EFFECTS OF PLUMBAGIN ON CYTOTOXICITY, TAMOXIFEN SENSITIVITY AND EPITHELIAL-MESENCHYMAL TRANSITION-ASSOCIATED METASTASIS IN ENDOCRINE RESISTANT BREAST CANCER CELLS

Mr. Nueng Sakunrangsit

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2014
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ผลของพลัมบาจินต่อความเป็นพิษของเซลล์ ความไวต่อยาทาม็อกซิเฟน และกระบวนการเปลี่ยนแปลงรูปร่างของเยื่อบุผิวซึ่งเกี่ยวข้องกับการแพร่กระจายในเซลล์มะเร็งเต้านมที่มีการดื้อยาต้านฮอร์โมน

นายหนึ่ง สกุลรังสิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	EFFECTS OF PLUMBAGIN ON CYTOTOXICITY, TAMOXIFEN SENSITIVITY AND EPITHELIAL-MESENCHYMAL TRANSITION-ASSOCIATED METASTASIS IN ENDOCRINE RESISTANT BREAST CANCER CELLS
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หนึ่ง สกุลรังสิต : ผลของพลัมบาจินต่อความเป็นพิษของเซลล์ ความไวต่อยาทาม็อกซิเฟน และกระบวนการเปลี่ยนแปลงรูปร่างของเยื่อบุผิวซึ่งเกี่ยวข้องกับการแพร่กระจายในเซลล์มะเร็งเต้านมที่มีการดื้อยาต้านฮอร์โมน (EFFECTS OF PLUMBAGIN ON CYTOTOXICITY, TAMOXIFEN SENSITIVITY AND EPITHELIAL-MESENCHYMAL TRANSITION-ASSOCIATED METASTASIS IN ENDOCRINE RESISTANT BREAST CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. พญ. วรรณรัศมี เกตุชาติ, 96 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของพลัมบาจินต่อฤทธิ์ทำลายเซลล์มะเร็งเต้านมของมนุษย์ที่ดื้อยาต้านฮอร์โมน MCF-7/LCC2 และ MCF7/LCC9 โดยวิธี MTT หลังจากนั้นใช้วิธี tamoxifen response และ combination index (CI) เพื่อตรวจสอบความสามารถในการเพิ่มความไวต่อยาต้านฮอร์โมน และศึกษาการแสดงออกของยีนในกลุ่ม NCoA3 ซึ่งมีความสำคัญในกระบวนการอยู่รอดของเซลล์ และการตอบสนองต่อยาทาม็อกซิเฟน เนื่องจากเป็นยีนที่ทำหน้าที่ร่วมในการแปลรหัสของยีนเป้าหมายของตัวรับเอสโตรเจน (ER target genes) นอกจากนี้เคยมีรายงานว่าพลัมบาจินสามารถยับยั้ง NF-kB ซึ่งมีผลเกี่ยวข้องกับกระบวนการเปลี่ยนแปลงรูปร่างของเยื่อบุผิวของเซลล์ (EMT) และการดื้อยาต้านฮอร์โมน เพื่อศึกษาการแสดงออกของยีนในระดับ mRNA ของยีน ER target genes ได้แก่ *Cyclin D1* และ *MYC* รวมถึงยีนที่เกี่ยวข้องกับ EMT ได้แก่ *E-cadherin*, *Vimentin* และ *Snail* โดยวิธี RT-PCR ต่อมาศึกษาผลของพลัมบาจินในการยับยั้งการเคลื่อนที่และลุกลามของเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมนโดยวิธี scratch และ invasion ตามลำดับ ผลการทดลองพบว่า (1) พลัมบาจินมีความเป็นพิษต่อเซลล์มะเร็งเต้านมที่ใช้ในการทดสอบ โดยมีค่าความเข้มข้นที่ทำให้เซลล์ตายร้อยละ 50 (IC_{50}) เท่ากับ 1.47, 1.22 ไมโครโมลาร์ สำหรับเซลล์ MCF-7 และ 1.69, 1.58 ไมโครโมลาร์ สำหรับเซลล์ MCF-7/LCC2 และ 1.24, 1.17 ไมโครโมลาร์ สำหรับเซลล์ MCF-7/LCC9 หลังจากได้รับสารเป็นเวลานาน 24 และ 48 ชั่วโมง ตามลำดับ (2) เมื่อให้พลัมบาจินที่ความเข้มข้น 0.75 ไมโครโมลาร์ ซึ่งเป็นค่าความเข้มข้นที่ทำให้เซลล์ตายน้อยกว่าร้อยละ 25 (IC_{25}) ร่วมกับยาทาม็อกซิเฟน สามารถเพิ่มความเป็นพิษและเสริมฤทธิ์ของยาดังกล่าวได้ โดยมีค่า CI เท่ากับ 0.56 และ 0.94 ตามลำดับ (3) ลักษณะการเปลี่ยนแปลงของเซลล์จากอิพิทีเลียลเป็นมีเซนไคมอลเซลล์ (EMT) พบได้ในเซลล์มะเร็งเต้านมที่มีการดื้อยาต้านฮอร์โมน (4) พลัมบาจินสามารถลดการแสดงออกของยีน *Cyclin D1, MYC*, *NCoA3, Vimentin* และ *Snail* ในขณะเดียวกันสามารถเพิ่มการแสดงออกของยีน *E-cadherin* อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม และ (5) พลัมบาจินยังสามารถยับยั้งการเคลื่อนที่ และลุกลามของเซลล์มะเร็งเต้านมที่มีการดื้อยาต้านฮอร์โมนที่ระดับความเข้มข้นที่ไม่มีความเป็นพิษต่อเซลล์ ผลจากการศึกษานี้เป็นรายงานแรกเกี่ยวกับฤทธิ์ทางเภสัชวิทยาของพลัมบาจินในเซลล์มะเร็งเต้านมของมนุษย์ที่มีการดื้อยาต้านฮอร์โมน รวมทั้งกลไกการออกฤทธิ์เบื้องต้นของพลัมบาจิน โดยพบว่าสามารถยับยั้งกระบวนการเปลี่ยนแปลงรูปร่างของเยื่อบุผิว แสดงให้เห็นว่าพลัมบาจินมีประสิทธิภาพในการนำไปพัฒนาเพื่อเป็นยาทางเลือกใหม่สำหรับผู้ป่วยมะเร็งเต้านมที่ล้มเหลวจากการรักษาด้วยยาฮอร์โมนบำบัดต่อไป

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สาขาวิชา	วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต	....
ปีการศึกษา	2557	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....

5574184930 : MAJOR MEDICAL SCIENCE
KEYWORDS: CYTOTOXICITY / SENSITIVITY / EPITHELIAL-MESENCHYMAL TRANSITION (EMT) / METASTASIS / ENDOCRINE RESISTANT-BREAST CANCER CELLS

NUENG SAKUNRANGSIT: EFFECTS OF PLUMBAGIN ON CYTOTOXICITY, TAMOXIFEN SENSITIVITY AND EPITHELIAL-MESENCHYMAL TRANSITION-ASSOCIATED METASTASIS IN ENDOCRINE RESISTANT BREAST CANCER CELLS. ADVISOR: WANNARASMI KETCHART, M.D., Ph.D., 96 pp.

This present study was aimed at investigating the growth inhibition effect of Plumbagin (PLB) on endocrine resistant breast MCF-7/LCC2 and MCF-7/LCC9 cells using the MTT method, followed by tamoxifen response assay and combination index (CI) analyses to identify the reversal of endocrine therapy by PLB of nuclear receptor coactivator 3 (*NCoA3*) involved in transcriptional activity of ER target genes. PLB has been shown to inhibit NF-kB which requires in EMT and endocrine resistance mechanism, so reverse transcriptase polymerase chain reaction (RT-PCR) was performed to determine the expression of ER target genes *Cyclin D1*, *MYC* and EMT-related genes including *E-cadherin*, *Vimentin*, and *Snail*. Furthermore, cellular migration and invasive capacities were also investigated in endocrine resistant breast cancer cells in the presence or absence of PLB. The results demonstrated that i) PLB has significantly cytotoxic activity with the IC_{50} values of 1.47, 1.22 μM for MCF-7 and 1.69, 1.58 μM for MCF-7/LCC2 and 1.24, 1.17 μM for MCF-7/LCC9 cells after 24 and 48 h of treatment respectively, ii) PLB synergistically enhanced cytotoxicity and restored tamoxifen sensitivity to resistant MCF-7/LCC2 and MCF-7/LCC9 breast cancer cells with combination index (CI) of 0.56 and 0.94 respectively, iii) EMT-like behaviors were observed in endocrine resistant breast cancer cells, iv) PLB decreased the expression of *Cyclin D1*, *MYC*, *NCoA3*, *Vimentin*, and *Snail*, while the expression level of *E-cadherin* was increased and v) PLB inhibited the motility and invasiveness of these resistant cells at non-toxic concentration. This study is the first report highlighted the pharmacological properties of PLB on endocrine resistant breast cancer cells and recognized the background molecular knowledge, especially PLB inhibited EMT process. Therefore, PLB will be a novel therapeutic target for ER-positive breast cancer patients who are unresponsive to endocrine therapy.

Field of Study: Medical Science — Student's Signature ....

Academic Year: 2014 — Advisor's Signature ....

ACKNOWLEDGEMENTS

This journey would have been impossible without the invaluable support of the kind of people around me. First and foremost, I wish to express my sincere thankful and appreciation to my advisor Dr. Wannarasmi Ketchart, Department of Pharmacology, Faculty of Medicine, for her special support, unforgettable efforts and excellent encouragement.

Besides my advisor, I am always grateful to all the wonderful my committee members, Dr. Vilai Chentanez, Chairman and Professor in the Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Assistant Professor Wacharee Limpanasithikul, Department of Pharmacology, Faculty of Medicine, Assistant Professor Dr. Amornpun Sereemaspun, Department of Anatomy, Faculty of Medicine, Chulalongkorn University and Associate Professor Rungravi Temsiririrkkul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, for their encouragement, interesting questions and insightful comments.

Additionally, I would like to give special acknowledgement to grants the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for their financial support.

Likewise, I am also sincere grateful to Dr. Roberts Clark from Georgetown University Medical Center, Washington, DC, USA, who generously provided endocrine resistant breast cancer cells MCF-7/LCC2 and MCF-7/LCC9 in this study.

Finally, I especially indebted to my family members, (too many to list here but you know who you are), all of whom were specifically supporting me spiritually throughout my life and particularly helpful, for their understanding, emotional assistance and encouragement.

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LIST OF ABBREVIATIONS

µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
AF-1	Activation function 1 (of the oestrogen receptor)
AF-2	Activation function 2 (of the oestrogen receptor)
AIB1	Amplified in breast cancer 1
AIs	Aromatase Inhibitors
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATCC	American type culture collection
bp	Base pair
CCND1	Cyclin D1
CDH1	E-cadherin
cDNA	Complementary deoxyribonucleic acid
CI	Combination Index
CO_2	Carbon dioxide
CCS	Charcoal dextran-stripped fetal bovine serum
DBD	DNA binding domain
ddH_2O	Double-distilled water
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulfoxide
dNTP	Nucleoside triphosphate
E2	17β-oestradiol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid

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EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Oestrogen receptor
ERE	Oestrogen response element
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
HER2	Human epidermal growth factor receptor 2
IC_{25}	Inhibitory concentration at 25% growth
IC_{50}	Inhibitory concentration at 50% growth
ICI 182,780	Fulvestrant
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor receptor 1
IMEM	Improved Minimum Essential Medium
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MCF-7	Human breast cancer cell lines
MCF-7/LCC2	Tamoxifen-resistant breast cancer cell lines
MCF-7/LCC9	Tamoxifen/fulvestrant-resistant breast cancer cell lines
MEM	Minimum essential medium
mg/mL	Milligram per milliliter
$MgCl_2$	Magnesium chloride
MMPs	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-tetrazolium bromide
MYC	V-myc myelocytomatosis viral oncogene homolog

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$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCoR	Nuclear receptor corepressor
nm	Nanometer
°C	Celsius degree
OD	Optical density
4-OHT	4-hydroxytamoxifen
PBS	Phosphate buffer saline
pH	Negative logarithm of hydrogen ion concentration
PI3K	Phosphoinositide 3-kinase
PLB	Plumbagin from Plumbago *indica*
RNA	Ribonucleic acid
RPM	Revolution per minute
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SEDRs	Selective oestrogen receptor downregulators
SERMs	Selective oestrogen receptor modulators
SP-1	Specificity protein 1
SRC	Steroid receptor coactivator
SRE	Specificity response element
TBE	Tris-borate-EDTA buffer
VIM	Vimentin

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CHAPTER I

INTRODUCTION

1.1. Statement and Significant of the problem

Breast cancer is one of the most commonly diagnosed illness and is the second leading cause of death in women worldwide [1]. The International Agency for Research on Cancer (IARC) has published in the World Cancer Report that breast cancer estimated to reach 1.7 million new cases per year [2]. In Thailand, the National Cancer Institute (NCI) reported that more than 5,500 new cases of breast cancer were identified each year and mostly found in the 45-59 age group which incidence was accounted for 25.6 per 100,000 females [3]. Approximately 75% of breast cancer was analyzed to express oestrogen receptor (ER) and/or progesterone receptor (PR). Endocrine therapy, Tamoxifen, a selective oestrogen receptor modulators (SERMs), has been used as the 'gold standard' and was accomplished to cure this patient group. Tamoxifen interacts to bind to oestrogen receptor and reduces transcriptional activity of ER target genes involved in tumourigenesis [4]. However, more than 50% of these patients develop acquired resistance after five years of treatment, characterized by tumour recurrence, and enhancement of metastatic potential breast cancer [5].

The metastatic initiation process includes cellular invasion which is potentiated by epithelial to mesenchymal transition (EMT), assisting tumour migration from primary cancer site spreading to other essential organs such as bone, lung, liver or brain respectively and finally leading cause to mortality [6]. Thus, developing the molecular targets essential for metastasis is critical in cancer biology and the studies provide a basis for novel therapeutic targets to treat metastatic disease is still challenging.

Currently, screening studies for the novel drugs from natural products showed various medicinal plants which were more toxic to destroy tumour than normal cells.

Accordingly, pharmacological properties of plumbagin (PLB), referred to 5-hydroxy-2-methyl-1,4-naphthoquinone, predominantly isolated from the root of *Plumbago indica* in Plumbaginaceae family was used in this study [7]. PLB was well documented that inhibition of several tumour cells including breast cancer was mediated through multiple signal transduction cascades. For example, the nuclear factor kappa-B (NF-κB) which regulate in cell survival and proliferation, epithelial-mesenchymal transition (EMT), drug resistance, invasion and metastasis [8-10]. Moreover, Hiscox et al. has been completely demonstrated that tamoxifen-resistant breast cancer (TAM-R) cells showed EMT-like characteristics, well-defined by the acquisition of some mesenchymal phenotypes and the reduction of epithelial phenotypes, which increased capability of breast cancer invasion and motility [11]. Furthermore, Huber et al. reported significantly exhibition of EMT during metastasis existed through NF-κB pathway [12].

Therefore, this study was proposed to discover the effect of plumbagin in human endocrine resistant breast cancer models of Dr. Roberts Clark's Laboratory [13, 14]. This study hypothesized that these findings provided basic molecular mechanism of the novel pharmacological activities of naphthoquinone plumbagin including the inhibition of tumour growth, EMT program and the ability to restore tamoxifen sensitivity in endocrine resistant breast cancer cells.

1.2. Objectives of the research

1) To investigate the effect of plumbagin on cell viability in human wild-type and endocrine resistant breast cancer cells.
2) To investigate the combination effect of plumbagin and tamoxifen in human endocrine resistant breast cancer cells.

3) To evaluate the effect of plumbagin on cell invasion and motility in human endocrine resistant breast cancer cells.
4) To determine the effect of plumbagin on regulation of mRNA expression of genes involved in cell survival, endocrine resistance and EMT-related metastasis.

1.3. Hypothesis

Plumbagin can induce cell death, enhance sensitivity of tamoxifen and decrease cellular invasion and migration via alteration of ER target genes and EMT biomarkers in acquired endocrine resistant breast cancer cells.

1.4. Expected benefit and Application

The results of antitumour activity of plumbagin on human endocrine resistant breast cancer cells and its ability to restore tamoxifen sensitivity will provide the preliminary data for further development of adjunctive or novel treatment for oestrogen receptor (ER)-positive breast cancer patients who develop resistance to hormonal therapy.

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1.5. Key Words

CYTOTOXICITY / SENSITIVITY / EPITHELIAL-MESENCHYMAL TRANSITION (EMT) / METASTASIS / ENDOCRINE RESISTANT-BREAST CANCER CELLS

CHAPTER II

LITERATURE REVIEWS

2.1. Understanding Breast Cancer Biology

The normal female breast is a complex organ consisting of several different cell-and tissue types. The two cellular components of mammary gland, the stromal component which is the supporting adipose tissue, fibroblasts, blood vessels, nerves and lymphatic vessels and the functional system of branching ducts and secretory lobules which are 15 to 20 lobes in each breast and each lobe is linked to nipple by branched ducts (**Figure 2.1a**). As illustrated in **Figure 2.1b-c**., the epithelial layer of cells ruled around each duct is responsible for producing milk. The glandular ducts are surrounded in stromal fibroblasts which this pattern is disrupted in breast cancer resulting in epithelial cell mass [4].

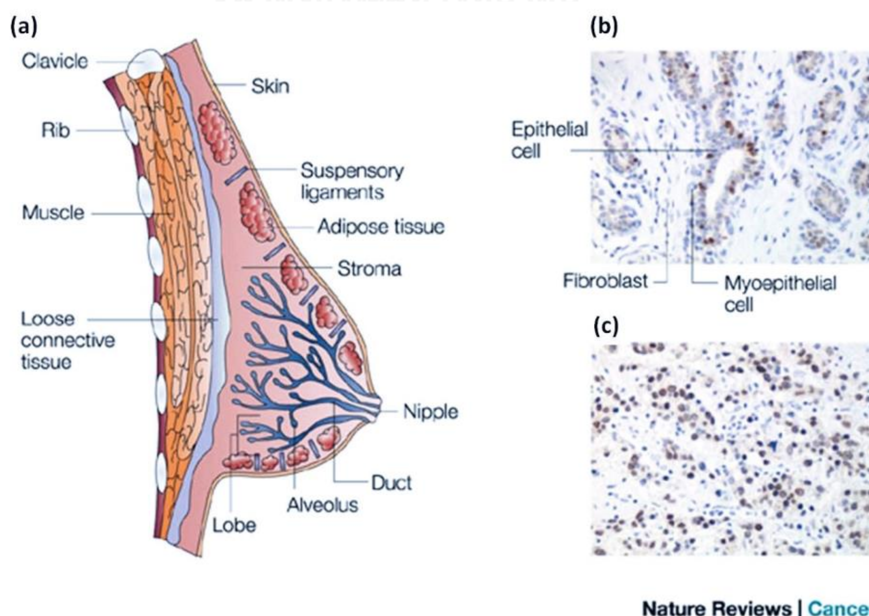

Figure 2.1 Structure and histology of carcinoma and normal breast [4].

The major risk factor of breast cancer is associated with exogenous oestrogens such as hormone therapy (HT). Reproductive factors including the first menstrual cycle, greater number of lifetime ovulatory cycles, deficiency of milk lactation, late menopause, and greater time between menarche and menopause also increase the risk of breast cancer [15]. Importantly, the reproductive risk factors suggest that a woman's lifetime exposure to oestrogens enhance breast cancer incidence. Diet, alcohol intake, and physical activity may involve or are studied to be risk factors of breast cancer but studies were still very controversy [16].

2.2. Trends in Breast Cancer Incidence

Breast cancer is the second leading cause of death and the most frequently diagnosed disease in females and can occur in men approximately 1% of total cases [1]. There were 232,340 new cases (28.7%) of breast cancer diagnosed worldwide in 2013. The prognosis is relatively poor, therefore it has a predominantly caused of mortality with 39,620 deaths (14.2%) per year (**Figure 2.2**). Nearly 75% of all cases were in women aged 45 or more and mortality rates were relatively high in the Caribbean, Southern and Central Africa, Northern and Western Europe, Australia, New Zealand and North and South America and Asian populations and North Africa. [2].

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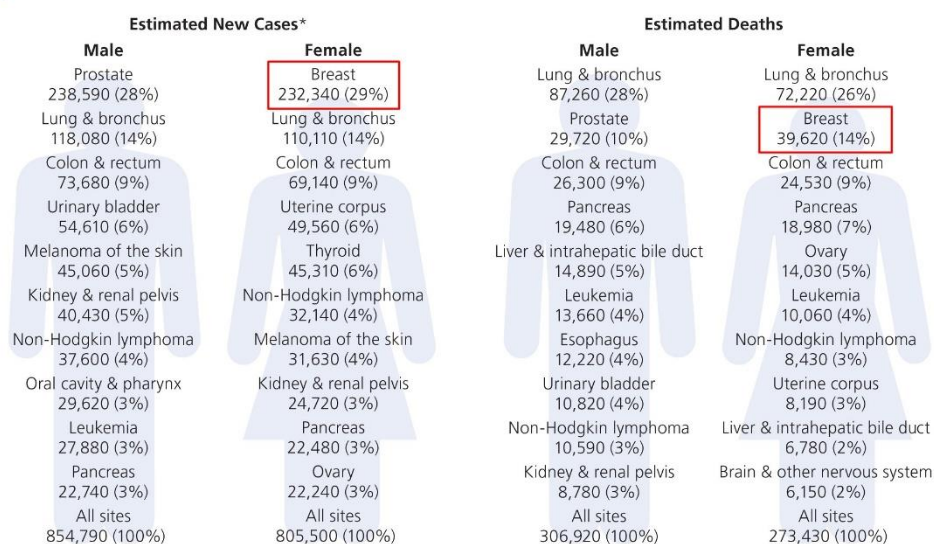

Figure 2.2. Breast cancer incidence and mortality. The highlighted area (red bordered boxes) illustrates amount of breast cancer cases and deaths estimated in 2013 [1].

2.3. Molecular Classification of Breast Cancer

Studies of female breast adenocarcinoma using gene expression profiling have identified several major breast cancer subtypes based on their hormone receptor expression. The molecular subtypes in the oestrogen receptor (ER)-positive cancer are the luminal A and luminal B groups. The HER2 and basal-like groups are the major molecular subtypes identified in oestrogen receptor (ER)-negative breast cancer. Other subtypes such as luminal C and normal-like breast groups have also been identified in few studies and were less well-described than the luminal A, luminal B, HER2, and basal-like types [17-19]. These breast cancer molecular subtypes differ with gene expression patterns, clinical appearances, prognosis and treatment as summarized in **Table 1**.

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Luminal A

The luminal A is the most common subtype of breast cancer, nearly 50–60% of the total. Based on their molecular profile expression includes ER, progesterone receptor (PR), Bcl-2 and cytokeratin CK8/18, an absence of HER2 expression, a low rate of proliferation measured by Ki67 and a low histological grade (**Figure 2.3**). This subtype of cancer patients have a good prognosis exhibited the relapse rate approximately which was 27.8% significantly lower than other subtypes that provided longer survival from disease relapse (average 2.2 years). The treatment for this subtype is mostly based on selective oestrogen receptor modulators (SERMs) such as tamoxifen which is the first line treatment, pure selective regulators of ER such as fulvestrant and aromatase inhibitors (AIs) [4, 17].

Luminal B

Tumours with the luminal B molecular profile were between 10% and 15% of all breast cancer cases. This subtype have more aggressive phenotype, higher histological grade and proliferative index and worse prognosis. Ki67 expression was used as a marker to distinguish between luminal A and B [19, 20]. Luminal A subtype has been defined as ER+/HER2- and low Ki67, while the luminal B subtype is tumours with ER+/HER2+ and high Ki67. The pattern of distant metastasis also differs, and although the bone is still the most common site of 30% recurrence, this subtype has a higher 13.8% recurrence rate in secondary sites such as livers.

Luminal B tumours have a worse prognosis than luminal A subtype for the treatment with tamoxifen and AIs, but they respond better to neoadjuvant chemotherapy by getting pathological complete response (pCR) in 17% of the luminal B tumours (7% in luminal A) [20]. Several clinical trials were performed to study inhibitory molecules involved in PI3K/AKT/mTOR signaling cascades at different levels to develop the treatment of luminal B groups [18].

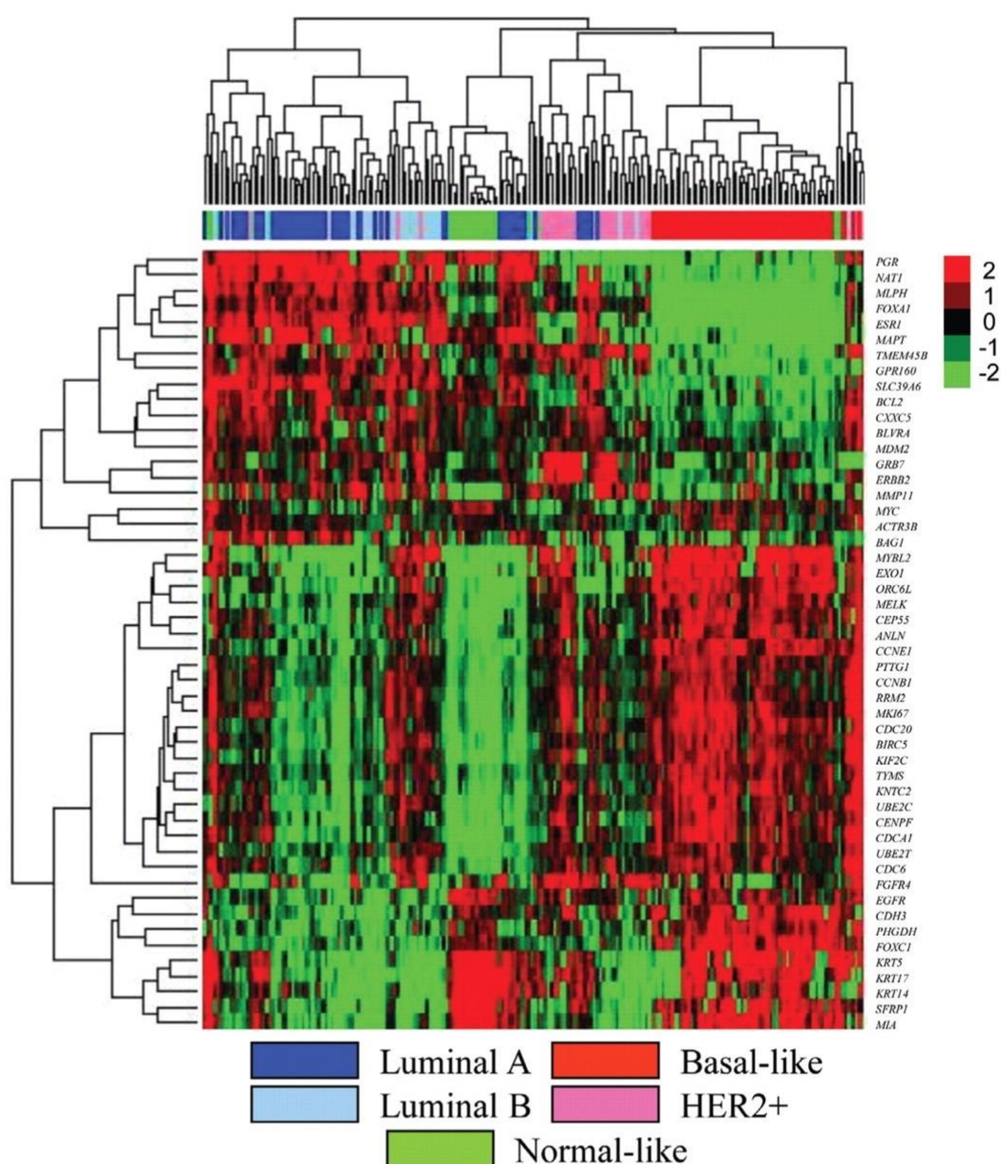

Figure 2.3. Molecular classification of breast cancer subtypes [18].

HER2 positive

Fifteen to twenty percent of all breast cancer patients responded to this subtype. They were described by a high expression of the HER2 gene (also known as ERBB2) and other genes associated with the HER2 pathway. From the clinical point of view, the HER2 subtype is a poor prognosis. This subtype as well as the basal-like subgroup have a high chemosensitivity with higher response rates than luminal subtypes (43% and 36% pCR vs 7% and 17% pCR, respectively) [18].

Basal-like

The basal-like subtype represents 10–20% of all breast cancer patients. The term basal-like derived from gene expression that commonly presents in normal breast myoepithelial cells, including high molecular weight cytokeratins CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR. The pattern of metastatic relapse is aggressive, mostly lung, lymph nodes and brains [19]. One of the most relevant features of this type of tumour is the absence of the three key receptors in breast cancer: ER, PGR and HER2 expression. Thus in clinical practice the terms basal-like and Triple Negative (TN) are often switched. They are not, however alike terms since a dissonance of up to 30% between the two groups has been described [17]. Efforts to identify the basal-like by the gene profile have led to the selection of five markers includes ER, PR, HER2, EGFR and CK5/6 [18].

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Table 1. Summarizes the gene expression patterns, clinical characteristics, treatment and outcome of breast cancer [18-22].

<table>
<tr><th></th><th colspan="4">Molecular subtypes</th></tr>
<tr><th></th><th>Luminal A</th><th>Luminal B</th><th>HER-2</th><th>Basal-like</th></tr>
<tr><td>Gene expression strategies</td><td colspan="2">High expression of hormone receptors and associated genes (luminal A>luminal B)</td><td>High expression of HER2 and other genes in amplicon
Low expression of ER and related genes</td><td>High expression of basal epithelial genes, basal cytokeratins
Low expression of ER and associated genes
Low expression of HER2</td></tr>
<tr><td>Clinical behaviors</td><td colspan="2">~75% of breast cancers
ER/PR positive
Higher histological grade (luminal B>luminal A)
Some overexpress HER2 (luminal B)</td><td>~15% of invasive breast cancers
ER/PR negative
More likely to be high grade and node positive</td><td>~10% of breast cancers
ER/PR/HER2 negative (triple negative, TN)
BRCA1 dysfunction (germline, sporadic)
Particularly common in African-American women</td></tr>
<tr><td>Treatment and prognosis</td><td colspan="2">Respond to endocrine therapy (but response to SERMs and AIs may be different for luminal A and luminal B)
Response to chemotherapy variable (greater in luminal B than in luminal A)
Prognosis (luminal A>luminal B)</td><td>Respond to trastuzumab (Herceptin)
Respond to anthracycline-based chemotherapy
Generally poor prognosis</td><td>No response to endocrine therapy or trastuzumab (Herceptin)
Appear to be sensitive to platinum-based chemotherapy and PARP inhibitors
Generally poor prognosis (but not uniformly poor)</td></tr>
</table>

2.4. Oestrogen and Oestrogen Signalling Pathway

The steroid hormone oestrogen (E_2) is synthesised from cholesterol through several enzymatic steps (**Figure 2.4**). E_2 is one of the most important steroid hormones of the adult females [23], and regulates many physiological processes and plays essential roles in the development, growth control, and differentiation of the normal breast tissues.

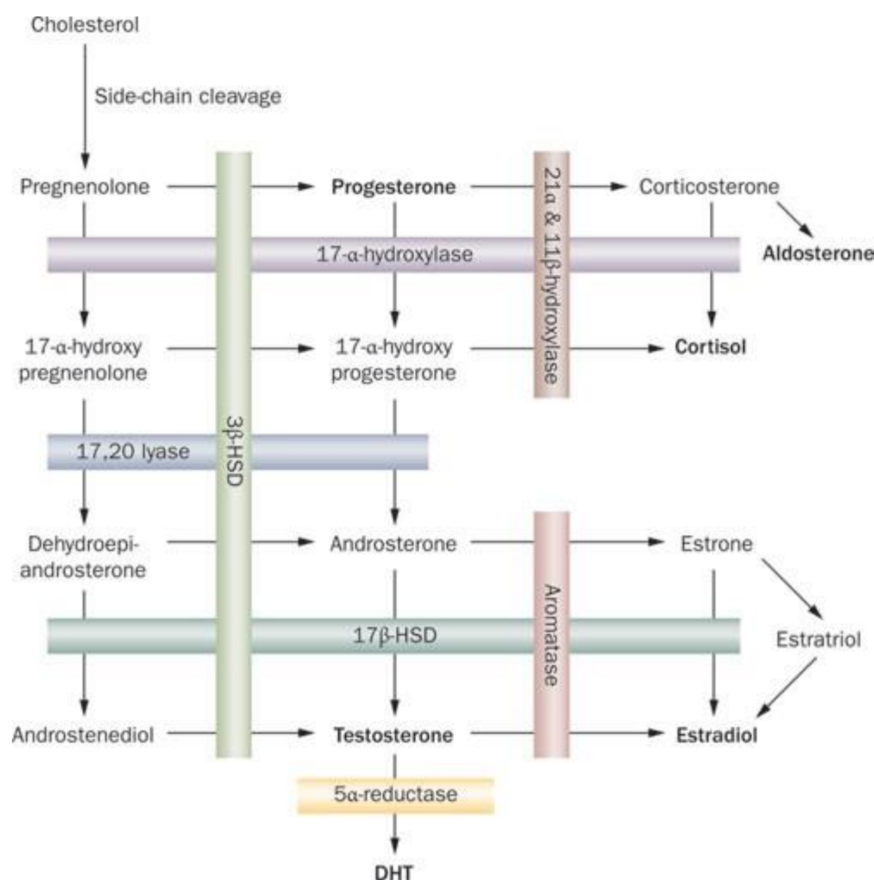

Figure 2.4. Oestrogen synthesis from cholesterol [23].

Oestrogen occurs in several forms, oestrone (E_1), 16-α-oestriol (E_3) and 17-β-oestradiol (E_2), where E_2 is the most biologically active hormone in breast tissue. E_2 is mostly produced in the ovaries, during the premenopausal period, and act as the classical endocrine action. In the postmenopausal period when the ovaries have ceased to produce E_2, the primary source of E_2 is from circulating androgens secreted

from the adrenal gland and ovaries. These precursors are then synthesised into E_2 via aromatase enzymes in neighboring tissues such as adipose, breast tissues, skin and muscle [19, 23]. Peripheral industrial E_2 acts locally in an intracrine or paracrine manner without being released in the blood circulation. The biological functions of E_2 are regulated by transcriptional activation through the ER [21].

2.4.1. Oestrogen Receptor (ER) Subtypes

Structurally, there are two ER isoforms in human, ERα and ERβ, which show distinct tissue-specific expression profiles and biological properties [24]. Since many studies about function of ER-β is still very controversy, also the term ER mostly denotes to ERα. In **Figure 2.5**., a schematic represents the structure of oestrogen receptors (ER) and 6 well-conserved functional domains A-F, particularly in the DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD). The N-terminal A/B domain of ER contains the ligand-independent but promoter and cell-type dependent transactivation function 1 (AF-1) which activates target genes by interaction between the components and the core transcription machinery. The C domain contains the DNA binding domain (DBD) and is the most strongly conserved region within nuclear receptors. This domain contains two zinc fingers, the first of which contains amino acids involved in the specific DNA binding (termed estrogen response elements; EREs). The second zinc finger contains residues by binding ultimately through hitching to other transcription factors such as SP-1 and AP-1 (called serum response element; SREs), which is responsible for the selectivity of binding to classical palindromic half-site EREs. The hinge D region is hyperflexible and tolerates the receptor to alter conformation to allow maximum ligand binding affinity. The C-terminal domain (labelled E/F domain) contains a ligand-binding domain (LBD), the second activation function domain

(AF-2), and has regions allowing receptor dimerization by a leucine-zipper machinery and interaction with transcriptional coregulatory proteins [25].

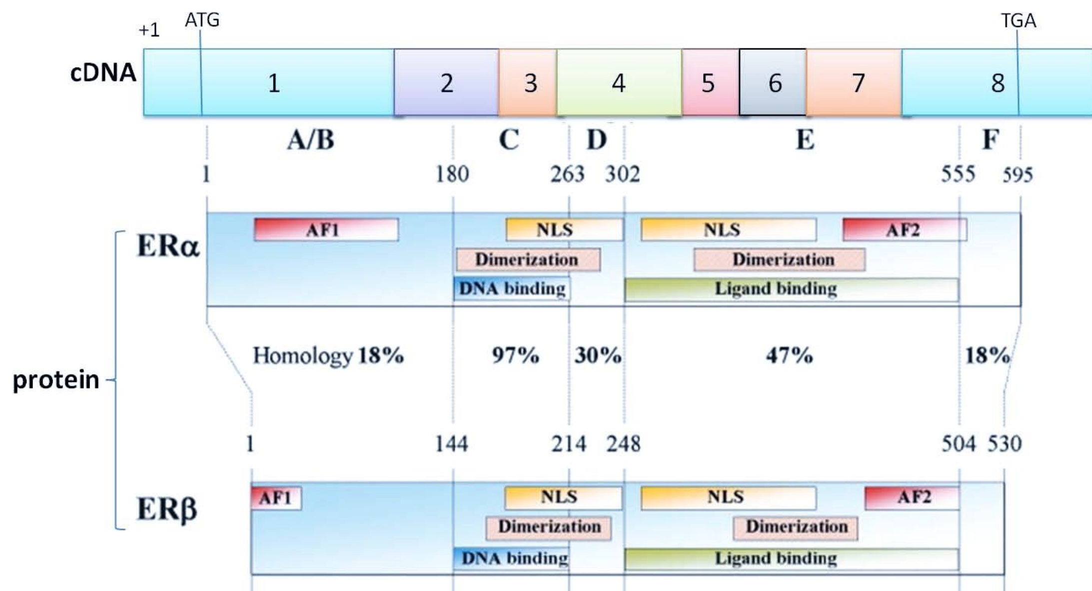

Figure 2.5. The functional domains encoded in ER-α and ER-β homology [24].

2.4.2. Genomic action of ER

The oestrogen receptor has at least two major machineries of action. ER can either undergo genomic or a non-genomic pathway. In the classical genomic of ER action (**Figure 2.6**), E_2 diffuses into the cell and binds to ER in the nucleus. This binding induces a conformational change of the receptor, which leads to the separation from heat shock protein-90 (HSP-90) and formation of receptor dimers or dimerization of ER. The activated ER then binds as a dimer to small palindromic DNA motifs known as oestrogen response elements (EREs) which are present in the promoter regions of ER target genes. The consensus ERE sequence is defined as 5’-GGTCA-NNN-TGACC-3’ where N denotes some nucleotide [25]. However, only a small number of the most E_2 inducible genes contain these consensus EREs. In most studies, irregular EREs or even partial EREs that are often separated by many base pairs mediate the

inducibility of oestrogen, either alone or in combination [21]. Promoter-bound ER recruits and forms a complex with coregulatory proteins, which function as intermediates between the receptor and the general transcriptional machinery and subsequently modulate transcriptional activity of oestrogen-responsive genes. Transcription of many genes is increased by oestrogen, while transcription of some others is repressed [26]. AF1 and AF2 are two different domains mediate transcriptional activity of ER. AF1 is constitutively active and its activity is hormone ligand-independent and regulated by phosphorylation, whereas AF2 is integral to the LBD and its activation is hormone dependency. The two activating domains can act synergistically and/or individually in a cell and promoter context specific manner [27].

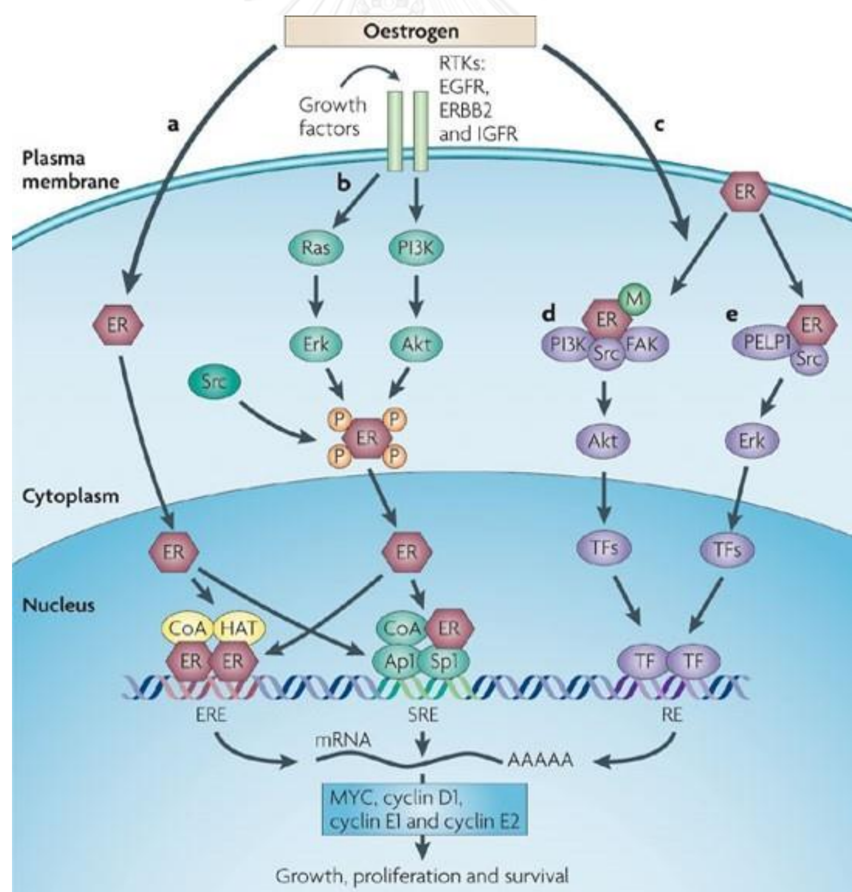

Figure 2.6. Genomic and non-genomic ER signalling pathways [27].

ER coregulatory proteins include coactivators, such as AIB1 and SRC3, which enhance ER transcriptional activity and corepressors, such as NCoR-1 and SMRT that decrease ER transcriptional activity [28, 29]. The coactivators usually bind ER when the receptor is bound by oestrogen. Coactivators enhance ER-driven transcription through different mechanisms including the enlistment of histone acetyl transferase (HAT) complex which helps relax the DNA, allowing gene transcription [27]. Furthermore, some of the coactivators themselves possess intrinsic histone acetyl transferase activity [28].

In contrast, corepressor proteins influence ER transcriptional activity at least in part through the recruitment of histone deacetylase complexes (HDAC) which allows DNA to wrap more tightly around the core histone proteins [29]. In fact, these coregulatory proteins may be indeed as important as ER itself in mediating transcriptional activity as well as prompting tumour growth. For instance, AIB1, is 65% overexpressed and 5-10% amplified in several breast cancers [28], suggesting its vital role in breast cancer. Additionally, reducing the level of AIB1 has been shown to inhibit ER-related gene transcription as well as tumour growth in cell lines [30].

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2.4.3. Non-classical genomic ER action

Besides the classical genomic mechanism of direct DNA binding (EREs), ER has also been shown to regulate gene expression at alternative regulatory DNA sequences such as AP-1, SP-1, and other poorly defined non-ERE sites. For instance, ER can indirectly modulate AP-1 response elements through its interaction with AP-1 transcription factors such as c-Fos and c-Jun which regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility and apoptosis [27].

In this event, ER mediates transcription via fastening interaction to specific promoter complexes through interaction between protein and protein rather than functions as the major transcription factor. These non-classical genomic ER signalling pathways, particularly at the AP-1 sites, could be important clinically. It has been shown that tamoxifen can act as an agonist on genes under the control of an AP-1 response element [16].

2.4.4. Nongenomic rapid ER activity

The nongenomic activity of ER results in elevated cell proliferation and survival signals through the activation of proliferation-related factors, such as growth factor receptors, MAPKs, PI3K, and Akt, in target cells. However, mechanisms by which E_2 activates membrane ER function are still not clear. One hypothesized machinery involves activation of growth factor cascades through direct interaction between activated membrane ER and growth factors. ER has been reported to bind to membrane signalling mediators including insulin-like growth factor receptor 1 (IGFR1), the p85 regulatory subunit of PI3K, Src and Shc [4]. In this case, E_2 activates the downstream signalling of other growth factors by binding to its membrane receptor, which leads to the activation of important molecules such as MAPK or Akt. [27].

2.5. Endocrine Therapies for Breast Cancer

Traditionally, Endocrine therapy is the gold standard in the management of oestrogen receptor (ER)-positive breast cancer patients group. Endocrine therapy, also called as antioestrogen (AE) or hormonal therapy, is a treatment considered based on the inhibiting the activity of ER pathway or reducing steroid hormone E_2 levels through (i) blocking the E_2 and ER interactions with selective oestrogen receptor modulators (SERMs); (ii) eliminating ER with selective oestrogen receptor degradators (SERDs); or (iii)

decreasing E_2 levels by aromatase inhibitors (AIs) [31]. The chemical structures of steroid hormone oestradiol (E_2) and two typical hormonal therapy drugs as illustrated in **Figure 2.7.**

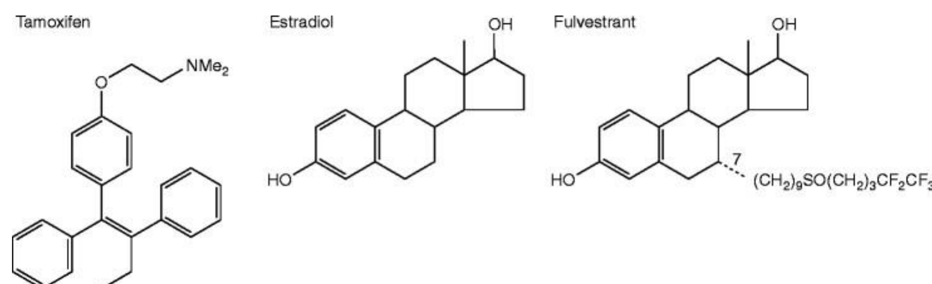

Figure 2.7. Chemical structures of endocrine therapy drugs [31].

2.6. Antioestrogens and Their Mode of Action

Meanwhile E_2 plays a critical role in activating breast cancer cell growth and proliferation upon binding with oestrogen receptors (ER), endocrine therapy is effective in treating ER-positive breast cancer patients by either removing E_2 or blocking binding of E_2 to cognate receptors. To completely eliminate oestrogen, ovariectomy (medical removal of ovaries) or ovarian ablation drugs is commonly used in premenopausal women whereas aromatase inhibitors are applied to postmenopausal breast cancer patients to prevent converting androgen to oestrogen. On the other hand, antioestrogen drugs which inhibit oestrogen action are often applied. The biological functions of E_2 are intervened by the activation of gene transcription via the oestrogen receptors (ERs). The FDA approved three different group for treatment of oestrogen-dependent breast cancers [27].

2.6.1. The Selective Estrogen Receptor Modulators (SERMs)

The ER activation process depends on the ligand and its mechanism of action. As illustrated in **Figure 2.8**., ER ligand agonist and antagonist complexes differ in receptor conformation, DNA binding, protein stability, and recruitment

of cofactors required in activation of gene transcription. E_2 promotes tumourigenesis by the dysregulation of cellular growth [32]. Selective Oestrogen Receptor Modulators (SERMs), such as tamoxifen (TAM), are used as a treatment for breast cancer by competitive binding to ER thereby blocking oestrogen–stimulated cellular proliferation. TAM is widely used clinically as chemoprevention and hormonal therapy for ER–positive breast cancer [33].

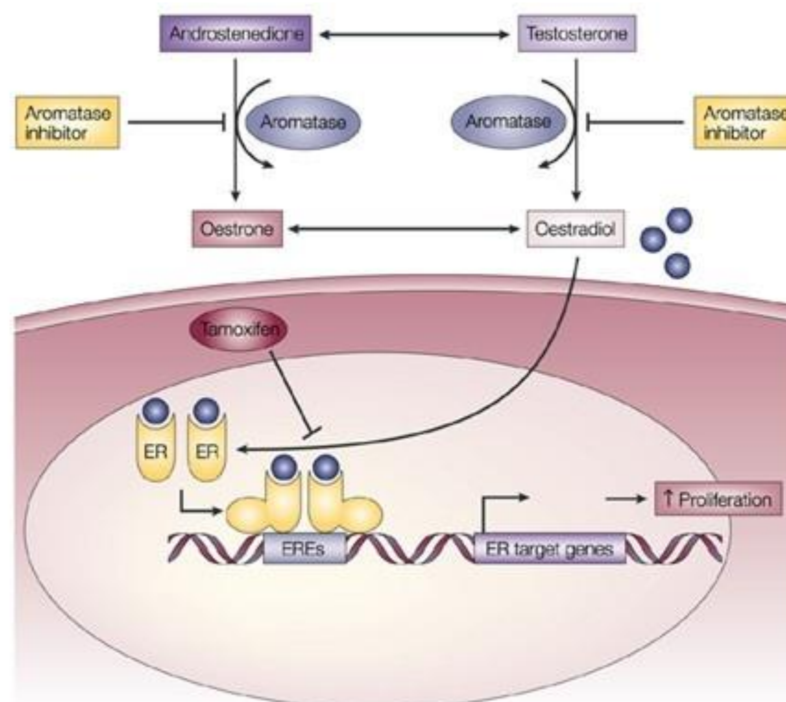

Figure 2.8. Antioestrogen action in breast cancer [33].

Tamoxifen acts competitively to the ligand binding domain of the ER and influence its folding. However, the antioestrogen AE/ER complex is still able to undergo dimerisation, although its ability to promote transcription is reduced [31]. Many studies on the antioestrogenic effect of tamoxifen have suggested that TAM causes cell growth arrest and apoptosis both in pre-clinical and clinical studies by affecting growth factors including down-regulation of TGFα, induction of stromal TGFβ-1 and a decrease in the production of the

potent mitogen IGF-1 [32]. Thiantanawat et al. studied both the in cell cultures and in animal models about signalling cascades by which tamoxifen utilizes its effect and their results indicated that the inhibition of cell proliferation of cells by inducing cell survival arrest in the G0/G1 phase associated with increased apoptosis [34].

2.6.2. The Aromatase Inhibitors (AIs)

The E_2 synthesis is decreased with AIs that effectively postponing oestrogen-dependent breast cancer cell existence through competing with the androgen substrate of aromatase (**Figure 2.8**). AIs can be classified into two major categories, steroidal and non-steroidal inhibitors, which block the aromatase enzymatic reaction through distinct machineries. Clinical use of AIs is limited to old adult women. In adolescent and young adult women, ovarian aromatase is lower gonadotropin regulation by luteinizing hormone (LH) and follicle stimulating hormone (FSH), which induce E_2 synthesis via aromatase but do not control peripheral aromatase expression [35]. Currently, FDA approved three third-generation AIs, anastrozole, exemestane and letrozole used clinically in the treatment of ER-positive breast cancer [33].

2.6.3. The Selective Estrogen Receptor Downregulators (SERDs)

In addition to SERMs, fulvestrant (Faslodex, ICI 182,780), a pure antioestrogenic agent that affords complete ER antagonism, is currently in clinical use for the treatment of aggressive breast cancer in postmenopausal women with recurrence or progression following E_2 antagonist including tamoxifen. Fulvestrant targets and degrades the ER thereby inhibiting oestrogen-dependent cascade through the ER [36]. Recently, Fulvestrant is also

accordingly considered second-line drug for oestrogen receptor (ER)-positive breast cancer group that have failed tamoxifen therapy [37].

2.7. Endocrine Resistance in Breast Cancer

Even if most patients respond to tamoxifen therapy many patients remain experience resistance to endocrine therapy either at the beginning of the treatment or after prolonged use. The mechanisms for this resistance are not completely elucidated but several suggestions have been reported.

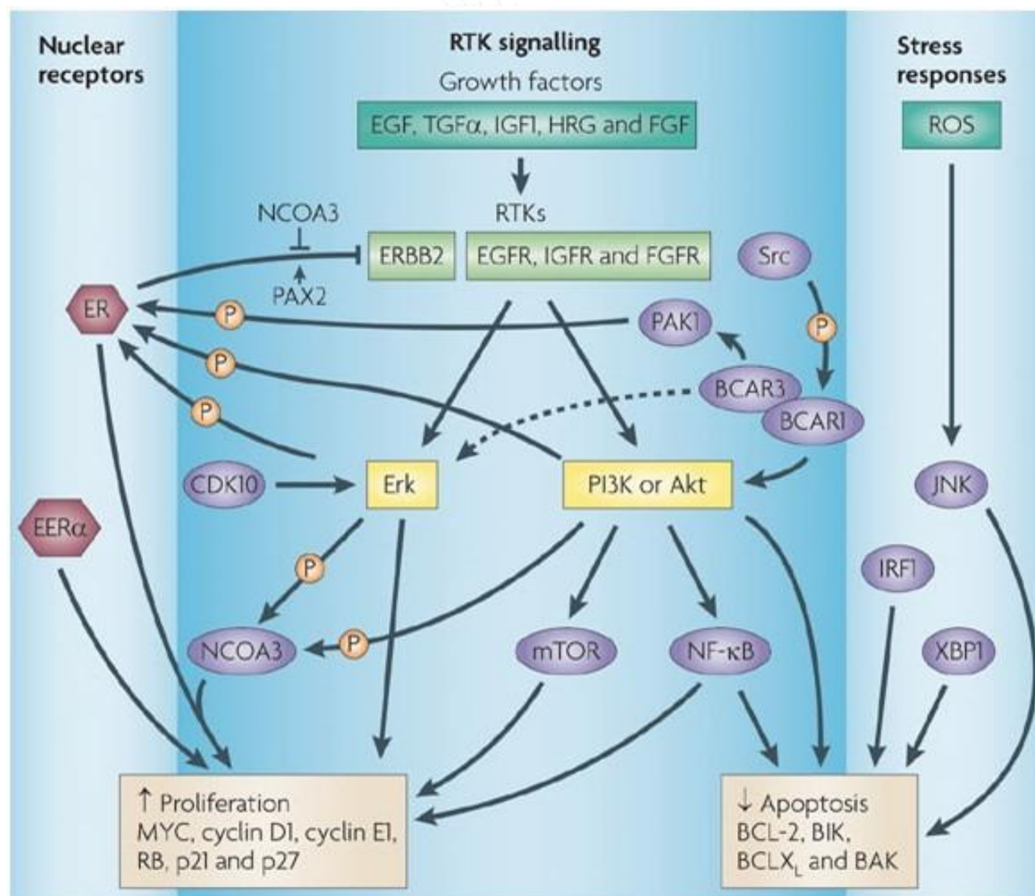

Figure 2.9. Mechanisms of endocrine resistance in breast cancer [27].

2.8. Mechanisms of Endocrine Therapy Resistance

Approximately 50% of ER-positive breast cancer patients can develop resistance to endocrine after five years of treatment [5]. Endocrine resistance is a complex phenomenon comprised of several mechanisms involved genomic and non-genomic factors. EGFR and HER2/neu crosstalk with ER signaling, reduced IGF receptor I expression, constitutively activated MAPK pathway in endometrial cancer cells, and oxidative stress are all associated with tamoxifen activity (**Figure 2.9**) [27].

2.8.1. ER and ER Coregulators

Co-regulators play an important role in mediating the transcriptional activity of ER. Because many of these may be present at rate restrictive levels in the nucleus, changes in their level of expression or activity can lead to alterations of ER signalling cascades [28]. In particular, overexpression of coactivators and downregulation of corepressors may contribute to tamoxifen resistance. An imbalance in the expression of coactivator and corepressor genes may impair TAM activity either by eradicating the E_2 agonist or by switching from the E_2 antagonist to E_2 agonist activity but few studies have been investigated the role of coregulators in tamoxifen resistance [38]. An imprecise expression of steroid receptor coregulators has also been involved in endocrine resistance. Numerous studies have demonstrated that increased coactivator expression including *NCoA3* can enhance the agonist tamoxifen activity *in vitro*. The nuclear receptor coactivators 3, *NCOA3*, (also termed *AIB1* (amplified in breast cancer) or steroid receptor coactivators 3, *SRC-3*) is an ER coactivator thought to be important in breast cancer. AIB1 is phosphorylated and activated by mitogen-activated protein kinases (MAPKs), and high levels of activated AIB1 may potentially reduce the antagonist effects of tamoxifen, especially in tumours that also overexpress the HER-2 receptor, a member of the epidermal

growth factor (EGF) receptor family that activates MAPKs [39]. *NCoA3* is overexpressed in nearly 50% of breast tumours, and enhances the agonist effect of tamoxifen in breast cancer *in vitro* models [30].

2.8.2. Cell Cycle Signaling and Apoptosis

However, the inhibitory effect of cellular proliferation by tamoxifen is associated with blockade of ER signalling pathway which regulation of the cell cycle processes. Principally, the cyclin-CDK complex, *Cyclin D1* is a critical role of a cell cycle control mechanism by phosphorylating molecules such as Retinoblastoma (RB) gene that permit the replication initiation [27]. MCF-7 breast cancer cells, have been studied that stimulation with E_2 increases the expression and phosphorylation of Cyclin D1 activity *in vitro*. Induction of pRB phosphorylation upon increase antioestrogens lead to downregulate the overall activity of the Cyclin D1-cdk4 complexes [40].

In addition, the nuclear phosphoprotein, *MYC* is also regulated by oestrogen and hormonal therapy. The role of *MYC* in cell cycle progression involved transcription regulation domain and this proto-oncogene product is required for the progression from G1-S phase [27]. Studies have shown that E_2 stimulates MYC mRNA and protein expression in breast cancer cells [41]. Further studies have revealed that the inhibition of *MYC* inhibits the oestrogen-stimulated growth of breast cancer cells, and E_2 rapidly activates the complex by preventing the inhibition by p21 which is well-known as CDK inhibitors or negative regulators of cell cycle progression and hastens from G1-S phase transition [42].

Endocrine therapy prevents the expression of oestrogen-responsive genes involved in inhibition of apoptotic effect consistent with the cell cycle. In normal breast growth E_2 stimulates proliferation by adapting gene expression

involved in cell survival [27]. There were increasing evidence demonstrated that the expression of apoptotic including the Bcl-2 family members are regulated by E_2 during normal breast progression and development [43]. *In vitro* studies have also shown that increasing tamoxifen (TAM) concentration can enhance the anti-apoptotic Bcl-2 family and reduced proapoptotic expressions [44]. Moreover, in the parental MCF-7 breast cancer cells exhibited increased of caspase 6, 7 and 9 activities after treatment with TAM resulting in improved intrinsic apoptotic cell death [45].

2.8.3. Growth Factor Receptor Pathways

ER may recruit rapid intracellular cascades by direct interaction with components of growth factor signalling. ER activation through the non-genomic pathway outside the nucleus resulting in phosphorylation, and activation of tyrosine kinase receptors, including IGF-IR, EGFR, and HER2, and also with cellular kinases and adaptor molecules such as c-Src and PI3K [27]. As illustrated in **Figure 2.9**., several of these interactions lead to the stimulation of downstream signalling kinases such as MAPK and AKT, which also have the potential to phosphorylate and thereby activate ER itself or its co-activator proteins [28]. The alteration these signalling cascades have the capacity to alter the therapeutic responses to endocrine agents including tamoxifen and hypothetically lead to the development of resistance [46].

2.8.4. Transcription Factors

Alternatively, ER can regulate gene expression via tethering to other direct DNA-binding transcription factors (TFs). As such, ER can bind to EREs that are near the response elements (REs) of the cooperating TFs, or ER can indirectly interact with DNA via tethering to TFs. In addition, E2-activated ER

localized in the cytoplasm or where ER can be methylated (M) at the cell membrane can interact with SRC, PI3K and G proteins (GPs) and stimulate non-genomic cascade. This signalling pathway mediated by protein kinase cascades resulting in the phosphorylation and activation of target TFs, which can thereby regulate target gene transcription through binding to response element (RE) sites. Finally, growth factor receptors, such as EGFR, HER2 (well-known as ERBB2) and insulin-like growth factor receptor (IGFR) after other growth factors (GFs) stimulation can activate ERK and AKT kinases, which can then phosphorylate and activate ER in an oesteogen-independent manner [27].

2.9. Epithelial-Mesenchymal Transition (EMT) and Metastasis

During the process of cancer metastasis, a minority of epithelial cells lose their basal cell polarity, detach from neighboring cells, scatter and obtain increased motility and are able to invade into the extracellular matrix.

2.9.1. The Basics of EMT

EMT process is facilitated by a morphological transformation into a fibroblastoid structure that has all the hallmark characteristics of EMT, Both processes share remarkable similarities, with feature phenotypic modifications. As illustrated in **Figure 2.10**, these include the loss of cell-cell adhesive molecule as resulting in reduction of E-cadherin (*CDH-1*) adherens junctions, occludins and claudins that in humans is encoded by the *OCLN*, CLDN of tight junctions respectively and down regulation of epithelial cytokeratins including CK8, CK18, and CK19 and up-regulation of mesenchymal proteins most especially vimentin (*VIM*) and fibronectin and sometimes alpha smooth muscle actin (α-SMA) accompanied by many other changes [47].

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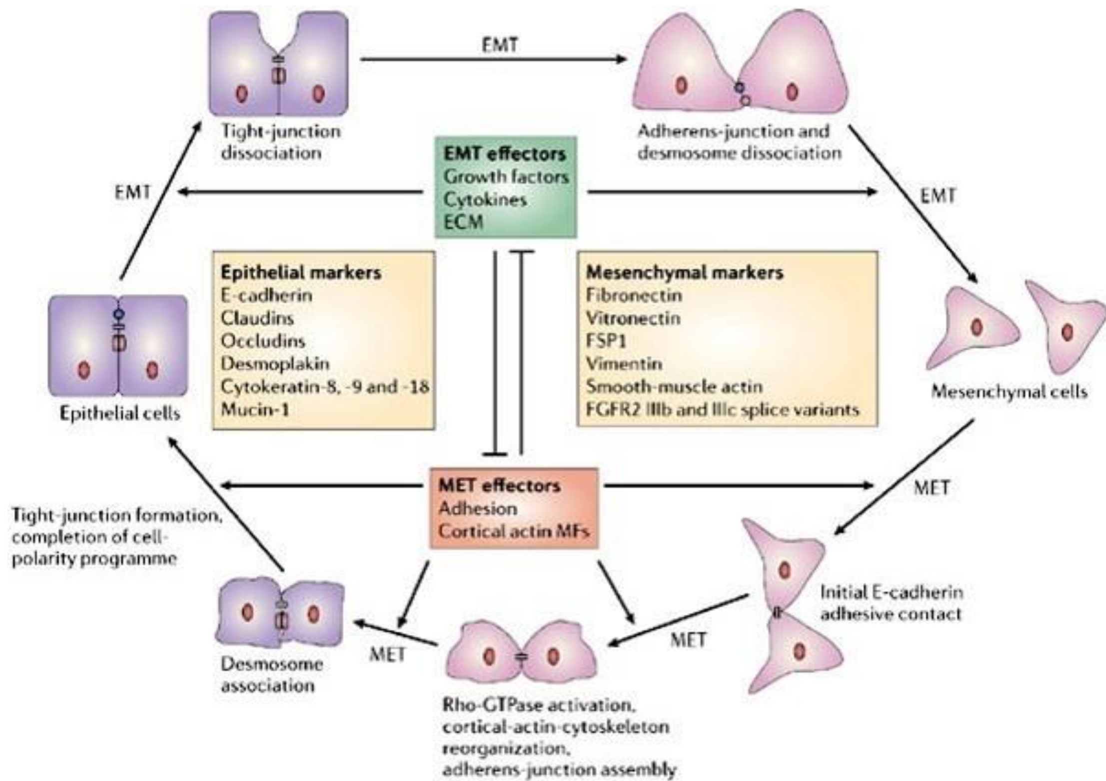

Figure 2.10. The Epithelial-Mesenchymal Transition (EMT) [47].

2.9.2. Mechanism Regulation of EMT

The transformation of epithelial cells into a mesenchymal-like form requires the contribution of a complex network of both intra– and extra–cellular signals. Amongst the various mediators are TGF-β, HGF, FGF, EGFR family members, IGF1 and 2, and PDGF [48]. An array of embryonic transcription factors such as the homeobox protein goosecoid (GSC), TCF3, the zinc-finger proteins SNAIL and SLUG (also termed SNAIL2), the basic helix-loop-helix protein TWIST1, the forkhead box proteins including FOXC1 and FOXC2 and the zinc-finger E-box-binding proteins ZEB1 and ZEB2 which known as SIP1 are produced by the activity of these growth factor pathways (**Figure 2.11**), each of which is capable, on its own, of inducing an EMT [49].

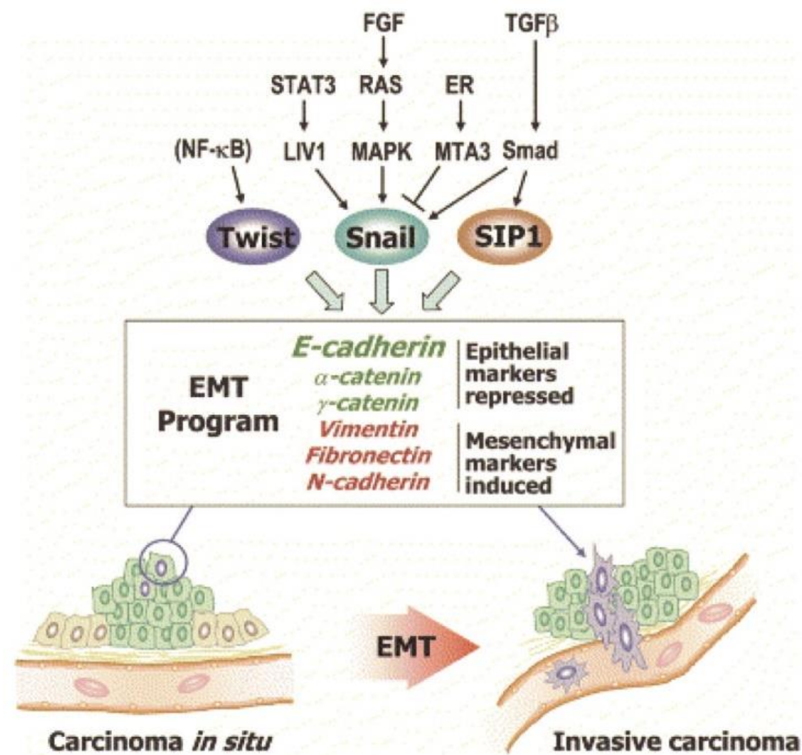

Figure 2.11. Signalling mechanisms of the EMT [50].

2.9.3. EMT in Carcinoma Invasion and Metastasis

Majority of breast cancer related deaths due to tumour invasive and metastatic capacities. Metastasis is a highly complicated and unspontaneous process that generally affects important organs such as lung, bone, liver or brain in the advanced platforms of carcinogenesis [6]. As illustrated in **Figure 2.12.**, It is well recognized that metastasis involved in distinct steps within cancer cells (a) the epithelial cells has rapidly proliferate rate (b) tumour cells are unlimited proliferation causing hypoxic condition and starting angiogenesis, (c) new blood vessels generating form pre-existing vessels are not well-organized resulting in cells can migrate away from the primary tumour site, invade through neighboring tissue and infiltrate through basement membrane that well-known as intravasation, (d) enter the lymphatic vessels or blood circulation, survive the situation of anoikis while they are detached from the tumour mass and in

circulation, (e) exit the lymphatic vessels or blood circulation at a distant organ which also called extravasation, (f) adapt and reprogram the surrounding stromal microenvironment, and finally form macrometastases [51].

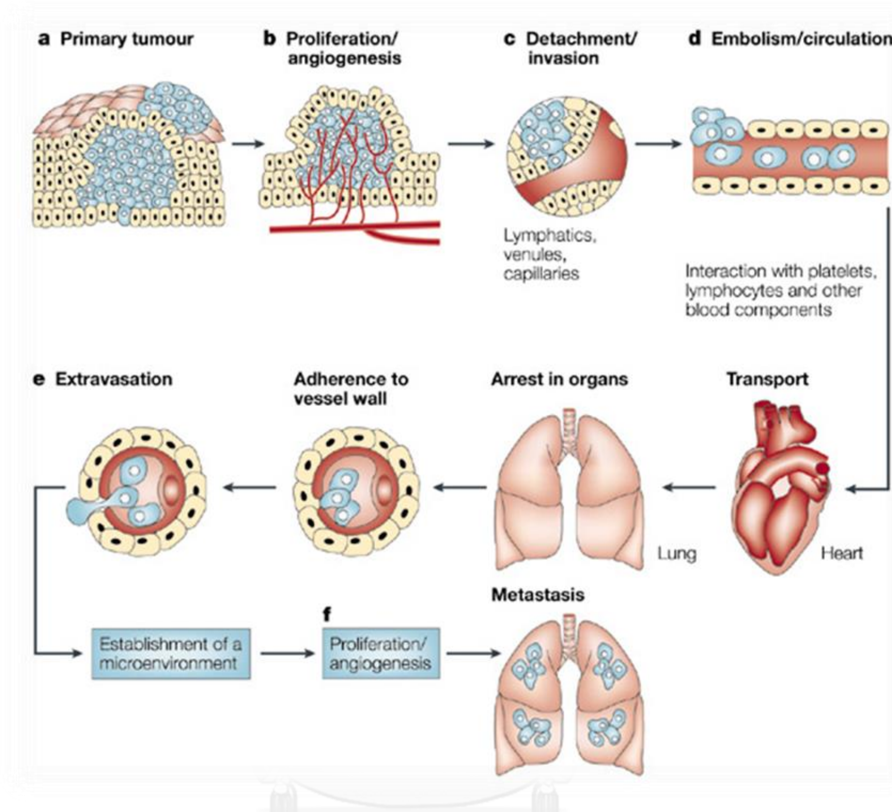

Figure 2.12. The metastatic process in breast carcinoma [51].

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Investigators have tried to understand the cellular and molecular bases of tumour metastasis are certainly challenged by the fact that metastasis is a complex, multistep biological process that most possible is controlled by several distinct genes and signalling cascades to individually step.

2.10. Plumbagin

Plumbagin was first isolated in 1829, a detailed study on its extraction, isolation, crystallization, solubility and chemical reactions were reported later by Thomson [52]. This study focused on Plumbagin (PLB) from *Plumbago indica* L. (**Figure 2.13**) showed original, phytochemicals and many potential therapeutic uses including the treatment of cancer which were also indicated efficacy and safety of PLB.

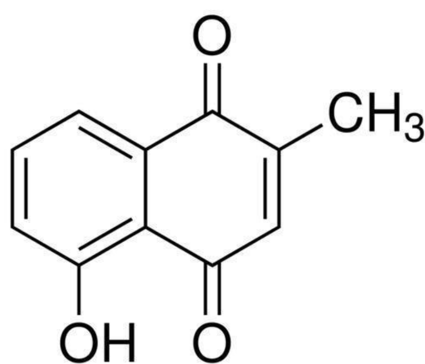

Figure 2.13. 2D structure of plumbagin [53].

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2.10.1. Origin of Plumbagin

Plumbago indica L. of Plumbaginaceae family is a medicinal plant or herb grown up to 1-1.5 meter tall. Stem is erect, trailing or climbing, simple or branched from the base. The leaves are simple, alternate arrangement, short petiole and the stipule is absent. The flowers are bisexual, regular and pentamery. The pedicel measures 0-1 mm long. The calyx is tubular in shape measuring 8-9 mm long, glandular and red in colour. The corolla tube measures 2.5-4.5 cm long. The lobes obovate in shape measuring 1.5-3 cm in diameter, apex rounded, mucronate, purple to red in colour; stamens free, exserted; ovary superior, ellipsoid-ovoid, 1-celled, style filiform, 5 stigma lobes, seeds absent [54].

2.10.2. Chemical Properties of Plumbagin

Plumbagin is the major bioactive compound (0.9-1% g.dw) present in the root of *Plumbago indica* L. Other naphthoquinones reported are 6-hydroxyure plumbagin, roseanone (binaphthoquinone), droserone, elliptinone and plumbagic acid lactone [55]. Polyphenols includes; flavonoids such as ayanin, azaleatin, arachidyl alcohol, myricetin-3,3',5',7-tetra methyl ether, ampelopsin-3',4',5',7-tetramethyl ether and carboxylic acid, plumbagic acid, roseanoic acid, were also isolated from the roots Alkaloids (α-naphthylamine), benzenoids (dinitrobenzene), aliphatic acids including myricyl palmitate, palmitic acid and steroids such as β-sitosterol and its glycoside are the other compounds which present in roots [56].

2.10.3. Pharmacological Effects and Mechanisms of Plumbagin Action

Biological effects of medicinal plant extracts from *Plumbago indica* as well as of plumbagin have been studied. It has previously demonstrated that PLB has multiple pharmacological effects including anti-bacterial [57], anti-fungal [58], anti-inflammatory [59], anti-proliferative [60], anticoagulants [61], anti-angiogenesis [62, 63], and anti-metastatic activities [64, 65]. The molecular underlying mechanisms of the anticancer effects of PLB modulate the NF-κB activation pathway, which in turn induces S-G2/M cell cycle arrest through inducing of p21 cyclin-dependent kinase (CDK) and modifying of Bcl-2 family which is anti-apoptotic proteins and dysregulating of pro-apoptotic proteins, the microtubule network disruption, DNA breakage and repair [66, 67], cell cycle arrest and generation of reactive oxygen species (ROS) [68-71]. Furthermore, PLB was also found to improve the radiosensitizing effects in both cell cultures and xenograft models [72]. Hsu et al. found that PLB has anticancer effect in

human non-small cell lung cancer A549 cells underwent G2/M phase arrest with increased levels of p21 and reduced amounts of Cyclin B1, Cdc2, and Cdc25C and triggered the mitochondrial apoptotic pathway indicated by a change in Bax/Bcl-2 ratios, resulting in mitochondrial membrane potential loss, cytochrome c release, and caspase 9 activation [69]. These study was correlated with Kuo et al. showed that PLB inhibited cell proliferation with increased the p21WAF1/CIP1 expression resulting in cell cycle arrest in S-G2/M phase [73].

Moreover, Shih et al. report revealed that PLB had indicated an inhibitory effect on cell migration and invasion of hepatic cancer HepG2 cells by decreasing the functional level of MMP-2 and u-PA activities. PLB action decreased the levels of MMP-2, u-PA and u-PAR mRNA and protein were vividly decreased, while elevated TIMP-2 and PAI-1 expression. In the nuclear levels of NF-κB, c-Fos and c-Jun were significantly decreased [9], this consisted with Manu et al. demonstrated the expression of chemokine receptor CXCR4 involved in cell invasion was downregulated in different types of tumor cells after treatment with PLB and was also found the level of CXCR4 expression in breast cancer BT474 cells which were ERBB2 overexpressed cells. Importantly, the downregulation of CXCR4 did not occur through proteolytic degradation of the receptor but rather through attenuation of the transcription. Moreover, decreasing of CXCR4 expression resulting in the inhibition of migratory and invasive ability in both breast cancer MDA-MB-231, which are triple negative subtype and gastric cancer AGS cells which induced by its cognate ligand SDF-1α/CXCL12 [64]. Furthermore, Santosh et al. demonstrated that PLB suppressed tumour necrosis factor (TNF)-induced NF-κB activation and other stimuli such as stress, hypoxia and lipopolysaccharide (LPS). PLB also inhibited

the constitutive NF-κB activation in cancer cells. Inhibiting of NF-κB activation correlated with progressive inhibition of TNF-induced activation of IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and the NF-κB-dependent reporter gene expression activated by TNF, TNFR1, and the p65 subunit of NF-κB. PLB was significantly decreased nuclear p65 binding and recombinant p65 to the DNA region both *in vivo* and *in vitro*. Conversely, PLB did not inhibit p65 binding to DNA when cells were transfected with the plasmid of p65 containing Cys-38 was replaced by serine. PLB downregulated of NF-κB-regulated gene involved in anti-apoptotic effect such as Survivin, IAP1, IAP2, Bcl-2, and Bcl-xL, proliferative effect including *Cyclin D1* and angiogenesis including MMP-9 and VEGF expression [74].

2.10.4. Plumbagin Safety and Toxicology

The ideal goals for discovery of anticancer therapeutic agents are more toxic against tumour cells than or less in normal cells. Aziz et al. reported that PLB was found to have no apoptotic-inducing effect on normal prostate epithelial RWPE-1 cells [75]. Ahmad et al. demonstrated that PLB also significantly inhibited the growth of breast cancer cells with no effect on normal epithelial breast tissues [76].

Consequently, PLB is able to be further investigated to use as a therapeutic agent for ER-positive breast cancer. Alternatively, the pharmacological properties of PLB on endocrine resistant cell lines and its machineries have rarely been reported. Therefore, this study was proposed to discover the effects of PLB in human endocrine resistant breast cancer cell survival, overcome resistance to tamoxifen and EMT-driven cellular invasion and metastasis.

CHAPTER III
MATERIALS AND METHODS

3.1. Chemicals and Reagents

The following chemicals and reagents were used in this study; Minimum essential medium eagle (MEM) (Gibco, USA), 0.25%Trypsin/EDTA (Gibco, USA), 0.4% Trypan blue solution (Sigma, USA), Fetal bovine serum (FBS) (Gibco, USA), Penicillin/Streptomycin (Gibco, USA), Fungizone (Amphotericin B) (Gibco, USA), MEM non-essential amino acid (Gibco, USA), Phenol red–free IMEM (Gibco, USA), Charcoal-dextran stripped FBS (CCS, Gibco, USA), Recombinant human insulin zinc solution (Gibco, USA), Dimethyl sulfoxide (DMSO) (Sigma, USA), Thiazolyl blue tetrazolium bromide (MTT, Sigma, USA), Growth factor reduced (GFR) Matrigel® basement membrane matrix (BD Biosciences, USA), 37% Formaldehyde solution (Sigma, USA), Crystal violet dye (Sigma, USA), TRIzol® Reagent (Invitrogen, USA), Diethyl pryrocarbonate (DEPC) (Molekula, UK), Ethanol (Merck, Germany), Chloroform (Merck, Germany), Isopropanol (Merck, Germany), Improm-II™ reverse transcription system (Promega, USA), Platinum® Taq DNA Polymerase (Invitrogen, USA), Deoxynucleotide (dNTP) solution mix (Vivastis, Malaysia), Oligo $(dT)_{15}$ primer (Vivastis, Malaysia), Recombinant RNasin® RNase inhibitor (Vivastis, Malaysia), 6X loading dye (Vivastis, Malaysia), VC 100 bp plus DNA ladder (Vivastis, Malaysia), Agarose LE grade (Vivantis, Malaysia), Ethidium bromide (ErBr) solution (Sigma, USA), 4–hydroxytamoxifen (Sigma, USA) and Plumbagin from *Plumbago indica* (MW = 188.18, Sigma, USA).

The catalog numbers of reagents and the name of equipments and instruments are showed in **Appendix A**. The detail of reagents and/or buffers used in this study as showed in **Appendix B**.

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3.2. Cell Lines and Cell Culture

The human breast adenocarcinoma MCF-7 cells were originally achieved from the American Type Culture Collection (ATCC, Cat. #HTB-22™) and two endocrine resistant breast cancer MCF-7/LCC2, MCF-7/LCC9 cells were generous donated from Georgetown University Medical Center, Washington, DC, USA, which is headed by Dr. Robert Clarke. MCF-7/LCC2 was developed as a stable variant of MCF7 cells that is resistant to tamoxifen (TAM), an oestrogen antagonist. In addition, MCF-7/LCC9 cells were developed resistant to fulvestrant (ICI), a pure antioestrogen, on crossed resistant to tamoxifen [13, 14]. Cells were routinely maintained in tissue culture flasks in modified Eagle's medium (MEM) containing phenol red (Invitrogen, USA) and supplemented with 5% heat inactivated fetal bovine serum (FBS) (Invitrogen, USA) and 100 units/mL penicillin and 100 µg/mL streptomycin (Life Technologies, USA), in a humidified incubator at 37°C containing 5% CO_2 in 95% air. During experiment 5 µL fungizone (Gibco, USA) was added to the culture medium in a sterile environment under a laminar airflow cabinet. Passage levels were in the range of 5 to 25 after thawing frozen cells from -196 °C in liquid nitrogen tank.

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3.3. MTT Cytotoxicity Assay

To determine the effect of plumbagin (PLB) on cell viability on wide-type MCF-7 and human endocrine resistant breast cancer MCF7/LCC2, MCF-7/LCC9 cells were performed by the methylthiazolyldiphenyl–tetrazolium bromide (MTT) assay as previously described [77]. The experiment is performed under the following protocol;

Principle

The cytotoxic activity of tumour cells are determined by using the 3–[4,5–dimethylthiazol-2yl]–2,5–diphenyltetrazolium bromide (MTT) assay as previously described. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell viability. MTT assay, first described by Mosmann [77], is based on the

ability of a mitochondrial succinate dehydrogenase enzyme, by generating reducing equivalents such as NADH and NADPH, from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a shady blue formazan crystals, which is largely waterproof to cell membranes, accordingly resulting in its assembly within healthy cells. The resulting intracellular purple formazan can be solubilized with DMSO and quantified by ELISA microplate reader. Meanwhile, MTT reduction can only occur in metabolically active cells and the level of activity is measured of the viability of the cells, the colour product will not act on dead cells. The reaction was illustrated in **Figure 3.1**.

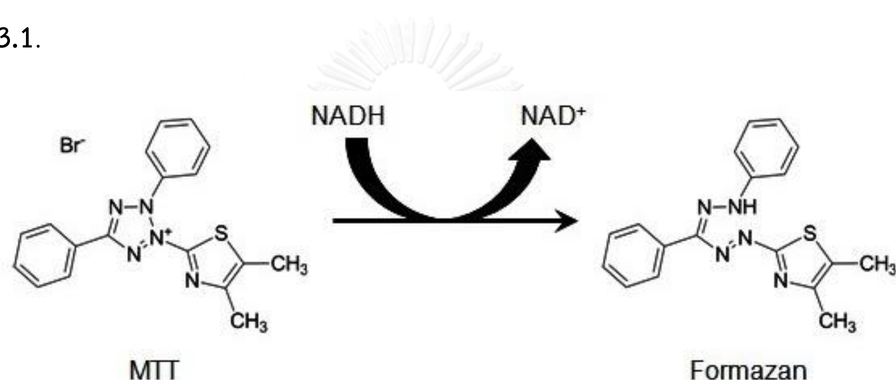

Figure 3.1. The principle of MTT assay [77].

Procedure

1. Cells were seeded in triplicate at a density of $5x10^3$ cells in complete MEM medium into each well of 96-well plates, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 hours.

2. After overnight, the cells were refreshed medium and added plumbagin (PLB) to wells in various concentrations. The exposure concentrations were determined as 0.5, 1, 1.5, 2.5 and 5 μM in 0.1% dimethyl sulfoxide (DMSO), and incubated in a humidified at 37°C containing 5% CO_2 in air for 24 and 48 hours respectively.

3. Prior to either 24 or 48 hour of PLB–treated, Cells were directly added 10 μL of MTT (5 mg/mL in PBS) solution to each well, including controls, then mixed gently tap the plate for a few seconds. The plates were foil-wrapped and returned to cell culture incubator for 4 hours.

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4. Following a 4 h incubation period with MTT, media was carefully removed, without disturbing the MTT formazan dyes in wells. The blue formazan crystals are formed in the cells dissolved with 100 µL DMSO and slightly shaken mixing the plate until the crystal violet was completely dissolved.

5. Measured the absorbance to each sample was measured at 570 nm with a background correction at 630 nm using a Multiskan® MS reader. The optimal density (OD) of the control group and the treatment group should be compared for quantitation of cell viability which calculated based on the following formula;

$$\% \text{ Cell Viability} = \frac{\text{Absorbance}_{sample} - \text{Absorbance}_{blank}}{\text{Absorbance}_{control} - \text{Absorbance}_{blank}} \times 100$$

3.4. Tamoxifen Response Assay

To determine the ability of plumbagin (PLB) to enhance sensitivity of metabolite 4-hydroxytamoxifen (4-OHT) was performed by using tamoxifen response assay. The experiment is performed under the following protocol;

1. Cells were plated in triplicate at a density of 5×10^3 cells in 96-well plates in phenol red-free IMEM supplemented with 5% charcoal dextran-treated FBS, and incubated at 37°C in a humidified containing 5% CO_2 for 24 h.

2. Medium was removed and refreshed. Cells were treated with combination of fixed concentrations of PLB (less than IC_{25}) and increasing concentrations of 4–OHT (0–10 µM) into each well of 96-well plates, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days.

3. Cells were washed twice with 1X PBS buffer solution and added tamoxifen alone, then incubated in a humidified at 37°C containing 5% CO_2 in air continued for 4 days.

4. Survival fractions were assessed by MTT method.

3.5. Combination Index (CI) Analysis

To determine whether the combination of plumbagin (PLB) and 4-hydroxytamoxifen (4-OHT) were synergistic, additive, or antagonistic effect, MTT cytotoxicity assays were completed for each individual agent and for combinations of varying concentration of 4-hydroxytamoxifen (0–10 μM) and a fixed micromolar of plumbagin (72-hour drug exposure) in triplicate and three independent experiments. The combination index (CI) technical method of Chou and Talalay was investigated based on the individual IC_{50} cytotoxicity level of wild-type MCF-7 and endocrine resistant MCF-7/LCC2, MCF-7/LCC9 breast cancer cells. The CI value is a quantitative measure indicating the type of interaction between treatment modalities: CI < 0.9 represents synergism, whereas 0.9 - 1.1 represents an additive effect, and CI > 1.1 represents antagonism [78]. The CI value for each experimental group was calculated using the following formula:

$$\text{Combination Index (CI)} = \frac{(C)_a}{(IC_x)_a} + \frac{(C)_b}{(IC_x)_b}$$

Where $(C)_a$ and $(C)_b$ in the numerator are the concentrations of compound a and b required in *combination* to produce 50% cell survival and $(ICx)_a$ and $(ICx)_b$ in the denominator are the concentrations of compound a and b required to *individually* produce 50% cell survival respectively.

3.6. Scratch Wound Assay

Principle

The scratch wound assay is a method to study the direction of cell migration in vitro based on the observation that upon creation of a new synthetically gap on a confluent cell monolayer so called ‘‘wound field’’. The cells on the edge of the newly generated gap will move toward the opening to close the wound until new cell–cell

arguments are happened again. As illustrated in **Figure 3.2.**, A wound is reached into a confluent monolayer of cells (**A**) involve making of a wound on monolayer cells (**B**), capture of images at the beginning and regular intervals during cell migration to close the scratch (**C**), and comparison of the images to determine % of cell migration (**D**).

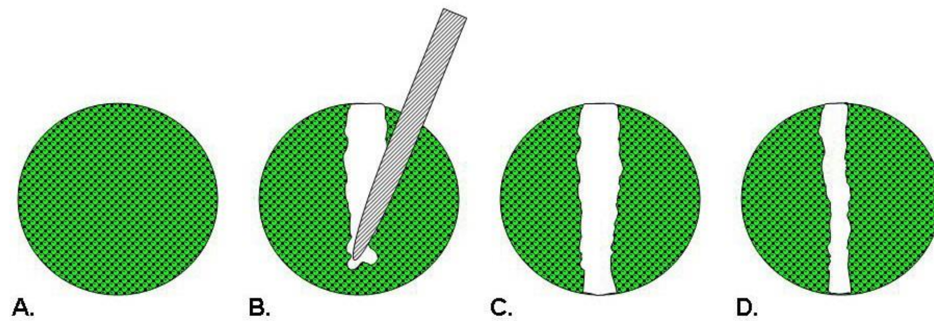

Figure 3.2. The scratch wound assay experiment [79].

Procedure

The metastatic properties of endocrine resistant breast MCF-7/LCC2 and MCF-7/LCC9 cells were evaluated by scratch wound-healing assay. The experiment is performed under the following protocol;

1. Cells at a density of $5x10^5$ cells were seeded into 6 well plate supplemented with 5% heat-inactivated FBS in MEM medium and cultured at 37°C in a humidified containing 5% CO_2 in an incubator.

2. After cells reached nearly 90–95% confluence, the cells monolayer was scraped in a straight line with a pipette tip to create a wound.

3. Both the smooth edges of the scratch and debrided cells were rinsed twice with 1X PBS buffer (pH 7.4) solution, then incubated cells without or with PLB (0.5-1 μM) in completed MEM medium for 24 h.

4. Migrated cells to wound surface was monitored by microscope and digital photograph. Set same alignments of a light microscope when shooting pictures for random fields of the scraped monolayer. The area of wound closure was quantitatively evaluated using ImageJ software v1.47 (NIH, USA).

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3.7. Matrigel Invasion Assay

The invasive ability of endocrine resistant MCF-7/LCC2, MCF-7/LCC9 breast cancer cells with the presence or absence of plumbagin (PLB) was investigated by using invasion chambers 6.5 mm in diameter with 8-μm pore size (BD Biosciences, France) according to manufacturer's procedure. The experiment is performed under the following protocol;

1. Growth Factor Reduced (GFR) Matrigel was thawed on ice at 4°C overnight.
2. GFR Matrigel was diluted 1:30 with serum-free MEM medium. The upper part of 24-well permeable support chambers were added by 40 μL of the diluted matrigel and incubated the transwell under the hood at room temperature (25°C) for 8 hours for solidifying matrigel. Keep product on ice and handle using aseptic method.
3. The matrigel-coated permeable plates were added warmed serum-free MEM medium and incubated at 37°C for an hour to dehydration.
4. Cells were trypsinized and seeded at a density of $5x10^4$ cells in 0.2 mL of serum–free medium with different concentration of PLB treatment onto the matrigel-coated upper chambers, bottom wells of the system were added by 0.6 mL MEM supplemented with 5% FBS which served as a chemo-attractant in this study and then incubated at 37°C for 48 h.
5. Supernatants were moved out both upper and lower chambers followed by fixed and stained coated permeable chambers with 3.7% w/v formaldehyde, 0.1% crystals violet solution respectively. Non-invaded cells were scraped off the top of the transwell with a cotton swab.
6. Invaded cells were counted in 5 random fields under a light microscope. The method was routinely carried out in duplicate and repeated three times for quantitative analysis. Data were presented as the average number of invading cells compared to the vehicle control from three independent experiments.

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3.8. RNA Isolation and Purification

Total RNA from wild-type MCF-7 human breast cancer and endocrine resistant MCF-7/LCC9 treated cells were isolated using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The experiment is performed under the following protocol;

1. Wild-type MCF-7 and MCF-7/LCC9 cells were collected by trypsinization and resuspension at a final concentration of 5×10^5 cells/mL in fresh medium with 5% FBS.

2. Aliquots of 2.0 mL cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.

3. Cells were treated with a difference of PLB concentrations (0.5 and 1 μM) using 0.1% DMSO as negative control for 24 hours.

4. Attached cells in the bottom were collected. Solutions were removed and added with 1 mL of TRIzol® reagent per well. Cells were lysed by pipetting the cells up and down several times.

5. Lysed cells were transferred to 1.5 mL eppendorf and incubated for 5 minutes at room temperature to certificate whole separation of the nucleoprotein complex.

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6. Phase separations were performed by adding 0.2 mL of chloroform per 1 mL of TRIzol® reagent used for homogenization. 1.5 mL eppendorf tubes were covered tightly and vertexed for 10 seconds. Then, the tubes were incubated for 2–3 minutes at room temperature.

7. The samples were centrifuged at 12,000 RPM for 15 minutes at 4°C. The mixtures were separated into a lower red phenol-chloroform phase, an interphase, and a monochrome upper aqueous phase. RNA remained totally in the aqueous phase. The upper aqueous phase was a half of the total volume.

8. The aqueous phase of the sample was transferred into a new eppendorf tube by putting the tube in a microtube rack and pipetting the solution out slowly.

While removing the aqueous layer, drawing the interphase or organic phase into the pipette tip was avoided.

9. Isopropanol was used to precipitate RNA. 0.5 mL isopropanol, HPLC grade, was added to the aqueous phase (0.5 mL isopropanol per 1 mL of TRIzol® reagent used for homogenization). The tubes were stored at -20℃ for 2 h and centrifuged at 12,000 RPM for 10 minutes at 4°C. The RNA was often invisible prior to centrifugation, and formed a gel-like pellet on the side and bottom of the eppendorf.

10. RNA pellets were exposed to RNA wash. The supernatant was removed from the eppendorf tubes, leaving only the RNA pellet. The pellets were washed with 1 mL of 75% ethanol (in 0.1% DEPC–treated water) per 1 mL of TRIzol® reagent used.

11. RNA pellet was temporarily vortexed and centrifuged the tube at 7,500 RPM for 5 minutes at 4°C. The wash was discarded.

12. RNA pellets were air dried for 5–10 minutes on ice. The RNA was not allowed to dry completely, since the solubility of pellets could defeat.

13. RNA pellets were resuspended in 0.1% DEPC–treated water 10–20 µL and mixed the solution up and down several times by a pipette tip.

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14. The RNA content and phenol contamination were determined by quantifying the absorbance at 260 and 280 nm (NanoDrop ND-1000, USA). The RNA concentration had ratio of A260/280 between 1.8 and 2.

15. The total RNA was stored at -80 ℃ until use to converse to cDNA.

3.9. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The mRNA expression of ER target genes including *Cyclin D1* and *c-Myc*, coactivator molecule, *NCoA3* and EMT biomarker such as E-cadherin (*CDH1*), Vimentin (*VIM*) and transcription factor Snail (*SNAI1*) were performed in PLB-treated MCF-7 and MCF-7/LCC9 cells using RT-PCR. The experiment is performed under the following protocol;

Conversion of RNA to primary cDNA

Total RNA samples were converted to cDNA using Improm-II™ reverse transcription system (Promega, USA) according to manufacturer's protocol.

1. 0.2 mL sterilized PCR tubes were placed on ice. The experimental RNA was thawed and kept any unused portion back to the -20°C freezer as rapidly as aliquot were taken.

2. Total RNA (1 μg/μL) combined with 1 μL of the Oilgo(dT)$_{15}$ primer was alighted and brought up to a final volume of 5 μL per reaction with DEPC-treated water was plated on ice followed by mixing and spinning down of each tube to shield bubbles and to keep original volume.

3. Each tube of RNA was tightly closed, then placed the thin-walled PCR tubes into a Mastercycler® thermocycler (PCR machine) and incubated at 70°C for 5 minutes followed by immediately chilled on ice for at least 5 minutes.

4. PCR tubes were vertexed and spun down for 20 seconds to collect drops and maintain the original volume. While the master mix solutions were preparing while tubes were kept on ice all the time.

5. The reverse transcription reaction mater mix was prepared by integrating the following components of the ImProm-II™ Reverse Transcription System in a sterile eppendorf. The master mix was prepared to allow 15 μL for each cDNA synthesis reaction to be performed. Volume was determined for each component, and

combined them in the order list. Master mix solutions were frequently vortexed and kept on ice prior to provide into the reaction tubes. The reverse transcription reaction master mix contained the following components;

1) Nuclease-Free Water (to a final volume of 15 µL)	7.3 µL
2) ImProm-II™ 5X Reaction Buffer	4.0 µL
3) 25 mM MgCl2 (final concentration 2.0 mM)	1.2 µL
4) dNTP Mix (0.5 mM each of dATP, dCTP, dGTP and dTTP)	1.0 µL
5) Recombinant RNasin® Ribonuclease Inhibitors (20 u)	0.5 µL
6) ImProm-II™ Reverse Transcriptase	1.0 µL
Total volume	**15 µL**

6. 15 µL of master mix solutions were added into each reaction tube on ice for a total reaction volume of 20 µL per tube.

7. The reaction tubes were placed into a Mastercycler® thermal cycler machine to generate cDNA using the following conditions; annealing step at 25°C for 5 minutes, extension step at 42°C for 90 minutes, and inactivated Reverse Transcriptase at 70°C for 15 minutes. The resultant cDNA were kept at -20°C to use as a template for the determination of mRNA expression.

Semiquantitative RT-PCR

1. Following components were added into a sterilized 0.2-mL PCR tube. The reaction volumes were 25 µL. PCR master mix solutions of constituents were prepared for several reactions.

1) Sterilized, UltraPure water (to a final volume of 24 µL)	19.05 µL
2) 10X PCR Buffer without Mg (1X PCR buffer)	2.5 µL
3) 10 mM dNTP mixture (final concentration 0.1 mM each)	0.25 µL
4) 50 mM MgCl2 (final concentration 2.0 mM)	1.0 µL

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5) 10 μM Forward Primer (final concentration 0.2 μM)	0.5 μL
6) 10 μM Reverse Primer (final concentration 0.2 μM)	0.5 μL
8) Platinum® Taq DNA Polymerase (1.0 unit)	0.2 μL
9) First stand cDNA Template	1.0 μL
Total volume	**25 μL**

2. PCR tubes were covered, mixed, and centrifuged briefly to collect drops.

3. Each of PCR tube was incubated in a Veriti® 96-well thermal cycler (Applied Biosystems, USA.) at 94°C for 2–3 minutes to completely denature the template and activate the reverse transcriptase.

4. PCR amplifications were performed 25–35 cycles as follows;

Denaturing	94°C for 30 seconds
Annealing	Tm for 30 seconds
Extending	72°C for 1 minute

5. The final product extensions were held at 72°C for 10 minutes.

6. PCR products were kept at 4°C after the cycling, when extension step is completed, the post-PCR amplicons were removed from a Veriti® 96-well thermal cycler and plated on ice followed by storing at -20°C until being used to determine the amount of amplification products by agarose gel electrophoresis.

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Table 2. Specific primers for RT-PCR and annealing temperature.

Genes	Primer sequences	Tm (ºC)	Product (bp)
GAPDH	F: 5’-GAG AAG GCT GGG GCT CAT TT-3’ R: 5’-AGT GAT GGC ATG GAC TGT GG-3’	57	231
CCND1	F: 5’-CTG GCC ATG AAC TAC CTG GA-3’ R: 5’-GTC ACA CTT GAT CAC TCT GG-3’	60	483
MYC	F: 5’-GCT TCT CTG AAA GGC TCT CCT-3’ R: 5’-CCA TTC CCG TTT TCC CTC TG-3’	56	295
NCoA3	F: 5’-AGC CAT CAG TGA AGG TGT GG-3’ R: 5’-ACT TGT GCA AAA TCC GGT GC-3’	57	482
CDH1	F: 5’-CGC ATT GCC ACA TAC A-3’ R: 5’-CGT TAG CCT CGT TCT CA-3’	57	502
VIM	F: 5’-CGC TTC GCC AAC TAC AT-3’ R: 5’-AGG GCA TCC ACT TCACAG-3’	57	662
SNAI1	F: 5’-CGA GTG GTT CTT CTG CGC TA-3’ R: 5’-TGG CTT CGG ATG TGC ATC TT-3’	58	585

Abbreviations; F forward primer, R reverse primer; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *CCND1*, Cyclin D1; *MYC*, v-myc avian myelocytomatosis viral oncogene homolog; *NCoA3*, nuclear receptor coactivator; *CDH1*, E-cadherin; *VIM*, vimentin; *SNAI1*, snail family zinc finger 1.

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Agarose gel electrophoresis

1. A gel was prepared in 1.5% agarose concentration by weighing 0.75 grams of agarose powder followed by transferring into an Erlenmeyer flask. 50 mL of 1X TBE solution was added in an Erlenmeyer flask and vigorously swirled to thoroughly mix agarose.

2. Erlenmeyer flask was taken into a microwave. Agarose was heated on medium-low temperature for a minute. At 1 minute, flask was removed from microwave and swirled. After 2 minutes of heating, flask was repeated heating and swirling procedure every 30 seconds until solution is clear.

3. A gel tray was placed into casting chamber followed by adding casting combs into appropriate slits.

4. Agarose gels were poured after cooling to 55–60°C. When pouring the gel, bubbles were avoided as this prevented current from flowing through the gel.

5. The gel was allowed to be cool for at least 45 minutes until it was set, when it was ready, it turned colorless and milky. Gels were fairly thin, approximately 0.625 to 1.25 cm in thickness.

6. Combs were carefully removed by pulling them upwards firmly and smoothly in a continuous motion.

7. Both gel and tray were plated into gel outfit, and wells were on the cathode (negative) end. Then, gel outfit was filled with 1X TBE to cover the entire gel.

8. A piece of parafilm was cut and placed a 2 µL drop of 6X loading dye onto the waxy side for each sample to be loaded.

9. PCR product samples were held on ice, 4 µL of a sample was pipetted and added the sample to one of the loading dye.

10. PCR products and loading dye were combined by pipetting the mixture up and down several times, then 5 µL of the mixture was loaded into a well.

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11. Samples were continually loaded into the rest of wells, then 1.5 µL of VC DNA ladder was added in the first well of each row as a marker.

12. The cover of the gel box was inserted and connected with the electrodes. The electrode cables were connected to the power supply, making sure the black (negative charge) and red (positive charge) ends are correctly connected.

13. A power supply was usually set up to a constant value at 100 volts (V) resistor rated for 2 watts (W) to run the gel for 50 minutes. The power pack was switched off to run when the bromophenol blue dye front was migrated at 3/4 of the gel.

14. A power wallet was turned off, the gels were transferred and placed into the appropriate tray filled with 400 mL of 1X TBE and 0.5 µg/mL ethidium bromide staining (EtBr) solution for 15 minutes followed by destaining gels in 1X TBE buffer for 30 minutes.

15. The band densities were measured by UV Transilluminator using Gel Doc™ XR+ System and Image Lab™ software 4.1 (Bio-Rad Laboratories, USA).

16. Experiments were done in triplicates and fold changes were analyzed by the relative quantity method. Experiments were performed in triplicates with a housekeeping gene GAPDH expression as an internal control.

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3.10. Morphological Analysis

To evaluate the morphology of wild-type MCF-7 cells and their endocrine resistant MCF-7/LCC2, MCF-7/LCC9 cells, each cultured line was trypsinized, washed and counted by a hemocytometer (Hausser Scientific, PA). Suspended cells were seeded at a density of 5×10^5 cells per well into 6 well plate in complete MEM medium and cultured at 37°C in a humidified incubator containing 5% CO_2. When the cells reached approximately 70-75% confluence, Cells were photographed using a light microscope (Carl Zeiss, Germany) equipped with a digital camera (Nikon D500).

3.11. Cell Counting

The viability of cells was evaluated with trypan blue dye exclusion. Whole cells (viable and dead cells) were trypsinzed and counted in 4 corner squares and calculated the average of each large square of a hemocytometer. The place with cover slip designates a total volume of 0.1 mm^3 (1.0mm x 1.0mm x 0.1mm) or 10^{-4} cm^3. Approximately 1 mL is equivalent to 0.1 mm^3. The total number of cells per mL was determined by the following calculations; % cell viability = [total viable cells (unstained) divided by total cells (viable + dead)] x 100. The percentage of cell viability equal to 95% or more was used for all experiments in this study.

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3.12. Statistical Analysis

Data analysis was performed using the statistical software SPSS 22.0 (SPSS Inc., Chicago, USA). All results were expressed as mean ± standard deviation (S.D.) of triplicate in at least three independent experiments. Statistical calculations between two groups were analyzed using the Student’s t-test. For comparing multiple groups, one-way analysis of variance (ANOVA) was used followed by the Least Square Difference (LSD) test. *P*-value less than 0.05 was considered statistically significant. All data were represented as (*) for $p < 0.05$, (#) for $p < 0.01$, and (†) for $p < 0.001$, respectively.

CHAPTER IV

RESULTS

4.1. Effect of plumbagin on cytotoxicity of wide-type MCF-7 and endocrine resistant breast cancer cells

To investigate the effect of plumbagin (PLB) on the growth of endocrine–sensitive and –resistant human breast cancer cell lines, cell viability study was performed by MTT method. Wild-type MCF-7/WT cells and endocrine resistant breast MCF-7/LCC2, MCF-7/LCC9 cells were treated with different concentrations of PLB (0–5 μM) for 24, 48 hours and 0.1% DMSO was used as the vehicle control. The growth inhibition bars indicated that PLB had potent cytotoxic activity in a concentration– and time–dependent manner As illustrated in **Figure 4.1**, with a half-maximal growth inhibitory IC_{50} values of 1.46 ± 0.09, 1.22 ± 0.02 μM for wild-type MCF-7 cells (**Figure 4.1A**) and endocrine resistant MCF-7/LCC2 and MCF-7/LCC9 cells showed cytotoxicity with IC_{50} values of 1.69 ± 0.02, 1.53 ± 0.11 μM and 1.24 ± 0.09, 1.16 ± 0.03 μM for 24, 48 h respectively (**Figure 4.1B-C**).

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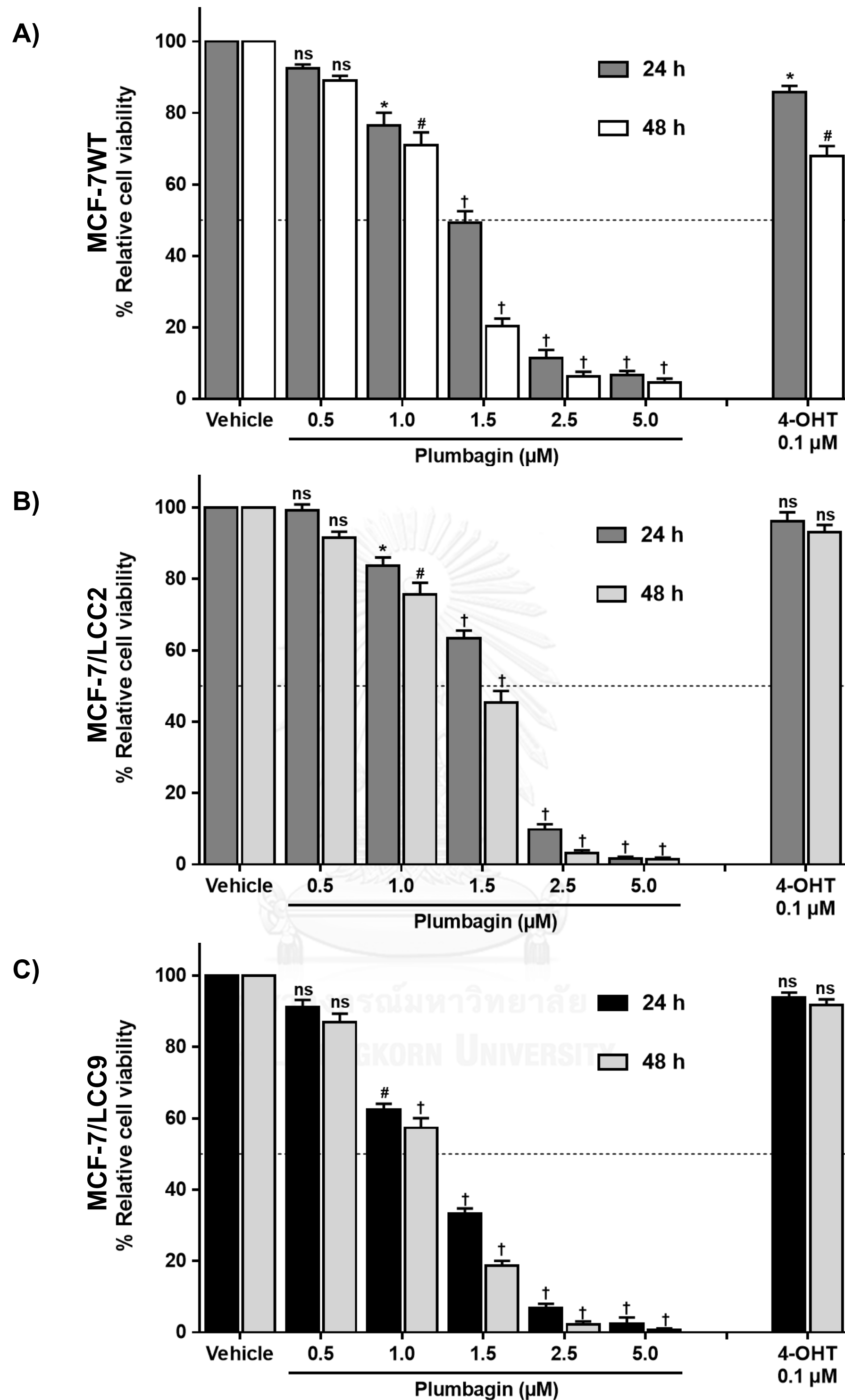

Figure 4.1. **PLB-induced cytotoxicity of wild-type MCF-7 and endocrine resistant breast cancer cell viability**. The percentage of viability of MCF-7 (A), MCF-7/LCC2 (B) and MCF-7/LCC9 cells (C) after treated with PLB at 0–5 μM for 24, 48 h. Each value represents the mean ± S.D. (*) for *P* < 0.05, (#) for *P* < 0.01, and (†) for *P* < 0.001; ns = not significant compared to the vehicle control (0.1% DMSO). Data are the representative of triplicates and three independent experiments (n = 3).

Table 3. The calculated growth inhibition IC_{25} and IC_{50} values of plumbagin (PLB)-induced cytotoxicity after 24 h of treatment in wild-type MCF-7 and endocrine resistant breast cancer cells

Cell lines	**Plumbagin (PLB)-induced cytotoxic activity**	
	IC_{25}	**IC_{50}**
MCF-7	0.96 ± 0.05 μM	1.46 ± 0.08 μM
MCF-7/LCC2	1.07 ± 0.06 μM	1.69 ± 0.02 μM
MCF-7/LCC9	0.80 ± 0.04 μM	1.24 ± 0.09 μM

Each value represented the mean ± S.D. Data are the representative of triplicates and three independent experiments (n = 3).

Table 4. The calculated growth inhibition IC_{25} and IC_{50} values of plumbagin (PLB)-induced cytotoxicity after 48 h of treatment in wild-type MCF-7 and endocrine resistant breast cancer cells

Cell lines	**Plumbagin (PLB)-induced cytotoxic activity**	
	IC_{25}	**IC_{50}**
MCF-7	0.89 ± 0.10 μM	1.22 ± 0.02 μM
MCF-7/LCC2	1.04 ± 0.03 μM	1.53 ± 0.11 μM
MCF-7/LCC9	0.69 ± 0.04 μM	1.16 ± 0.03 μM

Each value represented the mean ± S.D. Data are the representative of triplicates and three independent experiments (n = 3).

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4.2. Effect of plumbagin and tamoxifen combination on the growth of wide-type MCF-7 and endocrine resistant breast cancer cells

To investigate the ability of plumbagin (PLB) to restore tamoxifen sensitivity of wild-type MCF-7 and endocrine resistant breast MCF-7/LCC2, MCF-7/LCC9 cancer cells. Tamoxifen response assay was performed to determine the effect of either 4-hydroxytamoxifen alone or co-treatment with PLB after a 72–hours incubation which compared to 0.1% DMSO as the solvent control. The result demonstrated that a fixed non-toxic concentration of PLB (0.75 μM) revealed significantly enhanced sensitivity tamoxifen both in wild-type and resistant cells as illustrated in **Figure 4.2**.

A similar degree of anti-hormonal sensitization was observed with less than IC_{25} of PLB, with combination index (CI) values of 0.72 for the MCF-7 cells and 0.58, 0.94 for the MCF-7/LCC2, MCF-7/LCC9 cells respectively (**Table 5**; $P < 0.05$). Wide-type MCF-7 cells were more sensitive than the MCF-7/LCC2, MCF-7/LCC9 cells to tamoxifen alone, as evidenced with a lower IC_{25} value (**Table 4**). These data demonstrate that the level of ability to restore tamoxifen sensitivity by PLB had synergistic effect in human wild-type MCF-7 and tamoxifen-resistant MCF-7/LCC2 cells and exhibited additive effect in fulvestrant/tamoxifen-resistant MCF-7/LCC9 cells.

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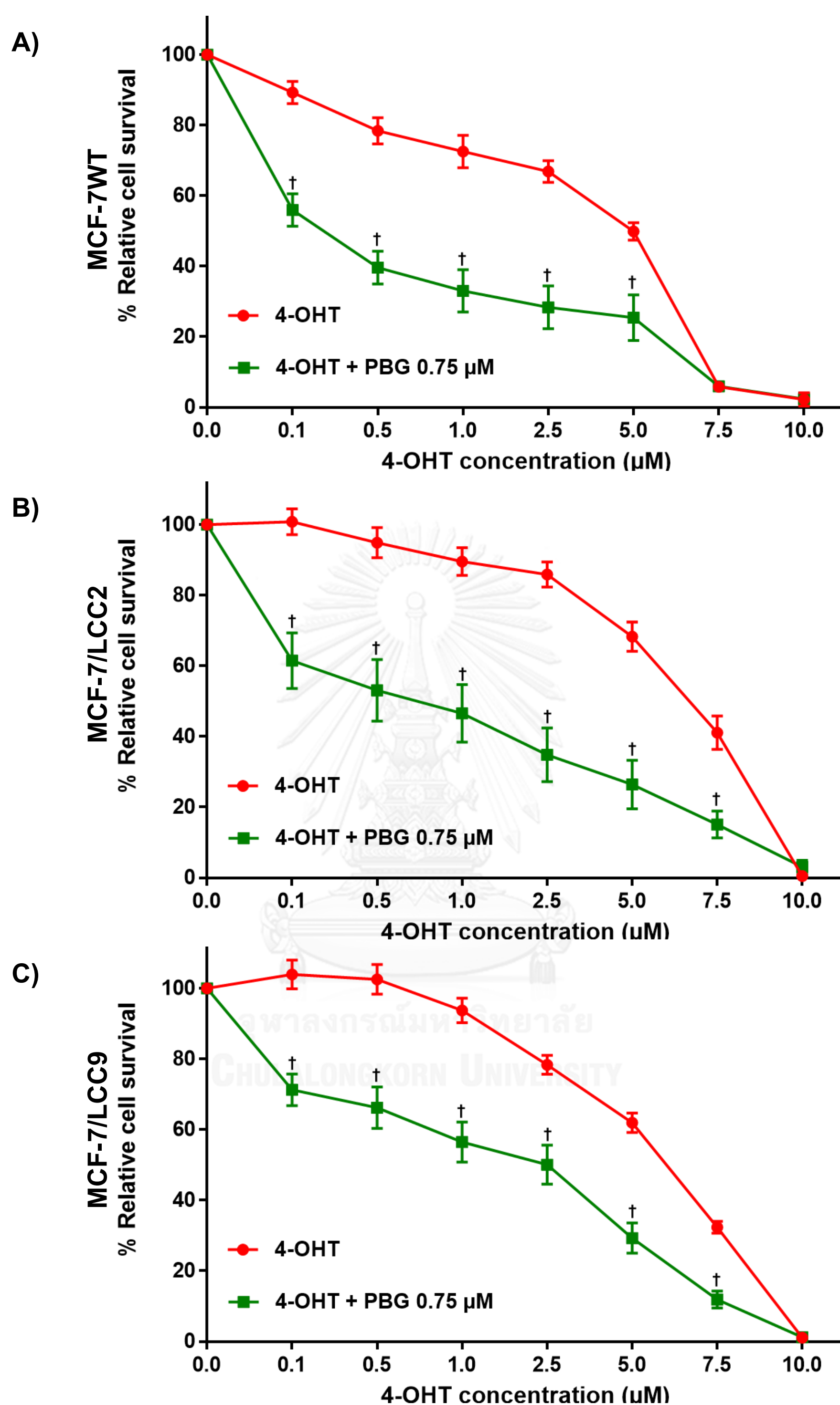

Figure 4.2. PLB enhanced tamoxifen cytotoxic activity in wild-type MCF-7 and endocrine resistant breast cancer cells. MCF-7 (**A**), MCF-7/LCC2 (**B**) and MCF-7/LCC9 (**C**) cells were incubated with 4-hydroxytamoxifen (4-OHT) alone (•) or co-treatment with 0.75 µM (▪) plumbagin for 72 h. Cell survival was assessed by the MTT assay. Each value represented the mean ± S.D. (†) for *P* < 0.001 compared to 4-OHT treatment alone. Data are the representative of triplicates and three independent experiments (n = 3).

Table 5. The growth inhibition (IC_{50}) and combination index (CI) values of 4-hydroxytamoxifen (4-OHT)-induced cytotoxicity. The inhibitory concentration IC_{50} values of either 4-OHT individually or co-treatment with 0.75 µM plumbagin, were used to calculate CI values of the combination treatment.

Cell lines	**4-OHT combined with PLB (0.75 µM)**		
	IC_{50}	**CI values**	**Description**
MCF-7	$0.50 \pm 0.08^{\#}$ µM	0.72	Synergism
MCF-7/LCC2	$0.72 \pm 0.21^{*}$ µM	0.58	Synergism
MCF-7/LCC9	$1.86 \pm 0.16^{*}$ µM	0.94	Additive

Where CI < 0.9, CI = 0.9-1.1 and CI > 1.1 indicate synergistic, additive and antagonistic effects, respectively. Each value represented the mean ± S.D. (*) for $P < 0.05$, (#) for $P < 0.01$ when compared to 4-OHT treatment alone. Data are the representative of three independent experiments (n = 3).

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4.3. Acquired endocrine resistant breast cancer cells exhibited EMT–like features.

To observe the morphology of endocrine–sensitive MCF7/WT and endocrine resistant (MCF-7/LCC2 and MCF-7/LCC9) cells was carried out using a phase contrast microscope of cells routinely maintaining in culture, the result observed that wild-type cells grew as little formation of pseudopodia and exhibiting shape of marginal cells was rounded, strongly packed clusters of epithelial cells compared to illustrate taken at 200x magnification (**Figure. 4.3A**). In contrast, endocrine resistant cells appeared flattened, spindle shaped mesenchymal-like feature and increased formation of pseudopodia that compared to illustrate taken at 200x magnification (**Figure. 4.3B-C**).

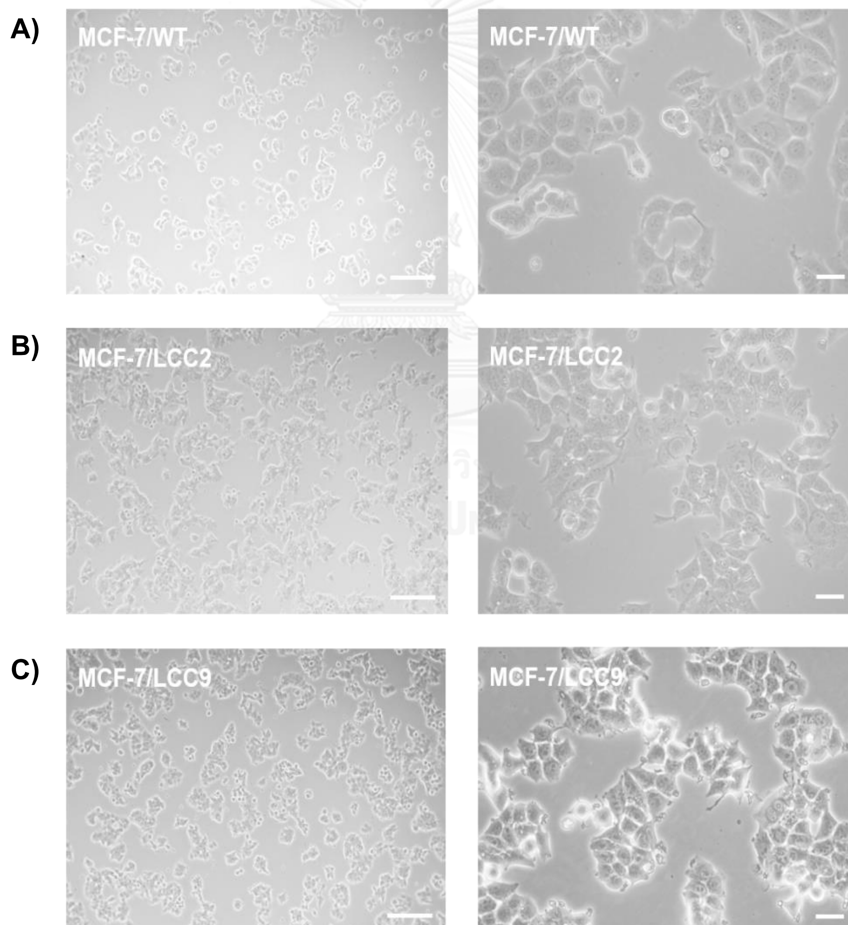

Figure 4.3. Morphological analysis of epithelial–mesenchymal transition (EMT)–like features in wild-type MCF-7 and endocrine resistant breast cancer cells. The representative photomicrographs of wild-type MCF-7 (**A**) and MCF-7/LCC2 (**B**), MCF-7/LCC9 cells (**C**) under a light microscope; left scale bar = 200 μm, right scale bar = 10 μm.

Principally, lacking of epithelial cell junction molecules and cell–cell adhesive interactions lead to compromise cell polarity, and receiving of mesenchymal phenotypes resulted in significantly enhanced aggressive, invasive and motile properties of several types of resistant tumour cells including breast cancer. To determine the expression of epithelial–mesenchymal transition (EMT)–associated genes and transcription factor of endocrine resistant breast cancer cells compared to wide-type cells.

Consistent with the results from morphological examination, RT-PCR analysis for EMT markers also demonstrated that endocrine resistant breast cells had EMT–like behaviors. As illustrated in **Figure 4.4**, E-cadherin (*CDH1*), epithelial phenotype-associated molecule was dramatically expressed in wild-type MCF-7 cells, while mesenchymal phenotype marker, vimentin (*VIM*) was predominantly upregulated in endocrine resistant MCF-7/LCC2 (data not shown) and MCF-7/LCC9 breast cancer cells. In addition, Snail mRNA expression levels were observed in both wild-type and resistant cells.

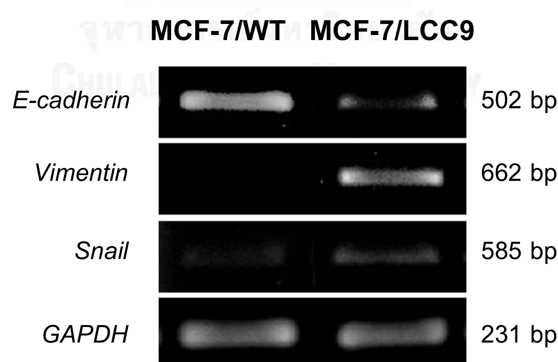

Figure 4.4. Baseline expression profiles of EMT biomarkers in wild-type MCF-7 and endocrine resistant breast cancer cells. Cells were harvested and isolated total RNA by TRIzol® reagent. 1 µg of total RNAs were converted to cDNA by Improm-II™ reverse transcription system and amplified with the specific primers of EMT markers by semi-quantitative RT-PCR analysis. The amount of PCR products was demonstrated by 1.5% agarose gel electrophoresis followed by quantifying with a Gel Doc™ EZ system and normalized to GAPDH expression.

4.4. Effect of plumbagin on alteration of mRNA expression involved ER target genes, coactivator and EMT markers

As mentioned previously, plumbagin (PLB) had a potent cytotoxic activity and restored endocrine sensitivity in wide-type MCF-7 and resistant MCF-7/LCC9 cell lines. The molecular regulations might be involved ER target genes and transcriptional coactivators. MCF-7 and MCF-7/LCC9 cells were treated with PLB (0.5–1 μM) for 24 h of treatment and 0.1% DMSO was used as the vehicle control. The treated cells were collected for extracting RNA, synthesizing cDNA and PCR amplifications of ER target genes such as *Cyclin D1*, *MYC* and nuclear receptor coactivatior *NCoA3* expression. Agarose gel electrophoresis was performed to measure the concentrations of PCR products and the housekeeping gene GAPDH was used as an endogenous control.

PLB at the concentration of 0.5 and 1 μM significantly downregulated *Cyclin D1* and *MYC* expression compared to the vehicle control as illustrated in **Figure 4.5**.

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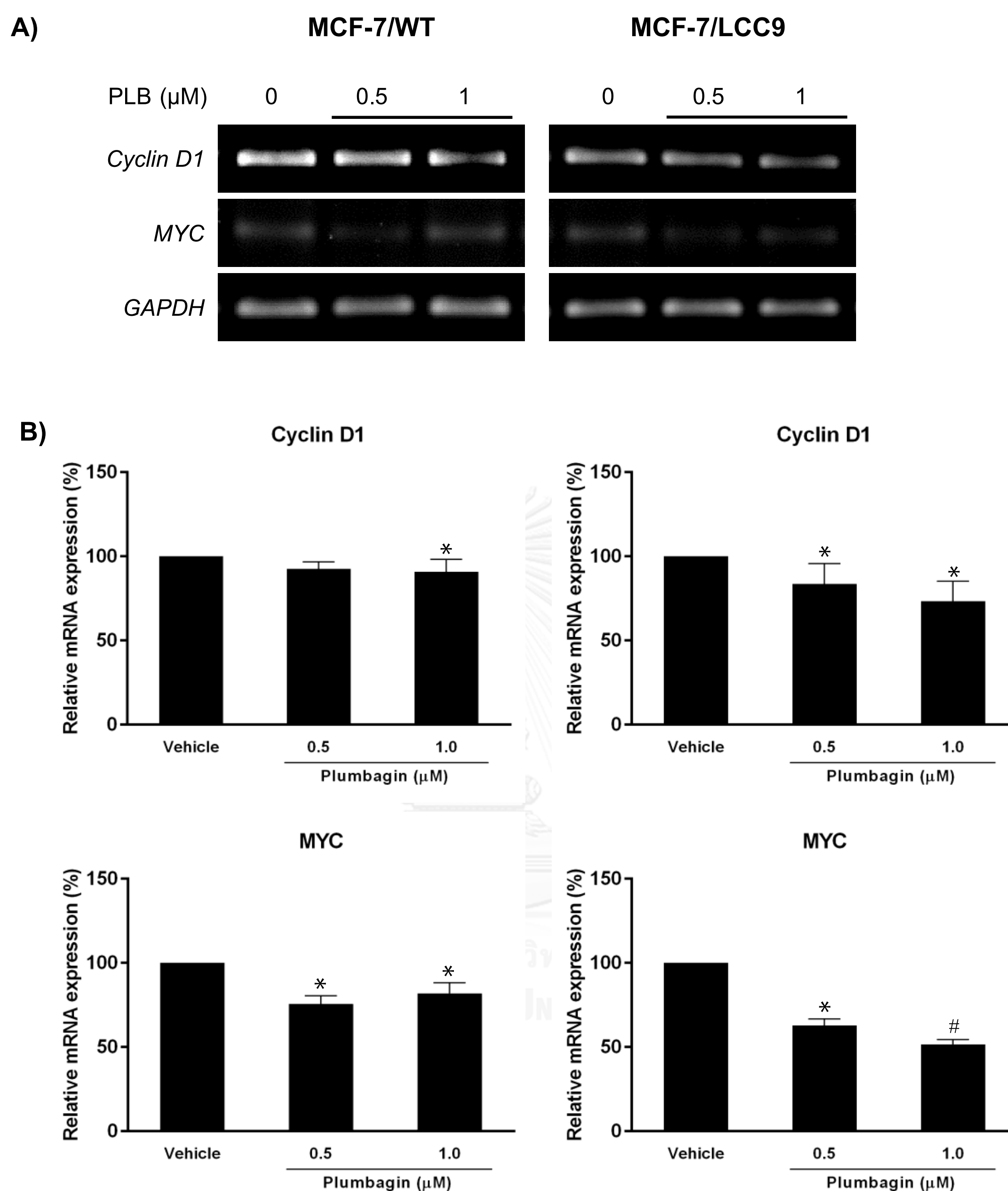

Figure 4.5. PLB-induced alterations of the expression of ER target genes in wild-type MCF-7 and endocrine resistant breast cancer cells. MCF-7 and MCF-7/LCC9 cells were treated with 0.5–1 μM of plumbagin (PLB) for 24 h. (**A**) The expression of ER target genes were assessed by RT-PCR. Figures were representative of PCR products of ER target genes. (**B**) Densitometric analysis of the PCR products of *Cyclin D1* and *MYC* normalized to GAPDH expression. Each value represented the mean ± S.D. (*) for $P < 0.05$, (#) for $P < 0.01$, and (†) for $P < 0.001$ compared to the vehicle control (0.1% DMSO). Data are the representative of three independent experiments (n = 3).

To further confirm the ability of PLB to inhibit cell survival and restore endocrine sensitivity via altering ER regulatory molecules, MCF-7 and MCF-7/LCC9 cells treated with 0.1% DMSO (vehicle group), 0.75 μM PLB (PLB group), 0.1 μM 4-hydroxytamoxifen (4-OHT group) or 0.1 μM 4-OHT combined with 0.75 μM plumbagin (4-OHT + PLB group) for 24 h were used to investigate the alteration of nuclear receptor coactivator (NCoA3) mRNA expression using RT-PCR.

As illustrated in **Figure 4.6**, the expression of NCoA3 in RT-PCR was presented in both wild-type and endocrine resistant breast cancer MCF-7/LCC9 cells. The *NCoA3* level was also reduced after treatment of PLB in MCF-7 and MCF-7/LCC9 cells at the concentration less than IC_{25} (0.75 μM) when compared to the vehicle control with 38% and 42% reduction respectively. Next, to compare between 4-hydroxytamoxifen (4-OHT)-treated cells and the vehicle control group, the expression of NCoA3 level was increased when compared to the vehicle control and was not significantly different in both of these cell lines in 4-OHT treatment. To compare between 4-OHT and PLB combination and vehicle control, *NCoA3* level was also dramatically reduced in both wild-type and resistant cells (47% and 24% reduction respectively, P = 0.012). Remarkably, the levels of *NCoA3* expression between 4-OHT treated group and 4-OHT with PLB treated group was significantly downregulated in MCF-7/LCC9 (P = 0.006, Student's t-test) and wild-type MCF-7 cells (P = 0.047, Student's t-test).

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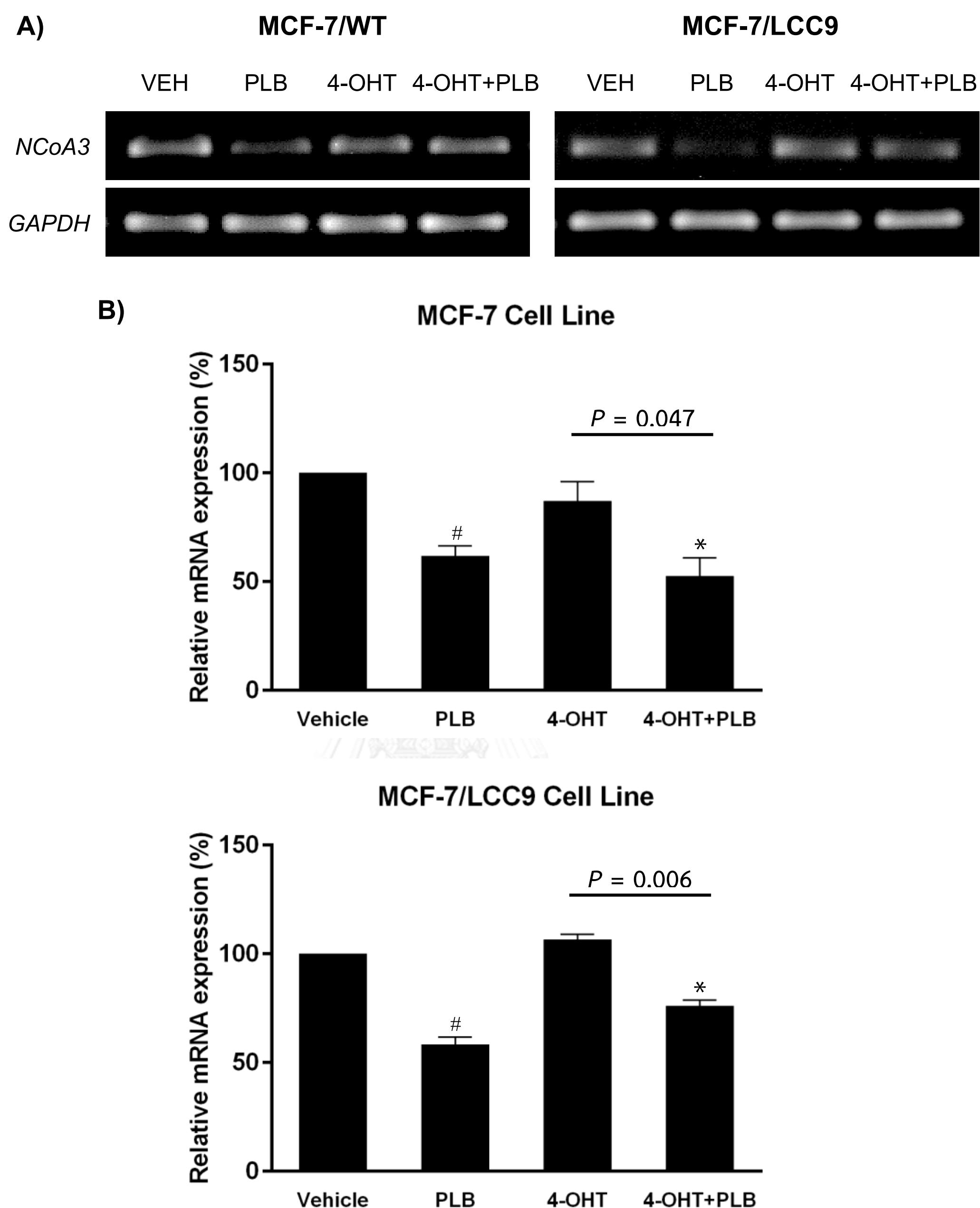

Figure 4.6. PLB decreased the expression of NCoA3 in wild-type MCF-7 and endocrine resistant breast cancer cells. MCF-7 and MCF-7/LCC9 cells were treated with 0.1% DMSO (vehicle group), 0.75 μM plumabgin (PLB group), 0.1 μM 4-hydroxytamoxifen (4-OHT group) or 0.1 μM 4-OHT combined with 0.75 μM plumbagin (4-OHT + PLB group) for 24 h. (**A**) Figures showed the representative of PCR products of NCoA3 normalized to GAPDH expression. (**B**) Densitometric analysis of the PCR products normalized to GAPDH expression. Each value represented the mean ± S.D. (*) for P < 0.05, (#) for P < 0.01 compared to the vehicle control (0.1% DMSO). Data are the representative of three independent experiments (n = 3).

To investigate whether PLB regulates expression of EMT-related genes and transcription factor, the mRNA expression levels of E-cadherin, Vimentin and Snail1 were determined using reverse transcription (RT-PCR). Wild-type MCF-7 and MCF-7/LCC9 cells treated with two different concentration of PLB and vehicle control (0.1% DMSO) was used to evaluate alterations in EMT markers. The results revealed that the expression of the epithelial phenotypic marker *E-cadherin* increased in wild-type MCF-7 cells compared to resistant cells (average 1.2 fold), while the mesenchymal phenotypic marker *Vimentin* presented only in MCF-7/LCC9 cells.

As illustrated in **Figure 4.7.,** PLB upregulated *E-cadherin* expression in a concentration-dependent manner by average 1.7 fold in MCF-7/LCC9 cells when compared to the vehicle control ($P < 0.001$, Student's t test) and average 1.2 fold in wild-type MCF-7 cells ($P = 0.002$, Student's t test), and significantly downregulated the expression of Vimentin mRNA levels in a concentration-dependent average 2.3 fold in MCF-7/LCC9 cells compared to the vehicle control ($P < 0.01$, Student's t test). In parallel, EMT-inducting transcription factors (*Snail1*) was increased expression in the resistant cells. However, *Snail1* was also dramatically reduced after PLB treatment in the concentration-dependent manner in both cell lines for 24 h of treatment as illustrated in **Figure 4.8**.

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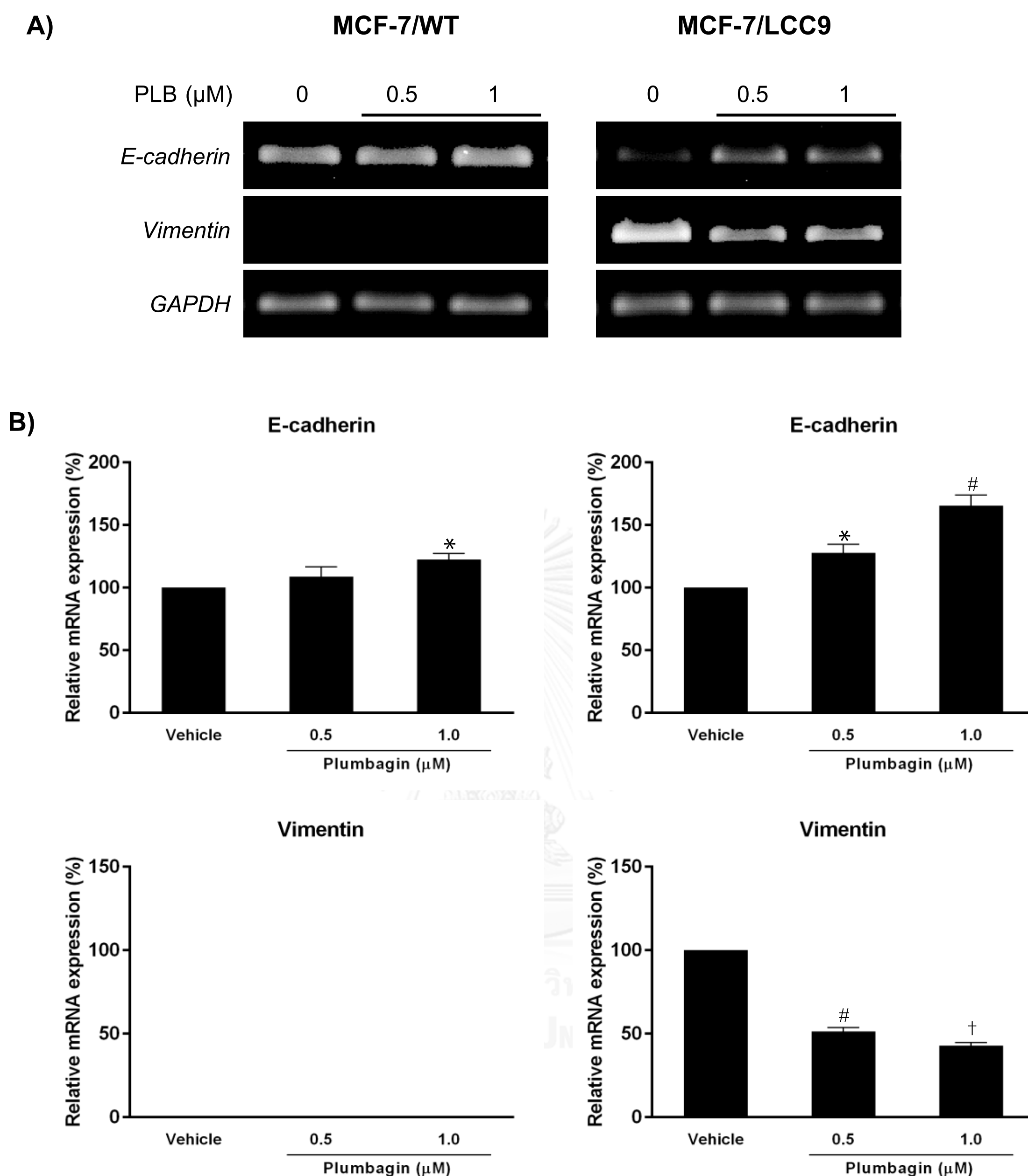

Figure 4.7. PLB altered the expression of EMT-related genes in wild-type MCF-7 and endocrine resistant breast cancer cells. MCF-7 and MCF-7/LCC9 cells were treated with 0.5–1 μM of plumbagin (PLB) for 24 h. (**A**) Expression of EMT-related genes were assessed by RT-PCR. Figures showed the representative of PCR products of EMT-related genes normalized to GAPDH expression. (**B**) Densitometric analysis of the PCR products of EMT-related genes includes; *E-cadherin* and *Vimentin*. Each value represented the mean ± S.D. (*) for P < 0.05, (#) for P < 0.01, and (†) for P < 0.001 compared to the vehicle control (0.1% DMSO). Data are the representative of three independent experiments (n = 3).

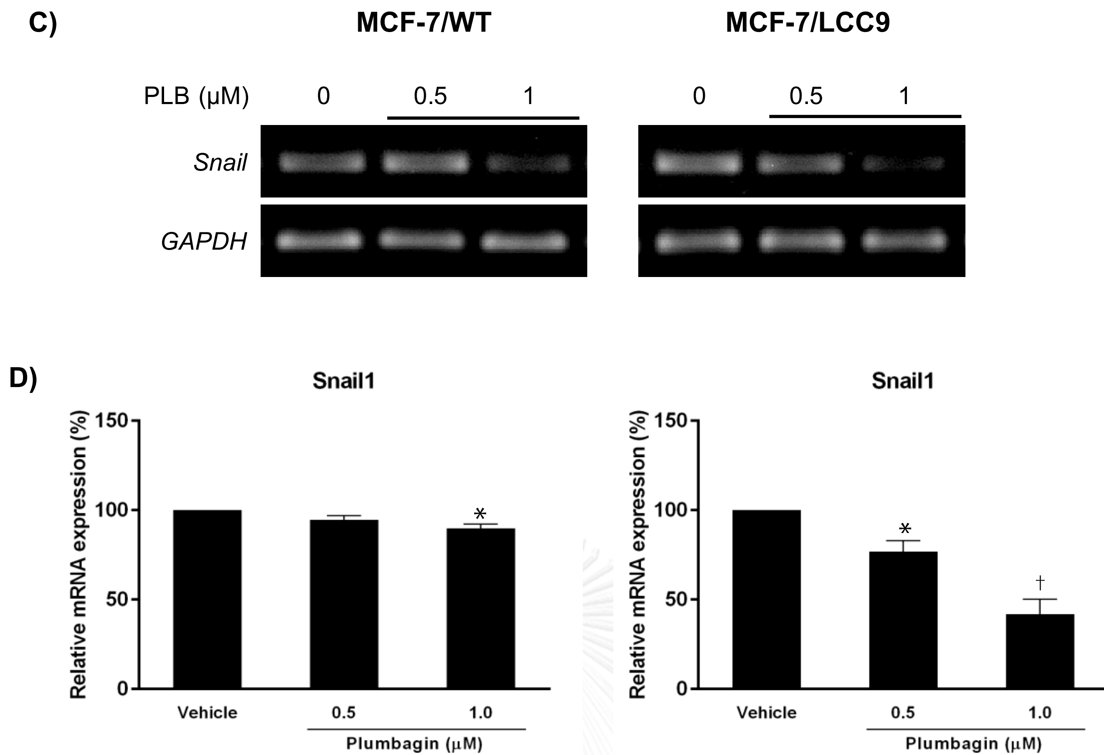

Figure 4.8. PLB altered the expression of transcription factor involved EMT in wild-type MCF-7 and endocrine resistant breast cancer cells. MCF-7 and MCF-7/LCC9 cells were treated with 0.5–1 μM of plumbagin (PLB) for 24 h. (**C**) Expression of EMT-related transcription factor was assessed by RT-PCR. Figures showed the representative of PCR products of transcription factor involved EMT normalized to GAPDH expression. (**D**) Densitometric analysis of the PCR products of transcription factor *Snail1*. Each value represented the mean ± S.D. (*) for $P < 0.05$, (†) for $P < 0.001$ compared to the vehicle control (0.1% DMSO). Data are the representative of three independent experiments (n = 3).

4.5. Effect of plumbagin on cellular invasion and migration in endocrine resistant breast cancer cells

It was previously reported that tamoxifen resistant breast cancer (TAM-R) cells were undergone EMT-like features, acquired mesenchymal phenotypes and loss epithelial characteristics which can be found to enhance migratory and invasive capacities of tumour cells [**22**]. Firstly, to investigate whether PLB could prevent cellular migration by scratch wound-healing assay. Wild-type MCF-7 and endocrine resistant breast cancer cells were seeded on 6-well plates and reached nearly 90–95% confluent. As illustrated in **Figure 4.8A-B**, Cells were mechanically scraped at least three straight lines with a sterile pipette-tip followed by treated without or with varying concentrations of PLB for 24 h and 0.1% DMSO was used as the vehicle control. The result demonstrated that PLB particularly inhibited cell migration in both cell lines by 49%-63% (**Figure 4.8C**; $P < 0.001$) when compared to vehicle control. Interestingly, endocrine resistant exhibited a dramatically higher in MCF-7/LCC2 (data not shown) and MCF-7/LCC9 cell migration (average 2.9 fold) compared to wild-type cells.

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A)

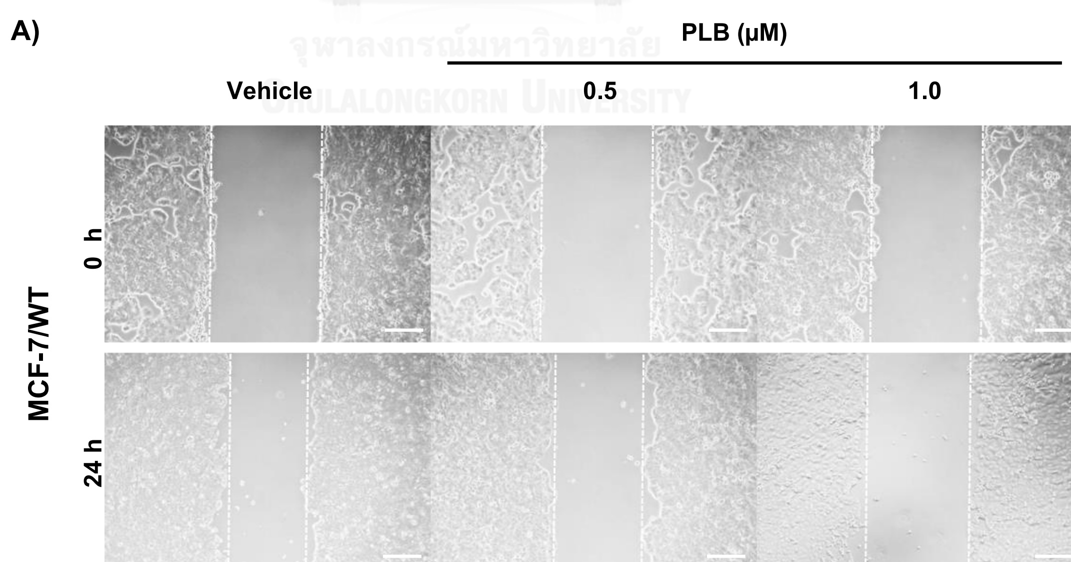

Figure 4.9. PLB attenuated cell migration of wild-type MCF-7 and endocrine resistant breast cancer cells. (A) The representative photomicrographs of confluent wild-type MCF-7 cells that were consistently scratched with a pipette-tip, and the migration of cells into the scraped wound after treated with different concentrations of PLB (0.5–1 μM) for 24 h. Dotted lines were illustrated the discarded area involved by the initial gap wound; scale bar = 200 μm. Data are the representative of three independent experiments (n = 3).

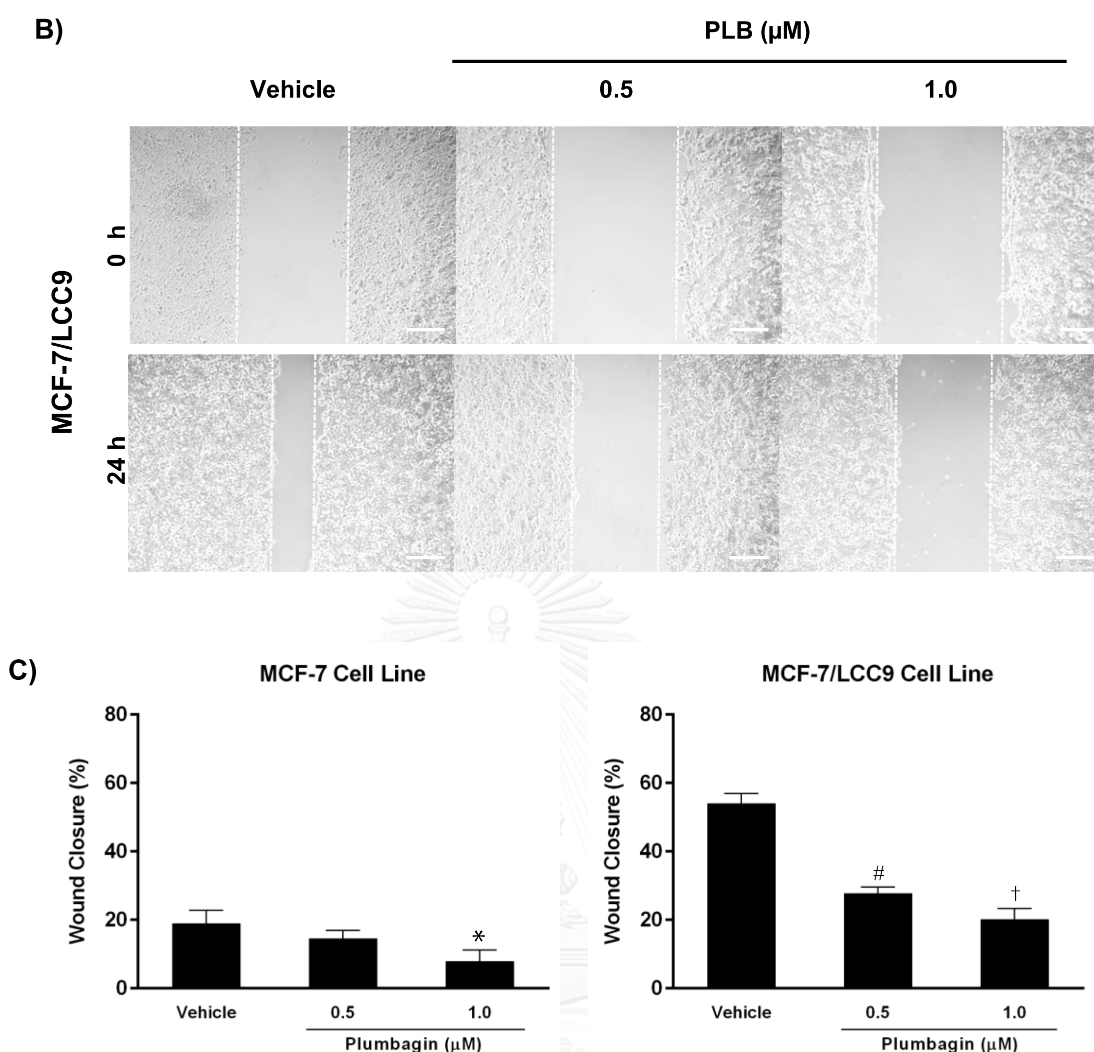

Figure 4.9. PLB attenuated cell migration of wild-type MCF-7 and endocrine resistant breast cancer cells. (**B**) The representative photomicrographs of confluent endocrine resistant MCF-7/LCC9 cells that were consistently scratched with a pipette-tip, and the migration of cells into the scraped wound after treated with different concentrations of PLB (0.5–1 μM) for 24 h. Dotted line illustrates the discarded area engaged by the initial gap wound; scale bar = 200 μm. (**C**) Quantitation of the distance of each scratched wound edge over time when comparing to the control group was expressed as a ratio of the migration distance in wild-type MCF-7 and endocrine resistant MCF-7/LCC9 cells. Each value represented the mean ± S.D. (*) for $P < 0.05$, (#) for $P < 0.01$, and (†) for $P < 0.001$ compared the vehicle control (0.1%DMSO). Data are the representative of three independent experiments (n = 3).

To further determine the effect of PLB on the ability of endocrine resistant breast cancer cell invasion was carried out by the growth factor reduced (GFR) matrigel-coated invasion chambers. MCF-7/LCC2 and MCF-7/LCC9 cells were seeded at desired cell density onto the top of each invasion chamber and cultured together with a fixed concentration of plumbagin (PLB group) in serum-free MEM medium or 0.1% DMSO (vehicle control) for 24 h incubation. The bottom chambers of 24-well culture plates were added MEM medium containing 5% heat-inactivated FBS which served as a chemoattractant in this study. As illustrated in **Figure 4.10,** the result of a fixed concentration of plumbagin (1 μM) significantly suppressed the invasive capabilities of acquired endocrine resistant breast MCF-7/LCC2 (*P* = 0.001, Student's t test) and MCF-7/LCC9 cells by 24% inhibition (*P* = 0.001, Student's t test).

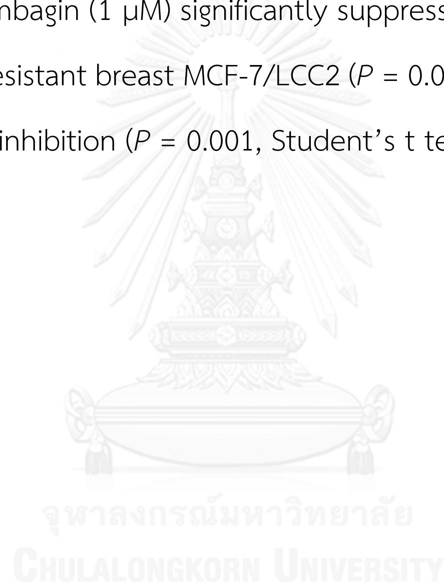
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Figure 4.10. PLB attenuated cell invasion in endocrine resistant breast cancer cells. (**A**) The representative photomicrographs of matrigel invasion of endocrine resistant MCF-7/LCC2 and MCF-7/LCC9 cells after treated without or with 1 µM plumbagin (PLB) for 48 h. Invaded cells were counted in five random fields after 0.1% crystal violet staining by a light microscope; scale bar = 100 µm. (**B**) Quantitation of the amount of invaded cells upon treatment with varying PLB concentrations was expressed as the percentage of the ability to invade through the matrigel-coated cell culture inserts of endocrine resistant MCF-7/LCC2 and MCF-7/LCC9 cells. Each value represented the mean ± S.D. (#) for $P < 0.01$ compared to the vehicle control (0.1%DMSO). Data are the representative of three independent experiments (n = 3).

CHAPTER V
DISCUSSION AND CONCLUSION

Although 75% of breast cancer patients whose oestrogen receptor expression remain respond to endocrine therapy such as tamoxifen (TAM), an antagonist in breast tissue. TAM has been widely used as the gold standard for ER-positive breast cancer treatment [31]. Several studies have demonstrated that more than 50% of these patients can develop resistance to tamoxifen or fulvestrant after five years of therapy resulting in decreasing overall survival, increasing disease recurrence, and leading to metastasis and mortality [5].

The discovery and development of novel compounds derived from several natural products with lower toxicity and excellent potential for cancer prevention or treatment are a major clinical challenge. Plumbagin (PLB), a naphthoquinone isolated from the roots of medicinal Plumbaginaceae plants, has been showed broad spectrum pharmacological properties such as antibacterial, antifungal, antiinflammatory, anti-apototic and antiproliferative activities against several tumour types including breast cancer in both pre-clinical and clinical studies [57-59, 66]. In this present study highlighted on investigating the impact of PLB on human wild-type MCF-7 and endocrine resistant breast cancer MCF-7/LCC2 and MCF-7/LCC9 cell survival, tumour migration and invasion and the potential role in inhibiting cancer metastasis.

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PLB (0–5 μM) inhibited the growth of wild-type and endocrine resistant MCF-7/LCC2, MCF-7/LCC9 cells in a concentration- and time-dependent with a half-maximal growth inhibitory IC_{50} values of 1.46 ± 0.09, 1.22 ± 0.02 μM for wild-type MCF-7 cells and 1.69 ± 0.02, 1.53 ± 0.11 μM for MCF-7/LCC2 cells and 1.24 ± 0.09, 1.16 ± 0.03 μM for MCF-7/LCC9 after 24, 48 h exposure respectively. These results are in-line with previously reported showing that low concentrations of PLB inhibit the viability of breast cancer cells, colorectal cancer cells, hepatic cancer cells, pancreatic cells, leukemic cancer cells as well as prostate cancer [64-69, 80].

To confirm this antitumour effect of PLB and investigate its effect on the capability to restored tamoxifen sensitivities, this study also demonstrated that different increasing concentration of 4-hydroxytamoxifen (4-OHT), an active metabolite of tamoxifen, and co-treatment with a fixed concentration lower than IC_{25} of PLB (0.75 μM) for a week strongly suppressed MCF-7/LCC2 and MCF-7/LCC9 cell survival. The result exhibited that PLB overcomes tamoxifen resistance in synergistic effect of MCF-7/LCC2 with combination index (CI) value of 0.58 (P = 0.001, one-way ANOVA), while MCF-7/LCC9 revealed additive effect of 0.94 ($P < 0.05$, one-way ANOVA). This *in vitro* experiment demonstrated that PLB has a potent cytotoxic activity and ability to enhance tamoxifen sensitivity on human endocrine resistant breast cancer cells.

The basic molecular mechanisms involved in the PLB action has been investigated to demonstrate that PLB is an effective cytotoxic agent that induced cell

death by inhibiting of ER target genes including *Cyclin D1* and *MYC* which are involved in cell cycle progression. The current study exhibited that PLB significantly decreased the expression of *Cyclin D1* against endocrine resistant MCF-7/LCC9 breast cancer cells in concentration-dependent manners, while the expression of these genes was little changed in wild-type MCF-7 cells. It was also showed that PLB downregulated the level of *MYC* expression in both of cell lines. Moreover, another study revealed that PLB inhibited human osteosarcoma cell growth by decreasing the level of *Cyclin E*, *Cyclin D1* expressions and nuclear factor-κB activities [70].

Previous studies demonstrated that tamoxifen resistant breast cancer (TAM-R) cells exhibited increased capacity of cellular invasion and motility [11]. The invasion is an important initiation step of metastatic process, which is enabled by EMT. In this context, this recent experiment also demonstrated that PLB induced a highly significant concentration-dependent inhibition of cell migration and invasion *in vitro* of the two endocrine resistant breast cancer MCF-7/LCC2 (data not shown) and MCF-7/LCC9 cells without inducing cell death. Interestingly, this finding recognized that endocrine resistant cells displayed over 2.9-fold higher migratory capacity when compared with wild-type cells ($P < 0.01$, Student's t-test), this consisted with previous reports of cellular invasion and migration studies indicated that PLB selectively inhibits breast tumor bone metastasis [65] and gastric cancer cell migration via altering CXCR4 chemokine receptor expression *in vitro* [64].

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The nuclear receptor coactivator *NCoA3* gene, also known as amplified in breast cancer 1 (*AIB1*), is a member of the p160 steroid receptor co-activator (SRC) family, plays vital role in assisting transcriptions of ER target genes. The *NCoA3* expression levels markedly increased in cancer cells such as breast, ovarian, pancreatic, lung, colorectal, and prostate cancers [81]. An increased NCoA3 expression is clinically significant since it has been correlated with resistance to endocrine therapy leads to poor clinical prognosis and decrease overall survival [30].

This study hypothesized that PLB has ability to restore tamoxifen sensitivity through regulation of NCoA3. This present result demonstrated for the first time that even fixed-low concentration of PLB can decrease NCoA3 expression. As illustrated in **Figure 4.6**., the expression of NCoA3 in RT-PCR was presented in both wild-type and endocrine resistant breast cancer MCF-7/LCC9 cells. The *NCoA3* level was also reduced after treatment of PLB in MCF-7 and MCF-7/LCC9 cells at the concentration less than IC_{25} (0.75 μM) when compared to the vehicle control with 38% and 42% reduction respectively. Next, to compare between 4-hydroxytamoxifen (4-OHT)-treated cells and the vehicle control group, the expression of *NCoA3* level was increased when compared to the vehicle control and was not significantly different in both of these cells in 4-OHT treatment. This finding is consistent with Lonard et al. that they demonstrated that 4-OHT was unable to elevate the steady-state level of *NCOA3* in breast cancer-derived T47-D and ZR-75-1 cell lines which endogenously expressed ER

and *NCOA3*. On the other hand, MCF-7 cells were treated with either E_2 or 4-OHT for 24 h, resulting in increased *NCOA3* expression when compared to untreated cells. These results showed that the ability of 4-OHT to impact the steady-state level of *NCOA3* is cell type specific [82]. However, to study the functions of coactivators of ER target genes in transcription rely on not only the level of gene expression, but also the recruitment of coactivators to the DNA plays vital role in the transcriptional process and this is beyond this recent study. To compare between 4-OHT and PLB combination and vehicle control, *NCoA3* levels was also preciously reduced in both wild-type and resistant cells (47% and 24% reduction respectively, P = 0.012). Remarkably, the levels of *NCoA3* expression between 4-OHT treated group, which previously observed in this gene expression was not statistically significant changes, and 4-OHT with PLB treated group was significantly downregulated in MCF-7/LCC9 (P = 0.006, Student's t-test) and wild-type MCF-7 cells (P = 0.047, Student's t-test).

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Taken together, gene transcription is closely regulated by several of protein complexes. At molecular mechanism of PLB action has not only mediated only by NCoA3 expression in endocrine resistance. Several mediators of transcriptional factors including *NCoA1* (SRC-1), *NCoA2* (SRC-2, TIF-1 or GRIP1) and other corepressor proteins such as *NCoR1* may involve in mediating transcriptional activity of ER target genes [28]. Thus, further study should be performed to investigate the function of other coregulators, in order to explain PLB machinery to increase tamoxifen sensitivity.

It has been shown that PLB can inhibit CXCR4 function in gastric cancer cells, resulted in the inhibition of cell invasion [64]. Moreover, tamoxifen resistant breast cells exhibit mesenchymal phenotypes which lead to increase invasive ability and cancer metastasis [11]. Therefore, this findings presented compelling evidence that PLB effectively inhibited EMT biomarkers in endocrine resistant breast cancer cells and altered both the epithelial and mesenchymal phenotypic profiles and decreased invasive capability of tumour cells. The result showed that a low concentration of PLB depleted the expression of vimentin mRNA levels, a mesenchymal marker of EMT process, in a concentration-dependent average 2.3 fold in MCF-7/LCC9 cells when compared to the vehicle control ($P < 0.01$, Student's t test) and significantly upregulated levels of E-cadherin expression, an epithelial cell marker, in a concentration-dependent manner by average 1.7 fold in MCF-7/LCC9 cells when compared to the vehicle control ($P < 0.001$, Student's t test) and average 1.2 fold in wild-type MCF-7 cells ($P = 0.002$, Student's t test). Another important finding is PLB can reduce the expression of *Snail1*, a transcription factor that plays a pivotal role in EMT of endocrine resistant breast MCF-7/LCC9 cells. The expression of Snail1 mRNA level was decreased more than 2.4 fold when compared to the vehicle control ($P < 0.001$, Student's t test). Therefore, this study demonstrated for the first time that PLB altered all types of EMT markers including epithelial, mesenchymal and transcriptional markers.

Summary of present study includes; (i) PLB, a naturally occurring naphthoquinone PLB has a potent cytotoxic activity both in tamoxifen-resistant MCF-7/LCC2 and fulvestrant/tamoxifen-resistant MCF-7/LCC9 cells; (ii) PLB can enhance cytotoxicity of 4-hydroxytamoxifen in MCF-7/LCC2 with synergistic effect and additive effect in MCF-7/LCC9 breast cancer cells; (iii) PLB downregulated the expression of ER target genes including *Cyclin D1* and *MYC*, decreased the expression of coactivator of ER transcription such as *NCoA3* and altered of EMT-associated genes (*E-cadherin*, *Vimentin*) and transcription factor *Snail1* expression and (iv) PLB also inhibited tumour migratory and invasive capacities in endocrine resistant breast cancer cells at a non-toxic concentration.

Even though PLB was primarily showed a potential role in alteration of EMT in this current research. However, this study is only demonstrated preliminary data in pharmacological activities of PLB. PLB has been reported to inhibit NF-κB that involved in cancer resistance or metastasis [64, 83]. NF-κB plays a critical role in tumour cell survival, invasion and metastasis which has been shown to mediate EMT through upregulation of TWIST [84]. Besides its role in enabling of NF-κB/TWIST, Aziz et al. shown that the STAT3 DNA-binding activity was inhibited by PLB treatment in prostate cancer cell lines as well as animal models [75]. Moreover, activator of transcription STAT signalling cascade, especially STAT-3, STAT-3, is often constitutively activated in several types of cancer cells including prostate, esophageal, colon, ovarian and breast

cancer. It has been shown to interact with NF-κB [85]. Increasing evidence support the insight that an activated STAT3 is the important contributor to invasion and migration of human breast and lung cancer *in vitro* [86].

The further study will focus more in depth of molecular mechanisms to investigate how PLB alters EMT markers. Therefore, the future will hypothesize that PLB can downregulate the crosstalk of NF-κB and STAT3 activation and important to know more about coactivator recruitment in tamoxifen sensitivity. On the other hand, this possible mechanism is not yet investigated in endocrine resistant breast cancer models. Therefore, the underlying mechanism of PLB action is warranted toward certifying these studies to completely elucidate in vitro. These results suggested that PLB is a novel potential agent for the treatment of ER-positive breast cancer patients who develop resistance to endocrine therapy.

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APPENDIX

APPENDIX A

APPENDIX A-1: Lists of the name and catalog numbers of reagents and chemicals

Name	Catalog Number	Company
Minimum Essential Medium Eagle (MEM)	61100-061	Gibco, USA
Improved Minimum Essential Medium Eagle (IMEM)	10373-017	Gibco, USA
0.25% Trypsin/EDTA	25200-056	Gibco, USA
0.4% Trypan blue solution	T8154	Sigma, USA
Fetal bovine serum (FBS)	10437-010	Gibco, USA
Penicillin/Streptomycin	15140-122	Gibco, USA
Fungizone	15290-018	Gibco, USA
Non-essential amino acid (NEAA)	11140-050	Gibco, USA
Charcoal-dextran stripped FBS	12676011	Gibco, USA
Recombinant human insulin zinc solution	12585-014	Gibco, USA
Dimethyl sulfoxide (DMSO)	472301	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	M2128-500MG	Sigma, USA
Growth factor reduced (GFR) Matrigel® basement membrane matrix	354230	BD Biosciences, USA
37% formaldehyde solution	415666	Sigma, USA

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Name	Catalog Number	Company
Crystal violet dye	C3886	Sigma, USA
Trizol® Reagents	15596-026	Invitrogen, USA
Diethyl pryrocarbonate (DEPC)	D5758	Sigma, USA
Ethanol	100983	Merck, Germany
Chloroform	102442	Merck, Germany
Isopropanol	101040	Merck, Germany
Improm-II™ reverse transcription system	A3800	Promega, USA
Platinum® Taq DNA Polymerase	10966026	Invitrogen, USA
dNTP Mix	U1515	Promega, USA
Oligo $(dT)_{15}$ Primer	C1101	Promega, USA
Recombinant RNasin® RNase Inhibitor	N2511	Promega, USA
6X loading dye	NM0410	Vivastis, Malaysia
VC DNA ladder	NL1403	Vivastis, Malaysia
Agarose, LE grade	PC0701-500G	Vivantis, Malaysia
Ethidium bromide (ErBr) solution	E1510-10ML	Sigma, USA
4–hydroxytamoxifen (4-OHT)	H7904-5MG	Sigma, USA
Plumbagin from *Plumbago indica* (MW = 188.18)	P7262	Sigma, USA

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APPENDIX A-2: Lists of the name and manufacturer of equipments and instruments

Name	Company name
Cellular level	
Forma™ Series II Water Jacketed CO_2 Incubator	Thermo Scientific
Purifier Logic Class II Biological Safety Cabinet	Labconco
Light Microscope	Carl Zeiss
Centrifuge 4235A	Alc
Graduated glass pipettes	Brand
Pipette controllers	Brand
Pipettes	Gilson
Clay Adams Cell Counter	Clay Adams
Hemacytometer	Hausser Scientific
Cover slips	Menzel Glaser
0.45 µm filters	Millipore
Disposable syringe	Millipore
T25 tissue culture treated flasks	Corning
6-well plates	Corning
24-well plates	Corning
96-well plates	Corning
Centrifuge tubes (15, 50 mL)	Corning
Cryogenic vials (2.0 mL)	Corning
Eppendorf (1.5 mL)	Corning
Pipette tips (10, 200, 1000 µL)	Corning
Plastic culture dishes (100 mm)	Corning
Transwell 24-well plates, 8 µm pore size	Corning
Glass bottles (100, 250, 500, 1000, 2000 mL)	Brand

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Name	Company name
Multiskan 347 Microplate reader	MTX Lab Systems
Vortex mixer G560	Scientific Industries
Microwave	LG
Molecular level	
Eppendorf Centrifuge 5810R	Eppendrof
Microlitre Centrifuges MIKRO 120	Hettich zentrifugen
Mastercycler Personal 5811 Thermocycler	Eppendrof
Veriti® 96-Well Fast Thermal Cycler	Applied Biosystems
Gel Doc™ EZ System	BioRad
Electrophoresis power supply	BioRad
Power adaptor	BioRad
pH-meter	Knick
Mini-Sub® Cell GT Systems	BioRad
Hettich Centrifuge MIKRO 200	Hettich zentrifugen
RNase-free microfuge tubes (0.2 mL)	Corning
RNase-free pipette tips (10, 200, 1000 µL)	Corning
Eppendrof (1.5 mL)	Corning
PCR tube rack with lid	VWR
UV Transilluminator	Microtek
Refrigerator	Toshiba

APPENDIX B

Buffers and Reagents

1. Incomplete MEM medium 1 liter

MEM powder	10.4	g
$NaHCO_3$	3.7	g
ddH_2O	900	mL

Adjust pH to 7.3 with 1 N HCl and 1 N NaOH

Add ddH_2O to 1 liter and sterilize by filtering through a 0.2 sterile membrane filter

2. Completed MEM medium 100 mL

MEM stock solution	94	mL
Fetal bovine serum	5	mL
Penicillin/Streptomycin	1	mL

Store the refrigerator temperature at or below 4°C

Warm the solution to 37°C before use

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3. DMEM stock solution 1 liter

DMEM powder	10.4	g
$NaHCO_3$	3.7	g
ddH_2O	900	mL

Adjust pH to 7.4 with 1 N HCl and 1 N NaOH

Add ddH_2O to 1 liter and sterilize by filtering through a 0.2 sterile membrane filter

4. Completed DMEM medium 100 mL

DMEM stock solution	94	mL
Fetal bovine serum	5	mL
Penicillin/Streptomycin	1	mL

Store the refrigerator temperature at or below 4°C

Warm the solution to 37°C before use

5. Completed IMEM medium 100 mL

IMEM medium, phenol red-free	93.9	mL
Charcoal dextran-stripped FBS	5	mL
Non-essential amino acid	1	mL
10^{-6} M Recombinant human insulin	0.1	mL

Store the refrigerator temperature at or below 4°C

Warm the solution to 37°C before use

6. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80.65	g
KCl	2	g
KH_2PO_4	2	g
Na_2HPO_4	11.5	g
ddH_2O	900	mL

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and sterilize by autoclaving

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7. Tris HCl 1 M pH 8.0 100 mL

Tris base	12.114	g
ddH_2O	80	mL

Adjust pH to 8.0 with HCl

Add ddH_2O to 100 mL and sterilize by autoclaving

8. EDTA 0.5M pH 8.0 100 mL

EDTA	18.612	g
ddH_2O	80	mL

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 mL and sterilize by autoclaving

9. 5x Tris-Borate-EDTA (TBE) Buffer 1 liter

Tris-base	54	g
Boric acid	27.5	g
EDTA 0.5M pH 8.0	20	mL
ddH_2O	900	mL

Add ddH_2O to 1,000 mL and sterilize by autoclaving

10. 1x TE Buffer 100 mL

Tris-HCl 1 M pH 8.0	1	mL
EDTA 0.5M pH 8.0	0.2	mL
ddH_2O	98.8	mL

Sterilize by autoclaving and store the room temperature at 25°C

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11. 0.1% Crystal violet staining solution 100 mL

Crystal violet dyes	0.05	g
ddH_2O	50	mL

Add ddH_2O to 100 mL and sterilize by filtering through a 0.2 sterile membrane filter

Store the room temperature at 25°C

12. 3.7% Formaldehyde solution 1 litter

Formaldehyde solution	100	mL
ddH_2O	900	mL

Sterilize by autoclaving and store the room temperature at 25°C

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VITA

Mr. Nueng Sakunrangsit was born in Bangkok on June, 1st, 1988. After completing his degree at high school in 2007, Nueng entered Thammasat University, Bangkok, receiving the degree of Bachelor of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University in March, 2010. During the following one years, Nueng was employed as a Teaching Assistant at the Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Bangkok. In May 2012, Nueng entered the degree of Master of Science in Medical Science, Major in Pharmacology, Faculty of Medicine, Chulalongkorn University.

Publication

1. Sakunrangsit N, and Ketchart W. (2014). Synergistic Cytotoxic Activity of Plumbagin and Tamoxifen in Endocrine-Resistant Breast Cancer Cell Lines MCF-7/LCC2 and MCF-7/LCC9. Thai Journal of Pharmacology. 36, 76-80.

Poster Presentation

1. Sakunrangsit N, and Ketchart W. (2014). Synergistic Cytotoxic Activity of Plumbagin and Tamoxifen in Endocrine-Resistant Breast Cancer Cell Lines MCF-7/LCC2 and MCF-7/LCC9. The 36th Congress on Pharmacology of Thailand. March 27-28, 2014, Bangkok, Thailand.

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