The combination effect of lapatinib and palbociclib in endocrine-resistant breast cancer cells

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ฤทธิ์ของลาปาทินิบและพัลโบซิคลิบต่อเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน

นายกรรธศรณ์ หอพัตราภรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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กรรธศรณ์ หอพัตราภรณ์ : ฤทธิ์ของลาปาทินิบและพัลโบซิคลิบต่อเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน. (The combination effect of lapatinib and palbociclib in endocrine-resistant breast cancer cells) อ.ที่ปรึกษาหลัก : รศ. ดร. พญ.วรรณรัศมี เกตุชาติ

ยาต้านฮอร์โมนยังเป็นหนึ่งในการรักษามะเร็งเต้านมที่สำคัญมากในปัจจุบัน โดยเฉพาะผู้ป่วยมะเร็งเต้านมที่มีตัวรับฮอร์โมน (ER-positive) ทั้งระยะต้นและระยะท้าย แต่ผู้ป่วยหลายรายนั้นสามารถเกิดการดื้อต่อยาต้านฮอร์โมนมะเร็งเต้านม และทำให้ตัวโรคนั้นลุกลามมากขึ้นได้ เซลล์มะเร็งเต้านมสามารถดื้อยาต้านฮอร์โมนนั้นได้จากหลายกลไกในเซลล์ หนึ่งในนั้นคือภาวะที่เซลล์เกิดการแสดงออกของ HER2 signaling proteins ที่มากขึ้นเช่น AKT และ ERK ซึ่งจะพบมากกว่าในเซลล์มะเร็งเต้านมที่ไม่ดื้อยาฮอร์โมน พัลโบซิคลิบเป็นยาในกลุ่ม CDK4/6 inhibitor และถูกใช้ในการรักษามะเร็งเต้านมในหลายข้อบ่งชี้โดยเฉพาะมะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน ลาปาทินิบเป็นยา dual tyrosine kinase inhibitor ที่ HER1 และ HER2 ใช้ในการรักษามะเร็งเต้านมที่มีการแสดงออกของ HER2 สูง มีการศึกษาการให้พัลโบซิคลิบร่วมกับลาปาทินิบในเซลล์มะเร็งศีรษะและลำคอ พบว่าการใช้ยาสองตัวร่วมกันเกิดผล synergistic ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งและลด phosphorylation ของโปรตีน ERK1/2 ได้ แต่ทว่ายังไม่มีการใช้ยาสองตัวคู่กันในเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมนที่เซลล์เกิดการแสดงออกของ HER2 signaling proteins มากขึ้น ผู้วิจัยจึงสนใจการนำยาสองตัวนี้มาใช้กับเซลล์นี้ การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์และกลไกการต้านมะเร็งของลาปาทินิบและพัลโบซิคลิบต่อเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน (MCF-7/LCC2 and MCF-7/LCC9) ทั้งการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง การยับยั้งการลุกลาม และกลไกในระดับโมเลกุล จากการศึกษาผู้วิจัยพบว่า การใช้ลาปาทินิบร่วมกับพัลโบซิคลิบที่ค่า IC_{50} สามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมนได้มากกว่าการใช้ยาเดี่ยว นอกจากนี้การใช้ลาปาทินิบร่วมกับพัลโบซิคลิบที่ความเข้มข้นของยาที่ต่ำกว่า IC_{50} สามารถยับยั้งการลุกลามของเซลล์มะเร็งเต้านมได้มากกว่ายาเดี่ยวอย่างมีนัยสำคัญ และพบว่าการใช้ยาสองตัวร่วมกันช่วยยับยั้งกลไก Epithelial-mesenchymal

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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KEYWORD: lapatinib, palbociclib, endocrine-resistant breast cancer cells, AKT, EPITHELIAL MESENCHYMAL TRANSITION (EMT), invasion

Kantasorn Horpratraporn : The combination effect of lapatinib and palbociclib in endocrine-resistant breast cancer cells. Advisor: Assoc. Prof. WANNARASMI KETCHART, M.D. Ph.D.

Hormonal therapy is necessary in hormone receptor-positive breast cancer patients. It is used in both early and advanced-stage patients. Unfortunately, many patients developed endocrine resistance later on after the treatment was initiated and this could progress the disease. Endocrine resistance results from many mechanisms, including HER2 signaling pathway. These resistant breast cancer cells exhibit more HER2 signaling proteins such as AKT and ERK, compared to wild-type hormone receptor-positive breast cancer cells. Palbociclib is a CDK4/6 inhibitor and is indicated in patients who developed tamoxifen resistance. Lapatinib is a dual tyrosine kinase inhibitor- HER1 and HER2 and is used in HER2-overexpressed breast cancer patients. Palbociclib combined with lapatinib was investigated in head and neck squamous cell carcinoma and this resulted in synergistic cytotoxic activity and a decrease in ERK1/2 phosphorylation. However, combining these two drugs has not been used in endocrine-resistant breast cancer cells whose tumors overexpressed HER2 after hormonal therapy. In this study, we investigated the combination effect of these two drugs in MCF-7/LCC2 and MCF-7/LCC9 breast cancer cells, including cytotoxic activity, anti-invasion, and the mechanism behind it. Lapatinib combined with palbociclib at IC_{50} showed a significantly increased cytotoxic activity. Moreover, the combination of these drugs resulted in higher anti-invasion activity than either single drug alone did. Lapatinib combined with

Field of Study: Medical Sciences Student's Signature .....

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LIST OF ABBREVIATIONS

μM Micromolar

N=3 Three independent experiments

AIs Aromatase inhibitors

AKT Protein kinase B

ANOVA Analysis of Variance

BMD Bone mineral density

BRCA1 Breast cancer type1

BRCA2 Breast cancer type1

CCND1 Cyclin D1

CCNE1 Cyclin E1

CDK Cyclin-dependent kinase

CI Combination index

cSCC cutaneous squamous cell carcinoma

CYP Cytochrome P450

DBD DNA binding domain

E2 Estradiol

ECD Extracellular Domain

ECM Extracellular matrix

EGFR Epidermal Growth Factor Receptor

EMT Epithelial-to-mesenchymal transition

ER Estrogen receptor

ERK Extracellular signal-regulated kinase

FBS Fetal bovine serum

GAPDH Glyceraldehyde 3-phosphate dehydrogenase 13

GnRH Gonadotropin-releasing hormone

GSK3β glycogen synthase kinase 3 beta

GTP Guanosine triphosphate-binding proteins

HER1/ErbB1 Human epidermal growth factor receptor 1

HER2/ErbB2 Human epidermal growth factor receptor 2

HER3/ErbB3 Human epidermal growth factor receptor 3

HER4/ErbB4 Human epidermal growth factor receptor 4

HNSCC HPV-negative head and neck squamous cell carcinoma

hr hour

IC_{50} 50% inhibitory concentration/Half maximal inhibitory concentration

IGF1 Insulin-like growth factor 1

kDa kiloDalton

LAP Lapatinib

MAPK Mitogen-activated protein kinase

mg Milligram

mg/kg Milligram per kilograms

miRNA microRNA

MMP2 Metalloproteinase 2

MMP7 Metalloproteinase 7

MMP9 Metalloproteinase 9

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NCoA3 Nuclear Receptor Coactivator 3

NCoR Nuclear receptor corepressor

PAL Palbociclib

PI3K Phosphoinositide 3-kinases

PgR Progesterone receptor

SEM Standard error of the mean

SERDs Selective estrogen receptor down regulators

SERMs Selective estrogen receptor modulators

Src Proto-oncogene tyrosine-protein kinase

TNBC Triple-negative breast cancer

TP53 Tumor protein 53

uPA Urokinase plasminogen activator

uPAR Glycolipid-anchored uPA receptor

VEGF Vascular endothelial growth factor receptor

CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

Breast cancer is one of the most common malignancies and occurs most frequently in one in eight to ten women [1]. There is an increasing trend in breast cancer incidence in Asia, Africa, and South America, and the mortality rate in these areas is rising [1]. Surgery and radiotherapy (Local therapies) are the first choices for patients with early-stage breast cancer. Apart from local therapy, systemic therapy still plays a vital role in almost all breast cancer patients, including early and advanced cancer patients [2, 3]. Endocrine therapy is crucial in hormone receptor-positive subtypes (luminal subtypes) [1]. However, many mechanisms can trigger endocrine resistance in breast cancer patients and are focusing on the estrogen receptor (ER) signaling pathway. Other growth factor receptors, such as HER2 and VEGF (angiogenesis factor), also impact resistance to endocrine therapy [4]. HER2 activation in endocrine-resistant breast cancer cells activates PI3K/AKT and MAPK/ERK pathways [5]. The signaling pathways promote cell proliferation and survival [6]. In MCF-7/LCC2 (tamoxifen-resistant breast cancer) and MCF-7/LCC9 (tamoxifen and fulvestrant-resistant breast cancer) cell lines, HER2 and its downstream signaling proteins such as pAKT and pERK1/2 were increased when compared to MCF-7 wild type cell line and the levels were similar to SKBR3 (HER2 overexpressed) cell line [5]. Patients with high levels of NCoA3 had resistance to tamoxifen and chemotherapy, leading to a worse prognosis [7, 8]. Epithelial-mesenchymal transition (EMT) is a cellular process necessary in metastasis. Cells lose their epithelial phenotypes and gain more mesenchymal phenotypes. The monolayer sheet cells initiate mobilization through EMT. This process is associated with downregulations of epithelial cell markers like E-cadherin & occludins and upregulations of mesenchymal markers such as vimentin and N-cadherin. Transcription factors like Snail, Twist, and Zeb1/2 are also known as regulators of the process [9]. Matrix metalloproteinases (MMPs) correlate with metastasis and aggressiveness of breast cancer cells. Especially MMP-9, it lyses the extracellular matrix (ECM) and basement membrane, leading to metastasis [10]. Once the ECM is destroyed, C-X-C chemokine receptor type 4

(CXCR4) binds with stromal cell-derived factor-1 (SDF-1 or CXCL12) and induces actin polymerization, which causes pseudopodia and leads to metastasis [11]. Wnt/β-catenin pathway also controls EMT and associates with primary and metastatic tumors [12]. Furthermore, endocrine-resistant breast cancer cells also exhibit overexpression of Wnt responsive genes [12]. Moreover, higher metastasis to lung and brain is higher if the patients are identified by the Wnt/β-catenin classifier [13].

Palbociclib, an orally selective CDK4/6 inhibitor, is developed and can be used in combination with fulvestrant to treat endocrine-resistant breast cancer patients [4]. Abemaciclib, another CDK4/6 inhibitor, was combined with fulvestrant and trastuzumab demonstrated longer PFS than trastuzumab and chemotherapy in HR-positive and HER2-positive metastatic breast cancer patients [14]. Moreover, a synergistic effect is seen when combining abemaciclib with HER2-directed therapies, especially in resistant HER2-positive breast cancer in transgenic mouse models [15].

Lapatinib is an oral dual tyrosine kinase inhibitor that blocks HER1 and HER2 tyrosine kinase activity by binding to the ATP-binding site of the intracellular domain of the receptor and resulting in tumor cell growth suppression [16]. In cutaneous squamous cell carcinoma (cSCC), lapatinib enhanced apoptosis of human cSCC cell lines, and the cSCC cell cycle was arrested in G2/M phase [17]. Lapatinib also interfered PI3K/AKT/mTOR pathway and reduced EMT via Wnt/ERK/PI3K-AKT pathway in human cSCC cells [17].

In HPV-negative head and neck squamous cell carcinoma (HNSCC), simultaneous inhibition of RB1 phosphorylation with Palbociclib and EGFR activity with lapatinib resulted in synergistic inhibitory effects on the HNSCC cell proliferation and suppressed ERK1/2 phosphorylation [18]. Prior clinical trials have shown that combining HER2-targeted therapy with an AI resulted in clinical benefits in patients with advanced HR-positive and HER2-positive breast cancer patients. Combining abemaciclib plus trastuzumab plus fulvestrant resulted in a better progression-free survival rate than chemotherapy plus trastuzumab [19-22]. However, clinical use of CDK4/6 inhibitor and HER2-targeted therapy in endocrine-resistant breast cancer patients whose tumors overexpressed HER2 after resistance to endocrine therapy has

not been included in any clinical guidelines. Thus, a potential research gap could be explored between the combination of lapatinib and palbociclib in endocrine-resistant breast cancer cells that overexpressed HER2, especially the MCF-7/LCC9 cell line.

1.2 Research question

- How combining lapatinib with palbociclib affects proliferation, HER2 signaling pathway (tamoxifen-resistant mechanism) and the EMT process (cell invasion/migration) in endocrine-resistant breast cancer cells?

1.3 Objective

The aims of this study are

- to study the anti-proliferation and anti-invasion effects of combining lapatinib and palbociclib in endocrine-resistant breast cancer cells.
- to study the molecular mechanisms of lapatinib and palbociclib, including anti-proliferation, anti-invasion, inhibition of HER2 signaling, and endocrine resistance in endocrine-resistant breast cancer cells.

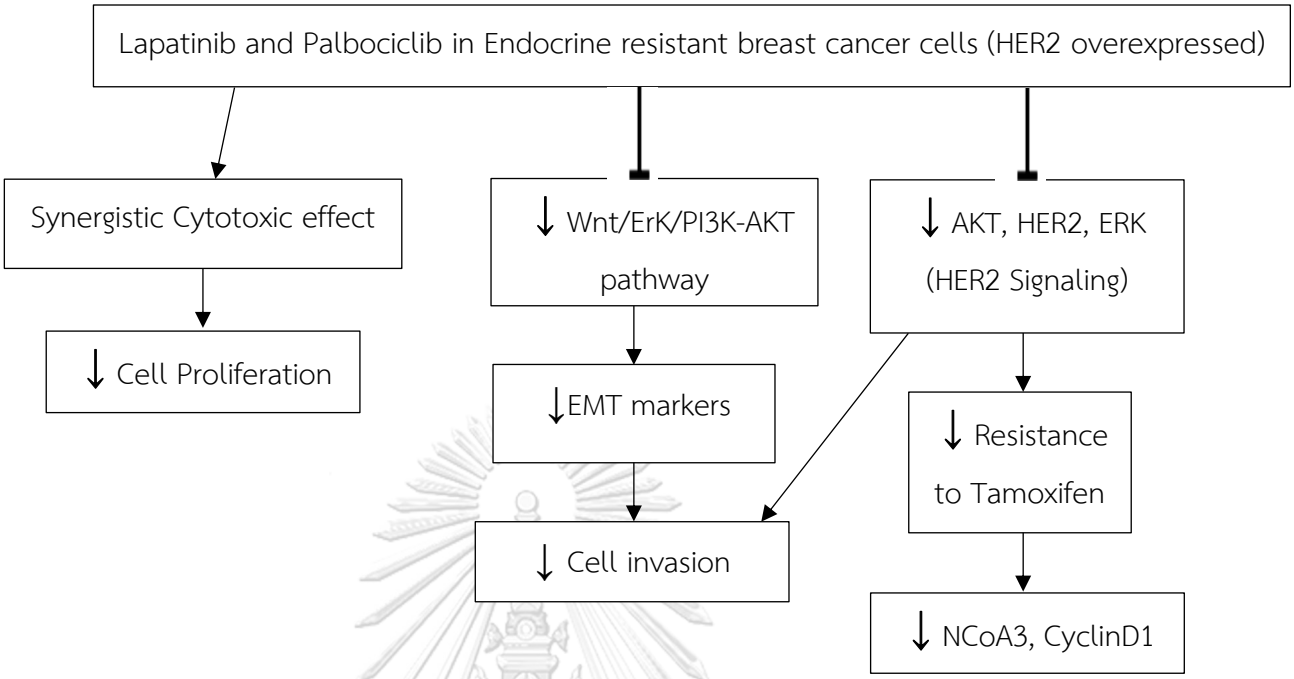
1.4 Hypothesis

- Lapatinib combined with palbociclib exhibits anti-proliferation and anti-HER2 activity through AKT, HER2, and ERK signaling pathways and reduces CyclinD1 and NCoA3 (tamoxifen-resistant mechanism) in endocrine-resistant breast cancer cells.
- Lapatinib combined with palbociclib exhibits an anti-migration/invasion effect through the EMT process in endocrine-resistant breast cancer cells.

1.5 Keywords

lapatinib, palbociclib, endocrine-resistant breast cancer cells, AKT, EMT, invasion

1.6 Conceptual framework



CHAPTER II REVIEW OF LITERATURE

2.1 Breast Cancer

The three most common cancers worldwide are colon, lung, and breast [1]. Particularly in women, breast cancer is the leading malignancy and occurs most frequently in one in eight to ten women. However, a decline in mortality rate is seen in North America and Europe due to early detection by mammograms and ultrasound and new emerging effective systemic therapies. On the contrary, there is an increasing trend in breast cancer incidence in Asia, Africa, and South America, probably caused by lifestyle changes and more developed screening programs in developing countries. Moreover, the mortality rate in these areas is rising [1].

2.2 Molecular subtypes of breast cancer

Breast cancer is clinically categorized into four molecular subtypes based on their cancer gene expressions (Luminal A, Luminal B, HER2-enriched, and basal-like (triple negative)). These subtypes are separated by the expression of Estrogen receptor (ER), Progesterone Receptor (PgR), and HER2 [1]. Ki67 also plays an essential role in investigating how aggressively breast cancer can proliferate [1]. ER and PgR-positive tumors can be treated by endocrine therapy. HER2-positive tumors can be treated by trastuzumab (targeted therapy) [1].

1) Luminal A breast cancer is steroid hormone receptor-positive (ER and/or PgR-positive, HER2-negative, and low proliferation (Ki67)). Luminal A is also the most common subtype and can be treated with estrogen receptor inhibitors like tamoxifen or aromatase inhibitors [1, 23].
2) Luminal B breast cancer is steroid hormone receptor-positive (ER and/or PgR-positive, and either HER2-positive or high proliferation (Ki67)). Adjuvant chemotherapy is added from only endocrine therapy (estrogen receptor inhibitors) [1].
3) HER2 subtype is ER and/or PgR-negative, HER2-positive. HER2-enriched tumors tend to be more aggressive and have a poorer prognosis than

Luminal A, but with the development of trastuzumab, the HER2 subtype prognosis is better [1, 23].

4) Basal-like cancer is more common in young African Americans and has a poorer prognosis than Luminal A. Furthermore, no targeted therapies are yet available (ER, PgR, and HER2-negative), so chemotherapy is always indicated, and BRCA testing is recommended [1, 23].

2.3 Treatment of Breast Cancer

1) Local therapy (Surgery and Radiotherapy)

Surgery and radiotherapy are the first choices for patients with early-stage breast cancer. There are mainly two surgical ways of breast cancer: total mastectomy and breast-conserving surgery. Many factors are used to consider which surgeries suit each patient, such as cancer's prognosis, patients' desire, etc. [24]. Total mastectomy with axillary lymph node dissection should be added with radiotherapy to reduce recurrence and mortality for patients with positive lymph nodes [25]. Radiotherapy should be added to every patient for breast-conserving surgery to reduce recurrence and deaths [26].

Fortunately, breast-conserving surgery is nowadays more practical because of the accomplishment of neoadjuvant drug therapies that can shrink the tumor's size [1].

2) Systemic therapy

Apart from local therapy, systemic therapy still plays an important role in almost all breast cancer patients, including early and advanced-stage cancer patients [2, 3]. Adjuvant tamoxifen helps reduce malignancy in the contralateral side of the breast [3]. All HER2-positive tumors should be treated with targeted therapy, trastuzumab (anti-HER2), and all basal-like tumors should receive adjuvant chemotherapy. Most clinical trials have proven that combining drugs is more effective than a single drug in all breast cancer diseases, both early and advanced

[3]. Endocrine therapy is crucial in hormone receptor-positive subtypes (luminal subtypes), which will be focused on later [1].

Endocrine Therapy

The mechanism of estrogen in breast cancer

Estrogen is essential in many body physiological processes, including the female reproductive, bone, central nervous, and cardiovascular systems. The imbalance of estrogen receptor signaling could lead to cancers in the breast, uterus, and ovary. So, anti-estrogens have become vital in treating breast cancer patients [4].

Selective estrogen receptor modulators (SERMs)

SERMS are anti-estrogens that compete with estrogen and have different effects on different tissues. For example, tamoxifen inhibits breast tissue proliferation, but it is an agonist for the uterus, the heart, and the bone. There are many SERMs in the market, which can be categorized based on their chemical structure. Triphenylethylenes (tamoxifen), benzothiophenes, phenylindoles, and tetrahydronaphthalenes are some examples [4].

Selective estrogen receptor degraders (SERDs)

SERDs, or the most known SERDS: Fulvestrant, are anti-estrogens that induce ER degradation and demolish the ER signaling pathway. Therefore, it acts as a universal ER antagonist and effectively treats tamoxifen-resistant breast cancers [4]. ER and PgR levels were reduced more than tamoxifen if treated with fulvestrant [4].

Aromatase Inhibitors (AI)

Aromatase inhibitors significantly reduce estrogen production in the body. Thus, aromatase inhibitors avoid stimulating ER-positive breast cancer cells [27]. It is the first-line drug in post-menopausal women only [3].

2.4 Endocrine-Resistant Breast Cancer

Many mechanisms can trigger endocrine resistance in breast cancer patients. The first one is metabolic resistance. Typically, tamoxifen will be converted to its active metabolites (4-hydroxytamoxifen and endoxifen) by CYP enzymes. The primarily responsible gene for this is the CYP2D6 gene, and 7% of patients are poor metabolizers of tamoxifen. Low levels of active metabolites resulted in a worse prognosis [4].

Alterations in ER and ER pathways could also change the outcome of endocrine therapy. Epigenetic silencing can result in ER loss and resistance to tamoxifen and fulvestrant. Moreover, the ligand-binding domain of ER could also mutate and lead to resistance to AIs [4].

Furthermore, the PI3K/Akt/mTOR pathway also correlates with resistance to hormonal therapy by activating ER in the absence of estrogen. PI3K activation lowers ER expression and results in tamoxifen resistance [4]. Other growth factor receptors, such as HER2 (an epidermal growth factor receptor (EGFR)), fibroblast growth factor receptor (FGFR), insulin growth factor receptor (IGFR), and vascular endothelial growth factor receptor (VEGFR) are also known to have an impact on resistance to endocrine therapy [4]. HER2 activation in endocrine-resistant breast cancer cells activates PI3K/AKT and MAPK/ERK pathways [5]. The signaling pathways promote cell proliferation and survival [6]. In MCF-7/LCC2 (tamoxifen-resistant) and MCF-7/LCC9 (tamoxifen and fulvestrant-resistant) cell lines, HER2 and its downstream signaling proteins such as pAKT and pERK1/2 were increased compared to MCF-7 wild type cell line and the levels were similar to SKBR3 (HER2 overexpressed) cell line [5]. Receptor tyrosine kinases (RTKs) also regulate MAPK, PI3K/Akt, and JAK/STAT signaling pathways. These pathways have a role in cancer angiogenesis and metastasis pathways. However, structural mutations, gene amplification, and alternate pathway activation made anti-RTK therapy struggle in breast cancer [28].

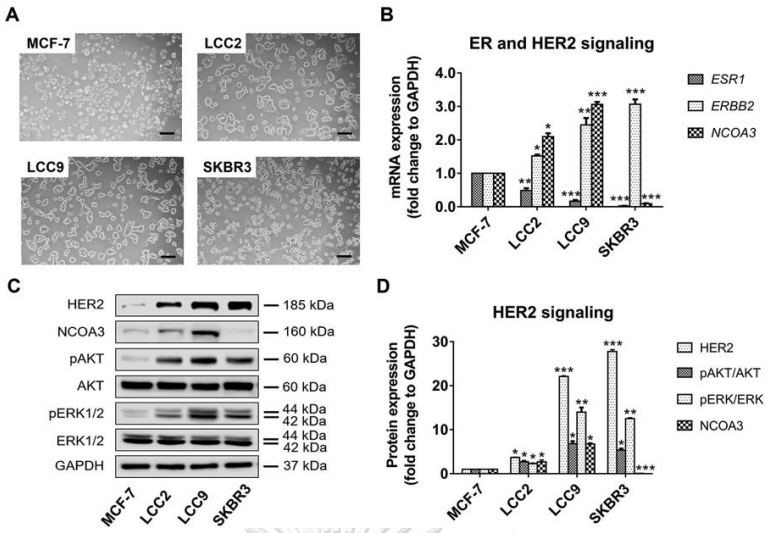


Figure 1 The expression of ER, NCOA3 and HER2 signaling in endocrine-resistant and HER2-overexpressed breast cancer cells [5]

As mentioned, tamoxifen acts as an ER antagonist in breast tissue but as an ER agonist in the bone, uterus, and cardiovascular system. These variations could be from diversities in the expression of coregulatory proteins [29]. The most known coactivator is the nuclear receptor coactivator 3 (NCoA3). Patients with high levels of NCoA3 had resistance to tamoxifen and chemotherapy, leading to a worse prognosis [7, 8]. From **Figure 1** above, MCF-7/LCC9 cells, tamoxifen and fulvestrant-resistant cells also overexpressed NCoA3 [30].

2.5 HER2 in Breast Cancer

Human epithelial growth factor receptor 2 (HER2, neu, ErbB2) is a tyrosine kinase receptor, a member of EGFR or ErbB family, including HER1, HER2, HER3, and HER4 [31, 32]. HER2/neu gene is located at q 21 of chromosome 17, encoding 185 kDa tyrosine kinase protein with 1255 amino acids [33]. The HER2 receptors consist of three specific domains: intracellular tyrosine kinase domain, transmembrane domain, and extracellular domain (ECD), which do not directly bind to identifiable ligands in contrast to other members in the family [34]. Among HER2/EGFR heterodimers, HER2/HER3 heterodimer is the most potent dimer to propagate signals [32, 34]. The

ECD may be cleaved by proteolytic mechanism and shed into blood circulation, leaving truncated transmembrane receptors that are 10-100-fold more oncogenic than full-length receptors [35]. Some studies suggested that serum ECD was a promising biomarker of treatment response, metastasis, and recurrence in HER2 overexpressed patients [35, 36].

After the activation of HER2 through dimerization, HER2 trans-auto-phosphorylates at tyrosine kinase residues which act as docking sites for other proteins, and then signal two main pathways – PI3K/AKT and MAPK pathways shown in **Figure 2** [31].

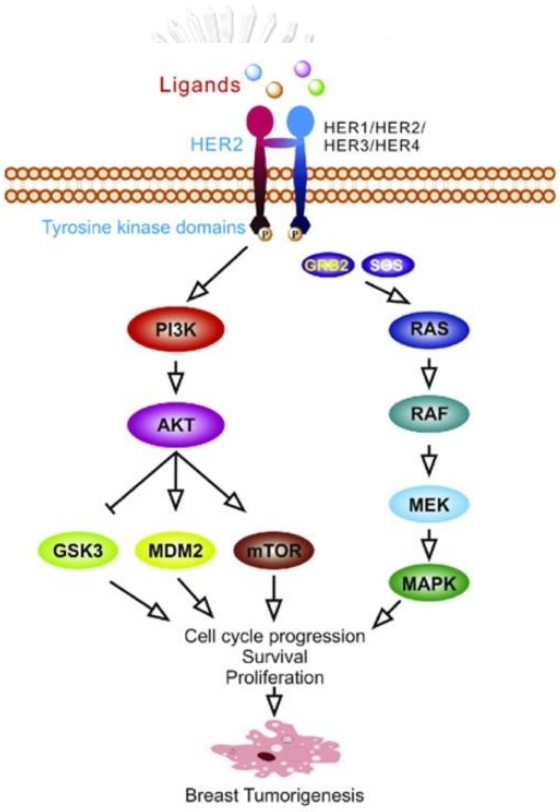


Figure 2 PI3K/AKT and MAPK pathways [37]

1. The PI3K/AKT pathway begins with the phosphorylated-RTK recruiting p85 and then p110, a regulatory subunit and a catalytic subunit of PI3K, respectively, forming an activated PI3K complex which then phosphorylates membrane PIP2 into PIP3. PIP3 docks AKT and PDK1 to the membrane and activates and stimulates AKT by PDK1 and mTORC2. The PI3K/AKT pathway, regulated by downstream signaling effectors such

as PTEN, mTOR, and NFkB contributes to the inhibition of apoptosis [33]. **(Figure 3,4)**

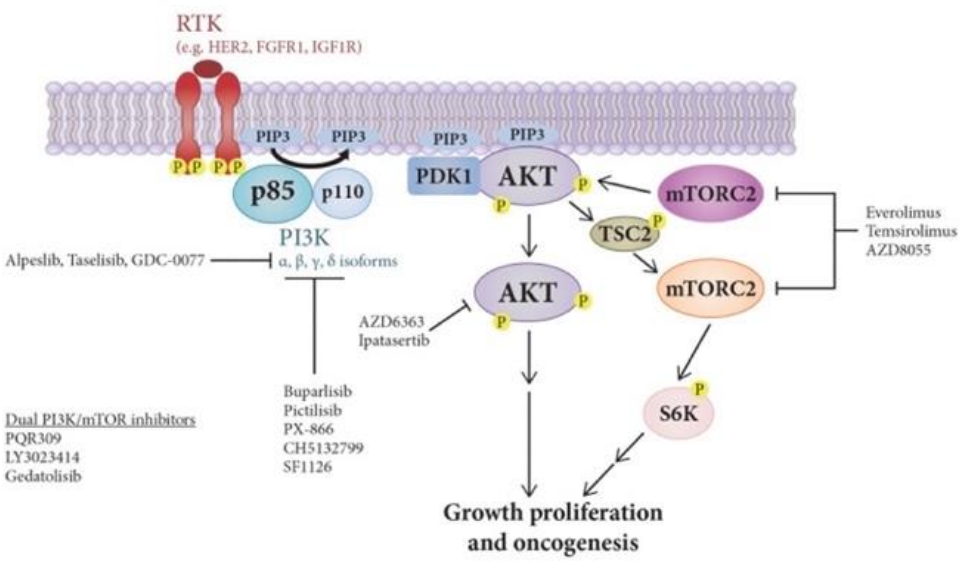


Figure 3 PI3K/AKT pathway [38]

2. The MAPK pathway starts with SH2 domain binding with phosphorylated-RTK. The SH3 domain, connected to the SH2 domain by non-functioning adaptor protein Grb2, activates Ras-GEF (SOS). SOS then replaces GDP from inactivated Ras in the membrane with GTP and turns into activated Ras. Ras activates Raf, which is then phosphorylated and turned into MAP kinase kinase (Mek) and MAP kinase (Erk) consecutively. The activation of MAPK pathway results in cell growth and differentiation [39].

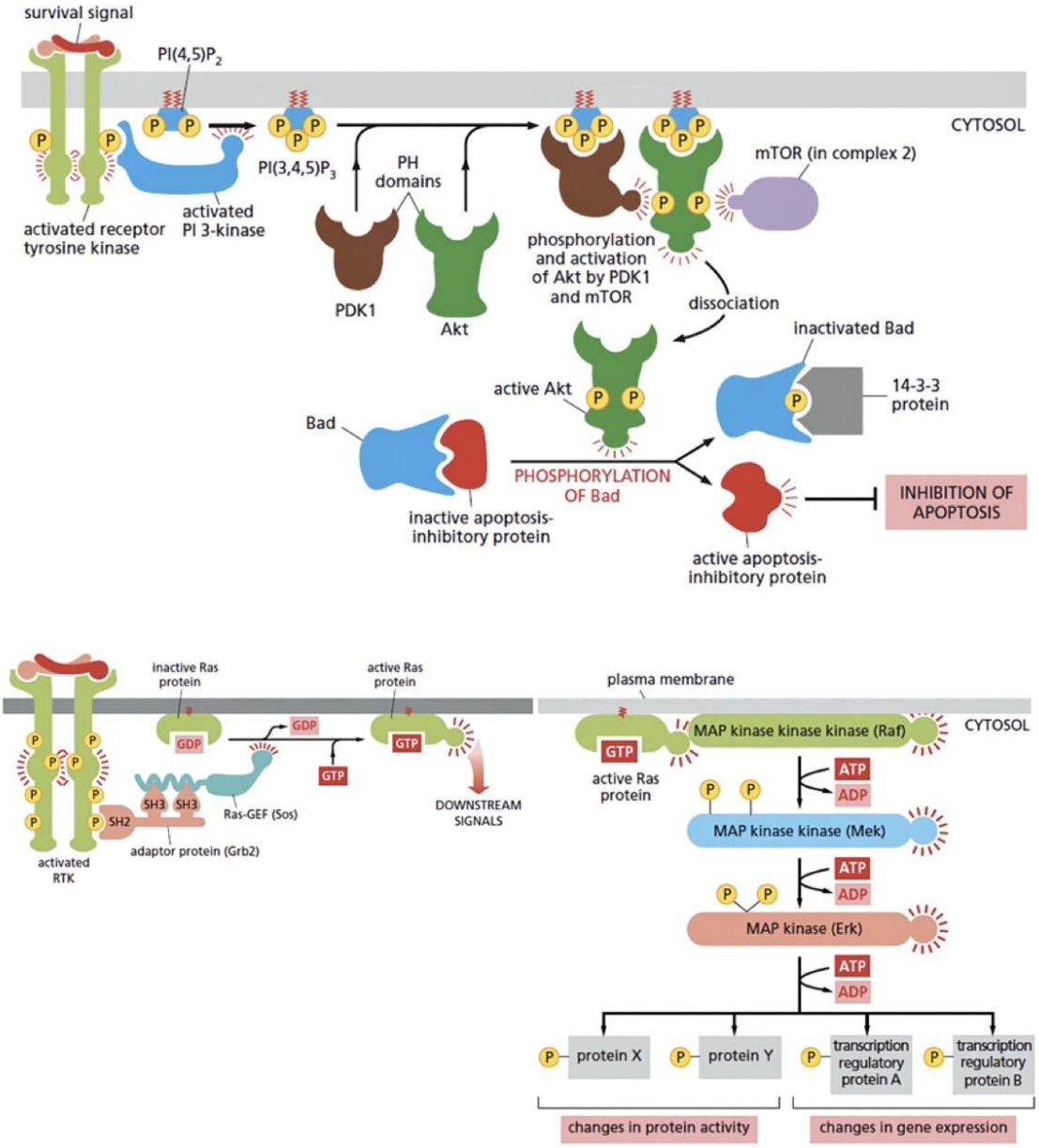


Figure 4 PI3K/AKT pathway and MAPK pathway [39]

As shown in the **Figure 5** below, Rb or retinoblastoma, a tumor suppressor protein, binds with E2F to prevent gene transcription. However, CDK4/6 can bind to CyclinD1 and together hyperphosphorylated Rb and lead to cell cycle progression. This process contributed to the resistance to anti-estrogens. Fortunately, Palbociclib, a CDK4/6 inhibitor, is developed and can be used in combination with fulvestrant to treat ER+ breast cancer patients [4].

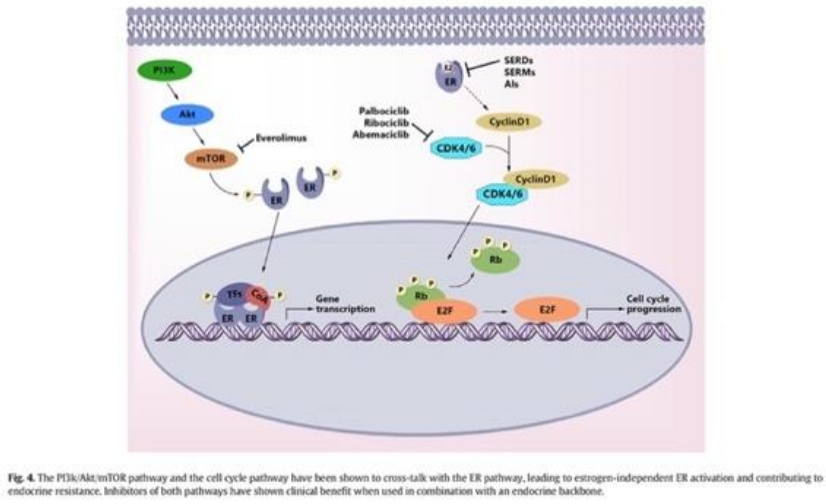


Fig. 4. The PI3k/Akt/mTOR pathway and the cell cycle pathway have been shown to cross-talk with the ER pathway, leading to estrogen-independent ER activation and contributing to endocrine resistance. Inhibitors of both pathways have shown clinical benefit when used in combination with an endocrine backbone.

Figure 5 The PI3k/Akt/mTOR and the cell cycle pathway contributing to endocrine resistance [4]

2.6 Metastasis

Metastasis is a process that starts from epithelial-mesenchymal transition (EMT),

local tissue invasion (ECM degradation), intravasation to the blood vessels, homing in the vessels, extravasation to other tissues, and metastatic niche formation at the new site [40].

Epithelial-mesenchymal transition (EMT) is a cellular process important in metastasis. Cells lose their epithelial phenotypes and gain more mesenchymal phenotypes. The monolayer sheet cells initiate mobilization through EMT. This process is associated with downregulations of epithelial cell markers like E-cadherin & occludins and upregulations of mesenchymal markers such as vimentin and N-

cadherin. Transcription factors like Snail, Twist, and Zeb1/2 are also known as regulators of the process [9]. Finally, EMT leads to invasion, migration, metabolic reprogramming, and apoptotic resistance [41].

Matrix metalloproteinases (MMPs) correlate with metastasis and aggressiveness of breast cancer cells. Especially MMP-9, it lyses type IV collagen and gelatin, which are the components of the extracellular matrix (ECM) and basement membrane and leads to metastasis [10]. Once the ECM is destroyed, CXCR4 becomes the important mediator to metastasis. CXCR4 binds with stromal cell-derived factor-1 (SDF-1 or CXCL12) and induces actin polymerization, which causes pseudopodia and leads to metastasis [11].

MCF-7 cells, wild-type ER-positive cells, lived in tightly packed cells and had high levels of E-cadherin (*CDH-1*, the epithelial molecule). MCF-7/LCC9 cells are more spindle in shape, have pseudopodia, and highly expressed vimentin (*VIM*) and *Snail* (mesenchymal biomarkers), which were the characters of mesenchymal cell type [30]. These changes show that the endocrine-resistant breast cancer cells underwent EMT changes [30].

Wnt/β-catenin pathway is also a vital pathway controlling breast cancer progression. The pathway regulates epithelial-mesenchymal transition (EMT). EMT associated with primary and metastatic tumors [12]. The Wnt pathway gene expression also upregulates cell proliferation, invasion and metastasis, and angiogenesis. Furthermore, endocrine-resistant breast cancer cells also exhibit overexpression of Wnt-responsive genes [12]. Moreover, higher metastasis of breast cancers to the lung and brain is higher if the patients are identified by the Wnt/β-catenin classifier **(Figure 6)** [13].

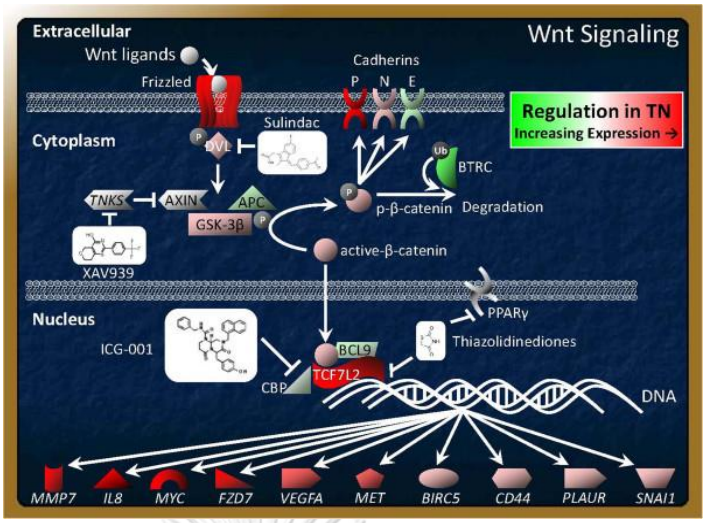


Figure 6 Wnt signaling in triple-negative breast cancer [13]

2.7 Palbociclib

Palbociclib, an orally selective CDK4/6 inhibitor, is developed and used in combination with fulvestrant to treat ER+ breast cancer patients [4]. The indications are patients with advanced or metastatic hormone receptor-positive patients with HER2-negative in pre-menopausal women or men with the progression of the disease after an endocrine therapy (used in combination with fulvestrant) ([42]. Palbociclib combined with fulvestrant showed a longer progression-free survival (PFS) rate than fulvestrant alone [43]. Another approved indication is in advanced or metastatic HR-positive and HER2-negative breast cancer in post-menopausal women or men as an initial endocrine-based therapy (combined with an aromatase inhibitor) [42]. Palbociclib combined with letrozole improved PFS compared to letrozole alone [44]. Currently, there are clinical trials using the inhibitor in other breast cancer subtypes (HER2-positive and triple-negative) and combinations with other targeted therapies [42, 44]. Myelotoxicity is seen in patients after receiving palbociclib, but it is well described and could be easily managed through dose reductions [14]. Abemaciclib, another CDK4/6 inhibitor, was combined with fulvestrant and trastuzumab. This combination demonstrated longer PFS compared with trastuzumab and chemotherapy in HR and HER2-positive metastatic breast cancer patients and also better anticancer activity *in vitro and in vivo* [14, 45]. Moreover, a synergistic effect is

seen when combining abemaciclib with HER2-directed therapies, especially in resistant HER2-positive breast cancer in transgenic mouse models [15]. CDK4/6 inhibitor decreased Rb phosphorylation and TSC2 phosphorylation and partially attenuated mTORC1 activity. This effect leads to feedback inhibition of upstream EGFR family kinases and delays tumor recurrence [15].

2.8 Lapatinib

Breast cancer with HER2 overexpression tends to be more aggressive, but with the development of HER2-targeted therapy, the HER2 subtype prognosis is better than before [1, 23]. Lapatinib is an oral reversible dual tyrosine kinase inhibitor that blocks HER1 and HER2 tyrosine kinase activity by binding to the ATP-binding site of the intracellular domain of the receptor such as Raf, PI3K, and PLC1 proteins and resulting in the inhibition of downstream signaling cascades – MAPK, PI3K/AKT, and PLC pathways. Thus, tumor cell growth is suppressed [16, 46]. In addition, lapatinib activates p38 MAPK, a class of MAPK that responds to stress stimuli and promotes cell killing in G1 phase, downregulates anti-apoptotic protein Bcl2 (a mediator in MAPK pathway), and reduces expression of surviving, which is an apoptosis inhibitor protein through PI3K/AKT pathway [36, 47]. In cutaneous squamous cell carcinoma (cSCC), lapatinib enhanced apoptosis of human cSCC cell lines and the cSCC cell cycle was arrested in G2/M phase [17]. Lapatinib also interrupted PI3K/AKT/mTOR pathway and decreased EMT via Wnt/ErK/PI3K-AKT pathway in human cSCC cells [17].

The most common side effect is diarrhea which is typically well tolerated. Lapatinib has been approved since 2010 for post-menopausal women with HR-positive and HER2-positive metastatic breast cancer in combination with letrozole [16]. Another indication is for patients with advanced or metastatic breast cancer with HER2 overexpression who progressed after an anthracycline, a taxane, and trastuzumab (in combination with capecitabine) [16].

2.9 Prior clinical trials

The eLEcTRA trial (Study of the Efficacy and Safety of Letrozole Combined with Trastuzumab in Patients with MBC), the TAnDEM trial (Trastuzumab in Dual HER2 ER-Positive MBC), and the EGF30008 trial demonstrated that combining HER2-targeted therapy to an AI resulted in clinical benefit [19-21]. The phase 2 monarcHER trial showed that in patients with advanced HR-positive and HER2-positive breast cancer patients, combining abemaciclib plus trastuzumab plus fulvestrant resulted in a better progression-free survival rate than chemotherapy plus trastuzumab with safe tolerability [22].

2.10 Rationale of this study: Combining CDK4/6 inhibitor and HER2-targeted therapy in endocrine-resistant breast cancer cells

ER and HER2 signaling interact with each other through many pathways, and co-expression of the two receptors resulted in resistance to hormonal therapy [48]. Targeting multiple pathways simultaneously may improve anti-cancer activity [49]. Chemotherapy's side effects are not as well tolerated as targeted therapy, so palbociclib combined with fulvestrant is used in endocrine-resistant breast cancer patients [42]. In MCF-7/LCC2 and MCF-7/LCC9 cell lines, HER2 and its downstream signaling proteins were increased compared to MCF-7 wild-type cell line, and the levels were similar to SKBR3 (HER2 overexpressed) cell line **(Figure 1)** [5]. Thus, adding lapatinib, an oral dual tyrosine kinase inhibitor that blocks HER1 and HER2 tyrosine kinase activity [16], could potentially suppress tumor cell growth. In HPV-negative head and neck squamous cell carcinoma (HNSCC), simultaneous inhibition of RB1 phosphorylation with palbociclib and EGFR activity with lapatinib resulted in synergistic inhibitory effects on the HNSCC cells proliferation and suppressed ERK1/2 phosphorylation [18]. Prior clinical trials in HR+ and HER+ breast cancer patients have shown that combining HER2-targeted therapy with an AI resulted in clinical benefits [19-21]. The phase 2 monarcHER trial showed that in patients with advanced HR-positive and HER2-positive breast cancer patients, combining abemaciclib plus trastuzumab plus fulvestrant resulted in a better progression-free survival rate than

chemotherapy plus trastuzumab [22]. However, clinical use of CDK4/6 inhibitor and HER2-targeted therapy in endocrine-resistant breast cancer patients whose tumors overexpressed HER-2 after resistance to endocrine therapy has not been included in any clinical guidelines and MCF-7/LCC2 and MCF-7/LCC9 cells do resemble these patients.

From the literature reviews above, a potential research gap could be explored between the combination of lapatinib and palbociclib in endocrine-resistant breast cancer cells, especially MCF-7/LCC9 cells.

- The combination of lapatinib and palbociclib could potentially inhibit HER2 signaling pathway (tamoxifen-resistant mechanism) in the endocrine-resistant cell lines.
- The combination of lapatinib and palbociclib could inhibit the EMT process (cell invasion) in endocrine-resistant cell lines.

CHAPTER III MATERIALS AND METHODS

3.1 Materials

Cell lines

- MCF-7 wild type, MCF-7/LCC2 (tamoxifen-resistant breast cancer cell) and MCF-7/LCC9 (tamoxifen and fulvestrant-resistant breast cancer cell) from Dr. Robert Clarke (Georgetown University Medical Center, Washington, DC, USA)

Drug

- Lapatinib 5 g (abcam) and Palbociclib 5 g (abcam)

Chemicals

- Minimum Essential Medium (MEM) (Gibco, USA)
- Penicillin-streptomycin (Gibco, USA)
- Fetal bovine serum (FBS) (HyClone, USA)
- Amphotericin B (Gibco, USA)
- 0.25% trypsin/EDTA (Gibco, USA)
- 0.4% trypan blue dye (Gibco, USA)
- Improved MEM (Gibco, USA)
- MEM Non-Essential Amino Acids (MEM NEAA) (Gibco, USA)
- Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma, USA)
- PBS

Equipments

- CO2 incubator (Thermo forma, USA)
- Lamina flow hood (ESSCO ,USA)
- Microplate reader (Perkin Elmer, Victor3, USA)
- pH meter (CG842 Schott, Scientific Promotion, Co., Ltd., Japan)
- Autopipette (BRAND., Germany) and Serological pipette (Gilson, USA)
- Centrifuge (Hettich, USA)
- Vortex mixer (Scientific Industries, USA)
- Gel electrophoresis (Bio-Rad, USA), thermocycler machine (Eppendorf, USA)

- Microscope (ECLIPSE TS100, Hollywood International, Co., Thailand)
- 96 and 6-well culture plates (Corning, USA)

3.2 Methods

3.2.1 Cell preparation

Prepared MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells in Minimum Essential Media (MEM) which was added with 5% fetal bovine serum (FBS), 1% penicillin & streptomycin and Amphotericin B. The cells were cultured in T25 flasks and put in the incubator which was controlled in a 5% CO_2 gas and 37°C environment. Media was changed routinely every 2-3 days. When approximately 80% of cell density was seen, cells were subcultured into a new passage.

3.2.2 Drugs preparation

Lapatinib and palbociclib, pure compounds, were dissolved in DMSO into a stock solution at 10 mM concentration, and the stock solution was kept at -20°C. When the drug was going to be used, it would be dissolved in complete media to get the specified concentrations.

3.2.3 Cell Viability assay (MTT assay)

1. Cells were cultured until 70-80% density and seeded into a 96-well culture plate with 5,000 cells per well or 50,000 cells per ml.

2. The plate was then incubated for 18 hours in an FBS-free media.

3. The cells were treated with lapatinib or palbociclib at 0-50 uM, and left for 48 hours. 4. Then, the investigator added 10ul of thiazolyl blue tetrazolium bromide (MTT) per well and left for 4 hours.

5. The investigator then removed the media and formazan crystals were seen and added with DMSO.

6. The plates were read by a microplate reader at 570 nm absorbance to find out IC50. 7. Cell viability was calculated by the formula below and IC50 was calculated by GraphPad Prism program which required a series of drug

concentrations and cell proliferation percentages to plot x-y by linear regression. IC50 was then estimated using the plotted straight line.

$$Percentage\ of\ proliferation = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}}\ x\ 100$$

For the combination of lapatinib and palbociclib, two drugs will be combined in a non-constant ratio of concentrations below IC50 to determine the combined cytotoxic effect. Then, its potency will be calculated by the CompuSyn program which uses the Chou-Talalay method. The result is combination index (CI): CI = 1 means additive effect, CI < 1 means synergism, and CI > 1 shows antagonistic effect in each drug combination.

3.2.4 Matrigel invasion assay

Matrigel assay is a functional assay for studying the ability of cell invasion.

1. The upper chambers of a transwell were coated with matrigel and incubated for 24 hours.
2. MCF7/LCC9 cells at the density of 50,000 cells/well were added to upper chambers in serum-free media, but lower chambers contained 5% serum media in 24-well plates.
3. Non-toxic concentrations (at two concentrations lower than IC50 of both drugs and two combined constant ratio concentrations at concentrations below IC50) of lapatinib and/or palbociclib solutions were added and then cells were incubated for 48 hours.
4. Next, the investigator scraped off the non-invaded cells from the upper chambers.
5. Fixed the invasive cells with 4% formaldehyde and stained with crystal violet dye.
6. Counted the invaded cells under a microscope (25 random fields). Cell invasion was calculated by the formula below.

$$Percentage\ of\ invasion = \frac{\text{Number of cells invading 22atrigel (Drug solution)}}{\text{Number of cells invading 22atrigel (Control)}}\ x\ 100$$

3.2.5 Western Blot Analysis

1. Cells were cultured in 6-well plates with different drug concentrations (two concentrations lower than IC50 of both drugs and two combined constant ratios at concentrations below IC50).

2. The western blot analysis procedure started with cell extraction. The cells were lysed by lysis buffer containing proteinase inhibitor cocktails.

3. The protein extracts were then loaded into acrylamide gels for gel electrophoresis

4. Gels were then transferred onto a nitrocellulose membrane.

5. The membrane blots were blocked by the blocking buffer (0.1% Tween 20 and 5% non-fat milk powder in Tris-buffered saline (TBS)).

6. Blots were incubated with primary antibodies for AKT, HER2, ERK, NCoA3, CyclinD1, and EMT markers (E-cadherin, Snail, and Vimentin) in 5% BSA in TBST buffer overnight (18-21 hours) at 4°C. GAPDH will be used as a loading control for every protein.

7. The membranes were washed three times with TBST and incubated with the secondary antibody.
8. Immunoblots were developed using ECL western blotting substrate and analyzed using a luminescent image analyzer.

3.3 Data Analysis and Statistics

Data analysis is demonstrated as mean and standard error from at least three independent experiments, each experiment was done in triplicates and analyzed by one-way ANOVA and Tukey post hoc test by GraphPad Prism 10. Microsoft Excel and GraphPad Prism were used for data analysis and graph processing. The statistical significance is accepted at P value ≤ 0.05.

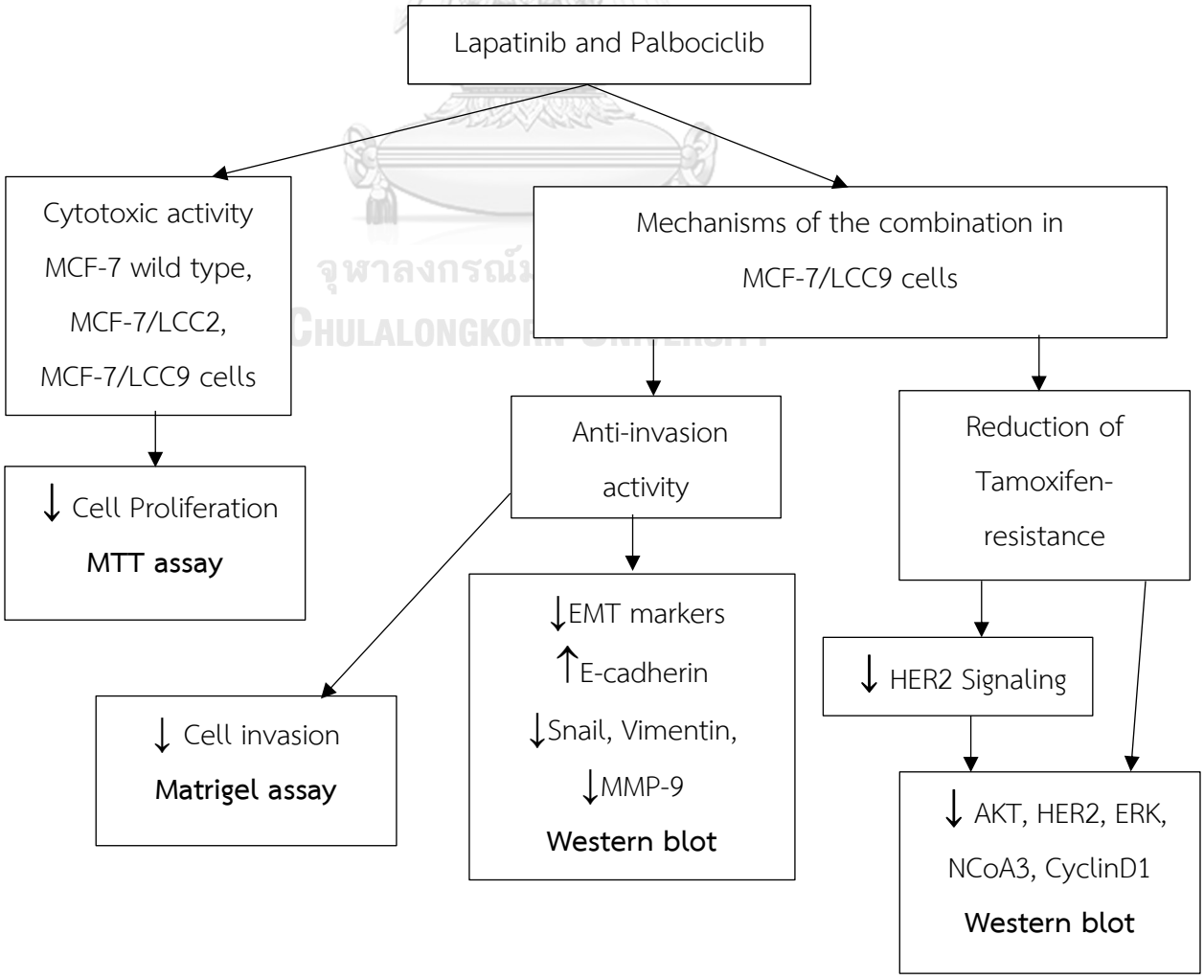
3.4 Expected Benefits and Application

The knowledge from this study would provide the preclinical data to support the use of lapatinib with palbociclib in further clinical studies or clinical trials to treat endocrine-resistant breast cancer. Lapatinib combined with palbociclib could be used for patients with endocrine-resistant breast cancer who overexpressed HER2. The quality of life of the patients could be improved.

3.5 Ethical Consideration

Ethical consideration was exempted from the Institutional Review Boards (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB Number: 419/66, COE Number: 037/2023) since the experiments were performed only in human cell lines. The cell lines are given by Dr. Robert Clarke (Georgetown University Medical Center, Washington, DC, USA) and are not obtained directly from animals or humans.

3.6 Experimental Design: *In vitro* experimental research



CHAPTER IV RESULTS

4.1 The anti-proliferation effect of lapatinib and palbociclib

4.1.1 The anti-proliferation effect of lapatinib in hormone-receptor positive (MCF-7) and endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines

MCF-7, MCF-7/LCC2, and MCF-7LCC9 breast cancer cells were treated with lapatinib (3.0-50.0 μM) for 48 hours. The cell viability was then investigated by MTT assay. Laptinib potentially decreased cell viability in all breast cancer cell lines ($p<0.05$) **(Figure 7)**. The half inhibitory concentrations (IC_{50} values) of MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells were 7.07± 0.29, 7.96±0.15, and 8.20±0.05 μM, respectively (**Figure 7)**.

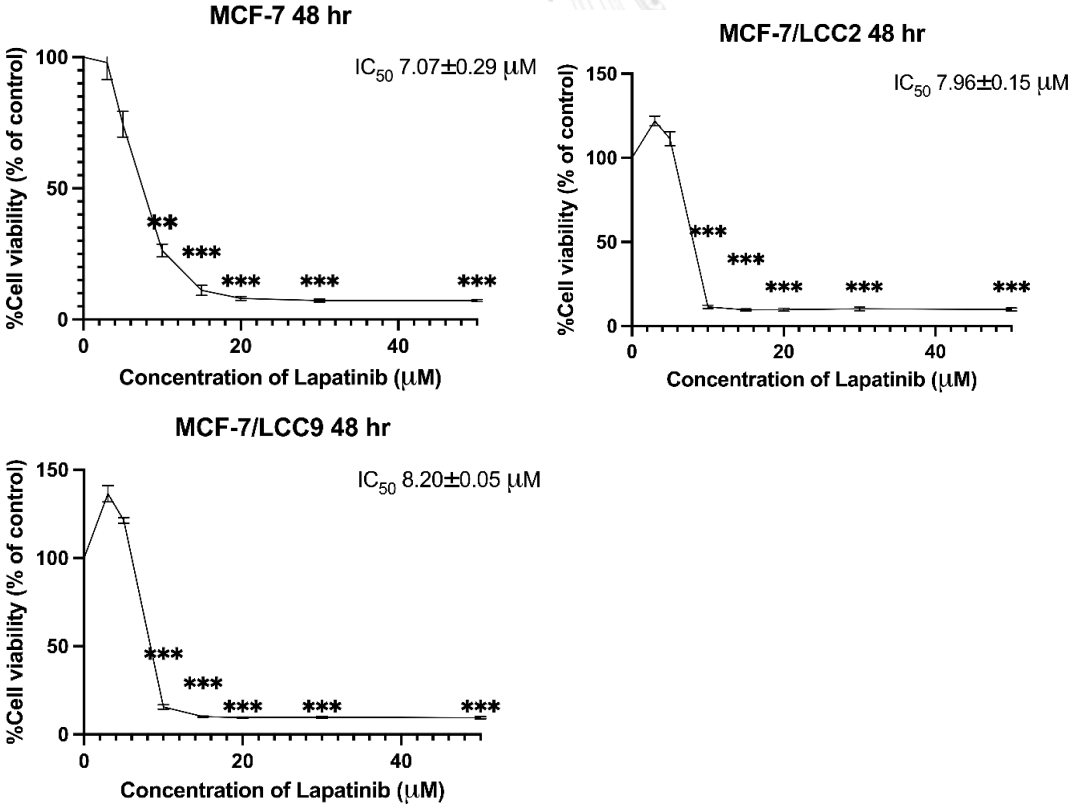


Figure 7 Cell viability after lapatinib treatment for 48 hours in wild-type hormone-receptor positive (MCF-7) and endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines. Statistical significance levels are indicated as *p <

0.05, **p < 0.01, and ***p < 0.001 compared to vehicle control. Each value was shown as mean ± SEM from 3 independent experiments (N=3).

4.1.2 The anti-proliferation effect of palbociclib in hormone-receptor positive (MCF-7) and endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines

MCF-7, MCF-7/LCC2, and MCF-7LCC9 breast cancer cells were treated with palbociclib (3.0-50.0 μM) for 48 hours. The cell viability was then investigated by MTT assay. Palbociclib potentially decreased cell viability in all breast cancer cell lines ($p<0.05$) **(Figure 8).** The half inhibitory concentrations (IC_{50} values) of MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells were 5.97± 0.67, 1.19±0.09, and 3.90±0.20 μM, respectively **(Figure 8)**.

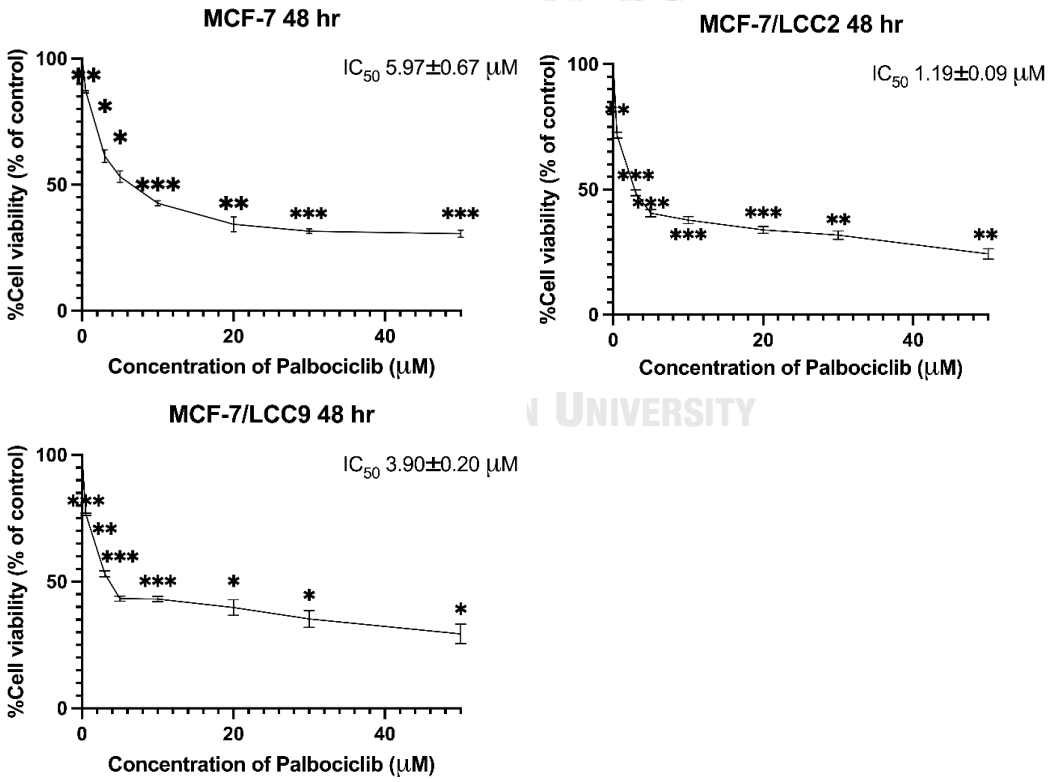


Figure 8 Cell viability after palbociclib treatment for 48 hours in wild-type hormone-receptor positive (MCF-7) and endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines. Statistical significance levels are indicated as *p <

0.05, **p < 0.01, and ***p < 0.001 compared to vehicle control. Each value was shown as mean ± SEM from 3 independent experiments (N=3).

4.1.3 The combined effect of lapatinib and palbociclib in endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines

After the calculation of IC_{50} values for each single drug, MCF-7/LCC2, and MCF-7LCC9 breast cancer cells were treated with the combined lapatinib and palbociclib in different concentrations (IC_{50} with IC_{50}, IC_{50}/2 with IC_{50}/2, IC_{50}/4 with IC_{50}/4) for 48 hours. The cell viability was then investigated by MTT assay. The cell viability of each drug combination is shown in **Figure 9.** In MCF-7/LCC2 cell lines, combining lapatinib and palbociclib at the half inhibitory concentrations of both drugs, resulted in the highest cytotoxic activity and is statistically significant compared to the control vehicle. Even though lapatinib at very low concentrations resulted in proliferative effects, combining lapatinib with palbociclib lowers that effect, and combining it at IC_{50} still resulted in the highest cytotoxic activity **(Figure 9)**.

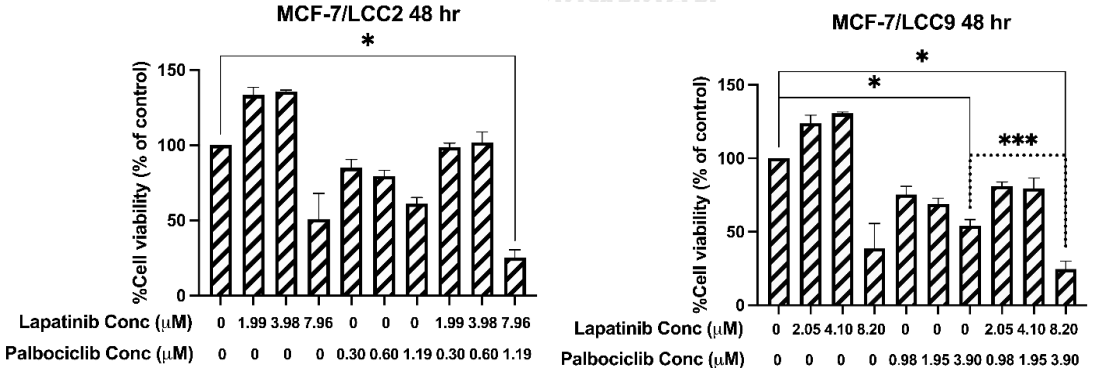


Figure 9 Cell viability after lapatinib and palbociclib treatment in different drug combinations for 48 hours in endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines. Statistical significance levels are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001 compared to vehicle control. Each value was shown as mean ± SEM from 3 independent experiments (N=3).

In MCF-7/LCC9 cell lines, lapatinib at very low concentrations also resulted in proliferative effects, combining it with palbociclib lowers that effect, and combining it at IC_{50} still resulted in the highest cytotoxic activity. Especially when combining lapatinib and palbociclib at the half inhibitory concentrations of both drugs, the cell viability was statistically significantly lower than those that were treated with IC_{50} of palbociclib. This demonstrated that combining both drugs at IC_{50} concentrations showed a synergistic effect and using a single drug alone (palbociclib is usually used in these cell lines but not with lapatinib) cannot compete with the combination **(Figure 10)**.

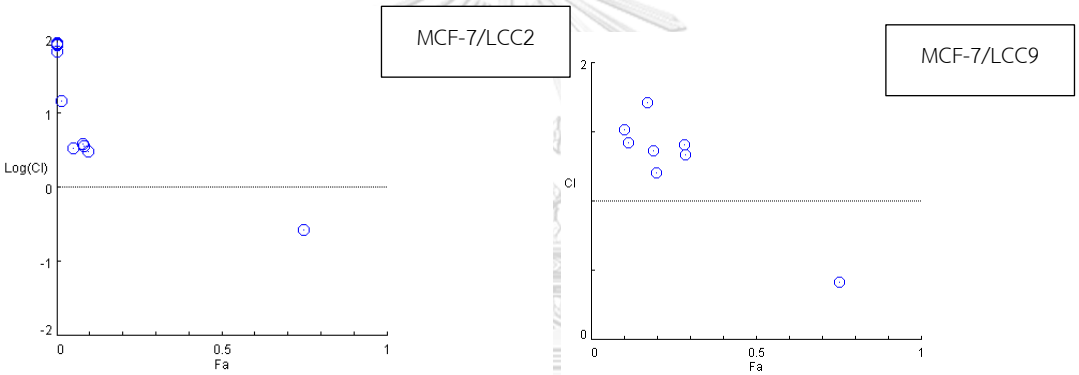
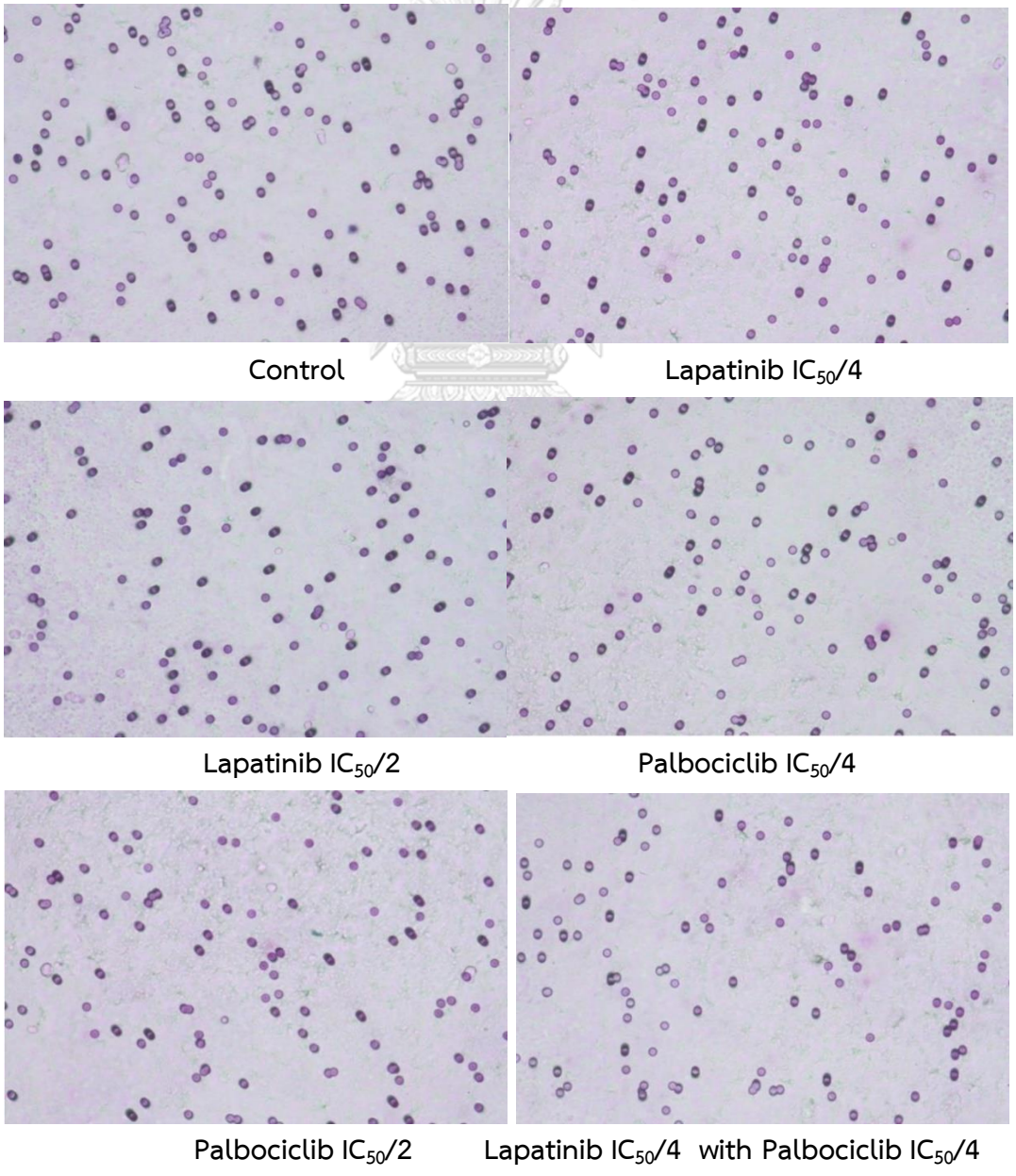


Figure 10 The combined effect of lapatinib and palbociclib in endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines. The combination index (CI) was generated from CompuSyn based on the Chou-Talalay Method. The graphs show combination index (CI) analysis; synergism (CI<1); additive effect (CI=1); and antagonism (CI>1). Combining lapatinib and palbociclib at the half inhibitory concentrations of both drugs resulted in a synergistic effect (CI<1) in both MCF-7/LCC2 and MCF-7/LCC9 breast cancer cell lines.

4.2 The anti-invasive effect of lapatinib and palbociclib in endocrine-resistant (MCF-7/LCC2 and MCF-7/LCC9) breast cancer cell lines

After the cell viability assay, the potential of combining both drugs was observed. Anti-invasion activity (metastasis) was investigated. Cells were treated with non-toxic concentrations ($IC_{50}/2$ and $IC_{50}/4$) of every single drug and the combination of both

drugs and put in the upper chambers of 28atrigel in an FBS-free (non-nutritious) environment. The lower chambers contained FBS and cells were attracted to them. All concentrations of either single or combined drugs could inhibit the invasion activity, but the non-toxic concentrations ($IC_{50}/2$) of both drugs resulted in the highest anti-invasion activity with the statistically significant level at $p<0.01$ in MCF-7/LCC9 breast cancer cell lines, while the same concentrations of a single drug alone did not show significant anti-invasion characteristics. The relative cell invasion percentage was reduced to 80.35% ± 2.44 **(Figure 11)**.



Control **Lapatinib $IC_{50}/4$**

Lapatinib $IC_{50}/2$ **Palbociclib $IC_{50}/4$**

Palbociclib $IC_{50}/2$ **Lapatinib $IC_{50}/4$ with Palbociclib $IC_{50}/4$**

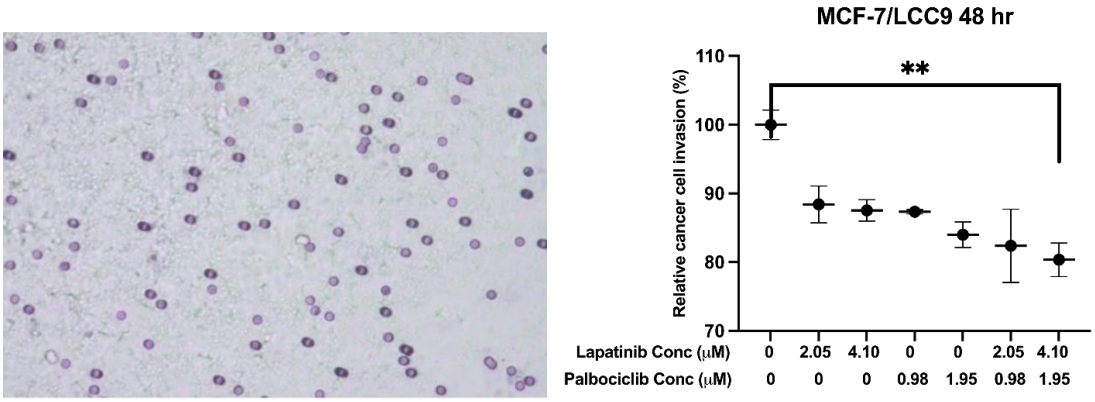


Figure 11 Pictures of MCF-7/LCC9 breast cancer cells captured from a microscope that were fixed in Matrigels to investigate the anti-invasive effect of different drug concentrations of lapatinib and/or palbociclib. The graph demonstrated the relative percentage of cell invasion compared to vehicle control in each drug concentration. Combining lapatinib and palbociclib at the non-toxic concentrations (IC50/2) of both drugs resulted in the highest anti-invasion activity in MCF-7/LCC9 breast cancer cell lines. Statistical significance levels are indicated as **p < 0.01 compared to vehicle control. Each value was shown as mean ± SEM from 3 independent experiments (N=3).

4.3 Lapatinib combined with palbociclib decreased AKT signaling pathway and EMT transcription factor

Western blot was performed after MTT assay and Matrigel assay to investigate the underlying mechanism. MCF-7/LCC9 cells were treated with lapatinib and palbociclib at different non-toxic concentrations ($IC_{50}/2$ and $IC_{50}/4$) **(Figure 12)**. Either lapatinib or palbociclib reduced the pAKT and Snail expression of both drugs. However, the combination of both drugs resulted in the highest suppression of both pAKT and Snail protein with statistical significance compared to control. Especially for Snail protein, lapatinib at $IC_{50}/2$ combined with palbociclib at $IC_{50}/2$ significantly decreased protein expression when compared to lapatinib at $IC_{50}/2$ alone. This finding suggests that the combination of both drugs is superior to using a single drug.

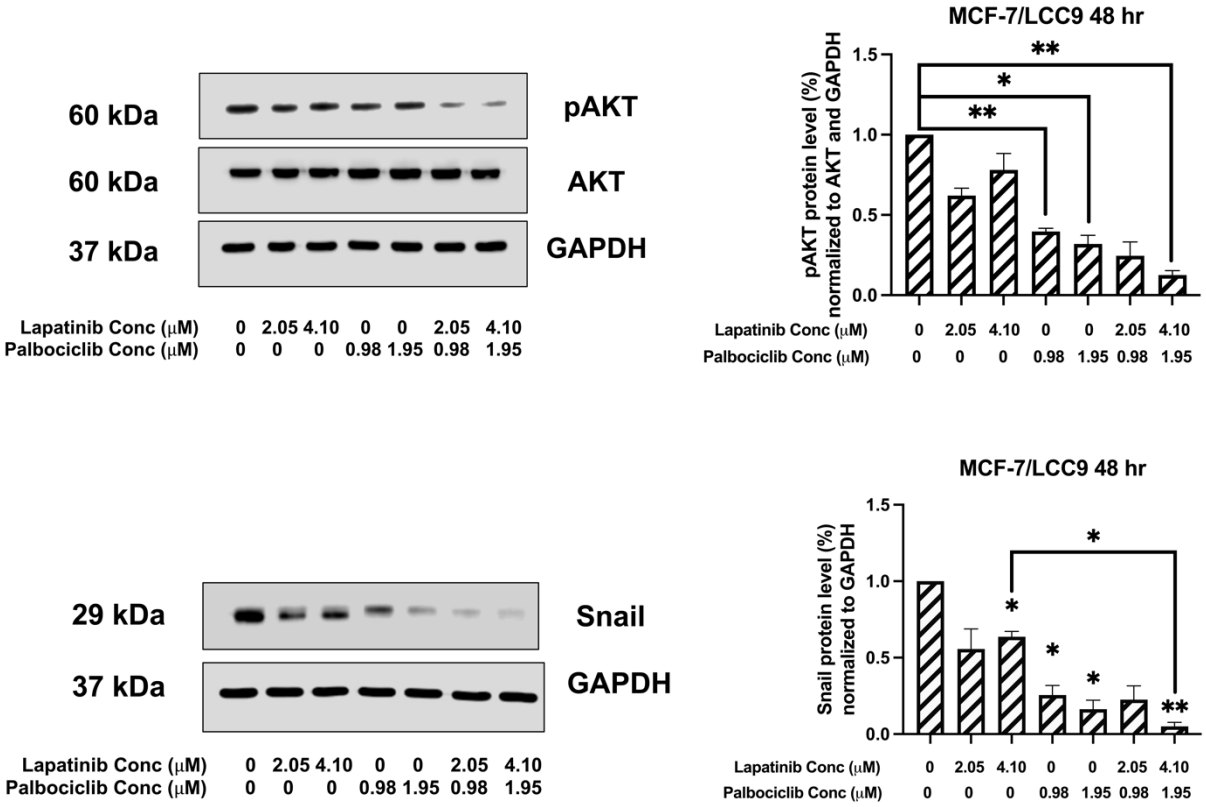


Figure 12 Western blots were performed to observe the amount of protein in each drug treatment. pAKT, AKT, and Snail were investigated and the results are interesting. The higher the concentrations of both drugs, the lower the expression of each protein. Statistical significance levels are indicated as *p < 0.05 and **p < 0.01 compared to vehicle control. Each value was shown as mean ± SEM from 3 independent experiments (N=3).

4.4 The combination effect of lapatinib and palbociclib on other signaling proteins

Western blot was performed in MCF-7/LCC9 cells that were treated with lapatinib and palbociclib at different non-toxic concentrations ($IC_{50}/2$ and $IC_{50}/4$) **(Figure 13, 14, 15)**. The results of the combination effects in some proposed signaling pathways were not significantly different as follows:

For tamoxifen-resistance signaling pathway proteins – NCoA3 and CyclinD1, the combination of lapatinib and palbociclib did not show potential inhibitory effects on these signaling pathway **(Figure 13)**.

For HER2 signaling pathway proteins (apart from AKT), pHER2 and pERK were investigated. The combination of lapatinib and palbociclib did not show potential inhibitory effects on these signaling pathways. **(Figure 14)**.

For EMT pathway and invasion activity (apart from Snail), MMP9, and Vimentin were investigated. The combination of lapatinib and palbociclib did not show potential inhibitory effects on these signaling pathway **(Figure 15)**.

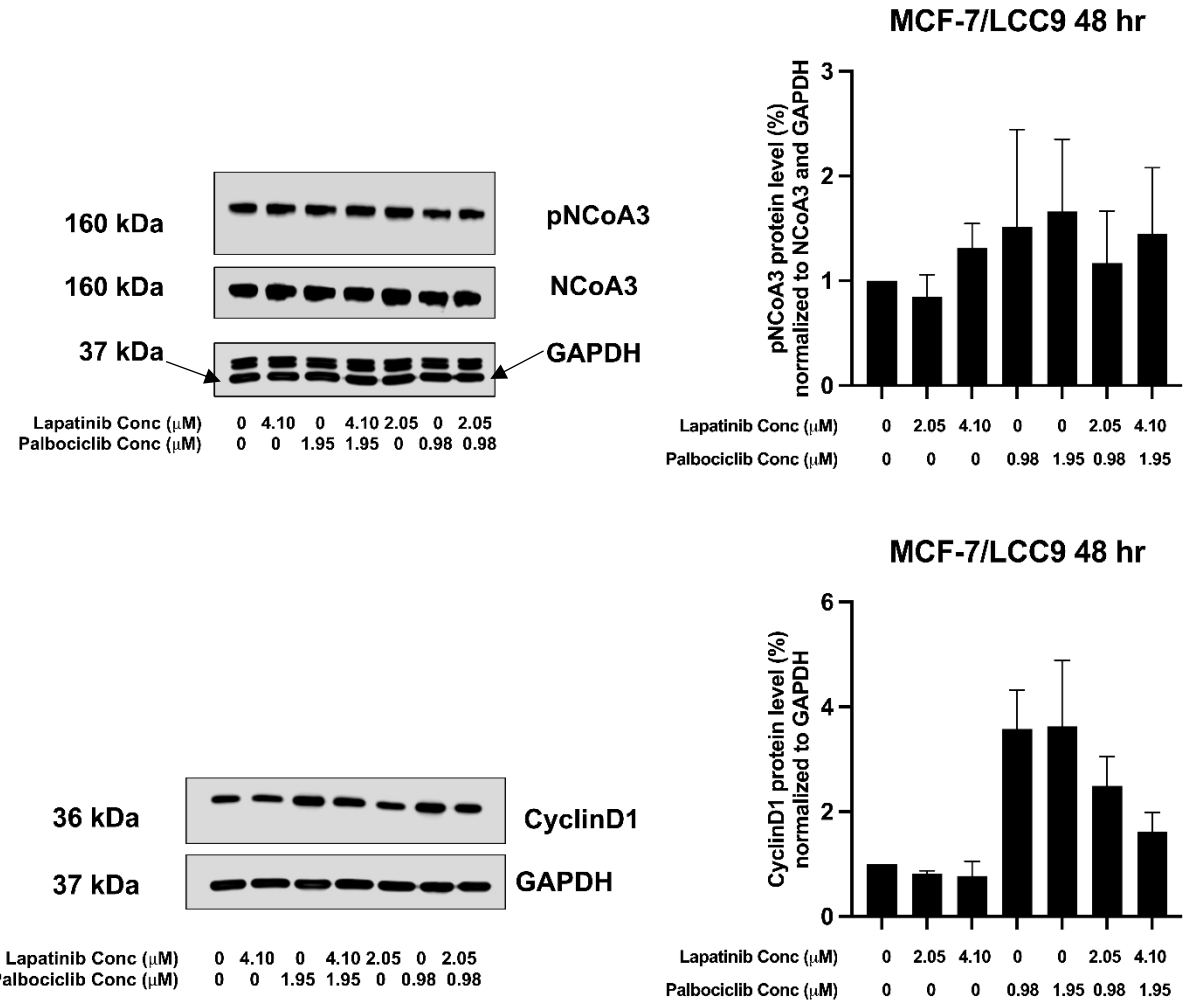


Figure 13 Western blots were performed to observe the amount of protein in each drug treatment for tamoxifen-resistance signaling protein. pNCoA3 and CyclinD1 were investigated. Each value was shown as mean ± SEM from independent experiments (N=2).

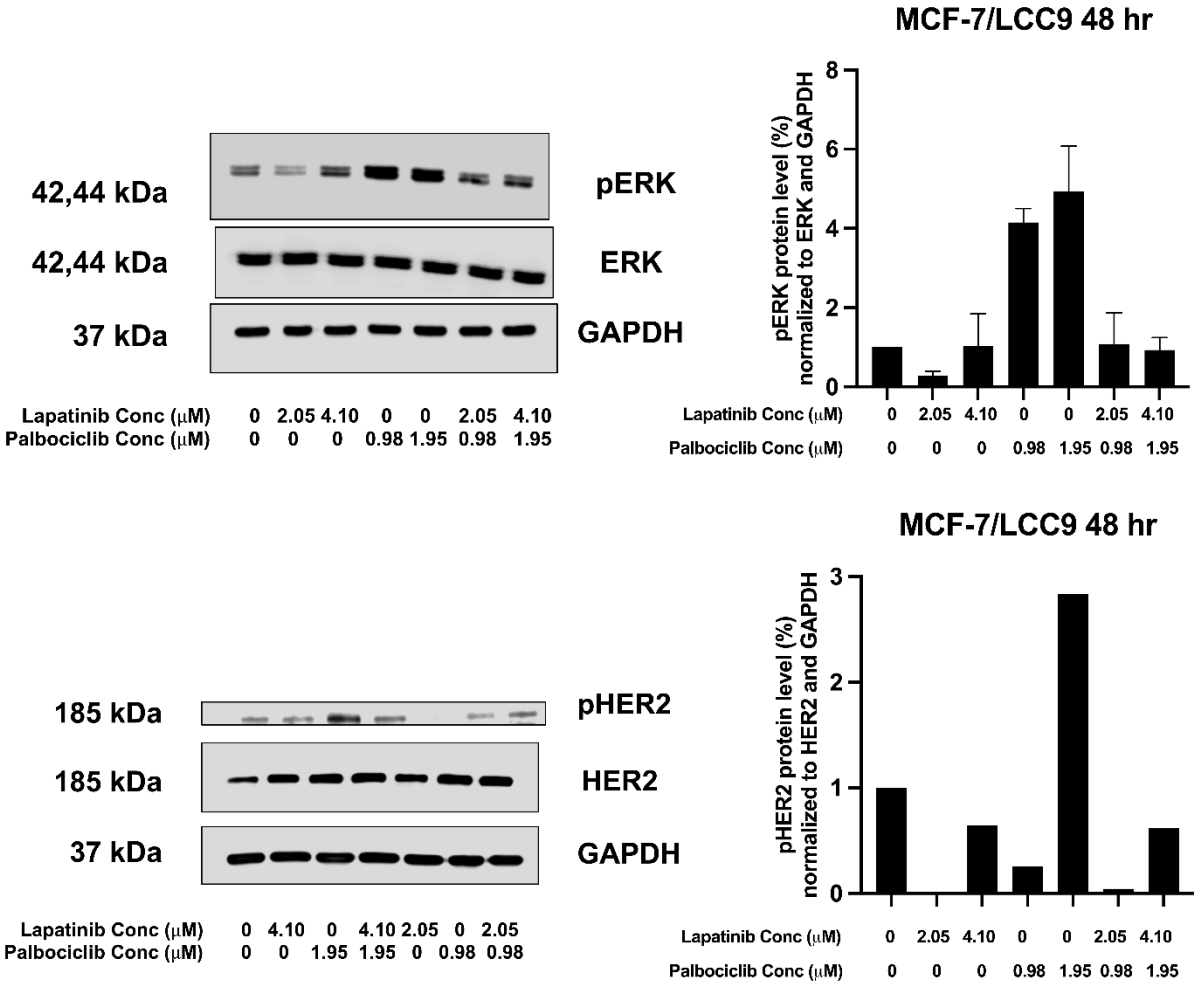


Figure 14 Western blots were performed to observe the amount of protein in each drug treatment for HER2 signaling pathway. pHER2 and pERK were investigated. Each value was shown as mean ± SEM from independent experiments (N=2 for ERK and N=1 for HER2).

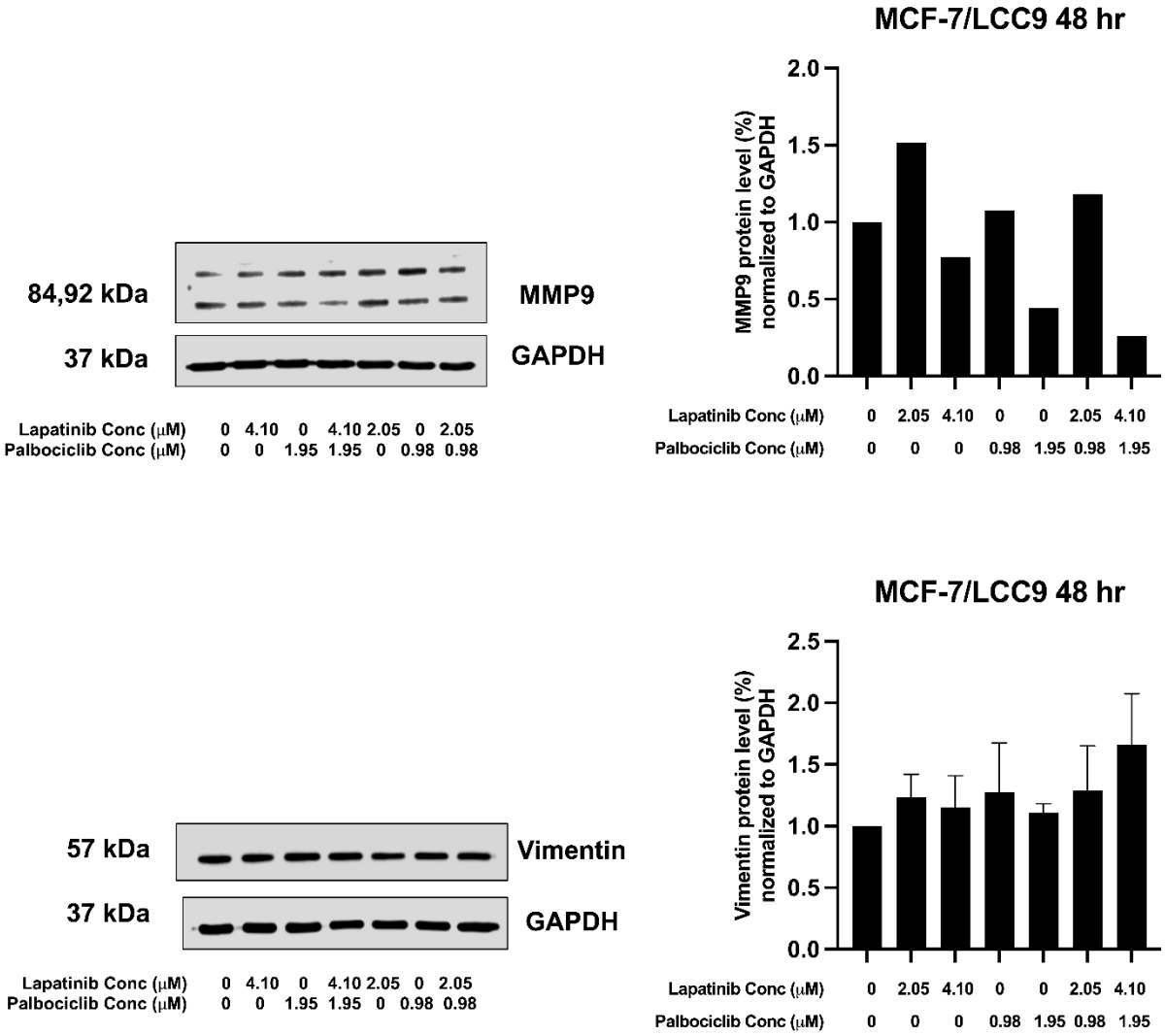


Figure 15 Western blots were performed to observe the amount of protein in each drug treatment for invasion protein pathway. MMP9 and Vimentin were investigated. Each value was shown as mean ± SEM from 3 independent experiments (N=3, except N=1 for MMP9).

CHAPTER V DISCUSSION AND CONCLUSION

Luminal A breast cancer is hormone receptor-positive and the most common subtype which can be treated with estrogen receptor inhibitors like tamoxifen or aromatase inhibitors [1, 23]. However, many mechanisms can trigger endocrine resistance in breast cancer patients. Growth factor receptors, such as HER2 and VEGF (angiogenesis factor), also impact resistance to endocrine therapy [4]. HER2 activation in endocrine-resistant breast cancer cells activates PI3K/AKT and MAPK/ERK pathways [5]. The signaling pathways promote cell proliferation and survival [6]. In MCF-7/LCC2 (tamoxifen-resistant breast cancer) and MCF-7/LCC9 (tamoxifen and fulvestrant-resistant breast cancer) cell lines, HER2 and its downstream signaling proteins such as pAKT and pERK1/2 were increased when compared to wild-type MCF-7 cell line and the levels were similar to SKBR3 (HER2 overexpressed) cell line [5]. Epithelial-mesenchymal transition (EMT) is a cellular process necessary in metastasis. This process is associated with downregulations of epithelial cell markers like E-cadherin and upregulations of mesenchymal markers such as Vimentin and Snail [9].

Palbociclib, an orally selective CDK4/6 inhibitor, is developed and can be used in combination with fulvestrant to treat endocrine-resistant breast cancer patients [4]**.** Lapatinib is an oral dual tyrosine kinase inhibitor that blocks HER1 and HER2 [16]. Lapatinib also interfered PI3K/AKT/mTOR pathway and reduced EMT via Wnt/ERK/PI3K-AKT pathway in human cSCC cells [17]. In HPV-negative head and neck squamous cell carcinoma (HNSCC), simultaneous inhibition of RB1 phosphorylation with Palbociclib and EGFR activity with lapatinib resulted in synergistic inhibitory effects on the HNSCC cells proliferation and suppressed ERK1/2 phosphorylation [18]. Prior clinical trials have shown that combining abemaciclib plus trastuzumab plus fulvestrant resulted in a better progression-free survival rate than chemotherapy plus trastuzumab [19-22]. However, clinical use of CDK4/6 inhibitor and HER2-targeted therapy in endocrine-resistant breast cancer patients whose tumors overexpressed HER2 after resistance to endocrine therapy has not been included in any clinical guidelines.

Therefore, in this study, the combination effects of lapatinib and palbociclib were investigated in endocrine-resistant breast cancer cells that overexpressed HER2, especially the MCF-7/LCC9 cell line.

First, we examine the cell viability of the wild-type MCF-7, MCF-7/LCC2, and MCF-7/LCC9 breast cancer cells that were treated with either lapatinib or palbociclib for 48 hours. Lapatinib potentially reduced cell viability in a single-digit micromolar range. Even though very low concentrations of lapatinib resulted in proliferative effects in MCF-7/LCC2, and MCF-7/LCC9 cells, its IC_{50} is still less than ten micromolar range which is considered a good candidate for clinical use. This could occur from the inhibition of HER1 and HER2 activities and the activation of other pathways through ER and HER2 crosstalk [48]. On the other hand, the proliferative effect of lapatinib did not occur in the wild-type MCF-7 cells and could be from less expression of HER2 signaling proteins and consequently, less crosstalk. Previous studies demonstrated that dual HER2-targeted therapy overcame HER2 single therapy in overall survival benefit [48]. Therefore, higher concentrations of lapatinib could highly inhibit cell proliferation.

For palbociclib, the anti-proliferative effect was high in the resistant cell lines as expected, since it is one of the drugs widely used for endocrine-resistant breast cancer patients. Its IC_{50} value is also less than ten micromolar range for all cell lines, wild-type MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells. Surprisingly, the IC_{50} value of palbociclib for wild-type MCF-7 is higher than both endocrine-resistant cell lines and this could be from the least proliferative nature of the cells, resulting in less CDK4/6 activation and less target-drug binding.

The combined cytotoxic activity in resistant cell lines indicated that their effects were cumulative when used at concentrations below the IC_{50}. However, when the two drugs were combined at the IC_{50}, they displayed a more potent cytotoxic effect on both MCF-7/LCC2 and MCF-7/LCC9 resistant cells, showing a synergistic effect. Consequently, the combination of these two drugs offers increased anti-proliferative effects in endocrine-resistant breast cancer cells.

Then, the anti-invasive property was observed for both drugs through the Matrigel invasion assay. All concentrations of either single or combined drugs could

inhibit the invasion activity, but the non-toxic concentrations (IC50/2) of both drugs resulted in the highest anti-invasion activity with statistically significant level at $p<0.01$ in MCF-7/LCC9 breast cancer cells, while the same concentrations of a single drug alone did not show significant anti-invasive characteristics. Therefore, combining these two drugs resulted in additional anti-invasive effects in endocrine-resistant breast cancer cells.

Phosphorylated AKT enhances the expression of mesenchymal markers and promotes epithelial-mesenchymal transition (EMT) in breast cancer cells that overexpress HER2 [50]. Typically, glycogen synthase kinase-3 beta (GSK3β) induces Snail to be unstabilized and leads to reduction of EMT pathway **(Figure 16)**. pAKT phosphorylate and inactivate GSK3β, resulting in Snail stabilization and increasing in breast cancer invasion activity [51]. Palbociclib, when used at non-toxic concentrations, can reduce phosphorylated AKT, but the combination of lapatinib and palbociclib greatly inhibited phosphorylated AKT. The level of Snail protein, a transcription factor that induces EMT, also decreased when the cells were treated with each drug individually. But when lapatinib and palbociclib were combined, they significantly suppressed Snail expression more effectively than when either drug was used alone. As a result, the combination of lapatinib and palbociclib effectively decreased Snail and phosphorylated AKT proteins in MCF-7/LCC9 cells, and this effect is in a concentration-dependent manner.

We did not observe the significant changes in other signaling proteins which are not in the AKT/GSK3β/Snail pathway. Therefore, that could be the reason behind those negative results, including NCoA3 and CyclinD1- the tamoxifen-resistance pathway, ERK and HER2 which are the upstreams family of HER2 signaling pathway and are not directly controlled by AKT, and also the invasion proteins – MMP9 and vimentin. GSK3β usually controls MMP7 [52], another MMP so the MMP9 was not significantly suppressed. Snail overexpression also results in induction of MMP7 transcription [53]. MMP-7, the MMP in GSK3β/Snail pathway [53], should be further investigated.

A synergistic effect is seen when combining abemaciclib with HER2-directed therapies, especially in resistant HER2-positive breast cancer in transgenic mouse

models (15). CDK4/6 inhibitor decreased Rb phosphorylation and TSC2 phosphorylation and partially attenuated mTORC1 activity. This effect leads to feedback inhibition of upstream EGFR family kinases and delays tumor recurrence (15). Lapatinib also interrupted PI3K/AKT/mTOR pathway and decreased EMT via Wnt/ErK/PI3K-AKT pathway in human cSCC cells (17). Consequently, these two drugs modified the AKT pathway, leading to a reduction in EMT and cell invasion, which has been validated by our findings.

From the research question, objectives, and hypothesis, all written problems have been addressed in this study. Combining lapatinib with palbociclib resulted in the synergistic anti-proliferation effect using the concentrations at IC_{50} of both drugs and the anti-invasive effect using non-toxic concentrations. The study also provides the underlying mechanism of the anti-invasive property of the combination, which is the regulation via the significant reduction of AKT signaling pathway and consequently decreased Snail, an important transcription factor of the EMT process in endocrine-resistant breast cancer cells. However, we did not observe the inhibitory effects of the combined treatment on HER2, ERK signaling, and tamoxifen-resistant molecules including CyclinD1 and NCoA3 as hypothesized. Therefore, we demonstrated the proposed mechanism **(Figure 16)** of the combination of these drugs and indicated the potential of the combination in further animal or clinical trials.

In summary, the combination of lapatinib and palbociclib (an anti-HER2 agent and CDK4/6 inhibitor), when used to treat endocrine-resistant breast cancer cells with HER2 overexpression after hormonal therapy resistance, offers promising preliminary data for clinical trials involving endocrine-resistant breast cancer patients. This is based on our research findings, which demonstrate superior anti-proliferative and anti-invasive effects, as well as a significant reduction in the EMT transcription factor and AKT levels when compared to using each drug individually.

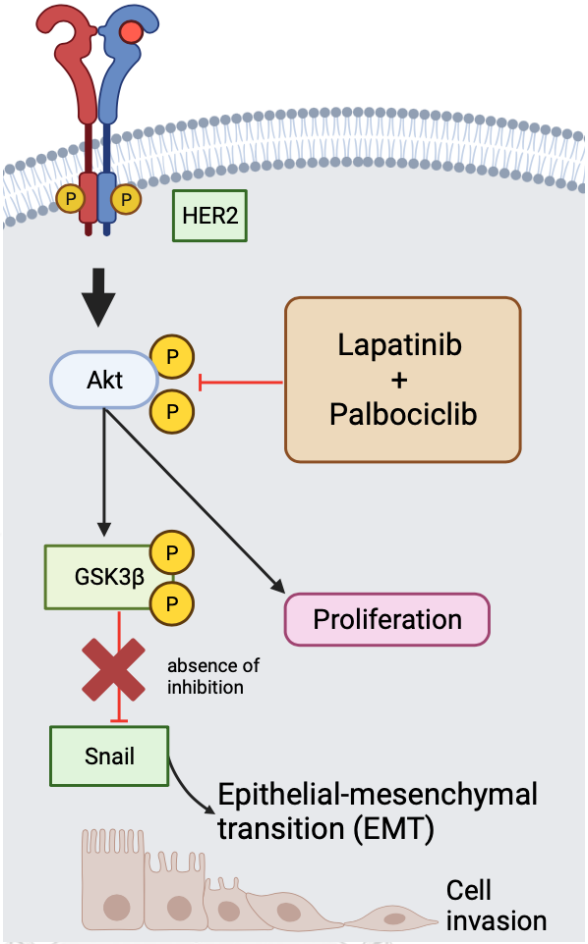


Figure 16 Proposed mechanism for the combination of Lapatinib and Palbociclib on endocrine-resistant breast cancer cells

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