRNA expression and CNA as predictive biomarkers of CDK4/6i response is needed.

Table 2.3.3 Frequent copy number alterations in breast cancer patients grouped by short CDK4/6i treatment (<2.3 months) and long CDK4/6i treatment (>10.3 months). Results between the two groups were not statistically significant. AMP= Amplification, HOMDEL= Homozygous deletion.

Short Duration of CDK4/6i Treatment			**Long Duration of CDK4/6i Treatment**		
Gene	**N (%)**	**CNA**	**Gene**	**N (%)**	**CNA**
CCND1	5/27 (18.5%)	AMP	*CCND1*	9/31 (29.0%)	AMP
RIT1	2/27 (7.4%)	AMP	*MYC*	5/31 (16.1%)	AMP
RB1	2/27 (7.4%)	HOMDEL	*NBN*	3/31 (9.7%)	AMP
CDKN2A	2/27 (7.4%)	HOMDEL	*CDKN2A*	3/31 (9.7%)	HOMDEL
FGFR2	1/27 (3.7%)	AMP	*RAD21*	3/31 (9.7%)	AMP
			AKT1	2/31 (6.5%)	AMP
			GNAS	2/31 (6.5%)	AMP
			RECQL4	2/31 (6.5%)	AMP
			MCL1	2/31 (6.5%)	AMP
			FGFR2	1/31 (3.2%)	AMP
			CCNE1	1/31 (3.2%)	AMP

Mutations

Patients who received CDK4/6i treatment for a longer duration had a higher frequency of *AKT1* (32.26%) mutations compared to the shorter CDK4/6i treatment duration group (18.75%) (Table 2.3.4). All the *AKT1* mutations seen in this dataset were activating $AKT1^{E17K}$ mutations. *AKT1* is a key regulator of the PI3K/AKT pathway which promotes cell growth and survival, and activation of *AKT* is associated with deregulated kinase activity. *GATA3* was also more commonly mutated in the long treatment duration group (19.35% vs 7.41%) (Table 2.3.4). In the long treatment duration group, all the *GATA3* mutations were driver mutations, with 5/6 mutations being truncating mutations and one missense mutation. In contrast, the *GATA3* mutations present in the short treatment group were in-frame or missense mutations of unknown significance. These findings suggest that *GATA3* driver mutations are correlated with longer CDK4/6i treatment, however due to the limited sample size, these findings should be interpreted with caution. *GATA3* is an ER associated gene and frequently mutated in luminal A breast cancer [19]. Due to the limited sample size in each group, none of the mutations were statistically significant between the two groups.

Table 2.3.4 Frequent gene mutations in the short (<2.3 months) versus long (>10.3 months) duration of CDK4/6i treatment in breast cancer patients.

Gene	Short CDK4/6i Treatment n=32, N (%)	Long CDK4/6i Treatment n=31, N (%)
AKT1	6/32 (18.75%)	10/31 (32.26%)
GATA3	2/27 (7.41%)	6/31 (19.35%)
MAP3K1	2/27 (7.41%)	4/31 (12.90%)
ARID1B	1/27 (3.70%)	3/30 (10.00%)
NOTCH1	NA	3/31 (9.68%)
MAP2K4	2/27 (7.41%)	3/31 (9.68%)

2.4 Discussion/Conclusions

This chapter addressed Objective 1 - PLB biomarker literature search (background) - and Objective 2 - analysis of publicly available databases to identify predictive biomarkers of response to CDK4/6i + AET treatment in breast cancer. Although CDK4/6i have been approved for use since 2015 and prescribed to many breast cancer patients within and outside the context of clinical trials, the number of publicly available databases containing clinical and molecular information regarding CDK4/6i treatment information remains very limited. From the three breast cancer databases containing treatment data, the AACR GENIE AKT cohort database was the only identified dataset that included CDK4/6i treatment information. This database was generated to answer a different question relating to *AKT1* mutations, and using it to identify potential CDK4/6i biomarkers comes with limitations [80]. I was unable to correlate the drug effect with regular reported outcomes as the data was limited to duration of CDK4/6i treatment rather than PFS. Unfortunately, not having PFS data in the GENIE database, makes it hard to draw any firm conclusions from this analysis.

From the AACR GENIE database, I found that heavily pretreated patients who experienced shorter duration of response to first and second lines of treatment were more likely to derive shorter benefit to a CDK4/6i. This observation could suggest that patients demonstrating resistance at any time of their treatment may be more likely to be CDK4/6i resistant. *RB1* homozygous deletion was seen in 7.4% of patients in the shorter CDK4/6i treatment duration group. This alteration is uncommon in HR+/HER2- breast cancer, but has previously been described as a mechanism of resistance to CDK4/6i [43]. CNAs within the cell cycle pathway, *CCND1* and *CDKN2A,* were not significantly associated with a specific treatment duration group. *AKT1* and *GATA3* were the most common mutations in both treatment duration

groups but were more frequently mutated in the long CDK4/6i treatment duration group. Both of these mutations have been correlated with a decreased response to AET [29,81], however the addition of CDK4/6i to AET-resistant cells has been previously reported to re-sensitize cells to AET [44].

I was unable to directly evaluate the biomarkers identified in the PALOMA trials because the GENIE database did not contain mRNA expression data. Complete and comprehensive clinical databases using standardized molecular data and detailed clinical information are needed to obtain robust biomarker results. Clinical trial data can be very informative for these types of analysis if molecular data has been collected for translational research.

In summary, no statistically significant CNA or mutation biomarkers were identified from the GENIE database. From the work outlined in this chapter, validation of the predictive biomarker candidates identified in the literature should include the following: p16, Rb loss, phosphorylated CDK4, MDM2, PTEN and FGFR1/2. As I will now outline in the subsequent chapters of my thesis, there is adequate justification to proceed with the aims and work outlined. Additional preclinical research is needed, and I chose to focus the identification of proteomic biomarkers in the panel of breast cancer cell lines.

Chapter 3- Defining optimal *in vitro* drug testing methods and analyses for CDK4/6i treatment evaluation

3.1. Background and Rationale

Various experimental assays have been used in the past to perform drug sensitivity testing *in vitro*. In general, cancer cell line models, both 2D and 3D, maintain genomic aberrations that are present in the tumours which they are derived from [82]. Immortalized cell line models proliferate indefinitely and provide a continuous supply of cells for experimental work. However, the reliance on *in vitro* testing using cancer cell line models has its limitations, including the inability to study the impact of tumour heterogeneity, the tumour microenvironment, and the impact of the immune system on drug response. Despite these limitations, cell line models are instrumental tools used in scientific research to evaluate drug sensitivity.

Our lab has successfully identified predictive biomarkers using *in vitro* drug testing on patient-derived cell line models [16]. From this work, we determined that selecting appropriate experimental designs were necessary to ensure accurate drug response evaluation and reporting. We found 4-day proliferation assays with Incucyte live-cell imaging were superior at capturing MEK inhibitor drug effect when compared to conventional 3-day IC50 experiments in our low-grade serous ovarian cancer cell lines [83].

Previous work has outlined the specific limitations using IC50 as assays for drug sensitivity testing. These are as follows: 1) results can be affected by the intrinsic differences in doubling times or cell line growth rates, 2) the dose ranges used for *in vitro* experimentation are often very high, significantly outside of the pharmacologic *in vivo* dosing range, and 3) experiments are run for a short period of time, without considering the mechanism of drug

action. Hafner *et al.* (2016) reported on these confounders that exist with conventional drug assays (IC50 values and area under the curve) that rely on experimental endpoint data to report drug sensitivity [84].

In preparation for my work on breast cancer cell lines I examined two papers that provided data on PLB sensitivity in breast cancer cell lines. Finn *et al.* (2009) evaluated drug efficacy in 47 breast cancer cell lines using 6-day cell counting experiments and Rapsé *et al*. (2017) evaluated 20 of the same breast cancer cell lines using a BrdU incorporation assay after 24 hours of PLB treatment [44,70]. When comparing cell line sensitives between the two papers, discrepancies in drug responses were reported. Two resistant cell lines, with IC50 values of >1000nM, reported in the Finn paper were classified as sensitive lines in the Raspé paper, with reported IC50 values of ≤25nM [44,70]. By grouping the cell lines into highly sensitive, intermediate sensitivity and resistant, 20% of cell lines were found to have discordant findings between the two studies. Pre-clinical biomarker identification relies on the accurate ranking of cell line treatment efficacy based on *in vitro* drug testing. These studies highlight how challenging it can be to obtain reproducible *in vitro* results and reflect the challenges of translating preclinical findings into clinical application.

Based on our laboratory's previous experience with *in vitro* drug testing and the discrepancies in PLB drug reporting highlighted above, we sought to optimize our experimental design for this project to best discriminate between PLB sensitive and resistant breast cancer cell lines. This was completed by optimizing the drug concentrations for testing to ensure pharmacologic drug doses were used for both PLB and TMX. Next, I increased the duration of the experiments to assess whether there were specific considerations that relate to the proliferation rates of breast cancer cell lines and the known cytostatic effect of PLB. I also

evaluated different measures of cell proliferation using Incucyte confluence and cell counting to determine the optimal method for measuring drug effect. Finally, I used a growth rate calculator to account for variability in growth rates and initial seeding densities. This chapter (Objective 3a) describes the methodologies and analytical considerations I used to classify responses to PLB +/- TMX in a large panel of breast cancer cell lines (Chapter 4), and later to search for proteomic biomarkers associated with differential drug response (Chapter 5).

3.2 Experimental methodology and analytical considerations

Breast Cancer Cell Lines

A panel of twenty well-characterized commercial breast cancer cell lines were acquired through collaborators (Table 3.2.1). Once received, all cell lines were authenticated by short tandem repeat (STR) profiling and screened for mycoplasma infection (Appendix A – Experimental Methodology). The selected panel of lines included all molecular subtypes of breast cancer, including nine luminal A (9/20=45%; BT483, CAMA-1, HCC1428, MCF7, T47D, MDA-MB-134, MDA-MB-175, MDA-MB-415, ZR-75-1), three luminal B (3/20=15%; BT474, MDA-MB-361, ZR-75-30), four HER2-amplified (4/20=20%; AU565, HCC202, MDA-MB-453, SKBR3) and four TNBC (4/20=20%; BT549, HCC38, MDA-MB-231, MDA-MB-468). The four TNBC cell lines are further classified into the basal-like subgroup. All cell lines were originally cultured in the ATCC recommended media, summarized in Table 3.2.1. Certain cell lines were grown in L-15 media that required incubation in an air-only incubator; however, it was not feasible to use these cell lines for Incucyte experiments, which were performed in a 5% CO_2 incubator. Thus, to simplify the experimental design and reduce the potential variability due to differences in culturing conditions, all cell lines were cultured in RPMI + 10% FBS and grown

at 37°C in a 5% CO_2 incubator for all drug treatment experiments. To ensure changing the media did not alter the expression levels of the known receptors (ER, PR and HER2), protein levels were confirmed by Western blot in all cell lines prior to any *in vitro* drug experiments (Figure 3.2.1, Appendix A – Experimental Methodology).

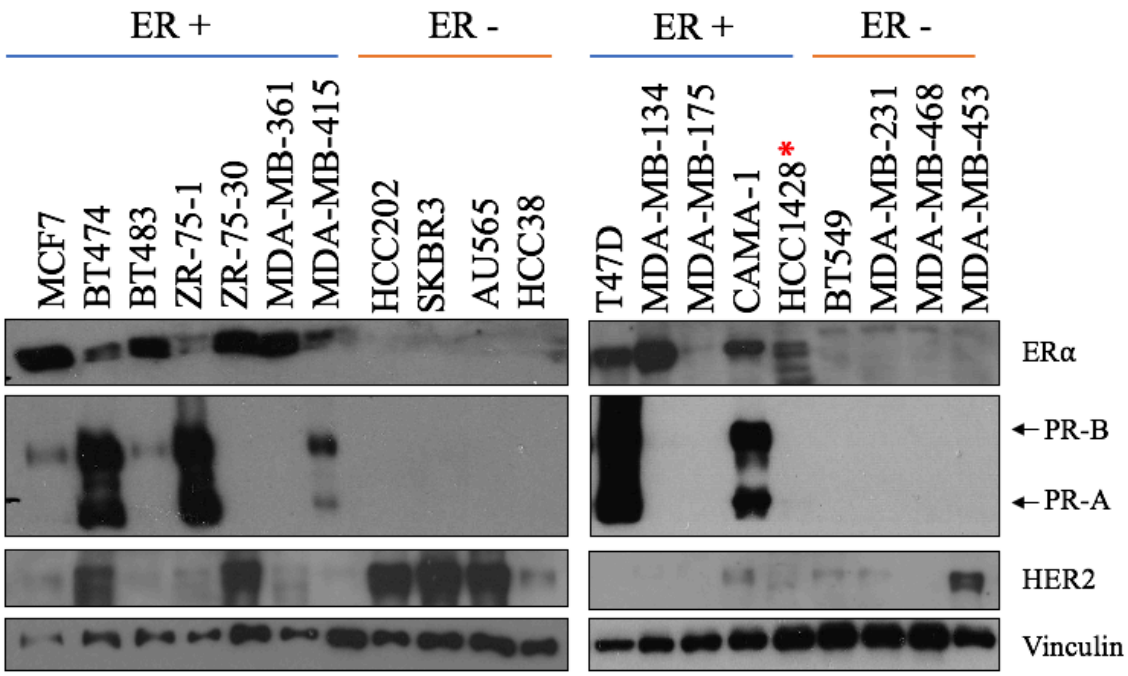

Figure 3.2.1 Western blot of estrogen and progesterone receptor and HER2 expression in twenty breast cancer cell lines. Two gels were run in parallel to evaluate all breast cancer cell lines, within each gel, cell lines were grouped by positive and negative estrogen receptor protein levels. *HCC1428 has PR expression with longer exposure. Cell lysates were prepared from sub confluent cells grown in RPMI +10% FBS.

Table 3.2.1 Twenty breast cancer cell line characteristics, cell culturing conditions and clinical features of tumors where the lines were derived. ER/PR/HER2 status was evaluated by Western blot. ER: Estrogen receptor; FBS: fetal bovine serum; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; TNBC: triple negative breast cancer.

Cell Line	ER Status	PR Status	HER2 status	Diagnosis	Age at Tumour Removal	Source	Ethnicity of Patient	Year Cell Line Was Established	Original Cell Culture Media	Culturing Media
AU565	-	-	+	HER2+	43	Pleural Effusion	White	1970	RPMI + 10% FBS	RPMI + 10% FBS
BT474	+	+	+	Luminal B	60	Primary Breast	White	1978	RPMI + 10% FBS	RPMI + 10% FBS
BT483	+	+	-	Luminal A	23	Primary Breast, Mammary gland	White	1978	RPMI + 20% FBS + 10µg/ml insulin	RPMI + 10% FBS
BT549	-	-	-	TNBC (Basal-like)	72	Primary Breast	White	1978	RPMI + 10% FBS + 10µg/ml insulin	RPMI + 10% FBS
CAMA-1	+	+	-	Luminal A	51	Pleural Effusion	White	1975	EMEM + 10% FBS	RPMI + 10% FBS
HCC1428	+	+	-	Luminal A	49	Pleural Effusion	White	1995	RPMI + 10% FBS	RPMI + 10% FBS
HCC202	-	-	+	HER2+	82	Mammary gland	White	1992	RPMI + 10% FBS	RPMI + 10% FBS
HCC38	-	-	-	TNBC (Basal-like)	50	Primary Breast	White	1992	RPMI + 10% FBS	RPMI + 10% FBS
MCF7	+	+	-	Luminal A	69	Pleural Effusion	White	1973	RPMI + 10% FBS	RPMI + 10% FBS
MDA-MB-134	+	-	-	Luminal A	47	Pleural Effusion	White	1973	L-15% 20% FBS	RPMI + 10% FBS
MDA-MB-175	+	-	-	Luminal A	56	Pleural Effusion	Black	1973	L-15 + 10% FBS	RPMI + 10% FBS
MDA-MB-231	-	-	-	TNBC (Basal-like)	51	Pleural Effusion	White	1973	RPMI + 10% FBS	RPMI + 10% FBS
MDA-MB-361	+	-	+/-	Luminal B	40	Metastatic site, Mammary gland	White	1975	L-15 + 20% FBS	RPMI + 10% FBS
MDA-MB-415	+	+	-	Luminal A	38	Pleural Effusion	NA	1975	L-15 + 15% FBS + 2mM GlutaMax + 10µg/ml insulin + 10µg/ml glutathione	RPMI + 10% FBS
MDA-MB-468	-	-	-	TNBC (Basal-like)	51	Pleural Effusion	Black	1977	RPMI + 10% FBS	RPMI + 10% FBS
MDAMB453	-	-	+	HER2+	48	Mammary gland; Pericardial Effusion	White	1976	L-15 + 10% FBS	RPMI + 10% FBS
SKBR3	-	-	+	HER2+	43	Pleural Effusion	White	1970	McCoy 5a + 10% FBS	RPMI + 10% FBS
T47D	+	+	-	Luminal A	54	Pleural Effusion	NA	1974	RPMI + 10% FBS	RPMI + 10% FBS
ZR-75-1	+	+	-	Luminal A	63	Ascites	White	1978	RPMI + 10% FBS	RPMI + 10% FBS
ZR-75-30	+	-	+	Luminal B	47	Ascites	Black	1978	RPMI + 10% FBS	RPMI + 10% FBS

Four-day Cell Counting Experiments

Breast cancer cell lines were plated in a 48-well plate at experimentally determined seeding densities (6,000-47,000 cells/well) to achieve 20% initial cell confluence 18-24 hours after seeding. Initial cell confluence was measured by live cell-imaging using Incucyte™ ZOOM 2016B and Incucyte® S3 instruments (Sartorius – Essen Biosciences, Ann Arbor, MI, USA). PLB and TMX concentrations were chosen based on the calculated steady-state patient serum levels described in the literature: 259nM and 108nM for PLB and TMX, respectively (Table 3.2.2) [5,51,85]. Cells were treated with DMSO (control, 1μL/mL), PLB (31.25nM-250nM), TMX (25nM-200nM) and three different drug combinations, being equal or lower than the maximum drug doses detected in the serum levels of these patients (250nM PLB + 100nM TMX, 62.5nM PLB + 50nM TMX, and 31.25nM PLB + 25nM TMX) to evaluate drug synergy. Experiments were run for 4-days, after the drug treatment was initiated, and re-scanned at the end of the experiment using Incucyte to determine the final cell confluence of all DMSO-control and drug-treated wells. Individual wells were trypsinized and counted using a BioRad TC-10 automated cell counter. Cell counting data was analyzed and visualized using Graphpad Prism8 software. Within each experiment, each drug condition had three technical replicates and three biological replicates for each cell line to confirm reproducibility of findings.

Table 3.2.2 Palbociclib and tamoxifen drug pharmacokinetics and calculated serum levels [5,85].
Palbociclib bioavailability, C_{max} and serum levels were determined in the PALOMA-1 trial [51]

Drug	Dose Administered	Bioavailability	Steady State	Cmax (ng/ml)	Molecular Weight (g/mol)	Serum Levels (nM)
Palbociclib	125mg	46%	8 days	116	447.533	259
Tamoxifen	20mg	>98%	3 months	40	371.5146	108

Cell Line Doubling Time

The doubling time of the cell lines was calculated using the DMSO control initial and final cell counts from the 4-day experiments. These calculated values were then divided over time to determine doubling time per hour.

Twelve-day Cell Counting Experiments

Similar to the 4-day experiments, cells were seeded at 20% confluence, as determined by Incucyte ™ ZOOM 2016B 18-24 hours after plating. Cell lines were treated 24 hr after seeding with DMSO (control, 1µL/mL), PLB (31.25nM-250nM), TMX (25nM-200nM) or a combination of PLB (31.25nM-250nM) + TMX (25nM-100nM). In some cases, the drug doses were selected based on cell line doubling times. Cell lines with a faster growth rate (MCF7, T47D, MDA-MB-415, MDA-MB-453, MDA-MB-468, BT549, HCC38, SKBR3, ZR-75-30, AU565, MDA-MB-231) were treated with a limited protocol, DMSO (1µL/mL), PLB (250nM PLB) and two PLB + TMX combination doses (250nM PLB + 100nM TMX, 62.5nM PLB + 50nM TMX). Live-cell images were captured using Incucyte at 6-hour intervals to determine change in confluence for the duration of the 12 days after treatment. On day 4 and day 8 the media/drugs were replenished. Individual wells were visually inspected, trypsinized and counted using the BioRad TC-10 automated cell counter at the end of the 12-day experiment. Proliferation curves were generated using Incucyte confluence data, both proliferation curves and cell counting data were visualized using Graphpad Prism8 software. Of note, MDA-MB-468 and BT549 experiments were ended early, on day 6, as these lines demonstrated complete resistance to all treatment conditions and had reached 100% confluence by day 4.

Endpoint/Outcome Measures

The outcome measures used for this work were 1) percent of control calculations and 2) growth rate calculations. The details of these outcomes are outlined below.

1: Percent of Control

Drug response to PLB, TMX and the combination was initially reported as percent inhibition compared to the DMSO control. Simply, this is calculated by dividing the number of treated cells by the number of untreated cells, from the cell counting data.

$$\%\ Control = \frac{\#\ of\ treated\ cells}{\#\ of\ untreated\ cells}\ x\ 100$$

Extrapolation of Cell Counting Based on Doubling Times

For the cell lines with a faster doubling time, it was noted that certain treatment conditions and the DMSO control reached 100% confluence before the end of the 12-day experiments. To correct for the growth rates that were inhibited due to space constraints in the well, I used cell counting data from the exponential cell growth phase of the DMSO control in the 4-day experiments. For certain lines, cell counts for each individual treatment were also corrected for using the 4-day growth rates. The treatment conditions that were corrected for are highlighted in Table 3.2.3. Of note, for the faster growing cell lines, 100nM TMX was not evaluated in the 12-day experiments as drug effect was captured in the 4-day experiments, therefore 4-day cell counting data was used for further analysis in 55% (11/20) of cell lines.

Table 3.2.3. Cell counting corrections for DMSO and treatment conditions that reached 100% confluence before the end of the 12-day experiments. Cell counts were corrected for using the growth rates from the 4-day cell counting data. The growth rate values for 100nM TMX in the fast-growing lines were calculated using 4-day cell counting data.

Cell Line	DMSO	250nM PLB	100nM TMX	Combination (62.5nM PLB + 50nM TMX and 250nM PLB + 100nM TMX)
BT483				
MDA-MB-361				
T47D	Corrected		4-day cell counting data used	
MDA-MB-134				
HCC202				
MDA-MB-175				
CAMA-1	Corrected			
MCF7	Corrected		4-day cell counting data used	
MDA-MB-453	Corrected	Corrected	4-day cell counting data used	
ZR-75-30			4-day cell counting data used	
BT474				
AU565	Corrected		4-day cell counting data used	
MDA-MB-415	Corrected	Corrected	4-day cell counting data used	
HCC38	Corrected	Corrected	4-day cell counting data used	Corrected
HCC1428	Corrected		Corrected	
ZR-75-1	Corrected	Corrected	Corrected	
SKBR3	Corrected	Corrected	4-day cell counting data used	Corrected
MDA-MB-231	Corrected	Corrected	4-day cell counting data used	Corrected
BT549	Corrected	Corrected	4-day cell counting data used	Corrected
MDA-MB-468	Corrected	Corrected	4-day cell counting data used	Corrected

2: Growth Rate (GR) Calculator

The GR calculator evaluates individual cell line growth rates using initial and final cell count data from the DMSO control and comparing the growth rate to the treatment conditions, thereby correcting for variability in proliferation rates seen between cell lines [84]. GR values were calculated using the equation below, where x(c) = treated cell count, x_{ctrl} = control cell count and x_0 = initial cell count:

$$GR(c) = 2^{\frac{\log_2(x(c)/x_0))}{\log_2(x_{ctrl}/x_0)}} - 1$$

Raw cell counting data and corrected cell counts were entered into grcalculator.org, and GR values were calculated for each drug concentration tested [84]. The interpretation of the GR values is as follows:

- between 1 and 0, signifies a partially cytostatic response,
- 0 indicates a complete cytostatic response,
- less than 0 signifies a cytotoxic effect,
-greater than 1 signifies that the drug treatment promotes cell growth.

3.3 Results – Optimizing assays for CDK4/6i efficacy in breast cancer cell lines

Cell Size and Cell Line Doubling Times

After reviewing preliminary PLB proliferation assay results, it was noted that PLB caused the cells to increase in size compared to the DMSO control. This morphology change was seen in 9/20 breast cancer lines (Figure 3.3.1 and Appendix C-C1). The cell count data was then used to determine the doubling times of each cell line. Cell lines were classified into slow (doubling time >8 hours), medium (doubling time 4<8 hours) and fast (doubling time <4 hours). Cell line growth characteristics, growth rates and cell size are reported in Table 3.3.1.

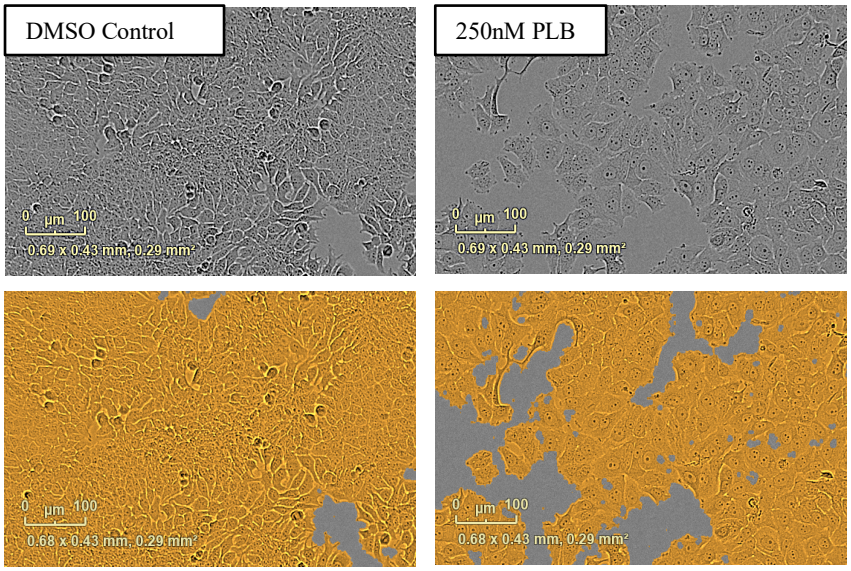

Figure 3.3.1 Morphology change seen with 250nM palbociclib treatment compared to the DMSO control in a representative cell line, MCF7, with and without Incucyte confluence mask.

Sensitivity Categorization Based on Experimental Duration: 4-day vs 12-day

From the 4-day cell counting experiments, treatment with 250nM PLB demonstrated some efficacy in 90% (18/20) of the cell lines (Table 3.3.1). There were several cell lines in which PLB showed limited efficacy and these cell lines uniformly had a slow doubling time. Recognizing the cytostatic effect of PLB and the long treatment duration in patients, I chose to run 12-day experiments with repeated drug dosing in all cell lines. From these 12-day experiments, PLB efficacy was more effectively demonstrated in the cell lines with slower doubling times. For example, in the slow growing BT483 cell line, the 250nM PLB treatment elicited a 50% growth inhibition compared to the control in the 4-day experiment, whereas in the 12-day experiment the cells were inhibited to 15% of the control (Figure 3.3.2). In contrast, in faster growing cell lines like ZR-75-1 the PLB and TMX drug effect showed minimal changes in drug efficacy with the longer experiments (Figure 3.3.2). Notably, when comparing PLB efficacy

ranking amongst all the cell lines the results are considerably different using the 12-day experimental protocol (Table 3.3.2).

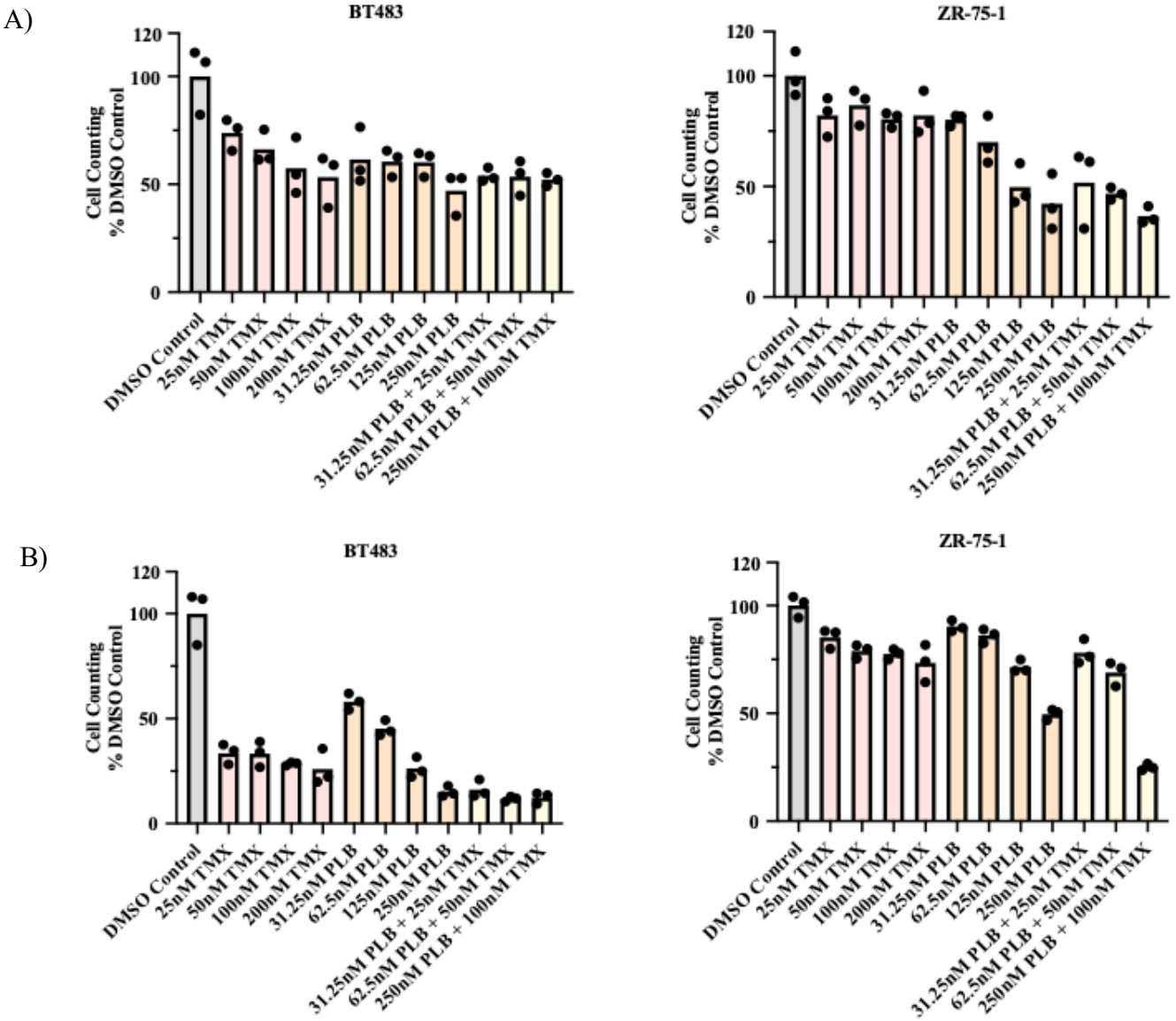

Figure 3.3.2 Representative cell counting graphs showcasing the effects of experimental duration, A) 4-day and B) 12-day, on palbociclib and tamoxifen drug response. Cell lines were seeded for 20% confluence and treated with DMSO, TMX [25-200nM], PLB [31.25-250nM] and combination of TMX + PLB [31.25nM PLB 25nM TMX, 62.5nM PLB + 50nM TMX, 250nM PLB + 100nM TMX]. Individual wells were trypsinized and counted using an automated cell counter at the end of the experiment. Drug response was calculated as a percent of the DMSO control.

Table 3.3.1 Effect of experimental duration on cell line sensitivity to 250nM palbociclib. Drug effect reported as precent of control, comparison of 4-day experimental duration and 12-day experiment. Cell lines were ranked by percent of control to 250nM PLB treatment from the 12-day experiments.

Cell Line	ER/PR/HER2 Status	Cell Size	Cell Speed (doubling time- hours)	% Control of 250nM PLB	
				4-day	12-day
T47D	+/+/-	Medium	Medium (5.7)	19.2	3.2
MDA-MB-361	+/-/+	Small	Slow (13.1)	35.7	8.0
MCF7	+/+/-	Medium	Fast (1.9)	22.1	12.8
BT483	+/+/-	Medium	Slow (22.8)	47.1	15.1
CAMA-1	+/+/-	Medium	Slow (9.5)	45.5	16.6
HCC38	-/-/-	Big	Medium (5.4)	34.8	18.0
HCC202	-/-/+	Small	Slow (17.8)	50.4	18.2
MDA-MB-134	+/-/-	Small	Slow (19.4)	60.0	18.6
MDA-MB-175	+/-/-	Small	Slow (8.4)	28.3	19.1
HCC1428	+/+/-	Medium	Medium (8.2)	49.6	24.1
BT474	+/+/+	Small	Slow (9.7)	71.0	27.2
AU565	-/-/+	Medium	Medium (6.6)	45.5	29.6
MDA-MB-415	+/+/-	Small	Slow (10.7)	40.2	32.9
ZR-75-30	+/-/+	Medium	Slow (13.9)	51.4	36.2
MDA-MB-453	-/-/+	Small	Medium (4.3)	25.6	39.4
SKBR3	-/-/+	Medium	Medium (6.5)	50.6	45.3
ZR-75-1	+/+/-	Medium	Medium (4.7)	42.7	49.7
MDA-MB-231	-/-/-	Medium	Fast (2.8)	46.3	61.2
MDA-MB-468	-/-/-	Medium	Medium (4.7)	100.0	91.7
BT549	-/-/-	Big	Fast (2.1)	89.6	100.0

Sensitivity Categorization Based on Endpoint Measure: Percent Inhibition vs. GR Calculator

Table 3.3.2 shows a comparison of PLB sensitivity rankings for the breast cancer cell lines using percent inhibition versus calculated GR values as an endpoint measure. As described in the methods, GR calculations account for variability in cell line doubling times, resulting in more accurate measures of drug efficacy. For simplicity, I highlight the ranking for PLB dosing of 250nM. Significant changes in rank order were seen when the 12-day GR values were compared to the 12-day percent of control ranking (Table 3.3.2). For example, using the GR calculations, BT483 (Luminal A) was ranked the most sensitive line to 250nM PLB treatment,

initially this cell line was reported as 4th most sensitive line to PLB using percent inhibition as the outcome measure (Table 3.3.2).

The cell line ranking results were compared to PLB sensitivity ranking as reported in the preclinical data published by Finn *et al.* (2009). The three top resistant cell lines were ranked similarly between our laboratory and the Finn report; however, significant ranking discrepancies were seen with the more sensitive lines (Table 3.3.2). Specifically, the top two PLB sensitive lines reported by Finn (MDA-MB-175 and ZR-75-30) ranked 6th and 10th with my GR ranking. Whereas the 2nd and 3rd most sensitive lines (MDA-MB-361 and T47D) in my ranking were ranked as 6th and 11th according to Finn's IC50 data.

Table 3.3.2 Breast cancer cell line characteristics and effect of different endpoint reporting on cell line sensitivity to 250nM palbociclib. Percent of control drug reporting compared to calculated GR values, and cell line ranking determined by IC50 values reported by Finn *et al.* (2009) [44]. Cell lines were ranked by 12-day GR value to 250nM PLB treatment.

Cell Line	ER/PR/ HER2 Status	Cell Size	Cell Speed	12-day % of control	12-day GR value	Finn *et al.* PLB IC50 Ranking [44]
BT483	+/+/-	Medium	Slow	15.1	-0.02	NA
MDA-MB-361	+/-/+	Small	Slow	8.0	0.11	6
T47D	+/+/-	Medium	Medium	3.2	0.19	11
MDA-MB-134	+/-/-	Small	Slow	18.6	0.21	4
HCC202	-/-/+	Small	Slow	18.2	0.29	5
MDA-MB-175	+/-/-	Small	Slow	19.1	0.38	1
CAMA-1	+/+/-	Medium	Slow	16.6	0.43	3
MCF7	+/+/-	Medium	Fast	12.8	0.55	12
MDA-MB-453	-/-/+	Small	Medium	39.4	0.55	10
ZR-75-30	+/-/+	Medium	Slow	36.2	0.56	2
BT474	+/+/+	Small	Slow	27.2	0.58	13
AU565	-/-/+	Medium	Medium	29.6	0.59	NA
MDA-MB-415	+/+/-	Small	Slow	32.9	0.62	7
HCC38	-/-/-	Big	Medium	18.0	0.64	8
HCC1428	+/+/-	Medium	Medium	24.1	0.70	NA
ZR-75-1	+/+/-	Medium	Medium	49.7	0.71	9
SKBR3	-/-/+	Medium	Medium	45.3	0.75	14
MDA-MB-231	-/-/-	Medium	Fast	61.2	0.77	15
BT549	-/-/-	Big	Fast	100	0.96	16
MDA-MB-468	-/-/-	Medium	Medium	91.7	1.00	17

3.4 Discussion

This work draws attention to the impact of different methodologies and analytical considerations on the classification of PLB sensitivity/resistance. I show that the design of the experiment will influence outcome measures which is of particular importance when trying to compare drug efficacy between different cell lines. It is also demonstrated that PLB drug effect is not well measured using 4-day experiments in cell lines with slower doubling times. The longer 12-day experiments better discriminate PLB drug effect, as these experiments provided sufficient time for the cells to replicate, thus allowing the true drug effect of PLB to be captured. This is of particular importance for cytostatic drugs, such as PLB and TMX. Furthermore, most targeted agents are given to patients on a daily basis for many months, so it makes intuitive sense that experiments of longer duration are of value when assessing novel targeted agents.

The experimental data can be confounded by variability in proliferation rates when the number of cells plated at the beginning of the experiment is not considered [84]. The GR calculator, which accounts for both initial and final cell counting data, corrects for variability in growth rates while calculating drug effect. This drug response reporting was found to be the most accurate in determining PLB efficacy in the panel of cell lines. Interestingly, of the top 7 sensitive cell lines, 6 of them had slower doubling times (4 luminal A, 1 luminal B, 1 HER2+), and I was unable to capture the high degree of sensitivity without considering the growth rate of the cells. Therefore calculating drug response on a per division-basis reduces the intrinsic variability that exists between cell lines by controlling for proliferation rates [84].

Using the 12-day cell counts with the GR calculator best captured PLB effects, however certain experimental factors should be considered. One limitation that exists with the current methodology is that all cell lines were grown in the same media, RPMI + 10% FBS. After

carefully evaluating growth rate and cell speed, growing cell lines in media that differs from their original composition may have impacted the intrinsic cell growth rate of individual cell lines. Another limitation is that by correcting the cell counts that reached 100% confluence in the long-term experiments, specifically correcting the PLB, TMX or combination counts, this assumes that there were no additive drug effects seen after multiple drug treatments.

Discrepant drug sensitivity rankings were seen when comparing my PLB ranking to previously published literature [44]. As previously discussed, the variability in experimental duration, 12-day for the reported drug sensitivities versus the 6-day experiment run by Finn *et al.* (2009) and differences in culturing media can confound reported drug sensitivity, resulting in inconsistent data [44]. Again, this highlights the need for accurate and consistent experimental methodologies to measure drug sensitivity. With the methodological considerations described in this chapter, I have identified a robust approach of evaluating drug sensitivity data in the breast cancer cell lines. This provides the basis for conducting the analysis of the reverse phase protein array (RPPA) data to identify proteomic biomarkers (Chapters 4 and 5).

Chapter 4- *In vitro* evaluation of palbociclib treatment alone and in combination with tamoxifen

4.1 Background and Rationale

CDK4/6i in combination with AET is now a standard treatment for HR+/HER2- advanced or recurrent breast cancer. Finn *et al.* (2009) observed notable synergistic activity when combining PLB with TMX to treat ER+ breast cancer cell lines [44]. As such, PLB was evaluated in combination with different AETs in the design of multiple clinical trials. These trials have shown that the addition of CDK4/6i to AET has resulted in improved patient outcomes [5]. PLB + AET treatment increased PFS times by 10, 13, and 6.6 months as compared to single AET alone as reported in the PALOMA-1, -2, and -3 trials, respectively. Because PLB and TMX are thought to act synergistically, in this aim (Objective 3b), I evaluated the effects of PLB treatment alone, TMX treatment alone, and PLB/TMX combinations on the panel of breast cancer cell lines. The precise nature of the synergism between PLB + TMX is poorly characterized and there is a need to better understand the interaction of these drugs *in vitro.*

4.2 Methods

A panel of 20 breast cancer cell lines, including all subtypes, were grown using the same cell culture media and conditions (RPMI + 10% FBS, 37°C, 5% CO_2). STR and Western blot analysis were performed to confirm cell line authenticity (Appendix A-Experimental Methodology) and molecular subtyping (ER+, HER2+ or TNBC) (Figure 3.2.1). Next, all cell lines were evaluated for response to single TMX, single PLB, and a combination of PLB + TMX in 48-well plates seeded at 20% initial confluence (number of cells ranging from 6,000 cells/well

to 47,000 cells/well depending on predetermined cell size for each cell line). Combination assays were performed as outlined in Chapter 3.2 and the methods are summarized in Figure 4.2.1. Experiments were run for 4-days (1-time treatment) and 12-days (3-time treatments) and cells in each well were counted and compared to the DMSO control. The doses of both PLB and TMX were selected based on calculated steady-state patient serum levels (Table 3.2.2). Single PLB doses ranged from 31.25-250nM, while single TMX doses ranged from 25-200nM. Three PLB + TMX combination doses were evaluated to assess drug synergy.

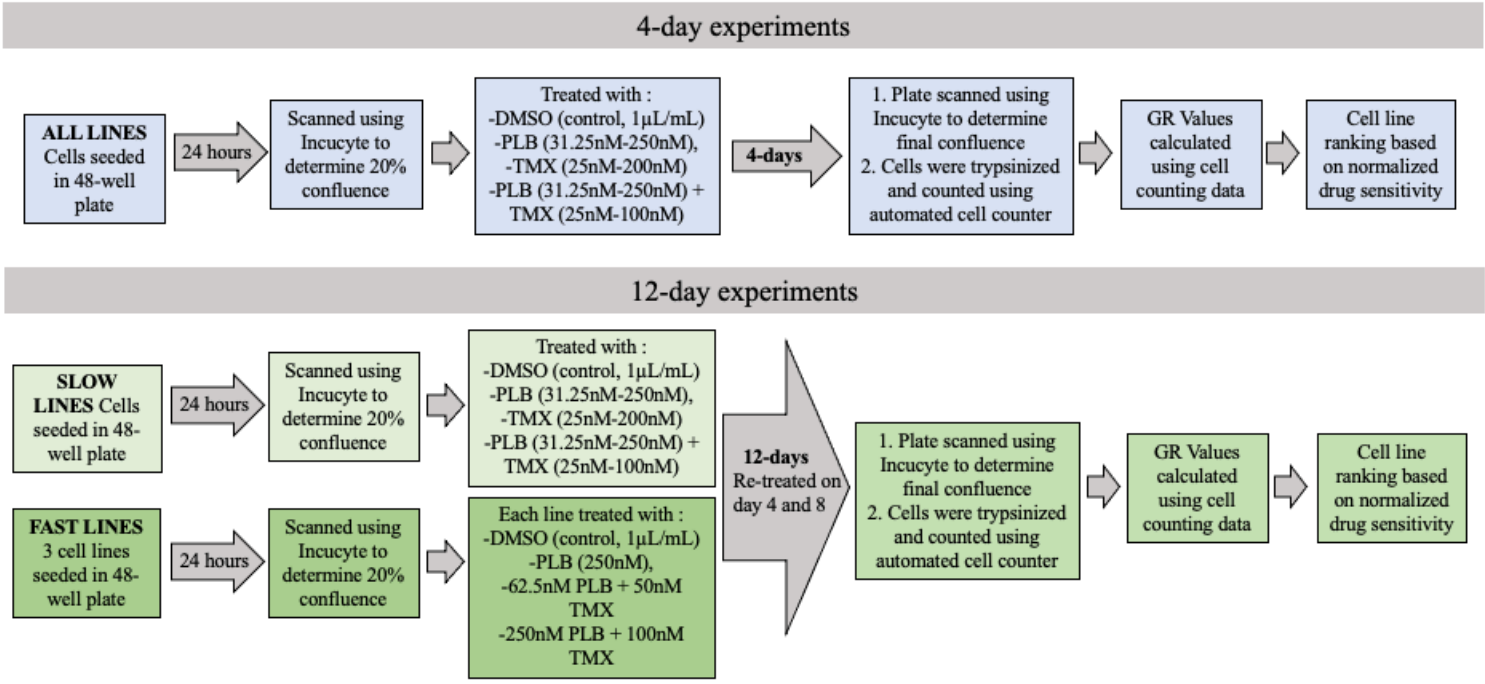

Figure 4.2.1 Flow chart of cell counting methodology for 4-day experiments for all 20 breast cancer cell lines and 12-day experiments for slower growing (n=9) and faster growing (n=11) cell lines.

Growth Rate Calculations

The twenty breast cancer cell lines were ranked based on drug response to single PLB, single TMX, and the PLB + TMX combination using the online growth rate (GR) calculator tool as outlined in Chapter 3.2 [84]. Briefly, the initial cell count and the final raw or corrected cell counts were entered into the growth rate calculator and a GR value was computed for each drug dose.

Drug Synergy Evaluation

1- CompuSyn

Drug synergy was calculated on the 4-day and 12-day experiments using CompuSyn software [86]. A minimum of two drug concentrations are needed for each single agent drug to accurately calculate synergy using CompuSyn [84]. As I treated the faster growing cell lines with a limited dosing protocol for the 12-day experiment, I could not calculate drug synergy for these lines. Using cell counting data, the fraction affected (% of control) was determined and entered into the CompuSyn software using a 'non-constant ratio'. The software computed a Combination Index (CI) value for each PLB + TMX combination dose. The interpretation of the CI values is as follows:

CI <1, synergistic drug combination effects,

CI =1, additive drug effects,

CI >1, antagonistic effects.

2- Comparing GR Values

GR values were calculated as described above, and they may be used as a method of assessing drug synergy. The GR values for the 62.5nM PLB + 50nM TMX and the 250nM PLB + 100nM TMX combinations were directly compared to the GR values of 250nM PLB to evaluate the combination effects.

4.3 Results

PLB and TMX Drug Efficacy Evaluation Alone and in Combination

GR values were calculated for PLB, TMX and PLB + TMX combination doses in the 4-day and 12-day experiments. The growth rate cell line ranking for 250nM PLB, 100nM TMX, 62.5nM PLB + 50nM TMX and 250nM PLB + 100nM TMX combinations are highlighted in Table 4.3.1. Two TNBC cell lines (2/20, 10%) were highly resistant to both single drugs and the combination treatment (BT549 and MDA-MB-468). PLB treatment alone showed some activity in 90% (18/20) of cell lines (GR value <0.8) (Table 4.3.1). A quarter of all cell lines (5/20=25%; BT483, MDA-MB-361, T47D, MDA-MB-134, HCC202) showed a high degree of cytostatic response to PLB (GR value <0.3), including four luminal A/B and one HER2+ line. Interestingly, these five cell lines were also more likely to exhibit a high degree of response to the PLB + TMX combination (GR value <0.3).

Table 4.3.1 Growth rate ranking of breast cancer cell lines to 250nM palbociclib, 100nM tamoxifen, 250nM palbociclib + 100nM tamoxifen and 62.5nM palbociclib + 50nM tamoxifen. Cell lines were ranked based on growth rate (GR) value when treated with 250nM palbociclib.

Cell Line	ER/PR/ HER2 status	Subtype	250nM PLB GR Value	100nM TMX GR Value	250nM PLB + 100nM TMX Combo GR Value	62.5nM PLB + 50nM TMX Combo GR Value
BT483	+/+/-	Luminal A	-0.02	0.24	-0.11	-0.11
MDA-MB-361	+/-/+	Luminal B	0.11	0.65	0.03	0.26
T47D	+/+/-	Luminal A	0.19	0.75	0.22	0.25
MDA-MB-134	+/-/-	Luminal A	0.21	0.67	0.14	0.30
HCC202	-/-/+	HER2+	0.29	0.78	0.25	0.66
MDA-MB-175	+/-/-	Luminal A	0.38	0.82	0.30	0.69
CAMA-1	+/+/-	Luminal A	0.43	0.77	0.19	0.40
MCF7	+/+/-	Luminal A	0.55	0.75	0.45	0.49
MDA-MB-453	-/-/+	HER2+	0.55	0.93	0.63	0.79
ZR-75-30	+/-/+	Luminal B	0.56	0.90	0.47	0.68
BT474	+/+/+	Luminal B	0.58	0.91	0.30	0.70
AU565	-/-/+	HER2+	0.59	0.98	0.58	0.76
MDA-MB-415	+/+/-	Luminal A	0.62	0.71	0.34	0.57
HCC38	-/-/-	TNBC (Basal-like)	0.64	0.96	0.72	0.75
HCC1428	+/+/-	Luminal A	0.70	0.86	0.43	0.70
ZR-75-1	+/+/-	Luminal A	0.71	0.90	0.55	0.87
SKBR3	-/-/+	HER2+	0.75	1.05	0.74	0.88
MDA-MB-231	-/-/-	TNBC (Basal-like)	0.77	1.01	0.80	0.88
BT549	-/-/-	TNBC (Basal-like)	0.96	0.99	0.96	1.01
MDA-MB-468	-/-/-	TNBC (Basal-like)	1.00	1.37	0.98	0.97

For single agent TMX treatment, 40% (8/20) of the cell lines exhibited some degree of cytostatic effects (GR value <0.8) and only the BT483 cell line showed a high cytostatic response (GR value <0.5). The efficacy of TMX within the ER+ lines was surprising, almost half of the ER+ lines (5/12, 41.7%; MDA-MB-175, ZR-75-30, BT474, HCC1428, ZR-75-1) did not exhibit a clear response to treatment (GR value >0.8). Only one ER- cell line appeared to be somewhat sensitive to TMX alone (GR value <0.8; HCC202), noting also that this was 1 of the 4 the HER2+ cell lines. In general, single agent PLB had a higher degree of efficacy than TMX as a single agent.

Synergistic Effects of PLB and TMX in Combination

CompuSyn combination indexes (CI) for the 4-day and 12-day experimental data are shown in Figure 4.3.3. As previously mentioned, the 12-day experiments were run on the ER+ cell lines with slower doubling times. In the 4-day experiments, 20% (4/20) of the cell lines showed marked synergy (HCC1428, MDA-MB-361, ZR-75-1, CAMA-1) (Figure 4.3.1A), with a CI value of <1 for all three tested PLB + TMX combination doses. All these lines demonstrated some degree of PLB sensitivity, while ZR-75-1 and HCC1428 demonstrated limited TMX sensitivity. In the 12-day experiments, a high degree of synergy was seen across all the tested combinations in the nine evaluated cell lines, with the exception of HCC202 (HER2+) where only the high combination dose resulted in a CI value of <1 (Figure 4.3.1B). When comparing the results of the 12-day experiments versus the 4-day experiments, synergy was seen in twice the number of cell lines (8 versus 4, 40% of all lines).

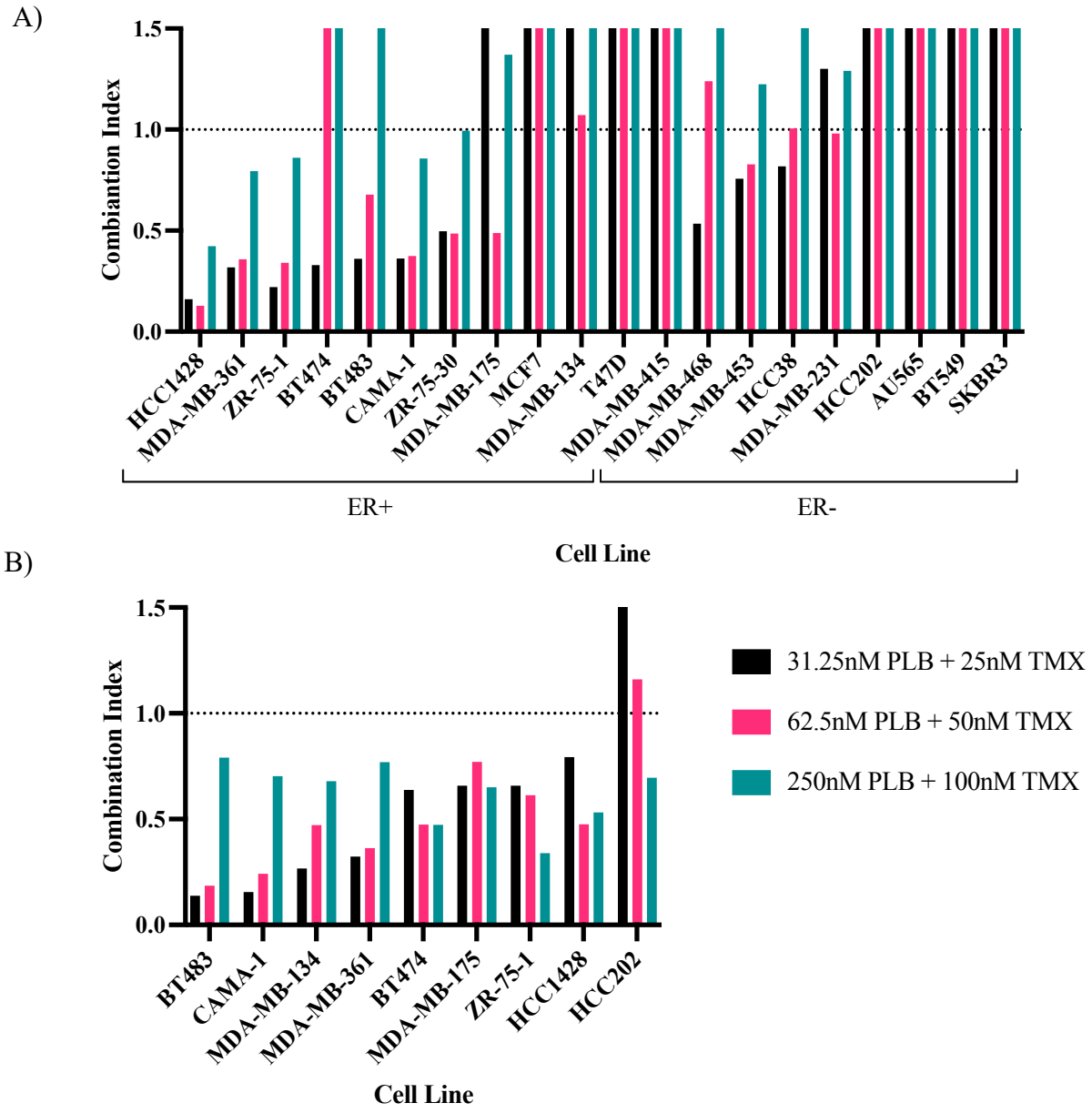

Figure 4.3.1 Drug synergism analysis of three tested combination doses of palbociclib + tamoxifen in breast cancer cell lines. A) Drug synergism of 4-day cell counting experiments, and B) drug synergism of 12-day cell counting experiments for the slower growing cell lines. Combination indexes (CI) were calculated using CompuSyn software, a CI value of <1.0 indicates synergism, while CI >1.0 indicates drug antagonism [86].

Synergy Evaluation Using GR Calculations

The high dose PLB + TMX drug combination (250nM PLB + 100nM TMX) was found to outperform the effects of the single 250nM PLB drug treatment, reducing the GR value over 0.15 points, in a total of five cell lines (5/20, 25%) (Table 4.3.1). Of these, only four lines exhibited a high degree of PLB growth inhibition (4/20 = 20%; BT474, MDA-MB-415, HCC1428, CAMA-1). These four cell lines belong to the ER+ or luminal A/B molecular subtype, accounting for a third (4/12, 33%) of all ER+ cell lines. The remaining synergistic line, ZR-75-1, demonstrated limited response to single treatment with PLB or TMX alone (GR value >0.7). The cell lines with a high degree of synergy determined by GR scores were also found to be synergistic using CompuSyn CI calculation (BT474, HCC1428, CAMA-1 and ZR-75-1).

4.4 Discussion

For the work described in this chapter, my aim was to accurately determine breast cancer cell line sensitivity to PLB and TMX as this is crucial for identifying proteomic biomarkers in Chapter 5. From the growth rate ranking, a cytostatic response was seen in 90% of the tested breast cancer cell lines when treated with a pharmacological dose of PLB (250nM). Similar results were seen in a study which reported GR values of PLB treated breast cancer cell lines [87]. These authors reported concordant cytostatic GR values (0-0.76) using 100nM PLB [87].

A degree of PLB efficacy was observed across all breast cancer subtypes. Luminal A and B subtypes were found to exhibit increased PLB cytostatic effects, compared to other subtypes. This finding was also reported by Finn *et al.* (2009) [44]. Interestingly, PLB efficacy was observed in 3/4 HER2+ cell lines but had limited or no effect in the TNBC subtype.

Response to TMX alone was limited to ER+ cell lines, with only one luminal A cell line exhibiting a highly cytostatic response to TMX. Within the ER+ cell lines, 41.7% of lines did not derive substantial benefit from 100nM TMX treatment. While the TMX dosing used may be somewhat lower than *in vitro* doses reported in literature, these results do reflect observed clinical response rates of 30-40% for metastatic breast cancer [24]. Nonetheless, considerable synergy using PLB and AET in combination was observed. A high combination index (CompuSyn CI) was observed in most of the ER+ lines tested in the 12-day experiments. This data supports substantial synergistic activity between PLB and TMX. Furthermore, I compared the efficacy of using PLB and TMX in combination using the CompuSyn index (CI) and changes in GR calculations. There was a substantial concordance between the GR values (differences in GR scores) and CompuSyn combination index in 4 of the 5 cell lines that demonstrated marked synergy. While using GR values to evaluate synergy is a novel approach, further exploration into this method is warranted. Previous *in vitro* combination testing performed by Finn *et al.* (2009) demonstrated high synergistic effects in both MCF7 and T47D cell lines when treated with varying doses of PLB (3.125 – 50nM) and TMX (312 – 5000nM) [44]. From the drug treatment data, PLB alone elicited a high degree of response in T47D, and the combination treatment did not increase efficacy. In the MCF7 line, the combination treatment elicited a similar drug response when compared to PLB alone.

Marked resistance to PLB, TMX and the PLB + TMX combination was observed in 10% of the tested cell lines, accounting for 50% of the TNBC subtype. These cell lines, MDA-MB-468 and BT549, have been previously classified as PLB resistant in multiple studies [44,70,87]. Within the cell line population, 40% of lines, representing all subtypes did not derive benefit

from PLB and TMX combination. This suggests that predictive biomarkers of PLB + TMX drug synergy and biomarkers of resistance to single PLB treatment would aid in patient selection.

Using stringent assay criteria, breast cancer cell line response to PLB alone and in combination with TMX has been evaluated. From these findings, I've ranked drug efficacy for PLB alone and combination therapy with PLB + TMX. Using the GR values in Table 4.3.1 I have defined GR cut-off values to discriminate PLB resistant cell lines from PLB sensitivity cell lines and similarly, cell lines that are resistant to the combination treatment. This is further described in the methods of Chapter 5.

In conclusion, I have established stringent efficacy comparisons in the cell lines using PLB and TMX alone and in combination. This work now enables stratification and grouping of the cell lines to perform the analyses for proteomic biomarker discovery as outlined in the next chapter.

Chapter 5- Discovery of novel predictive biomarkers for palbociclib treatment

5.1 Background and Rationale

Due to tumour heterogeneity, drug efficacy may differ for each individual cancer. In the absence of using predictive biomarkers, patients are given a drug and then observed prospectively to determine if the drug works over time. Predictive biomarkers help assess the potential benefit that a drug may have before the patient receives treatment. Using biomarkers to select patients who are likely to benefit from a specific drug therapy improves patient outcomes, reduces toxicity in turn improving a patient's quality of life, and reduces financial toxicity to the health care system. In breast cancer, the expression levels of ER (as measured by IHC) and HER2 amplification (as measured by FISH or IHC as a protein-based surrogate of amplification status), are examples of biomarkers used clinically to guide breast cancer treatment by identifying subsets of patients who are more likely to benefit from AET and HER2-targeted therapies, respectively [88]. As described in Chapter 1, there are no predictive biomarkers for PLB treatment in clinical use, and the extensive biomarker research done thus far has not resulted in clinically validated biomarkers (highlighted in Chapter 2). Currently, any patient with advanced/metastatic ER+/HER2- breast cancer is a candidate to receive PLB. As described previously, clinical evidence indicates that a substantial proportion of patients being treated with PLB do not achieve a meaningful benefit from this treatment [9–11]. Additionally, as reviewed in Matutino *et al.* (2018), preclinical studies and ongoing clinical trials have observed PLB efficacy in ER- and HER2+ breast cancers [39]. This suggests that there may be additional patient populations who could benefit from PLB, however are currently not candidates for treatment. Thus, the identification of PLB predictive biomarkers in breast cancer represents a substantial unmet need.

PLB + fulvestrant and PLB + letrozole combination biomarkers have been evaluated in the PALOMA trials (highlighted in Chapter 2); but was generally limited to genomic and transcriptomic data [67,68]. Our laboratory has previously had success with identifying predictive biomarkers in low-grade serous ovarian cancer cell lines using proteogenomic approaches [16,17]. Furthermore, our lab has previously shown that proteomic profiling can be used to identify predictive biomarkers and may discriminate better than transcriptomic profiling [17]. In terms of defining the scope of this project appropriate for my thesis, I decided to focus my efforts on identifying proteomic predictive biomarkers of PLB alone and in combination with TMX in breast cancer cell lines. I had access to multiple RPPA datasets for screening and comparative purposes making this objective feasible.

Utilizing the drug sensitivity rankings determined from the assay refinement (Objective 3a – Chapter 3) and drug evaluation/synergism studies (Objective 3b – Chapter 4) (Table 4.3.1), I aim to identify markers of resistance of PLB and PLB + TMX in the panel of breast cancer cell lines. My aim for this work (Objective 3c) is to specifically identify biomarkers of resistance - or of limited response - to PLB or PLB + TMX. I used two approaches for this: 1) To evaluate the proteins regulating the CDK4/6 pathway and correlate expression of these cell cycle proteins with drug resistance, and 2) Analyze protein expression in the RPPA datasets to identify proteins associated with drug resistance.

5.2 Methods

Western Blot

Protein levels of common cell cycle proteins (Rb, CDK2, CDK4, CDK6, cyclin D1, cyclin E1, p16, p27) were evaluated by Western blot in the twenty breast cancer cell lines (Figure 5.3.1) as described in Appendix A- Experimental Methodology. Western blot band intensities were quantified using Image J (Appendix E-E1).

Cell Line Sensitivity Ranking

To compare cell lines with distinct responses to single PLB and PLB + TMX combination treatments, cell lines were grouped as sensitive and resistant based on the GR values determined in Chapter 4. The cut off for the PLB sensitivity/resistant grouping was relative based on the range of response seen within the 20 cell lines from Chapter 4 (Table 5.2.1A). A GR value of 0.58 was used to group PLB sensitive and resistant lines. BT549 and MDA-MB-468 cell lines were excluded for the RPPA analysis as these lines were completely resistant to all drug treatments, confounding comparison.

For the PLB + TMX combination sensitivity grouping, all ER-negative cell lines were excluded from the RPPA analysis as patients with ER- breast cancer would not be offered TMX as a treatment option. After removing the ER- cell lines from the analysis it was noted all the resistant lines were excluded, and the GR values for the remaining 12 ER+ cell lines were <0.55. As an alternative way to classify response to the combination, cell lines were grouped based on the efficacy of the 250nM PLB + 100nM TMX combination compared to 250nM PLB, as determined in Chapter 4. Cell lines that exhibited high synergistic effects (>0.15 difference between 250nM + 100nM TMX combination GR value and PLB GR value) were grouped

together and compared to the cell lines where the combination elicited limited synergistic activity or similar effects to 250nM PLB alone (<0.15 GR difference) (Table 5.2.1B).

Table 5.2.1 Breast cancer cell line grouping for RPPA analysis. A) Sensitive and resistant cell line grouping based 250nM palbociclib growth rate values and B) cell line grouping based on synergy to PLB + TMX combination. Combination effects were determined by direct comparison of GR values for combination and single PLB treatment. Sensitive lines are highlighted in orange and resistant lines are highlighted in blue.

A)

Cell Line	PLB GR Ranking
BT483	-0.0223
MDA-MB-361	0.111
T47D	0.189
MDA-MB-134	0.209
HCC202	0.292
MDA-MB-175	0.381
CAMA-1	0.429
MCF7	0.545
MDA-MB-453	0.546
ZR-75-30	0.562
BT474	0.575
AU565	0.589
MDA-MB-415	0.62
HCC38	0.639
HCC1428	0.7
ZR-75-1	0.714
SKBR3	0.746
MDA-MB-231	0.768

B)

Cell Line	Difference in GR Values (Combo vs 250nM PLB)
BT474	-0.28
MDAMB415	-0.28
HCC1428	-0.27
CAMA-1	-0.24
ZR-75-1	-0.16
MCF7	-0.10
BT483	-0.09
ZR-75-30	-0.09
MDAMB361	-0.08
MDAMB175	-0.08
MDAMB134	-0.07
T47D	0.03

Reverse Phase Protein Array

RPPA is an antibody based high-throughput proteomic assay to quantitate protein expression in a selected panel of samples [16]. Three RPPA datasets were obtained. The first database was received personally from Dr. Gordon Mills which included 14 of the cell lines within my panel and contained protein expression data on 440 proteins. Cell lysates were prepared by the MD Anderson Functional Proteomics RPPA Core Facility and the antibodies used are listed on their website [89]. RPPA data was normalized and converted to log1.25.

The second and third RPPA datasets are publicly available datasets from the Cancer Cell Line Encyclopedia (CCLE) and MD Anderson Cell Line project (MCLP). Both datasets were downloaded from https://tcpaportal.org/mclp/#/download. The CCLE database contained 19/20 breast cancer lines in my analysis, data on BT549 was missing from this dataset. The CCLE dataset analyzed 214 proteins, 182 of these were also analyzed in the Mills dataset [90]. The MCLP dataset included all 20 breast cancer cell lines and analyzed 94 proteins for all lines, with up to 234 proteins quantified for some of the cell lines. The data in both datasets were normalized and converted to log2.

The SPSS statistics tool was used to analyze differential protein expression between breast cancer cell lines with distinct responses to single PLB and the PLB + TMX combination [91]. Protein expression between sensitive and resistant groups were compared using a non-parametric Mann-Whitney U test and a p-value of <0.05 was considered statistically significant. Proteins that were evaluated in multiple databases but not identified as a statistically significant hit in more than one database were excluded. Hierarchical clustering of the differentially expressed proteins was performed using Euclidean distance and average linkage of median centered data. Heat maps of clustered proteins were visualized in Java TreeView [92].

5.3. Results

Cell Cycle Protein Levels by Western Blotting

As highlighted in Chapter 1, aberrations within the cell cycle pathway, specifically at the G1/S phase are common in breast cancer. Western blot was performed to evaluate if the expression profiles of cell cycle proteins that are directly involved within this portion of the cell cycle correlate with sensitivity/resistance to PLB +/- TMX (Figure 5.3.1). The two lines completely resistant to PLB (MDA-MB-468 and BT549) identified in Chapter 4, displayed a distinct cell cycle expression profile, with high expression of p16 and Rb loss. Interestingly, MDA-MB-468 and BT549 had low levels or loss of cyclin D1, respectively, whereas the remaining lines had moderate-high expression (with the exception of HCC202) (Figure 5.3.1). Uniquely, ZR-75-1, expressed low Rb levels but had moderate levels of cyclin D1, and this line exhibited limited response to PLB (Appendix E-E2). Expression of p16 was seen in 12/20 cell lines, however neither basal p16 expression nor p16 loss were correlated with PLB response according to GR scores. P27 was expressed in 6 lines: 4 ER+ and 2 ER-. The protein levels of other cell cycle markers were not linked to PLB sensitivity as determined by GR values.

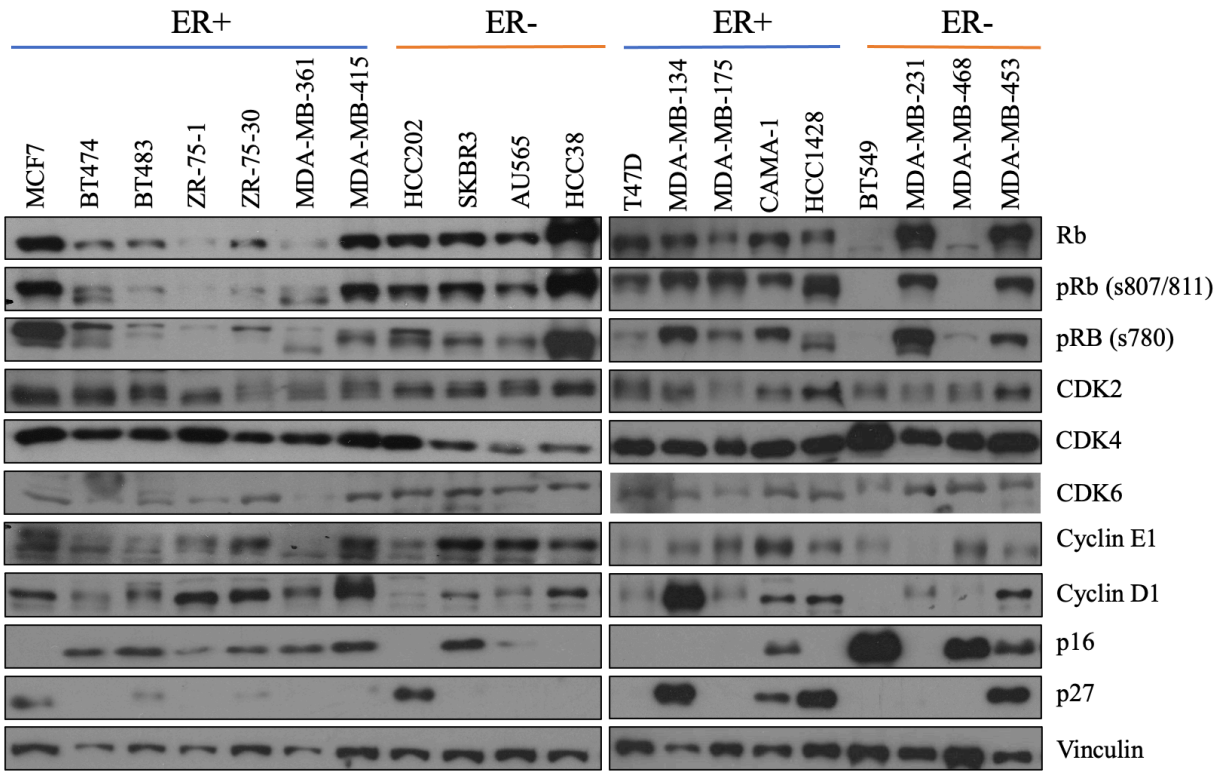

Figure 5.3.1 Western blot of common cell cycle proteins in twenty breast cancer cell lines. Two gels were run in parallel to evaluate all breast cancer cell lines, within each gel, cell lines were grouped by positive and negative estrogen receptor expression. All cell lines were grown in RPMI media and lysates were prepared from sub confluent cells.

Reverse Phase Protein Array Database Analysis

Breast cancer cell lines were categorized into sensitive and resistant groups to single PLB, and PLB + TMX combination treatment. The top 11 PLB sensitive (GR value <0.58) and bottom 7 PLB resistant (GR value >0.58) lines were compared at the protein level using the three available RPPA datasets (Table 5.2.1A). From the RPPA analysis, ten, five, and five statistically significant protein hits were seen in the Mills, CCLE, and MCLP datasets, respectively. Heat maps of these differentially expressed proteins in PLB-sensitive versus resistant cell lines are shown in Figure 5.3.2. Of these hits, BRAF, PKCα, Caveolin-1, GAB2, and GATA3 were seen across 2 of the datasets and PTEN was a significant hit in all 3 datasets. From the RPPA analysis,

decreased PTEN, GATA3, BRAF and GAB2 expression were correlated with PLB resistance. In contrast, increased levels of PKCα and Caveolin-1 were correlated with PLB resistance. These differentially expressed proteins suggest the PI3K and RAS pathways play a role in PLB response.

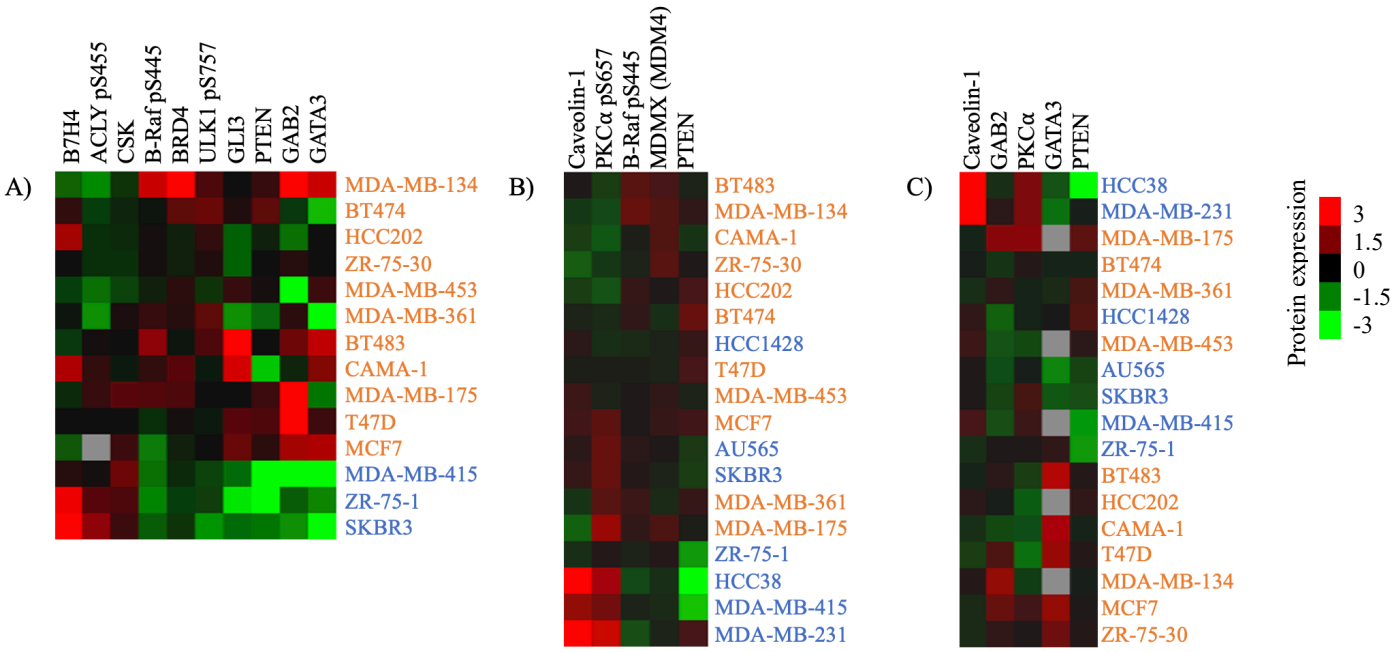

Figure 5.3.2 Statistically significant RPPA candidates in palbociclib sensitive versus resistant breast cancer cell lines. Heat maps for each dataset A) Mills, B) CCLE, C) MCLP were generated using JavaTree [92]. Statistically significant proteins identified in multiple databases and unique proteins from individual databases were included. RPPA data was normalized and converted to log1.25 for the Mills dataset and log2 for the CCLE and MCLP datasets. Hierarchical clustering was performed using Euclidean distance and average linkage of median centered data. Red and green indicate increased and decreased protein expression, respectively, grey squares indicate missing data. Cell lines highlighted in orange were grouped as palbociclib sensitive and blue lines were grouped as palbociclib resistant.

Using the combination grouping highlighted in Table 5.2.1B, protein expression was analyzed in the three RPPA datasets. From the RPPA analysis, 12 statistically significant hits were identified in the Mills dataset (Figure 5.3.3). GAB2 was the only differentially expressed protein seen in the MCLP dataset, and AR was the only candidate from the CCLE dataset, these two candidates were also identified in the Mills dataset, shown in Figure 5.3.3. High GAB2

expression and low AR expression were correlated with the cell lines that did not benefit from the combination treatment. Interestingly, p16 was identified as a protein candidate, suggesting lower expression may be predictive in identifying cell lines that derive limited response to the combination treatment.

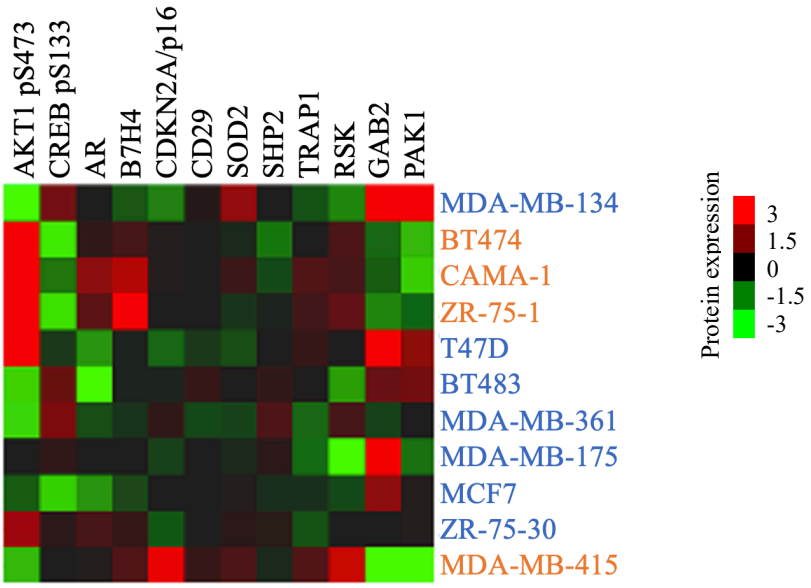

Figure 5.3.3 Statistically significant RPPA candidates of PLB + TMX synergy.
Proteins identified in multiple databases and unique proteins from the Mills dataset were included. RPPA data was normalized and converted to log1.25. Hierarchical clustering was performed using Euclidean distance and average linkage of median centered data, and heat map was generated using JavaTree [92]. Red and green indicate increased and decreased protein expression, respectively. Cell lines highlighted in orange were grouped as highly synergistic to PLB + TMX combination and blue lines were grouped as having limited or no synergistic response to the combination.

5.4. Discussion

The identification of robust predictive biomarkers of PLB or PLB + TMX resistance will help identify patients who are unlikely to derive a meaningful benefit from this treatment and could be offered alternative treatment options. As highlighted in Chapter 2, no predictive biomarkers were identified using available online databases containing clinical and molecular data on CDK4/6i treated patients. To further investigate potential biomarkers of response to PLB

and PLB + TMX combination, a proteomic approach was used to evaluate distinct expression between the sensitive and resistant cell lines.

A distinct cell cycle profile was observed in the two TNBC resistant cell lines: loss of Rb and p16 overexpression. While this signature was only seen in the TNBC subtype within the breast cancer cell line panel, Palafox *et al.* (2022) found p16 overexpression and heterozygous *RB1* loss to be predictive of CDK4/6i (ribociclib) resistance in ER+ breast cancer cell lines, xenografts and patient samples [71]. Upon further validation, using Rb loss and p16 overexpression as biomarkers of PLB resistance may help identify patients who are unlikely to derive benefit from PLB or other CDK4/6i.

The RPPA analysis identified thirteen distinct proteins that were differentially expressed in the PLB sensitive versus resistant cell lines. Of these candidates, five (PTEN, BRAF, PKCα, Caveolin-1, GAB2, GATA3) were identified in 2 or more of the RPPA datasets, further validating these proteins. The expression of GAB2, PTEN, and PKCα have been previously described to alter the cell cycle. PKCα has been reported to downregulate cyclin D1, specifically eliciting anti-proliferative effects at the G1/S phase of the cell cycle, thereby decreasing the reliance of cell proliferation at this point of the cell cycle [93]. Additionally, loss of GAB2 reduces cell-cycle progression resulting in a decrease of proliferation, and loss of PTEN has previously been described as a mechanism of acquired resistance to CDK4/6i in ER+ breast cancer [94,95]. Association of the RAS and AKT pathways with PLB resistance warrants further evaluation, and the predictive value of the individual protein candidates identified in the RPPA analysis will be further explored as part of the future directions of this project.

The RPPA analysis identified twelve differentially expressed proteins when comparing cell lines with high combination synergy with cell lines resistant to the combination. Negative

p16 expression was correlated with the cell lines that did not derive synergistic benefit from the combination. Four of the cell lines in this group (T47D, MCF7, MDA-MB-134, MDA-MB-175) had negative p16 expression by Western blot analysis whereas all lines in the synergy group showcased a degree of p16 expression (with the exception of HCC1428, which was not included in the RPPA analysis using the Mill's dataset) (Figure 5.3.1 – Appendix E-E1). Differential expression of the androgen receptor (AR) was identified between the PLB + TMX combination groupings. The predictive value of AR and response to CDK4/6i is currently being evaluated in the TNBC subtype, however these findings suggest AR expression may also hold predictive value in ER+ subtypes [39].

While the overarching goal of this project was to identify biomarkers of resistance, I did evaluate other potential biomarkers that may be predictive of PLB sensitivity using my PLB GR ranking. Interestingly, I found that cell lines that proliferated slower were more likely to be sensitive to PLB, suggesting a population with a lower proliferation rate (determined by Ki67 score) may benefit from CDK4/6i (Appendix E-E1). Recently, abemaciclib, a CDK4/6i, was approved for use in early stage breast cancer for patients at a high risk of recurrence and selection criteria included using a Ki67 score of ≥20% [96]. While the predictive value of Ki67 in metastatic breast cancer remains inconclusive, using a Ki67 of ≥20% cut-off could potentially exclude patients who would respond to a CDK4/6i. Additional evaluation of Ki67 as a biomarker for CDK4/6i (abemaciclib and PLB) response is warranted.

The *p16 (CDKN2A)* gene which encodes the p16 protein, a natural CDK4/6i, is frequently inactivated in breast cancers. Inactivation of *p16* commonly occurs through DNA methylation and copy number deletion [97]. The predictive value of *p16* promoter methylation as a biomarker of PLB response has been evaluated in multiple cancer types by Li *et al.* (2019),

where they found that methylated *p16* cells were more likely to be sensitive to PLB [97]. Within the cell line panel, only two lines (T47D and MDA-MB-134) had known *p16* methylation, and both ranked within the top 5 PLB sensitive lines (Appendix E-E2). These findings suggest that *p16* methylation may hold predictive value in selecting a subset of patients who are more likely to respond to PLB.

Other genetic alterations were considered to identify potential biomarkers. Increased p53 expression was more common in the PLB resistant grouping, seen in 6 of the 9 top resistant lines (Appendix E-E2). After reviewing the Wellcome Sanger Institute database for previously published drug sensitivities within the breast cancer cell line panel (summarized in Appendix E-E2) a trend was seen where cell lines with high PLB sensitivity were more likely to also be sensitive to PI3K and AKT inhibitors [98]. Of the top 8 PLB sensitive lines, 6 of them were sensitive to PI3K and AKT inhibitors, whereas the 7 most resistant lines had no previously documented sensitivity to these inhibitors. As PI3K and AKT inhibitors are associated with high toxicity profiles in the clinic, these findings suggest that tumours with a reliance on the PI3K pathway may benefit from PLB treatment which has a more tolerable toxicity profile [99].

In the clinical setting, all ER+/HER2- metastatic patients are candidates to receive CDK4/6i + AET treatment. Using these criteria in the breast cancer panel, 56% (5/9) of the ER+/HER2- cell lines would derive benefit from the combination treatment. Using the potential biomarker findings from this chapter, I have developed specific screening criteria to be considered when selecting patients for single PLB treatment (Figure 5.4.1). All breast cancer patients would be screened to determine p16 and Rb expression, which could be done by immunohistochemistry staining. Patients with overexpression of p16 and/or loss of Rb would likely be resistant to PLB, therefore should be offered an alternative treatment option. For the

remaining patients who express low or normal levels of p16 and have functional Rb, they would potentially benefit from PLB treatment, however further patient selection is needed. From the RPPA data, evaluating for increased expression levels of PKCα, Caveolin-1, or decreased expression PTEN, GATA3, BRAF or GAB2 could identify patients who are unlikely to respond to PLB. Additionally, patients with methylated *p16* and prior sensitivity to PI3K and AKT inhibitors would be selected to receive PLB treatment. Low Ki67 levels and p53 expression may also help select for patients to receive PLB. Any remaining patients who had not been selected out based on the criteria above should still receive PLB treatment. As this biomarker research was discovery based, all the identified biomarkers require further evaluation in a validation cohort.

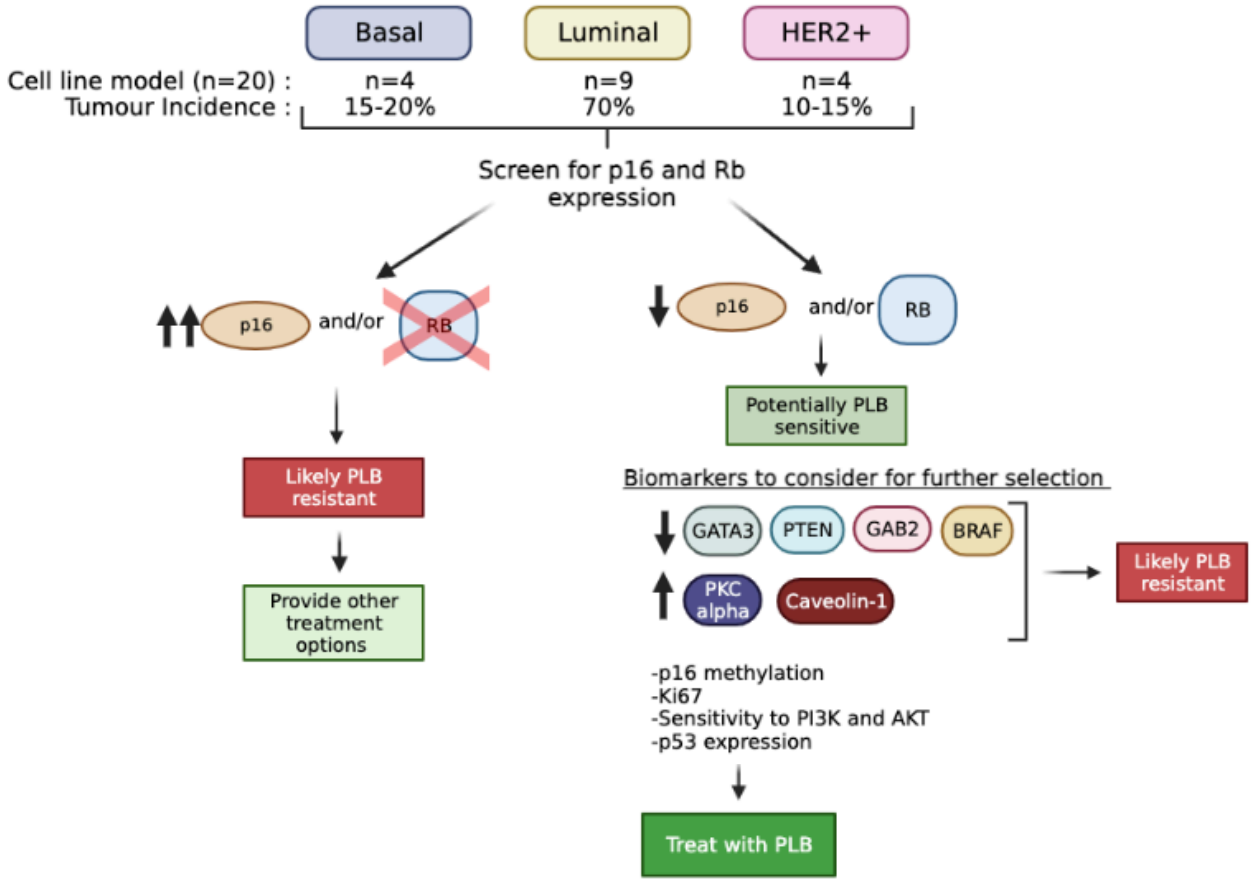

Figure 5.4.1 Proposed screening criteria for selecting patients to receive palbociclib treatment.

A limitation of this work is that the previously mentioned biomarkers were identified using breast cancer cell lines. While these are robust models for drug sensitivity and allow for biomarker identification, they only represent a subpopulation of the patients. Due to the challenges of developing 2D models, the lines that get established resemble only a selection of cells present within a patient's tumour. Additionally, the tumours from which these cell lines were derived came from different sources (i.e., pleural effusion, primary breast, ascites) and the age of the patients at tumour removal ranged from 23-82 (Summarized in Table 3.2.1). Additionally, the RPPA datasets are reliant on data generated from different laboratories that may have cultured the cell lines in different conditions. Ideally, additional RRPA analysis and multi-omics will be performed using samples prepared by our laboratory to confirm these findings. The highlighted biomarkers from this project require further validation using 3D cell line models, mouse models or patient samples.

Chapter 6 – Conclusion and future directions

Conclusions

This project provides a foundation of PLB +/- TMX drug sensitivity data on a panel of twenty breast cancer cell lines. The overarching goal of this project was to identify biomarkers that are predictive of resistance to PLB and PLB + TMX combination. In recent years, PLB in combination with AET has become the standard of care for treating ER+/HER2- metastatic breast cancer. While previous CDK4/6i biomarker research has been conducted, no predictive biomarkers beyond ER positivity have been clinically validated [15,67,68]. With the increasing costs of targeted therapies such as PLB, the financial burden on the healthcare system is not sustainable. To ensure breast cancer patients receive effective treatments, biomarker research is of great importance.

Using a carefully validated experimental design, which accounted for the variable growth rate observed within the panel of breast cancer cell lines, I found that PLB elicited cytostatic activity in all breast cancer subtypes. Luminal A cell lines were found to be the most sensitive to both PLB alone and the PLB + TMX combination, whereas the TNBC subtype was resistant or derived limited response to the tested drugs. Cell lines with distinct response to PLB were compared using RPPA datasets and from this I identified thirteen differentially expressed proteins between PLB sensitive and resistant cell lines. These proteins included PKCα, PTEN, BRAF, GATA3, and GAB2 as potential predictive biomarkers of PLB resistance. Additionally, tumour characteristics such as proliferation rate of the tumour, methylation status of the *p16* promoter, and prior sensitivity to AKT/PI3K inhibitors may also help select patients who are most likely to response to PLB. Twelve protein biomarkers of PLB + TMX resistance were identified, including p16, GAB2 and AR.

Table 6.1 Biomarkers of palbociclib and palbociclib + tamoxifen response identified from literature and RPPA analyses to be considered for further validation.

Literature	PLB Proteomics	Synergy Proteomics
CDK4 pT172	ACLY pS455	AKT1 pS473
CCNE1	B7H4	AR
ERBB3	B-Raf pS445	B7H4
FGFR1/2	BRD4	CD29
p16	Cavelolin-1	p16
Rb	CSK	CREB pS133
PTEN	GAB2	GAB2
	GATA3	PAK1
	GLI3	RSK
	MDMX	SHP2
	PKCα	SOD1
	PTEN	TRAP1
	ULK1 pS757	

From this project I have identified notable potential biomarkers that should be considered for further validation highlighted in Table 6.1. The literature review in Chapter 2 highlighted multiple biomarkers that have been previously explored, from these seven have been selected that warrant further validation. The RPPA analysis in Chapter 5 identified thirteen and twelve potential biomarkers of PLB and PLB + TMX resistance, respectively. Additional evaluation of these discovery-based biomarkers using a validation cohort to determine their predictive value is warranted.

Future Directions

Further validation of the identified RPPA hits by Western blot and functional shRNA knock-down experiments will be performed to determine if a change in drug response is seen when compared to the PLB response seen in the unmodified cell line. The development of a stable PCKα knock-down in a PLB resistant cell line is currently ongoing to evaluate the predictive value of this protein. Additional proteomic biomarker work will be completed using mass spectrometry to further validate the identified RPPA protein candidates in this project. Potential biomarkers will also be analyzed in patient samples to elucidate the predictive value of these markers.

Future projects will include the evaluation of the other two approved CDK4/6i, ribociclib and abemaciclib, in the panel of breast cancer cell lines using cell counting experiments and growth rate calculations. Additionally, PLB will be evaluated in combination with other AETs (letrozole and fulvestrant), to determine which combination elicits the highest degree of synergy. I hope that translational research will continue to inform treatment decisions using a personalized medicine approach to improve treatment outcomes for breast cancer patients, ultimately ensuring the right drug is being given to the right patient.

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Appendix

Appendix A- Experimental Methodology

Cell Line Authentication

Short tandem repeat (STR) analysis was performed on all breast cancer cell lines to authenticate each line, with the exception of HCC1428 which was purchased directly from ATCC. Genewiz Inc analyzed 16 markers/loci (D3S1358, D21S11, D18S51, Penta E, Penta D, D8S1179, FGA, D5S818, D13S17, D7S820, D16S539, vWA, TH01, AMEL, TPOX, CSF1PO). The STR results were compared to the ATCC STR database to confirm the identity of each cell line.

Western Blot Analysis

Breast cancer cell lines were plated in 100mm dishes and grown to 60-85% confluent. Plates were scraped using in-house lysis buffer made up of 20mM Bicine (pH 7.5) 0.6% CHAPS, Aqueous Inhibitor mix (40mM sodium fluoride, 17mM beta glycerophosphate, 1mM sodium orthovanadate, 2mM EDTA, 10mM EGTA), Phos-Stop phosphatase inhibitor (Roche, Cat. No. 4906845001), and Protease inhibitor cocktail (Sigma Aldrich, Cat. No. P8340). Cells were snap frozen at -80 degrees for 20 minutes, thawed and pelleted at 10,000 rpm for 15 minutes at 4 degrees. Supernatant was collected and used for Western blot analysis. Protein extracts were separated by SDS-PAGE on 6-15% polyacrylamide gels and transferred to nitrocellulose membranes (BioRad: 0.2µM membrane pores, Cat. No. 1620112) at 100V for 2 hours and probed with primary antibodies.

Primary antibodies against PR (CS-8757), Rb (CS-9309), pRb (s780) (CS-9307), pRb (s807/811) (CS-8516), Cyclin E1 (CS-4129), Cyclin D1 (CS-2922), CDK4 (CS-12790), CDK6 (CS-3136) were obtained from Cell Signaling; p27 (SC-528), CDK2 (SC-748) were obtained from Santa Cruz Biotechnology; ERα (MA5-14501), HER2 (MA5-14509) were obtained from Thermo Fisher Scientific and p16 (ab108349) was obtained from Abcam. Vinculin (V9131, Sigma) and α-tubulin (T5168, Sigma) were used as protein loading controls. Secondary antibodies, goat-anti-mouse (A9917) and goat-anti-rabbit (A0545) were purchased from Sigma and were used accordingly. Western blots were imaged using Immobilon HRP reagent (Cat. No. WBKLS0500, Millipore, Etobicoke, ON, Canada) and developed by autoradiograph.

Appendix B- Chapter 1 Appendices

B.1. Clinical trials evaluating palbociclib in A) HER2-enriched and B) triple-negative breast cancer patients.

Clinical Trial Name	Trial Phase	Trial Duration	Trial status	Disease Characteristics	Drug Combination	Estimated Completion Date
NCT03530696	Phase 2	2018-ongoing	Active	HER2+ MBC	T-DM1 +/- PLB	Dec-24
NCT02530424 (NA-PHER2)	Phase 2	2015-ongoing	Active	ER+, HER2+	Palbociclib + trastuzumab + pertuzumab +/- fulvestrant.	Preliminary results collected 2019.
NCT02448420 (PATRICIA, PATRICIA II)	Phase 2	2015-ongoing	Active	HER2+ MBC	Trastuzumab + palbociclib +/- letrozole	Aug-23
		2019-ongoing	Recruiting	HER2+ MBC	Trastuzumab + palbociclib + AET vs Physicians Choice	Aug-23
NCT02947685 (PATINA)	Phase 3	2017-ongoing	Active	HR+, HER2+ MBC	Chemotherapy + anti-HER2 for 4-8 cycles then randomized to anti-HER2 + AET vs anti-HER2 + AET + palbociclib	May-23
NCT03054363	Phase 1/2	2017-ongoing	Active	HR+ HER2+ MBC	Tucatinib + palbociclib + letrozole	Mar-23
NCT02774681	phase 2	2016-2020	Terminated	ER- HER2+ MBC with brain metastases	Palbociclib	N/A
NCT03304080	Phase 1/2	2017-ongoing	Active	HR+ HER2+ MBC	Anastrozole + palbociclib + trastuzumab + pertuzumab	Jul-23
NCT04858516 (neoPEHP)	Phase 2	2021-ongoing	Not yet recruiting	Neo adjuvant treatment ER+ HER2+	Palbociclib + exemestane + trastuzumab + pyrotinib	Apr-25
NCT03709082	Phase 1/2	2018-ongoing	Active	Refractory ER+ HER2+	Palbociclib + letrozole + T-DM1	Oct-25
NCT05076695 (NeoTPPF)	Phase 2	2021-ongoing	Recruiting	Neoadjuvant treatment ER+ HER2+	Trastuzumab + pyrotinib + palbociclib + fulvestrant	Oct-24
NCT03644186 (TOUCH)	Phase 2	2018-ongoing	Active	HR+ HER2+ early BC	Palbociclib + letrozole + HER2 blockade (trastuzumab or pertuzumab) vs paclitaxel + HER2 blockade.	Nov-22
NCT04334330	Phase 2	2020-ongoing	Recruiting	ER/PR+ HER2+, with brain metastasis	Palbociclib, trastuzumab, pyrotinib and Fulvestrant	Dec-24
NCT04778982	Phase 2	2022-ongoing	Recruiting	Male or female HER2+	KN026 + palbociclib + fulvestrant	Dec-24
NCT04360941 (Pavement)	Phase 1	2020-ongoing	Recruiting	Locally advanced or metastatic AR+ or HER2+ breast cancer	Palbociclib + avelumab	Jan-24

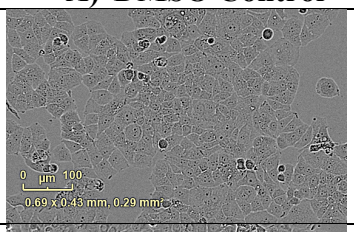
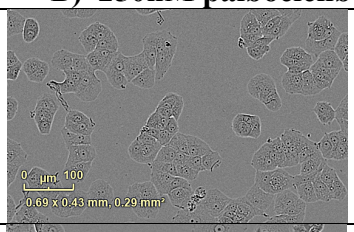
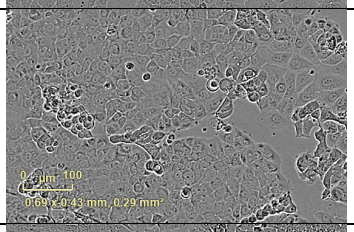
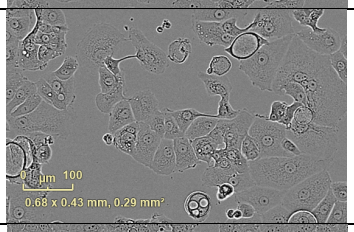
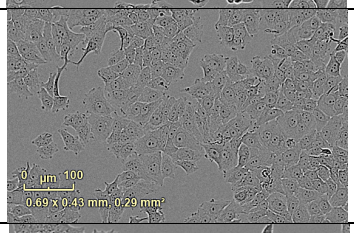
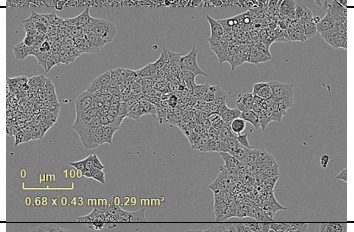
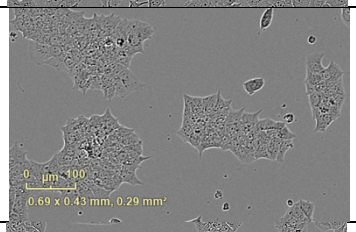
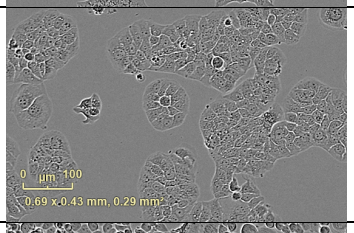
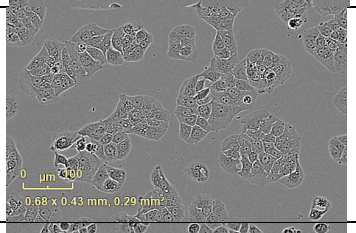
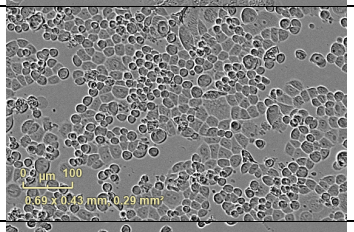
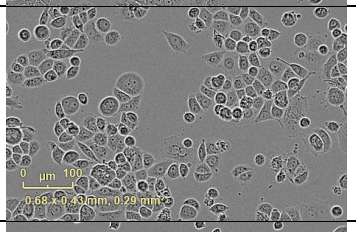
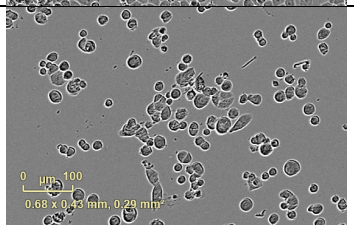
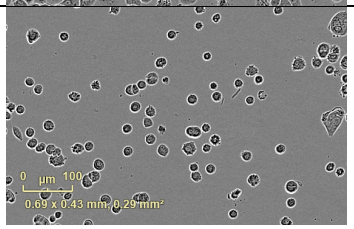
Clinical Trial Name	Trial Phase	Trial Duration	Trial status	Disease Characteristics	Drug Combination	Estimated Completion Date
NCT05067530 (CAREGIVER)	Phase 2	2021-ongoing	Not yet recruiting	Untreated metastatic TNBC	Chemotherapy (paclitaxel or carboplatin) + palbociclib vs palbociclib alone vs chemotherapy alone	Dec-26
NCT04494958 (PALBOBIN)	Phase 1/2	2020-ongoing	Recruiting	Advanced TNBC with hyperactivation of ERK and or CDK4/6	Palbociclib + binimetinib	Aug-23
NCT02605486	Phase 1/2	2015-ongoing	Active	AR+ Metastatic TNBC	Palbociclib + bicalutamide	23-Nov
NCT04360941 (Pavement)	Phase 1	2020-ongoing	Recruiting	Locally advanced or metastatic AR+ or HER2+ breast cancer	Palbociclib + avelumab	Jan-24

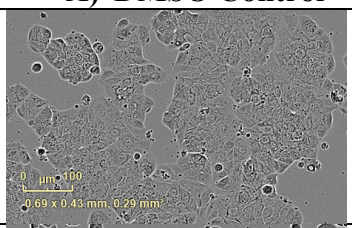
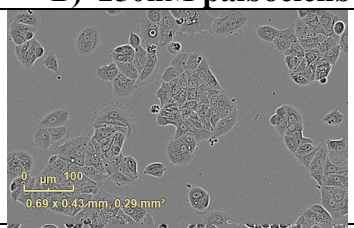
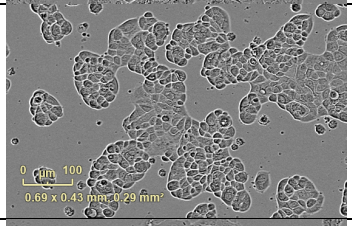
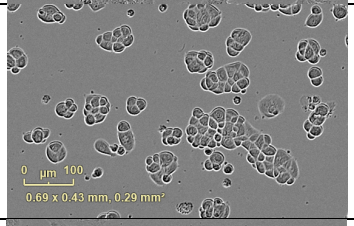
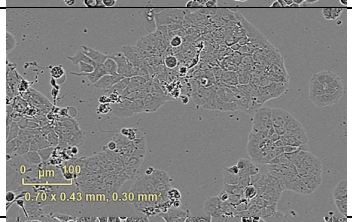
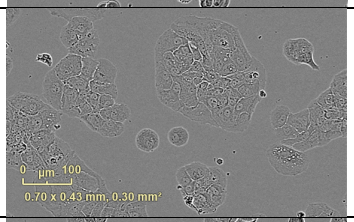
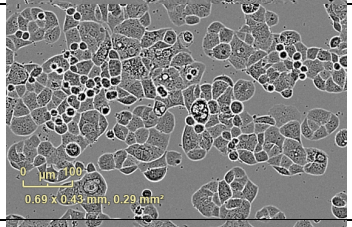
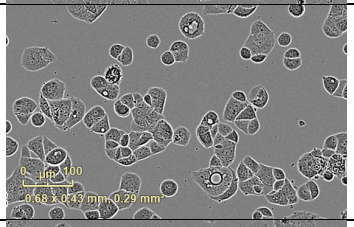
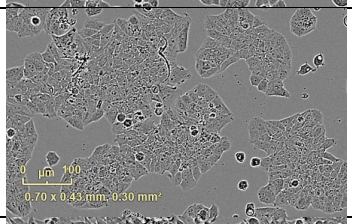
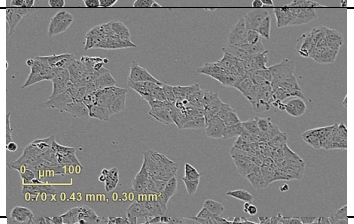
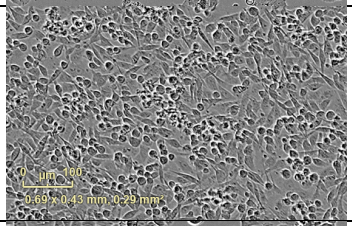
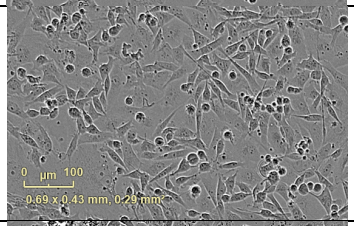
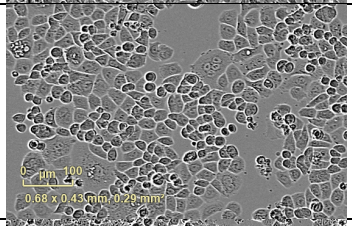
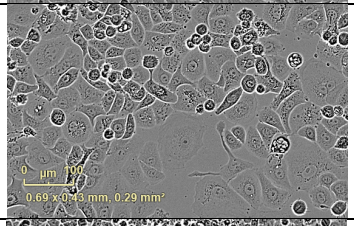
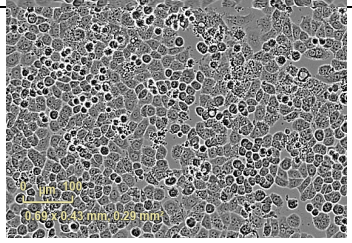
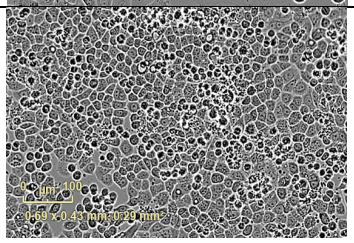
Abbreviations: AET, anti-estrogen therapy; AR, androgen receptor; ER, estrogen receptor; HER2, human epidermal growth factor 2; MBC, metastatic breast cancer; TNBC, triple negative breast cancer.

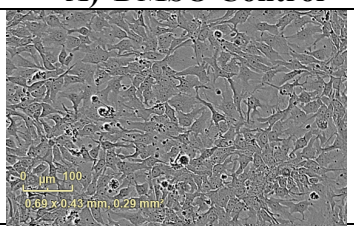
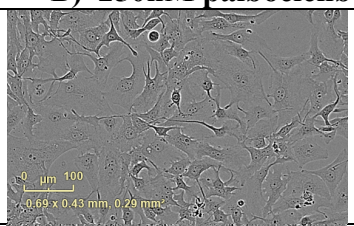
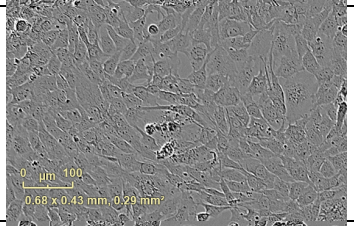
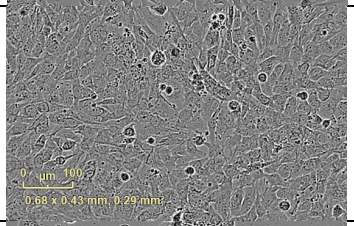
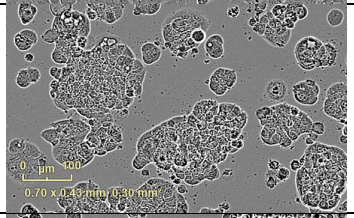
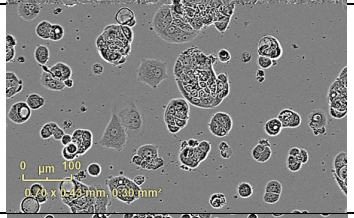
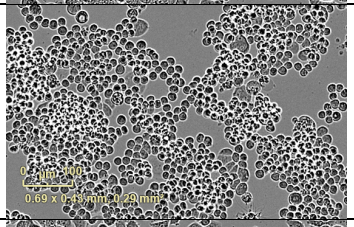
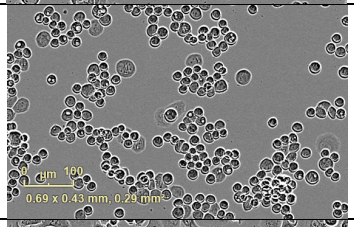
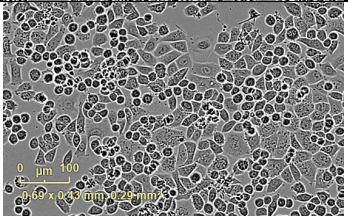
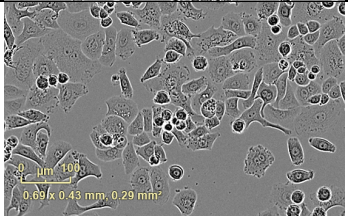
Appendix C- Chapter 3 Appendices

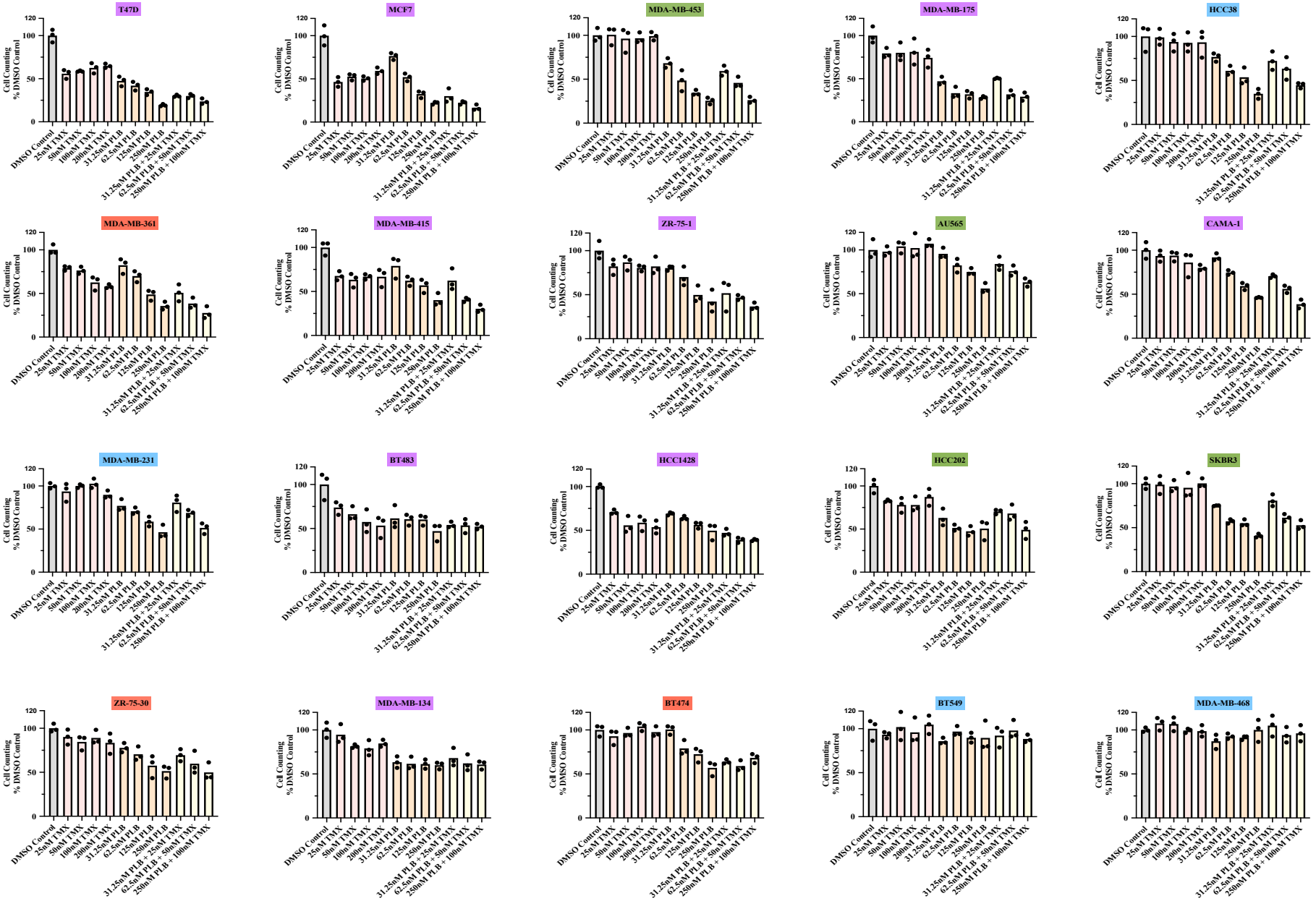
C.1. Live cell images of A) DMSO control and B) 250nM palbociclib treatment for 20 breast cancer lines.

Cells were seeded in 48-well plate and images were obtained at 10X magnification after 90 hours. A morphology change with 250nM palbociclib treatment was seen in 9/20 cell lines.

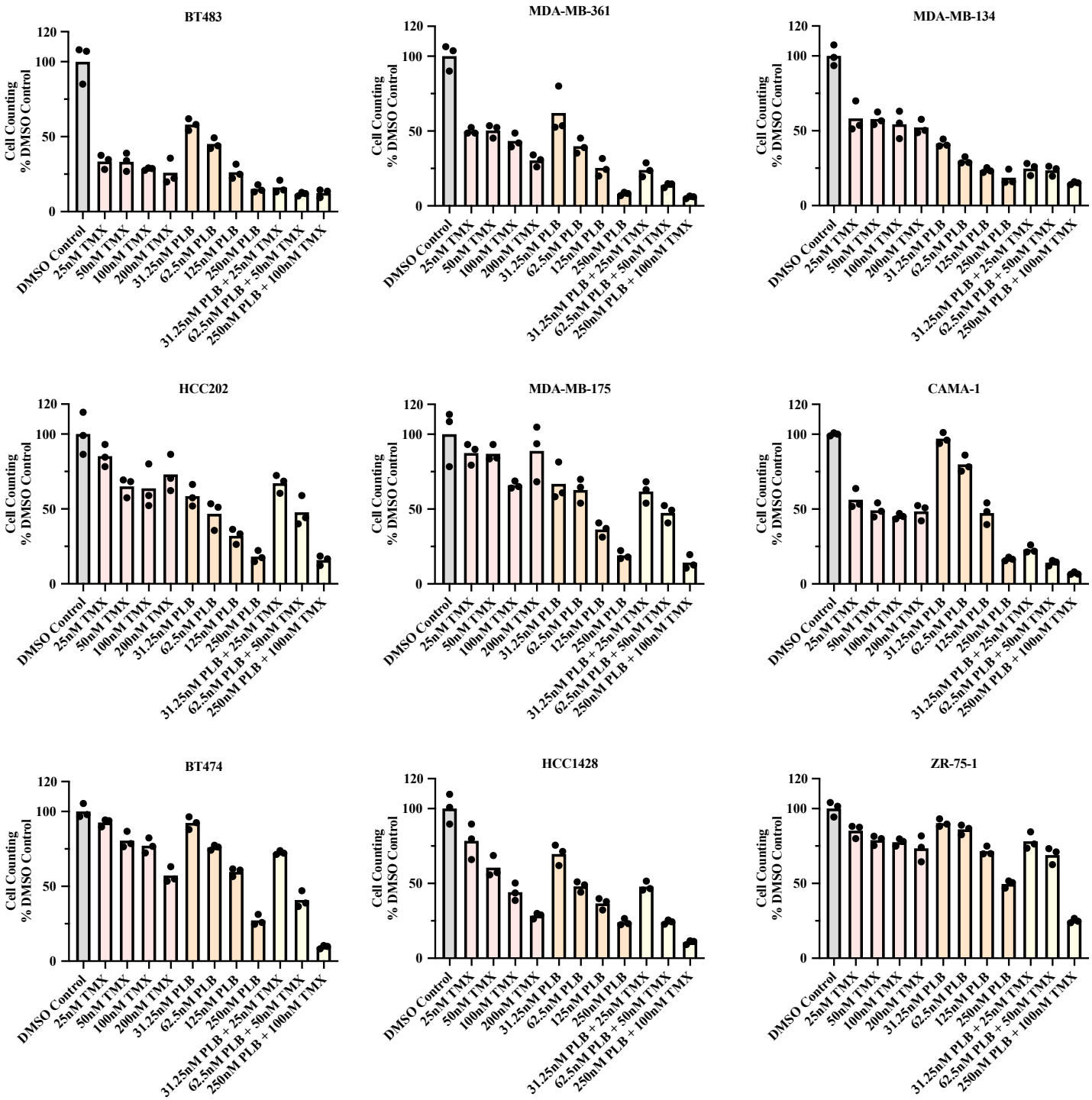
Cell Line	A) DMSO Control	B) 250nM palbociclib
T47D		
ZR-75-1		
MCF7		
BT474		
HCC1428		
CAMA-1		
MDA-MB-134		

Cell Line	A) DMSO Control	B) 250nM palbociclib
MDA-MB-175		
BT483		
MDA-MB-415		
ZR-75-30		
MDA-MB-361		
MDA-MB-231		
AU565		
MDA-MB-468		

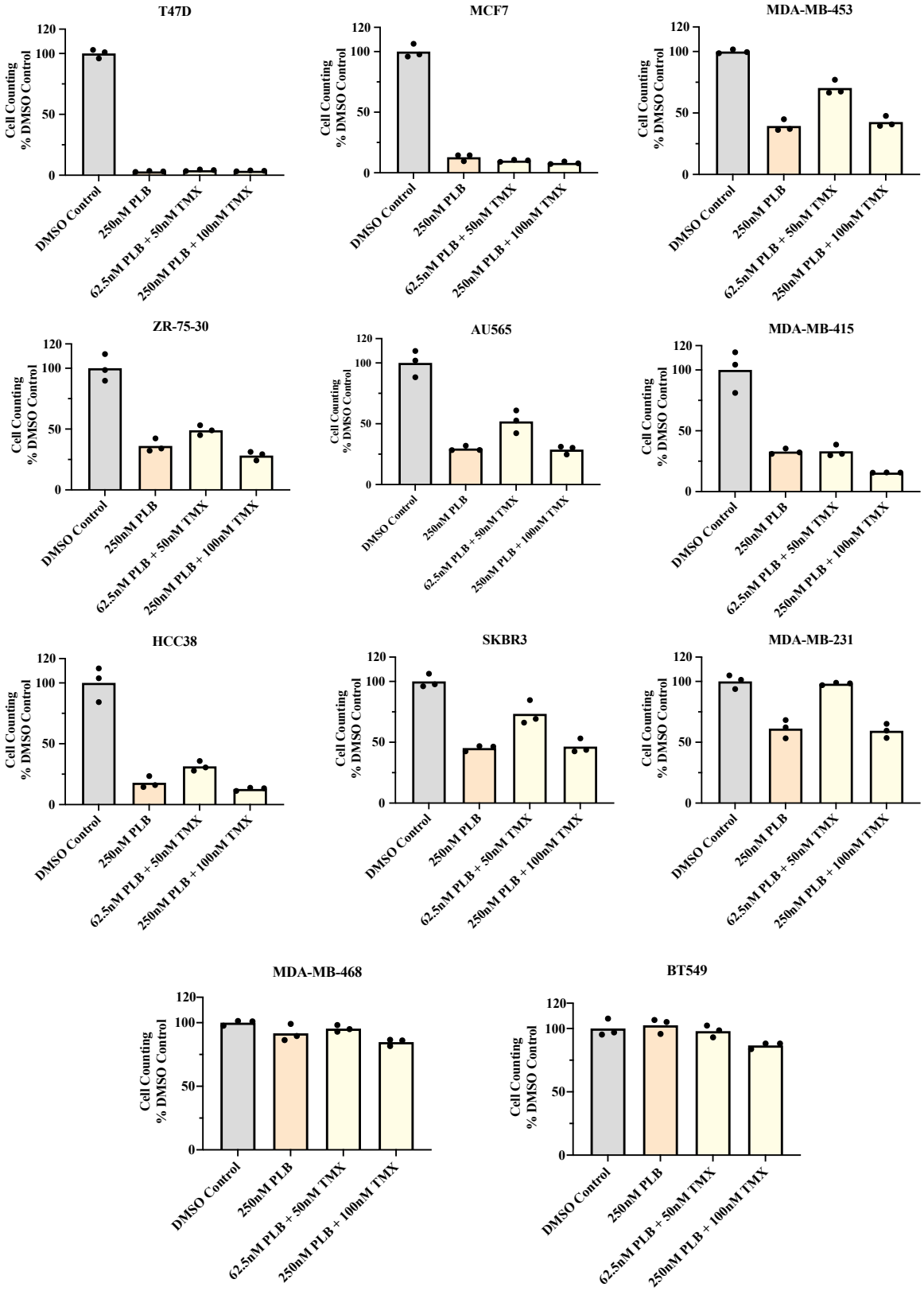
Cell Line	A) DMSO Control	B) 250nM palbociclib
HCC38		
BT549		
HCC202		
MDA-MB-453		
SKBR3		



D.1. Breast cancer cell counting graphs from 4-day palbociclib + tamoxifen experiments. Graphs were organized top left to bottom right by response to 250nM palbociclib reported as percent inhibition of control. Cell lines were seeded for 20% confluence and treated with DMSO, TMX [25-200nM], PLB [31.25-250nM] and combination of TMX + PLB [31.25nM PLB 25nM TMX, 62.5nM PLB + 50nM TMX, 250nM PLB + 100nM TMX], individual wells were trypsinized and counted using an automated cell counter after 4 days. Drug response was calculated as a percent of the DMSO control. Breast cancer subtype highlighted by title color, luminal A: purple, luminal B: red, HER2+: green, TNBC: blue.



D.2.1 Breast cancer cell counting graphs from 12-day palbociclib + tamoxifen proliferation assays. Graphs were organized top left to bottom right by response to 250nM palbociclib based on final growth rate calculations. Cell lines were seeded for 20% confluence and treated on day 0, 4 and 8 with DMSO, TMX [25-200nM], PLB [31.25-250nM] and combination of TMX + PLB [31.25nM PLB 25nM TMX, 62.5nM PLB + 50nM TMX, 250nM PLB + 100nM TMX], individual wells counted, and drug response was calculated as a percent of the DMSO control.



D.2.2 Breast cancer cell counting graphs from 12-day 250nM palbociclib and palbociclib + tamoxifen combination proliferation assays. Graphs were organized top left to bottom right by response to 250nM palbociclib based on final growth rate calculations. Cell lines were seeded for 20% confluence and treated on day 9, 4 and 8 with DMSO, PLB [250nM] and combination of TMX + PLB [62.5nM PLB + 50nM TMX, 250nM PLB + 100nM TMX], individual wells counted, and drug response was calculated as a percent of the DMSO control.

Appendix E- Chapter 5 appendices

Appendix E1. Western Blot quantification of cell cycle proteins.

Quantification of protein band intensity produced by ImageJ across all breast cancer cell lines. Each value was standardized to vinculin or α-tubulin housekeeping control. Cell lines were ranked by PLB sensitivity determined by 250nM PLB GR value. Heat map was generated using conditional formatting in excel.

Cell Line	ER/PR/ HER2 Status	PLB GR Ranking	Quantified Band Intensity									
			Rb	pRb (s807/s811)	pRb (s780)	CDK2	CDK4	CDK6	Cyclin E1	Cyclin D1	p16	p27
BT483	+/+/-	1	0.21	0.12	0.17	0.96	0.97	0.49	0.27	0.68	0.73	0.31
MDA-MB-361	+/-/+	2	0.08	0.24	0.19	0.72	0.72	0.09	0.26	0.78	0.60	0.02
T47D	+/+/-	3	0.67	0.72	0.29	0.82	1.05	1.34	0.22	0.30	0.00	0.17
MDA-MB-134	+/-/-	4	1.00	1.91	1.60	0.82	1.75	1.76	1.01	2.02	-0.01	2.84
HCC202	-/-/+	5	0.57	0.54	0.40	0.70	1.02	0.36	0.34	0.18	-0.01	1.29
MDA-MB-175	+/-/-	6	0.35	0.87	0.38	0.25	0.87	0.88	0.74	0.35	0.00	0.03
CAMA-1	+/+/-	7	0.91	0.78	0.72	0.41	1.21	0.61	1.33	0.37	0.28	0.63
MCF7	+/+/-	8	0.63	0.80	0.92	0.71	1.15	0.84	0.56	0.58	0.00	0.70
MDA-MB-453	-/-/+	9	1.20	0.88	0.62	0.85	1.43	0.48	0.37	0.55	0.79	1.30
ZR-75-30	+/-/+	10	0.19	0.11	0.14	0.53	0.70	0.24	0.35	1.03	0.45	0.11
BT474	+/+/+	11	0.22	0.35	0.60	0.81	0.88	0.71	0.35	0.36	0.42	0.02
AU565	-/-/+	12	0.38	0.37	0.21	0.57	0.27	0.14	0.61	0.28	0.08	0.01
MDA-MB-415	+/+/-	13	0.47	0.49	0.21	0.72	0.88	0.22	0.54	1.39	0.70	0.06
HCC38	-/-/-	14	1.16	1.06	1.07	0.73	0.41	0.47	0.46	0.57	0.00	0.00
HCC1428	+/+/-	15	0.37	0.72	0.31	1.26	0.93	0.52	0.45	0.66	0.00	0.84
ZR-75-1	+/+/-	16	0.05	0.04	0.03	0.62	1.18	0.17	0.41	0.95	0.20	0.02
SKBR3	-/-/+	17	0.49	0.54	0.22	0.51	0.48	0.12	0.66	0.27	0.53	0.03
MDA-MB-231	-/-/-	18	0.88	0.60	0.86	0.46	0.83	0.40	0.04	0.25	0.00	0.01
BT549	-/-/-	19	0.05	0.01	0.05	0.49	1.11	0.23	0.20	0.02	1.89	0.02
MDA-MB-468	-/-/-	20	0.04	0.01	0.09	0.56	0.65	0.45	0.40	0.10	1.17	0.00

E.2 Basic expression profile and molecular features of a panel of breast cancer cell lines, including drug sensitivity data.

Cell Line	250nM PLB GR Value	100nM TMX GR Value	250nM PLB + 100nM TMX GR Value	Subgroup	Protein expression (Western blot)					BRCA1 mutation [100]	BRCA2 mutation [100]	*p16* methylation [70,97,101,102]	Cell size	Cell speed	Doubling Time (hours)	Top 5 sensitive drugs (IC50) [98]	Top 5 resistant drugs (IC50) [98]
					ER	PR	HER2	EGFR [103]	p53 [103]								
BT483	-0.02	0.24	-0.11	Luminal A	+	+	-	-	+	-	-	Unmethylated	Small	Slow	22.8	BIRC5, p38/JNK2, mTORC1-2, BRD 2-4, **PI3K**	EGFR, RNA pol, Kinase i, RNA heli A, IGFR1
MDA-MB-361	0.11	0.65	0.03	Luminal B	+	-	+/-	-	-	-	N1657S	Unmethylated	Small	Slow	13.1	Acetalax, **PI3K**, AKT1-3, CHEK1-2/CDK2, HDACi	BIRC5, ERK1-2, Anthracycline, Mitoxantrone, IKK1-2
T47D	0.19	0.75	0.22	Luminal A	+	+	-	-	+	-	-	Methylated	Medium	Fast	5.7	TGFB1, PIM1-3, ER, IGFR1	BIRC5, RNA pol, BCL2/XL, broad spectrum KI, proteosome
MDA-MB-134	0.21	0.67	0.14	Luminal A	+	-	-	-	-	-	-	Methylated	Small	Slow	19.4	NA	NA
HCC202	0.29	0.78	0.25	HER2+	-	-	+	-	-	-	-	Wildtype	Small	Slow	17.8	AKT1-2, **PI3K**, VEGFR, HDACi	Docetaxel, CDK4/6i, 5-Fluorouracil, FGFR1-3, Kinase i
MDA-MB-175	0.38	0.82	0.30	Luminal A	+	-	-	-	-	-	-	Unmethylated	Small	Slow	8.4	EGFR, **AKT1**-3, MCL1, **PI3K**	IGFR1, ER, DNA PK, MET/ALK
CAMA-1	0.43	0.77	0.19	Luminal A	+	+	+	-	+	-	-	Unmethylated	Small	Slow	9.5	ER, **AKT1**-3, **PI3K**, PORCN	IGFR1, Topotecan, JAK2-3, CHEK1-2, MRCKB
MCF7	0.55	0.75	0.45	Luminal A	+	+	-	-	-	-	-	Deletion	Medium	Fast	1.9	ER, IAP, TAF1, MTORC1, **PI3K**	WEE1/PLK1, MET, JAK2-3, mTORC1-2
MDA-MB-453	0.55	0.93	0.63	HER2+	-	-	+	-	-	-	-	Unmethylated	Small	Fast	4.3	Dihydrorotenone, Acetalax, BRD2-4, BRPF1-2, ERK1-2	TNSK1-2, BCL2/BCL-XL, SYK, IGFR1, EGFR

Cell Line	**250nM PLB GR Value**	**100nM TMX GR Value**	**250nM PLB + 100nM TMX GR Value**	**Subgroup**	**Protein expression (Western blot)**					**BRCA1 mutation** [100]	**BRCA2 mutation** [100]	***p16* methylation** [70,97,101,102]	**Cell size**	**Cell speed**	**Doubling Time (hours)**	**Top 5 sensitive drugs (IC50)** [98]	**Top 5 resistant drugs (IC50)** [98]
					ER	**PR**	**HER2**	**EGFR** [103]	**p53** [103]								
ZR-75-30	0.56	0.90	0.47	Luminal B	+	-	+	-	-	-	M2322I	N/A	Medium	Slow	13.9	EGFR, BTK, ABL, TP53 activation, S6K1	CHEK1-2, WEE1/PLK1, MRCKB, ERK1-2, TNKS1-2
BT474	0.58	0.91	0.30	Luminal B	+	+	+	-	+	-	S3094	Wildtype	Small	Slow	9.7	BTK, EGFR, **PIK3**, TP53 activation, MCL1	MRCKB, CHEK1-2, topotecan, proteosome, TOP1
AU565	0.59	0.98	0.58	HER2+	-	-	+	+/-	-	-	-	N/A	Medium	Fast	6.6	BTK, TNKS1-2, PMRT5, EGFR, MCL1	IGFR1, BRD2-4, MEK1-2, Dihydrorotenone
MDA-MB-415	0.62	0.71	0.34	Luminal A	+	+	-	-	+	-	-	N/A	Medium	Slow	10.7	**PI3K**, AKT1-3, TERT, MTORC1	WEE1/PLK1, IGFR1, CHEK1-2, TOP1, IKK1-2
HCC38	0.64	0.96	0.72	TNBC (Basal-like)	-	-	-	+	+	-	-	Deletion	Big	Fast	5.4	Proteosome, BCL-XL, gemcitabine, CHEK1-2/CDK2, IAP	ERK1-2, USP1/UAF1, Leflunomide, VEGFR, CDK2
HCC1428	0.70	0.86	0.43	Luminal A	+	+	-	-	-	-	-	N/A	Medium	Slow	8.2	MCL1, HDACi, ABL, BRD2-4, CDK1	PI3K, CDK9, CDK2, AKT1-3, ROCK2
ZR-75-1	0.71	0.90	0.55	Luminal A	+	+	-	-	-	-	-	Partially methylated	Medium	Fast	4.7	NA	NA
SKBR3	0.75	1.05	0.74	HER2+	-	-	+	-	+	-	-	Unmethylated	Medium	Fast	6.5	NA	NA
MDA-MB-231	0.77	1.01	0.80	TNBC (Basal-like)	-	-	-	+	+	-	-	Deletion	Medium	Fast	2.8	Fludarabine, APK1-2, ATR, Kinase i, CDK9	BCL2/BCL-XL, Docetaxel, S6K1, USP1/UAF1, AKT1-3/ROCK2
BT549	0.96	0.99	0.96	TNBC (Basal-like)	-	-	-	+/-	+	-	-	Unmethylated	Big	Fast	2.1	ROCK1-2, DNAPK, MRCKB, TNKS1-2, CDK1	HDACi, TAC1, ERK1-2, DNA alkylating agent, PORCN
MDA-MB-468	1.00	1.37	0.98	TNBC (Basal-like)	-	-	-	+++	+	-	-	Unmethylated	Medium	Fast	4.7	Sinularin, TAF1, ERK1-2, VEGFR, BRPF1B	JAK1, MRCKB, Cytarabine, IGFR1, FGFR1-3