The effect of 1’ acetoxychavicol acetate on cell proliferation, metastasis, and angiogenesis in human endocrine-resistant breast cancer cells

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ผลของ 1’ อะเซทอกซีชาวิคอล อะซิเตต ต่อการเจริญเติบโต การแพร่กระจาย และการสร้างหลอดเลือดใหม่ ในเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมน

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ความเป็นมา: ผู้ป่วยมะเร็งเต้านมชนิดที่มีการแสดงออกของตัวรับเอสโตรเจนโดยส่วนใหญ่จะมีการพยากรณ์โรคที่ดี แต่ร้อยละ 30 ของคนไข้เหล่านี้โรคสามารถพัฒนาจนเกิดการดื้อต่อการรักษามาตรฐานได้โดยการกระตุ้นผ่าน PI3K/AKT, ERK1 / 2 และ NFκB ซึ่งมีความสัมพันธ์กับการทำงานของตัวรับเอสโตรเจน งานวิจัยนี้ทำศึกษานี้ผลของสาร 1’ อะเซทอกซีชาวิคอล อะซิเตต (ACA) ซึ่งเป็นสารประกอบเอสเทอร์ที่เป็นเมตาโบไลต์จากเหง้าข่าที่มีฤทธิ์ยับยั้ง NF-kB ในเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมน

วิธีการวิจัย: สารจากพืชได้ทำการแยกและทำให้บริสุทธิ์ด้วยกระบวนการโครมาโตกราฟีและพิสูจน์โครงสร้างโดยวิธีการ nuclear magnetic resonance และ mass spectroscopy จากนั้นทดสอบฤทธิ์ต้านการเจริญแบ่งตัวของเซลล์มะเร็งของ ACA ใน MCF7 / LCC2 และ MCF7 / LCC9 โดยกระบวนการ MTT และทดสอบในสัตว์ทดลองโดยใช้ปลาม้าลายที่ได้รับการปลูกเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมน MCF7/LCC9 การทดสอบฤทธิ์ต้านการรุกรานของ ACA ใช้วิธีการทดสอบคือ matrigel invasion assay จากนั้นทำการศึกษากลไกการออกฤทธิ์ของ ACA โดยศึกษาผลของ ACA ต่อการแสดงออกของยีนส์และโปรตีนในเซลล์มะเร็งเต้านม ด้วยกระบวนการ real-time PCR และ western blotting

ผลการทดลอง: ACA สามารถยับยั้งการเจริญแบ่งตัวของเซลล์ MCF7, MCF7 / LCC2 และ MCF7 / LCC9 แบบขึ้นกับความเข้มข้นสารและเวลาที่ได้รับสาร ซึ่งการออกฤทธิ์นี้เกี่ยวข้องกับการลดลงของระกับโปรตีนตัวรับ HER2, estrogen receptor coactivator (NCOA3), pERK1/2, pAKT และการลดลงของยีนส์ที่เกี่ยวข้องการการเจริญแบ่งเซลล์คือ *CCND1* และ *C-myc* อย่างมีนัยสำคัญทางสถิติ รวมถึงผลการทดลองในปลาม้าลายพบว่ากลุ่มที่ได้รับ ACA สามารถลดพื้นที่ของก้อนมะเร็งได้อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ผลการต้านการรุกรานเซลล์มะเร็ง พบว่า ACA ยับยั้งการรุกรานเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนได้ และสามารถลดการแสดงออกของยีนส์ *CXCR4* และ *uPA* ได้ นอกจากนี้ ACA ยังสามารถลดการแสดงออกของยีนส์ VEGF และ FGF2 ซึ่งทำหน้าที่เกี่ยวข้องกับการสร้างหลอดเลือดใหม่ของเซลล์มะเร็งได้และเซลล์ที่ได้รับสาร ACA นั้นถูกเหนี่ยวนำให้เกิดการตายแบบ apoptosis ผ่านการเพิ่มขึ้นของระดับโปรตีน JNK / SAPK และ cleave-PARP และการลดลงของโปรตีนที่ยับยั้งการเกิดกระบวนการ apoptosis คือ BCL2 และ MCL1

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Nalinee Pradubyat : The effect of 1’ acetoxychavicol acetate on cell proliferation, metastasis, and angiogenesis in human endocrine-resistant breast cancer cells. Advisor: Asst. Prof. WANNARASMI KETCHART, MD, Ph.D. Co-advisor: Prof. CARLO PALMIERI, Ph.D., FRCP,Asst. Prof. Chalermchai Mitrpant, M.D., Ph.D.,Dr. ATHINA GIANNOUDIS, Ph.D.

Background: Oestrogen receptor (ER)-positive breast cancer patients have a good prognosis, but 30% of these developed resistance through hyperactivation of PI3K/AKT, ERK1/2, and NFκB, which interconnected with ER signalling. In this study, the effects of 1’ acetoxychavicol acetate (ACA), a plant-metabolite acetate ester compound with an NF-kB inhibitory activity, were investigated in a panel of endocrine-resistant breast cancer cells.

Methods: Plant material was purified by chromatographic methods and followed by structural identification using nuclear magnetic resonance and mass spectroscopy. *In vitro* and *in vivo* antiproliferative effects of ACA were studied in MCF7/LCC2 and MCF7/LCC9 endocrine-resistant cells using the MTT assay and the zebrafish xenograft model respectively. Anti-invasion of ACA was performed by the matrigel invasion assay, while its mechanism of action was elucidated through molecular docking simulation, real-time PCR, and western blotting. Western blot analysis was also used to investigate its effect on apoptosis.

Results: ACA inhibited the proliferation of MCF7, MCF7/LCC2, and MCF7/LCC9 cells at a concentration- and time-dependent fashion. This was associated with down-regulation of HER2 receptor, estrogen receptor coactivator (NCOA3), pERK1/2, pAKT, and proliferative markers (*CCND1*, *C-myc*). While *in vivo*, significant reduction in the tumour mass of ACA-treated zebrafish engrafted MCF7/LCC9 groups was observed compared to the control treatment. Furthermore, the anti-invasive effects of ACA were confirmed *in vitro* by the matrigel invasion assay and with reduction in the expression levels of *CXCR4*, *uPA* and proangiogenic factors, *VEGF* and *FGF2* in ACA-treated cells compared to untreated control. The repressed expression of uPA and FGF2 was also validated by molecular docking analysis. Moreover, ACA-treated cells exhibited lower expression levels of the anti-apoptotic BCL2 and MCL1 proteins in addition to increase JNK/SAPK expression and enhance PARP cleavage, indicating apoptotic cell induction by ACA.

Conclusion: This research suggested that the ACA inhibited cell proliferation and induce apoptosis in breast cancer cells through HER2/MAPK/ERK1/2 and PI3K/AKT pathways. In addition, the anti-angiogenic and anti-invasive activity of ACA was through the downregulation of VEGF, FGF2, uPA, and CXCR4.

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ABBREVIATIONS

ACA	:	1’ Acetoxychavichol acetate
AMPK	:	AMP-activated protein kinase
CaSki	:	Epidermoid cervical carcinoma
CCND1	:	Cyclin D1
CDK	:	Cyclin-dependent kinase
CD31	:	Cluster of differentiation 31
CD44	:	Cluster of differentiation 44
c-Myc	:	MYC proto-oncogene
COX-2	:	Cyclooxygenase-2
CREB	:	cAMP response element-binding protein
CXCR4	:	C-X-C chemokine receptor type 4
EATC	:	Ehrlich ascites tumour cells
EBV	:	Epstein−Barr virus
EGFR	:	Epidermal growth factor
ER	:	Estrogen receptor
ERBB2/HER2	:	Human epidermal growth factor receptor 2
ERK1/2	:	Extracellular signal-regulated protein kinase 1/2
FAK	:	Focal adhesion kinase
FGF2	:	Basic fibroblast growth factor 2
FOXO3a	:	Forkhead box O protein 3a
GTPases	:	Guanosine triphosphatases
GLI	:	Glioma-associated oncogene homolog

GRIP1	:	Glucocorticoid receptor interacting protein-1
GSH	:	Glutathione
GSK3β	:	Glycogen synthase kinase-3 beta
GST	:	Glutathione S-transferase
HepG2	:	Human hepatocellular carcinoma cells
HGF	:	Hepatocyte growth factor
HIF-1α	:	Hypoxia-inducible factor 1 alpha
HRE	:	Hypoxia- response element
HSC-2	:	Oral squamous carcinoma
HMEC	:	Normal human mammary epithelial cell
IAP1	:	Inhibitor of apoptosis protein-1
IAP2	:	Inhibitor of apoptosis protein-2
IEC6	:	Rat intestine epithelial cells
IGF1R	:	Insulin Like Growth Factor 1 Receptor
IL-6	:	Interleukin 6
IP3	:	Inositol 1,4,5-trisphosphate
JNK ½	:	c-Jun N-terminal kinase1/2
MAPK	:	Mitogen-activated protein kinase
MEM	:	Minimal essential medium
MMP-2	:	Matrix metalloproteinase-2
MMP-9	:	Matrix metalloproteinase-9
MTT	:	Methylthiazolyldiphenyl tetrazolium bromide
NCOA3	:	Nuclear Receptor Coactivator 3
NFkB	:	Nuclear factor kappa B

NICD	:	Notch intracellular domain
NQO1	:	NAD (P)H: quinone oxidoreductase 1
OCT	:	Octamer-binding transcription factor.
PAK1	:	p21-activated kinase 1
PBS	:	Phosphate buffered saline
PC-3	:	Human prostate cancer cells
PIP2	:	Phosphatidylinositol 4,5-bisphosphate
PI3K	:	Phosphoinositide 3-kinases
PLC	:	Phospholipase C
PKC	:	Protein kinase C
PKCθ	:	Protein kinase Cθ
PRC2	:	Polycomb repressor complex 2
PTEN	:	Phosphatase and tensin homolog deleted on chromosome 10
Rb	:	Retinoblastoma
SCC	:	Oral squamous cell carcinoma
SHP-1	:	Src homology region 2 domain-containing phosphatase 1
Src	:	Proto-oncogene tyrosine-protein kinase
SDF-1	:	Stromal-derived-factor-1
TGF-β	:	Transforming growth factor beta
uPA	:	Urokinase plasminogen activator
VEGF	:	Vascular endothelial growth factor
EZH2	:	Zeste homolog

CHAPTER 1 INTRODUCTION

1.1 Background

According to the National Cancer Registry (NCR) in 2017, 1.762 million new cancer cases and approximately 606,880 deaths were predicted in the United States (1). The disease causes a substantial issue for patients and healthcare systems with up to 10% of cancer costs in total (2). In 2019, prostate, pulmonary, and colorectal cancers (CRCs) estimate for 42% of cases in male, prostate cancer alone estimating around 1 in 5 new diagnosed-cases (1). In the female, the three most common cancers are breast, lung, and colorectum, which represent 50% of all new diagnosed-cases; breast cancer alone considers for 30% of total new cancer diagnoses in women (1). In 2020, 1,806,590 new cancer cases and around 606,520 cancer deaths are projected to occur in the US (3). Nevertheless, the therapeutic practice that had been implemented in the therapy of breast cancer in the Western for the past 30 year-time and in Europe have increased, the age-standardised, 5-year relative survival rates was from 73% to 83% during 1992 and 2008 (4). One major issue regarding the medical treatment of breast cancer in Europe is the enormous cost in contemporary financial circumstances of healthcare system (5). The system to predict and diagnose using biomarkers could ameliorate the therapy and the cost, however, the process to efficiently develop precise and promised biomarkers were quite difficult (5). Further challenges for cancer therapy were the new cancer drug targets to fight against cancer-resistant types as well as the bench to bedside efforts to implement the knowledge acquired from translational research to the patients (5).

Around 70% of breast cancer patients express oestrogen receptor alpha (ERα) and are classified as ER-positive (+) therefore, ER signalling plays a crucial role in the pathogenesis of breast cancer (6). Consequently, therapeutic management aims to reduce the ER-ligand oestrogen or to inhibit ER signalling (6). Tamoxifen is the first-line adjuvant therapy for early and advanced ER positive breast cancer in pre- and post-menopausal women (7). Tamoxifen is the first-line choice for hormonal therapy in both early and advanced breast cancer patients for more than 30 years (8). The tamoxifen-adjuvant treatment has significantly improved disease-free survival and diminished mortality rate of breast cancer patients (9). In some cases, the use of tamoxifen is for neoadjuvant therapy for women with a high risk of breast cancer occurrence, aiming for prophylaxis the disease, but the concern of risks from adverse drug reaction outweighs the advantages is still required more information to be confirmed (10, 11). Also, around one-half of advanced-stages breast cancer patients do not adequately respond to tamoxifen treatment since the first time (*de novo* resistance) (12, 13). Additionally, some patients respond sufficiently to tamoxifen at first, and after the use of tamoxifen for 5-10 years the disease may relapse and cancer cell develop resistance to tamoxifen (acquired resistance) (12, 13). The *de novo* and acquired resistance of cancer to tamoxifen treatment can be caused from various molecular mechanisms and molecules, including single nucleotide polymorphisms (SNPs) of cytochrome P450 2D6 (CYP2D6), the loss of ER expression and function, altered expression patterns of co-regulatory proteins, overexpression of human epidermal growth factor receptor 2 (HER2/neu), hyperactivation of a phosphatidylinositol 3-kinase (PI3K) signalling pathway as well as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation (14-16).

Fulvestrant, a later anti-oestrogenic drug in the selective oestrogen receptor downregulators class, has been recently revealed the clinical efficiency in patients relapsed disease after the treatment of tamoxifen and aromatase inhibitors (AIs) (17). In this regard, more translational studies have been carried out for supporting the clinical potential of finding the regimen to address hormonal-resistant breast cancer (17, 18).

1.2 Breast cancer

Breast cancer is the second leading cause of global women-cancer mortality (19). Statistically, 10 per cent of female in the UK and the US experienced the disease in their lifetime (19). The incidence of breast cancer was higher in the western, and many risk factors involved in the pathogenesis such as non-adjustable and modified risk factors (20). Non-adjustable risk factors related to breast cancer are gender, age, and family history (20). The non- adjustable risk factors are difficult to control (20). On the other hand, the modified risk factors such as dietary lipid and alcoholic intake, obesity especially in postmenopausal age, hormonal exposure are the most interest regarding breast cancer prophylactic strategy (21). More interestingly, endogenous and exogenous endocrine factors, mammographic density and previous benign disease are also the non-favourable causes of the disease (22). The mutation of genetic factors such as the breast cancer gene (BRCA) 1 and 2, tumour protein (P53), phosphatase and tensin homologue deleted on chromosome ten (PTEN), and overexpression of HER-2 antigen are considerable issue for breast cancer patients (19). In addition, the proliferative marker Ki-67 is also significant to determine for the prognosis of disease progression (23). Therefore, breast cancer cannot be seen as a

single disease. Breast cancer can be classified into 4 major types by histopathological staining based on the expression of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) or the lack of these (24). However, breast cancer heterogeneity and the identification of additional molecular markers makes this classification a more complicated story, yet necessary for recommended distinct therapeutic management or clinical outcomes (24).

1.2.1 Molecular Subtypes of Breast Cancer

The last decades, several studies highlighted the heterogenicity of breast cancer and led to the identification of distinct molecular subtypes (5, 25, 26). The detection of breast tissue molecular subtypes is crucial in terms of the first-line treatment regimen. There are three primary receptors to be determined for the identification of breast cancer subtypes which are ER, PR and HER2 receptors. Breast cancer molecular subtypes can be distinguished into four main subtypes which are;

1. Luminal A breast cancer is hormone-receptor-positive (ER and/or PR positive), and HER2 negative. This type of breast cancer could represent a low level of Ki-67 protein, which describe the low aggressiveness that bears a good prognosis compared to other types (27).

2. Luminal B breast cancer is also hormone-receptor-positive (ER and/or PR positive), and either HER2 positive or HER2 negative and it is more common for the luminal B subtype to express high Ki-67 protein level. This type of breast cancer shows a more aggressive and poorer prognosis compared to the luminal A subtype (27).

3. HER2-enriched breast cancer is hormone-receptor-negative (ER and PR negative), and HER2 positive. HER2-enriched tumours were found approximately 20-30% of all breast tumours and were more aggressive than the luminal subtypes (28). With regards to the overexpression of the HER2 receptor, the HER2-targeted therapy; trastuzumab, and T-DM1 or ado-trastuzumab emtansine and pertuzumab, and tyrosine kinase inhibitors; lapatinib have been approved to give effectiveness for the patients (27).

4. Triple-negative (TN) or basal-like breast cancer is hormone-receptor-negative (ER and PR negative), and HER2 negative and commonly be detected the mutation of breast cancer gene 1 (BRCA1). The phenotype of the TN is mainly basal-like tumour as it shows various degree of basal-like biomarkers (29, 30). Clinically, this type of breast cancer was the poorest prognosis compared to other subtypes (31).

Table 1.2.1.1. Molecular subtypes of breast cancer (32-34)

Subtypes	**ER**	**PR**	**HER2**
Luminal A	+	+/-	-
Luminal B	+	+/-	+
HER2-enriched	-	-	+
Triple negative	-	-	-

1.2.2 The ER structures and signalling pathways

Oestrogens are steroid hormones that regulate growth, differentiation, and function in a broad range of many tissues such as breast, uterus, cardiovascular system, brain and urogenital tract of both males and females (35).

Oestrogens are the group of sex hormone that has a broad range of function regarding the regulation of female characteristics and the proliferation and differentiation of tissues, including breast, cardiovascular system, brain and urogenital tract of both male and female (35). Apart from that oestrogens are very crucial in the development process of mammary gland as of their potent mitogenic activity, the level of oestrogens; oestradiol (E_2) in particular and its signalling through ERs are involved in the tumourigenesis of breast tissue (35). In order to exhibit the oestrogenic response, E_2 will bind to the ER, which is a steroid receptor located in the cytoplasm to drive the ligand-induced transcription of ER-regulated genes (35). There were two main superfamilies of ERs that have been specified, ERα (in 1986) and ERβ (in 1996) (36). ERα and ERβ were transcribed by distinct genes on chromosome 6 and 14, ERβ was identified to co-expression with ERα; however, its roles on breast tumours and malignancy was still controversial (37, 38).

ERα and ERβ have a high level of homology in their DNA-binding domain (DBD; 97% amino acid identity) and ligand-binding domain (LBD; 59% amino acid identity) (37). The structure of ERα and ERβ composed of six domain-shared structure; domain A - F **(Figure 1.2.2.1)**, Domain A/B (non-ligand binding domain) at the N-terminus was the location of the activation function 1 (AF1), responsible for the transcriptional activity of the ER without E_2 binding (37). Domain C was the DBD comprised of two zinc-finger structure which was crucial for ligand-ER complex

dimerisation and the binding of the ligand-ER complex to the specific consensus sequence of the DNA in the nucleus (37). Domain D was the joint between domain C and E that was important for the nuclear localisation of the E_2-ER complex into the nucleus (37). Domain E (LBD) was the part for E_2 to bind with ER and composed of a second nuclear localisation signal, a dimerisation locality and a twelve-helix region required for ligand binding (37). The E domain was also the location of activation function 2 (AF2) was another section that involved in ligand-dependent activation of ER. At the end of the C-terminus, there was a domain F which was the region-mediated AF1 and AF2 (37).

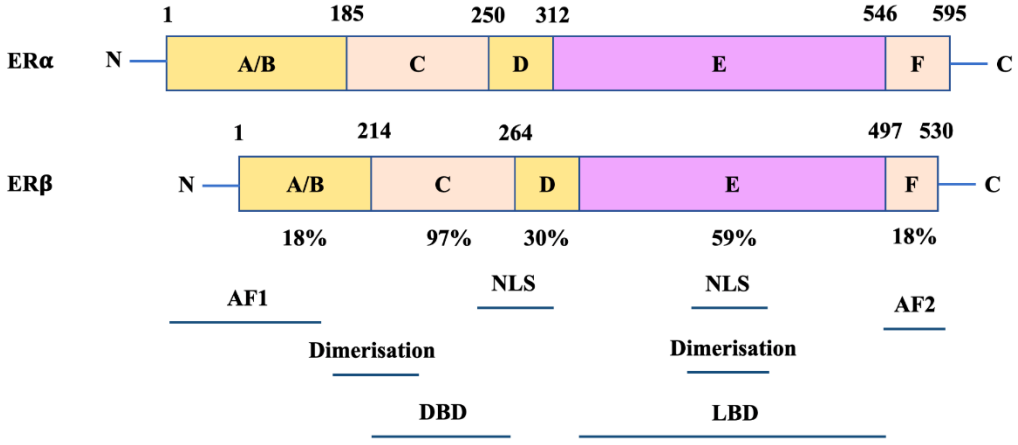


Figure 1.2.2.1. Schematic representation of the functional domains of human ERα and ERβ (37)

There are two distinct mechanisms of ligand-dependent activation of ERα signalling related to cell survival and proliferation, which are the classical genomic and non-classical genomic signalling pathway (37). The classical genomic pathway is initiated since E_2 bind to the binding site of ERα in the cytoplasm, the binding induces the conformational changes of the receptor and driven the removal of chaperone

proteins (heat shock protein 90; Hsp90) allowing the dimerisation of the receptor and the trans-localisation into the nucleus. This ligand-ERα complex will then bind to the consensus sequence of the DNA called oestrogen-responsive element (ERE) (37). The binding of the ligand-ERα complex can bind directly to the ERE (classical-genomic pathway) or indirectly through the protein-protein interaction with the activation protein 1 (AP1) or special protein 1 (SP1) sites (nonclassical-genomic pathway) at the promoter region (steroid-responsive element; SRE) of targeted genes, resulting in the recruitment of ER co-regulatory proteins; coactivators or corepressors and drives or represses the transcriptional activity of ER-regulated genes **(Figure 1.2.2.2a - b)** (37).

There are also the non-genomic pathways of ER signalling via activation of Ras/Raf/ERK1/2 and PI3K/AKT pathways through the binding of growth factors with the receptor of tyrosine kinases (RTKs) and mediate the genes, including cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors (39). These signalling the E_2-ER complex is not translocation into the nucleus itself, instead ERK1/2 or AKT act as the transcription factor (39). Apart from that, E_2 can bind to membranal ERα and activate the PI3K pathway, which will then activate the AKT to act as the transcription factor and translocation to the nucleus then bind with a responsive element (RE) of the DNA to drive the transcription of the genes **(Figure 1.2.2.2 c - d)** (37, 39).

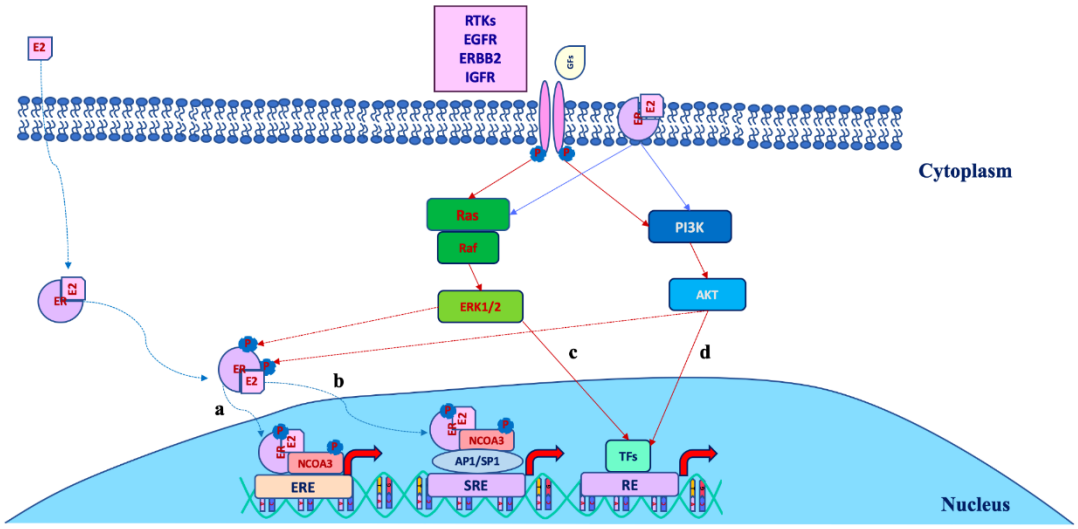


Figure 1.2.2.2. The ER signalling pathways. The three distinguish pathways of ER; classical genomic (a), non-classical genomic (b) and the non-genomic (c and d) pathways of ER (39).

1.2.3 Hormonal treatment of breast cancer

The treatment of breast cancer was based on several key considerations, including molecular subtypes, disease stage, tumour histology, and menopausal status (40). In the early stage of breast cancer patients are recommended for mastectomy and radiotherapy, however, systemic therapy is also recommended for almost all patients, and it is also prime for advanced-stage breast cancer patients (5, 40).

There are three drug classes of hormonal therapy for hormone receptor-positive breast cancer. Firstly, selective oestrogen receptor modulators (SERMs), the prototype of this group is tamoxifen (19). Secondly, selective oestrogen receptor down-regulators (SERDs), fulvestrant is the prototype of the group. Finally, aromatase inhibitors (AIs), anastrozole is the prototype of this class (19).

1.2.3.1 Selective Estrogen Receptor Modulators (SERMs)

The major drugs in this group are tamoxifen (TAM, the prototype) and raloxifene. These two drugs have both the oestrogenic agonist and antagonist depending on the tissue subtypes (21). TAM and raloxifene work by binding to the ER and trigger or inhibit the signalling pathways of the ER (21). Tamoxifen citrate is the first drug launched in this group, which is the prodrug required the phase I metabolism of the liver to be an active metabolite (21). Raloxifene, the second drug of this group, also functions as both oestrogenic agonist and antagonist, but the main different activity from TAM is that raloxifene does not possess the agonistic effect at endometrium (21). TAM was recommended to use as first-line adjuvant therapy after the mastectomy or radiation in both male and female with metastatic breast cancer and decrease the risk of ductal carcinoma *in situ* (DCIS) in women as well as diminish the disease occurrence of women with a high risk of breast cancer (21). However, there was a concern raised TAM usage, which was the risen risk of uterine or endometrial cancer. (41).

Table 1.2.3.1.1. Adverse drug reactions of tamoxifen (42)

Organ systems	**Adverse drug reactions of tamoxifen**
Cardiovascular	Vasodilatation, flushing, hypertension, peripheral edema
Endocrine & metabolic	Hot flash, fluid retention, menstrual disease, weight loss, amenorrhea
Neuromuscular & skeletal	Weakness, arthritis, arthralgia
Genitourinary	Vaginal discharge, vaginal hemorrhage

1.2.3.2 Selective Estrogen Receptor Down-Regulators (SERDs)

This class is a pure anti-oestrogenic activity through the enhance of internalisation and degradation of the ER and PR; thus, this class is more potent than SERMs (19). Fulvestrant (ICI182,780) is the first drug and the prototype of this group, which works via the binding of ER and produce no intrinsic activity (antagonistic effect) with a 100-times higher affinity compared to TAM (19). Unlike TAM, fulvestrant has no stimulatory effect on the uterus; therefore, it reduces the adverse events of uterine or endometrial cancer (19). Fulvestrant was approved for postmenopausal women with hormone receptor (HR)-positive metastatic breast cancer when disease progression after the endocrine therapy (19). It was also approved for the combined treatment with palbociclib (a cyclin-dependent kinase 4/6 inhibitors) for breast cancer patients with HR positive, HER2 negative and advanced or metastatic breast cancer (MBC) whose the disease has progressed after the endocrine therapy (43-45).

Table 1.2.3.2.1. Adverse drug reactions of fulvestrant (43)

Organ systems	**Adverse drug reactions of fulvestrant**
Central nervous system	Fatigue, headache, hot flash
Hematologic & oncologic	Anemia, bleeding disorders
Hepatic	Increased liver enzymes; AST, ALT, ALP

1.2.3.3 Aromatase Inhibitors (AIs)

The drug class is preferable for postmenopausal breast cancer patients more than other endocrine therapy (46). There are two types of AIs which are type I steroidal drugs (formestane and exemestane) which are the mimic of androgen substrates to competitively and irreversibly inhibit aromatase enzyme (47). The type II, non-steroidal inhibitors (anastrozole and letrozole) mechanistic work by reversibly binds to aromatase enzyme; consequently, it blocks the oestrogen biosynthesis from the precursor androgens (47). It is reported to be as effective as or superior to TAM for the first-line treatment of metastatic breast cancer (48, 49).

Table 1.2.3.3.1. Adverse drug reactions of anastrozole (50)

Organ systems	**Adverse drug reactions of anastrozole**
Cardiovascular	Angioedema
Endocrine & metabolic	Hot flash, vaginal haemorrhage, vulvovaginal dryness
Neuromuscular & skeletal	Bone loss and bone fractures, muscle and joint pain, arthritis

1.2.4 Resistance to tamoxifen

Cancer resistance is the pivotal issue for almost all cancer diseases, tamoxifen-resistant breast cancer, however, can occur at the first time of tamoxifen exposure (*de novo* or intrinsic resistance) or after the use of tamoxifen treatment for about five to ten years (acquired resistance) (51). Most HR positive breast cancer patients (around 70%) favourably respond to TAM. However, around 30% of the patients do not

respond to TAM after 15 year-time of usage. (9, 52). The resistant mechanism of breast cancer can be involved with several pharmacological and molecular bases (37).

1.2.4.1 Pharmacokinetic issues

The metabolism of tamoxifen is genetically related to tamoxifen-resistant breast cancer. Hepatic metabolisms basically metabolise drugs into more hydrophilicity to promote the excretion process (37). However, tamoxifen is the prodrug that required phase I metabolism of the liver to biotransform tamoxifen into a more potent metabolite for a therapeutic level (37). There are two main hepatic cytochromes P450 isoforms; CYP3A4 and CYP2D6 which metabolise tamoxifen into N-desmethyl tamoxifen and 4-hydroxytamoxifen, respectively (37). Subsequently, N-desmethyl tamoxifen undergoes the secondary metabolism mainly via CYP2D6 while 4-hydroxytamoxifen catalyses mainly through CYP3A4 to form an active metabolite called 4-hydroxy-N-desmethyltamoxifen, so-called endoxifen. The binding affinity of the metabolites (4-hydroxytamoxifen and endoxifen) to the ER binding site are higher than the parent tamoxifen (37). Additionally, the plasma concentration of endoxifen is 5 - 10 times greater than tamoxifen (37). Endoxifen and 4-hydroxytamoxifen both are about 100 times more potent than tamoxifen in terms of anti-oestrogenic activity (37). Hence, the variation of CYP genes, especially the CYP2D6 gene, can promote tamoxifen-resistant breast cancer (53).

1.2.4.2 Cancer stem cells (CSC)

Cancer stem cells (CSC) are the significant cause of cancer resistance. Breast cancer, in particular, can develop resistance and more aggressive leading to therapeutic failure and increased mortality in the patients (54). The current therapy can eradicate the bulk of tumours; however, the subpopulation of breast cancer stem cells cannot be all eradicated (54). Therefore, cancers maintained and differentiated into tumour cells (54). Furthermore, CSC promotes breast cancer resistance to the treatment through presumed mechanisms such as enhanced renewal ability, anti-apoptosis, and increase efflux pump of anticancer agents (55).

1.2.4.3 Loss of ER expression and function

According to the tamoxifen's mechanism of action, the expression of ER has negatively relevant to its activities as of a decrease in ER signalling and function (56). This downregulation of ER was the cause for *de novo* resistance to tamoxifen (56). There was reported that around 90% of HR negative breast cancer did not respond to hormonal treatment (56). However, most studies identified that hormonal-resistant breast cancer patients have expressed ER at the beginning and responded effectively with the treatment; once ER downregulation has occurred, cancer cells are not sensitive to the treatment (56). Therefore, this indicated that developing resistance of breast cancer to tamoxifen can alter from ER positive into ER negative phenotype (56). The downregulation of ER can be identified into two significant mechanisms, firstly, the decrease in transcriptional level of ER gene and secondly, the population remodelling to produce ER-negative cells from heterogeneous ER positive tumour cells (56).

1.2.4.4 Altered expression patterns of coregulatory proteins

Tamoxifen's mechanisms of action diversely depend on the tissues, for example, in the breast, tamoxifen acts as an antagonist, but it has the agonistic action on uterus, cardiovascular system, and bone (37). The emphasised mechanism for these actions is the recruitment of coregulatory proteins (coactivators and corepressors) which can mediate the ER transcriptional activities (37). The well-established coactivator of ER is amplified in breast 1 (AIB1), also named as nuclear coactivator 3 (NCOA3), steroid receptor coactivator 3 (SRC3), and thyroid hormone receptor activator molecule 1 (TRAM-1) (37). NCOA3 considered being a proto-oncogene, and more than 30% of breast tumours overexpress; moreover around 5 - 10% of breast tumours are genetically amplified (37).

Coregulatory proteins compose of the transcriptional complex at the promoter region which can drive (coactivators) or block (corepressors) the transcriptional activities of ER-regulated genes (57). This transcriptional activity is mediated by coregulatory proteins mostly represents the ligand-bound ER (57). In breast cancer patients, approximately 60% of the tumours express NCOA3 (57). Also, the clinical samples analysis suggested that the overexpression of NCOA3 closely related to the non-response of tamoxifen treatment (57). The activity of NCOA3 can be regulated via post-transcriptional modifications such as methylation, phosphorylation, acetylation, ubiquitination, and sumoylation (58). NCOA3 can be phosphorylated at many different positions by several extracellular signalling molecules, for example, steroid hormones, growth factors, cytokines, ERK1/2, MAPK, and IKKs (59-61).

The nuclear receptor corepressor 1 (NCOR1), has also related to tamoxifen-resistant breast cancer (62). Tamoxifen-bound ER can recruit the NCOR1 leading to

histone-deacetylase complexes and repress the ER-regulated genes (63). By knocking down the NCOR1 in breast cells, tamoxifen tends to show the agonistic action instead of anti-oestrogenic action (63). Therefore, the downregulation of NCOR1 associated with the sensitivity of breast cancer to tamoxifen (56, 62-65).

1.2.4.5 Growth factors receptors/kinase signal transduction pathways in endocrine-resistant breast cancer

The ER signalling is a complex network and crosstalk with the many growth factor pathways (56). Many downstream molecules of membrane receptor of tyrosine kinases (RTKs) such as EGFR, HER2, IGF1R can regulate ER signalling pathways resulting in promoting gene expression, cell growth, proliferation, and survival as well as the signalling molecules such as farnesyl transferase, mTOR or Raf are also involved in the responsiveness of breast cancer to tamoxifen (56). Apart from that membranous ER could stimulate growth factor signalling; in turn, this can also phosphorylate cytoplasmic ER and its coregulator proteins (66). Moreover, the phosphorylation of ER structure of serine residues (S118, S168 in particular) at domain AF-1 can be activated by the upstream MAPK/ERK1/2 and PI3K/AKT which promote the ligand-independent ER function, triggering the transcriptional activity of the genes (67). Also, the overexpression of EGFR or activated AKT leads to the phosphorylation of S167 at AF-1 domain which can enhance the interaction between ER and NCOA3 in the presence of tamoxifen (67). Ultimately this interaction caused the resistance of breast cancer to tamoxifen (67). The signalling pathways of growth factors promote the phosphorylation of ER, causing the resistance of breast cancer. Similarly, many conducted researches showed that the overexpression of coactivator NCOA3 related to tamoxifen-resistant breast cancer through the phosphorylation of

the EGFR/HER2/MAPK-dependent pathway in HER2 overexpressing MCF-7 (37). Confirmedly, there was the downregulation of ER expression in transfected HER2/ER positive breast cancer cells (37). HER2 is a kind of receptor tyrosine kinase and a member of the EGFR family. The overexpression of HER2 interruptedly associated with breast cancer phenotype via ER genomic pathways (68). ER can be phosphorylated at S118 and 167 at the AF-1 domain by the downstream molecules of HER2 signalling (MAPK and AKT, respectively) (68). PI3K/AKT is a significant downstream molecule of the HER2 pathway which frequently mutated and be the cause of hormonal-resistant breast cancer (68). PI3K/AKT can also activate and stimulate the transcriptional activity of ER regulated genes in both oestrogen-dependent and -independent pathways in ER positive breast cancers (68). Furthermore, it closely links with cell-cycle progression and cell survival (68).

Regarding ER positive breast cancer, PI3K-mediated AKT can phosphorylate ERα on the ligand-independent pathway (69). Also, AKT can phosphorylate ERβ and trigger the transcriptional activity and coactivator recruitment; however, the impact of ERβ on breast cancer resistance to anti-oestrogen is rarely understood (70). The overactivation and overexpression of constitutively active AKT can induce both tamoxifen and fulvestrant resistance in an oestrogen-independent pathway, whereas the inhibition of AKT can lead to tamoxifen-sensitivity restoration (71, 72). The overactivation of AKT also causes the resistance of standard chemotherapy such as doxorubicin (73). Although AKT overexpression was suggested to be a significant cause of endocrine resistance of breast cancer, there were studies showed that AKT was not upregulation in acquiring resistance to the aromatase inhibitors such as

letrozole (74-76). Also, this confirmed in both *in vitro* aromatase-expressing MCF-7 cell line (MCF-7/Ca) and xenografts model (76).

1.2.4.6 Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2)

Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2) is an ATP-binding cassette (ABC) transporter (77). It was suggested to be one cause of multidrug resistance for various chemotherapeutic drugs as well as targeted therapeutic drugs (77). The first discovered of BCRP-caused drug resistance was doxorubicin-resistant breast cancer cells (77). BCRP gene transcription was found to be regulated by hypoxia inducible factor 1α (HIF1 α), ER, and peroxisome proliferator-activated receptor (PPAR) (78-80). Apart from that PI3K/AKT signalling played a crucial role in both transcriptional and post-translational levels of BCRP expression (81, 82). Also, the expression BCRP of both gene and protein were found to express higher in tamoxifen-resistant MCF7 than wild-type MCF7 (55).

1.2.4.7 Nuclear factor kappa-light-chain-enhancer of activated B cells; NFκB its role in endocrine resistant breast cancer

NFκB is one of the majority causes of initiation and progression of many cancers (83). Regarding hormone-independent breast cancers, NFκB closely associated with the aetiology, progression, and aggressiveness via the mediation of targeted genes such as cyclin D1, urokinase-type plasminogen activator (uPA), and C-X-C chemokine receptor type 4 (CXCR-4) (84-86). The activity of ER and NFκB was reciprocally antagonism, and many conducted pieces of research have suggested that attenuation of ER functions by anti-oestrogens can promote NFκB-driven breast

tumour progression through driven NFκB transcriptional activity (83). On the other hand, inhibition of NFκB activation can downregulate ER expression or reduce ER activities and causing the rise of ER-negative or ER-irresponsive cell populations that are generally resistant to hormonal therapy (87). Having said that NFκB and ER negatively crosstalk function, many studies indicated that NFκB and ER synergistically transcribed and translated genes and proteins that drove in breast tumour aggressiveness such as multidrug resistance proteins and prosurvival factors (83, 87). Furthermore, NFκB can also be activated through growth factor signalling pathways such as MAPK and PI3K pathways (83). Therefore, the crosstalk between NFκB and ER involved MAPK and PI3K were reconciled for hormonal-resistant breast cancers (83).

The interaction between ER and NFκB in breast cancer has been the issue of numerous studies (88). Overall, the activation silhouettes of both transcription factors of ER and NFκB are inversely related (88). In 2000, Biswas *et al.* demonstrated that activation is found to be predominant in ER-negative breast tumours, and directly correlated with the expression of ErbB2/HER2 (89). Likewise, several studies have suggested that the levels of NFκB DNA binding in breast cancer patients negatively correlated to cellular ER amount and ER target gene expression (88, 90). The analysis of gene expression data of all breast cancer molecular subtypes from 78 breast cancer cell lines using the PAM50 algorithm has shown an inverse association between the expression of NFκB mediated genes and the ER activity (91, 92). It has been confirmed that ER activity and NFκB activity were mechanistically correlated by the data from cell line studies and in human tissues (91, 92). In ER positive breast cancers, T47D and MCF7 cells experiments showed that Toll-like receptor (TLR) 9-

mediated attenuation of ER activity, and by adding the NFκB inhibitor BAY11-7082 the activity of ER was reverse and the proliferation was increased (93). Also, the study in human retinal pigment epithelial cells identified that TLR4 could also mediate the activation of NFκB which will then trigger the transcription of NFκB-targeted genes (93). In contrast, with the presence of estradiol (E_2), the transcriptional activity of TLR4-mediated NFκB activation was inhibited. Admittedly, the binding of NFκB to the DNA was prevented by ER (94). Therefore, this showed that the inhibitory interaction between the transcription factors of NFκB and ER was reciprocal (94, 95). The data from Pratt *et al* revealed that the ER positive/E_2-independent MCF7/LCC1 cell which derived from ER positive/E_2-dependent MCF7 showed the elevation of NFκB activation via the withdrawal of E_2 induced the DNA binding of NFκB and the expression of Bcl-3, a coactivator of NFκB and also play role as a proto-oncogene to regulate the transcriptional activity of NFκB in both *in vitro* and *in vivo* studies (96). Another supported data in 1997 performed in ER positive-MCF7 breast cancer cell, human cervical adenocarcinoma (Hela) cell, and human osteosarcoma (Saos-2) cells revealed that ER blocked the binding of c-Rel and RelA to bind at the promoter of IL-6 (97). Shreds mentioned above of evidence were the information of crosstalk between NFκB and ER by inhibition of NFκB DNA binding through the interaction of ER with Rel homology domain of NFκB (98). In microglia cells, the activation of ER can inhibit RelA nuclear translocation via the ER nongenomic pathways through PI3K (98). In MCF7, 17β-estradiol can inhibit the activation of NFκB via increasing the level of p105 subunit; consequently, the nuclear translocation of NFκB complex was blocked as the presence of ankyrin repeats at the C-terminus of p105 (99). Interestingly, *in vivo* study in mouse's splenocytes, the

nuclear translocation of all NFκB family members was not affected by the ER signalling, but only the harbouring RelA, RelB and c-Rel transactivation domain that affected (100). Another crosstalk between ER signalling and NFκB activation was the reciprocation of ER with transcriptional activators or repressors, which can lead to a low potent induction of NFκB transcriptional activity. In MCF7 and primary osteoblasts, for example, ER can compete NFκB to bind to with transcriptional co-activators (such as cAMP response element-binding protein, CREB) or ER can recruit co-repressors (such as glucocorticoid receptor-interacting protein 1, GRIP1) to NFκB complexes (101, 102). Another mechanism linked the negative correlation of the activation of NFκB with the ER signalling activation was that the induction of c-Rel activity by protein kinase Cθ (PKCθ) and the activated AKT (103). Subsequently, forkhead box O protein 3a (FOXO3a) was activated and abated the synthesis of ER (103). The enhancement of zeste homolog 2 (EZH2), a member of the polycomb repressor complex 2 (PRC2) and regulates gene expression via trimethylation of lysine 27 on histone 3 by TNF-α then trigger the NFκB-dependent signalling (104). Research performed in breast cancer showed that silencing EZH2, the ER expression was two-fold higher than usual (105-107). This mechanism can imply that NFκB activation could be involved with the ER silencing (107). Ultimately, ER expression can be repressed by NFκB (more specifically RelB) via BLIMP1, a zinc finger protein that inhibits ER transcription (**Figure 1.2.4.7.1)** (105, 106).

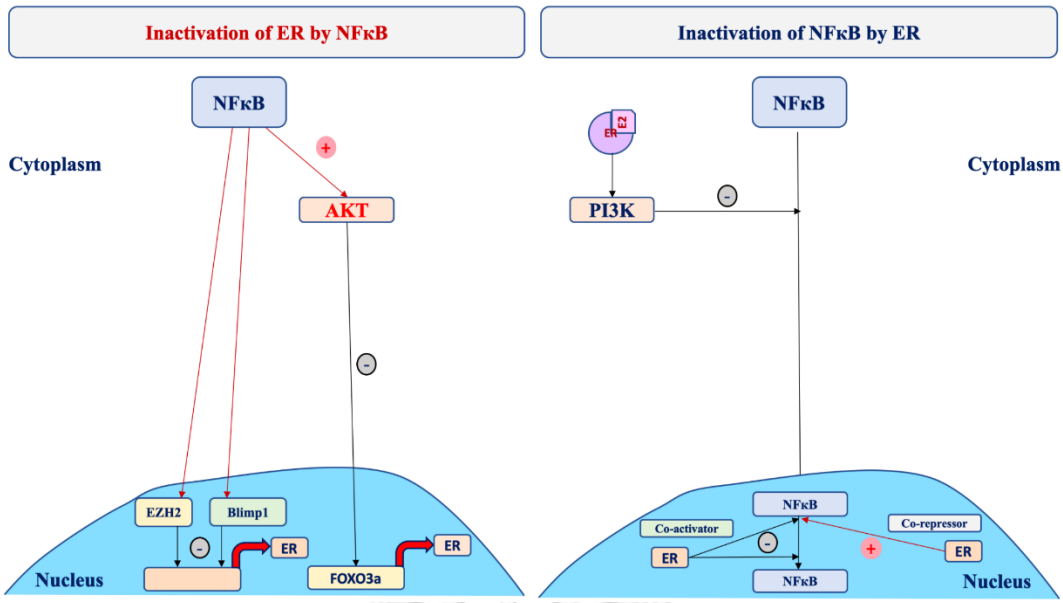


Figure 1.2.4.7.1. Transrepression of oestrogen receptor and NFκB (107)

Evidence confirmed that in ER positive tamoxifen-resistant breast cancer had a higher level of NFκB activity compared to ER positive tamoxifen-sensitive MCF7 (108). Indeed, tamoxifen-resistant MCF7 found the hyperactivation of AKT, which increased IκB phosphorylation and NFκB DNA binding (109). All in all, the data available for the crosstalk of NFκB activity and ER suggested that the activation of NFκB activity affected the sensitivity of endocrine/hormonal therapy on ER positive breast cancer cells (107). However, the data on the proposed mechanisms and hypotheses of this relationship was still to be clarified. The trans-repression of ER by NFκB can promote a mechanism of acquired resistance of ER positive breast cancer due to the loss of ER expression and function later when the subpopulation of tumours cell resistance to the endocrine therapy (107). This mechanism can also contribute to endocrine-resistant breast cancer via growth factor receptor signalling (107). On the

other hand, trans-repression of NFκB by ER can explain better for the resistance of ER positive breast cancer to SERDs, AIs and the withdrawal of oestrogen as the attenuated ER activation (107). This resulted from aromatase inhibition or oestrogen withdrawal can promote the releases of NFκB from the ER-mediated inhibition, leading to NFκB-driven tumour progression (107).

1.2.4.8 Cell cycle regulators; CyclinD1

Cyclin D1 is one of a significant cell cycle machinery contributed to tumour progression as well as the progression of ER positive breast cancer (110). Mainly, at the G1 phase, cyclin D1 plays significant roles in mediates the phosphorylation of tumour suppressor Rb through CDK4/6 and sequesters the CDK inhibitors p21 and p27 (111-113). Cyclin D1 is one of the ER targeted genes in ER non-classical genomic pathway where the induction of cyclin D1 transcription was driven by ER/AP-1 and ER/Sp1 transcription complex bind with the SRE segment of the DNA (35). Conversely, anti-oestrogen drugs such as tamoxifen can inhibit the expression of cyclin D1 via inhibition of ER signalling (8). Furthermore, AKT is also one of the molecules that can indirectly affect the expression of cyclin D1 by phosphorylation and inhibition of GSK3β activity, in turn, promote the degradation of cyclin D1 (114). Stable overexpression of cyclin D1 in ER positive breast cancer cell led to breast cancer resistance to both tamoxifen and fulvestrant (115). Also, breast cancer acquired resistance to tamoxifen expressed the upregulation of cyclin D1 (116). Likewise, cyclin D1 was significant for cell proliferation in tamoxifen-resistant breast cancer and cell cycle repression by tamoxifen (117).

Studies have revealed that there was an upregulation of cyclin D1 in tamoxifen-resistant MCF7 overexpressing HER2 (118). Noticeably, crosstalk between cyclin D1 and growth factor signalling in hormonal-resistant breast cancer (119). Similarly, overexpression of cyclin D1 has also led to PAK1 overexpression in tamoxifen-resistant MCF7 (120). As well as overexpression of BCAR3 can induce the activity of cyclin D1 promotor found in anti-oestrogen resistant MCF7 (121). Treatment with trastuzumab, a HER2 directed antibody in xenografts has downregulated the expression of cyclin D1 and inhibited tumour growth (122).

Confirmed role of cyclin D1 in clinical endocrine resistance type of breast cancer is still developing, in the clinical studies published that around 50% of breast cancer patients overexpress cyclin D1 (111, 123). Cyclin D1 amplification was shown to relate with worse survival in luminal A and B breast cancer patients and more aggressive cancer in luminal A breast cancer patients (124). Also, the high expression of cyclin D1 was associated with irresponsive to tamoxifen in breast cancer patients (125). Not surprisingly, decrease the expression of cyclin D1 was reported to be a good prognostic marker for breast cancer patients (126).

1.2.4.9 Roles of metastasis and angiogenesis in breast cancer: C-X-C chemokine receptor type 4 (CXCR4), Hypoxia inducible factor 1 alpha (HIF-1α), and Vascular endothelial growth factor (VEGF)

C-X-C chemokine receptor type 4 (CXCR4), also identified as fusin or CD184, is an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1 also called CXCL12) (127). CXCR4 belongs to the superfamily of the seven-transmembrane domain heterotrimeric G protein-coupled receptors displayed on the cell surface of various types of cancer cells, including oral cancer, esophageal cancer,

gastric cancer, colon cancer, liver cancer, pancreatic cancer thyroid cancer breast cancer ovarian cancer, prostate cancer, lung cancer, renal cancer, brain cancer, melanoma, and leukemia (128-140). The binding of SDF-1 to CXCR4 can trigger various molecular signalling involved in gene transcription, cell survival and proliferation, migration, metastasis, angiogenesis, chemotaxis, tumourigenesis, and cancer progression (141, 142) **(Figure 1.2.4.9.1)**.

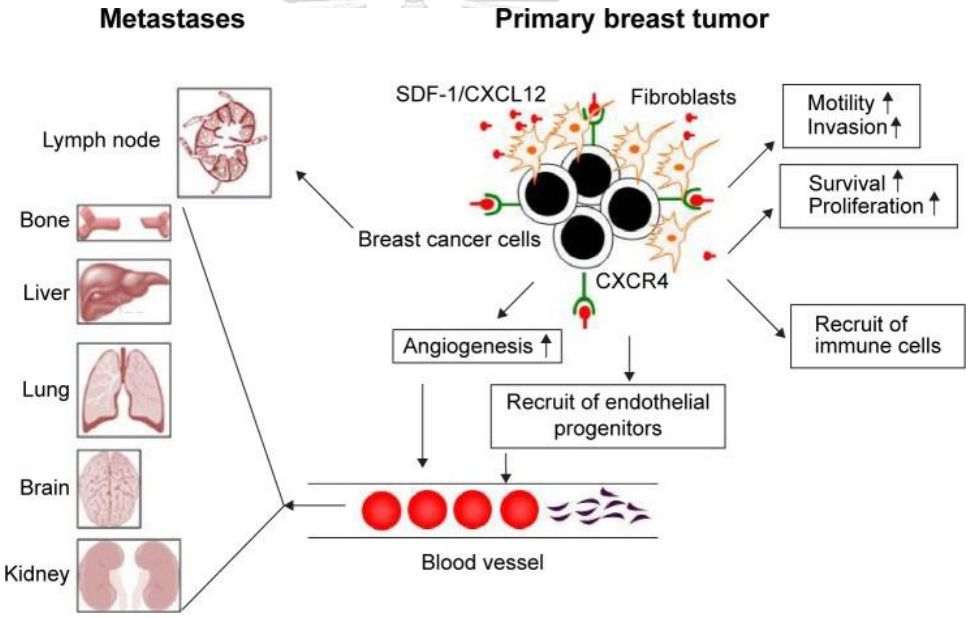


Figure 1.2.4.9.1. Potential roles for CXCR4 in breast cancer (143). CXCR4 can be driven its transcriptional activity via HIF, resulting in promoting cell proliferation, invasion, and angiogenesis in cancer cells.

In breast cancer, the level of CXCR4 expression was very low in breast epithelial cell but highly expressed in ductal carcinoma (143). CXCR4 transcription mechanism was related to hypoxia-inducible factor 1 alpha (HIF-1α) and HIF-2, as the promotor of CXCR4 possessed hypoxia response element (HRE) (143). Therefore, HIF can drive the transcriptional activity of CXCR4 (143). In normoxia state, Von Hippel Lindau (VHL) degraded HIF protein by hydroxylation leading to ubiquitination of HIF resulting in repression of CXCR4 transcription and translation (144). In hypoxia state, a lack of VHL (generally occurred in various cancer cells) (144). HIF protein was stabilised and translocated into the nucleus which then drove the transcription and translation of CXCR4, subsequently CXCR4/CXCL12 pathway was activated (144). The stabilised HIF-1α led to the transcriptional activity of HIF-targeted genes such as CXCR4 and vascular endothelial growth factor (VEGF), which caused the aggressive breast cancer cells regarding metastasis and angiogenesis (143, 144). Moreover, the expression of VEGF can occur during normoxia state and can be upregulated by the HIF-1 independent mechanism such as the activation of HER2/Neu and PI3K/AKT pathway through CXCR4 regulated phosphorylated AKT, in turn, increased VEGF transcription and translation (145). VEGF was a crucial angiogenic factor, the level of VEGF in both breast cancer cell lines and clinical breast cancer patients related to cancer progression (146). VEGF can promote the metastasis of breast cancer cells via two modes of actions; a paracrine mode in which increases vascular permeability by mediating rearrangement of actin and changing gap junction resulting in enhancing angiogenesis and tumour cell extravasation and an autocrine via stimulation of CXCR4 expression (147).

According to NFκB related CXCR4 signalling, there was an NFκB binding site located at CXCR4 promotor (148). Therefore, when the ligand such as hepatocyte growth factor (HGF) stimulated the receptor, p65 and p50 subunit of NFκB then move into the nucleus and bind to CXCR4 promotor, stimulating CXCR4 transcription and mediating tumour metastasis and invasion (148). The signalling of SDF-1/CXCR4 activated the MAPKs signalling to promote chemotaxis and proliferation, induced phospholipase C (PLC)/protein kinase C (PKC)-Ca^{2+} signalling regulated cell migration and affected PI3K/AKT advancing cell survival. (149, 150). This shows a positive feedback loop between CXCR4 and the signalling pathways mediating tumourigenicity of cancer cells **(Figure 1.2.4.9.2)** (143). Also, SDF-1/CXCR4 signalling and EGFR/HER2-neu signalling can promote invasive signs and metastatic growth of breast, prostate, and ovarian cancers (151-153).

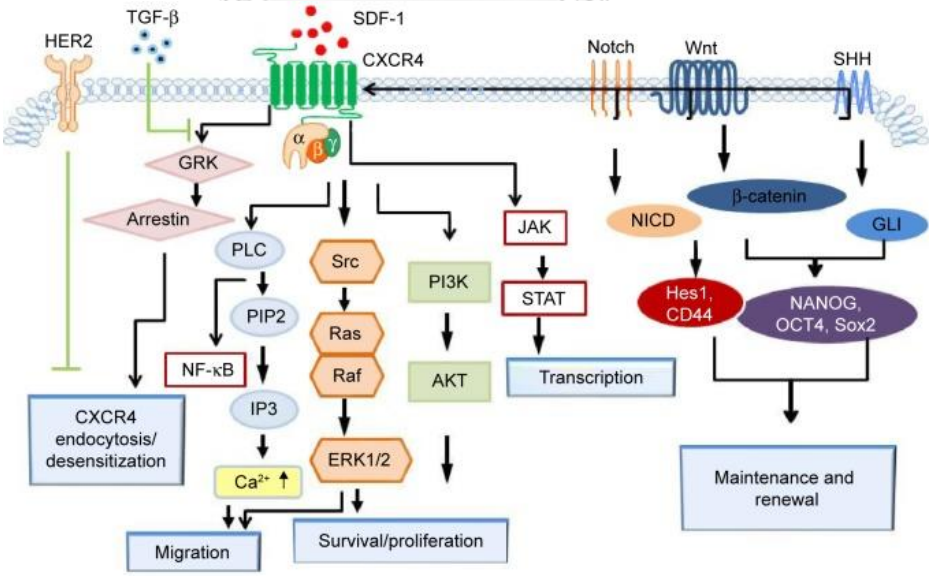


Figure 1.2.4.9.2. CXCR4-mediated feedback loop of multiple signalling pathways (143)

1.3 The galangal compound: 1’ acetoxychavicol acetate (ACA)

1.3.1 Background of ACA

Alpinia galanga (L.) Willd (Zingiberaceae) (greater galangal) is generally known by several names such as galangal, greater galangal, Java galangal and Siamese ginger (154). They were generally used as a cooking spice, especially in Thai and Indonesian cuisine. In the past, galangal has been used to address many diseases, such as eczema, bronchitis, and stomach disorder (155). 1’ Acetoxychavicol acetate (ACA), $C_{13}H_{14}O_4$ is a natural compound from rhizomes and seed of the ethnomedicinal plant *A. galanga* (156). The molecular weight of ACA is 234.25 g/mol and the density is 1.122 g/cm^3. The rhizome of *A. galangal* was edible and was popular in terms of herbal medicine in Southeast Asia (157). Galangal contained abundant flavonoids and volatile oils, and different parts of galangal possessed distinct pharmacological activities (157). The critical part provided major bioactive compounds for galangal was the rhizomes which were reported to have antimicrobial activity, anti-oxidative effect, immunostimulant and expectorant action (158). Natural ACA possessed structure-activity relationships (SARs) in two key features as its pharmacophore, which were the para substitution of the 1’ -acetoxyl group at the benzene ring and linear ethyl and propyl chain carbonates (159).

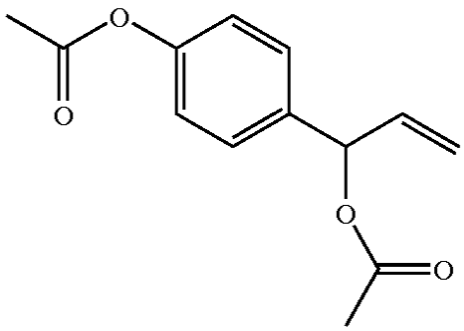


Figure 1.3.1. Structure of ACA from Palitapongarnpim et al (160)

1.3.2 Roles of ACA in cancers

ACA has been reported the anti-oxidant and anti-inflammatory activities in many studies (161). It showed the capability to block the accumulation of cellular lipid due to the downregulation of PPARγ and C/EBPα and promote the phosphorylation of AMP-activated protein kinase (AMPK) in rat xenograft model (161). Regarding growth inhibitory effects, ACA can induce the accumulation of tumour cells in the G1 phase resulting in the downregulation of phosphorylated Rb, an increase of total Rb, and a decrease of phosphorylated $p27^{kip1}$ (162).

ACA exhibited cytotoxic activity in various tumour cell lines, which were MCF7, HepG2, oral squamous carcinoma (HSC-2 and HSC-4), and epidermoid cervical carcinoma (CaSki) with IC_{50} value range between 5 and 50 μM with no toxicity to normal human mammary epithelial cell (HMEC) cell tested up to 80 μM (163). Tumour cells were all dead via apoptosis after 30 hours using 40.0 μM detected by flow cytometry analysis of annexin-V and PI co-staining (163).

In term of antiproliferative activity, ACA reported to have antiproliferation in four myeloid leukemic cell lines (NB4, UF-1, HL-60, and K562,) (164). ACA induced apoptosis as well as the impairment of mitochondrial transmembrane potential (ΔΨm) and activation of caspase-9, consequently activated death signalling through mitochondrial oxygen stress pathway in NB4 cell (164). While the apoptotic induction of ACA in HL-60 cell caused by DNA fragmentation, caspase-3 activation, and PARP cleavage (165). Also, ACA has been reported the apoptotic induced antitumour activity in other cancer types such as Ehrlich ascites tumour cells (EATC), and hepatocellular carcinoma cells (HepG2) (166, 167). ACA can trigger the activity of caspase-8 subsequently activated the Fas-mediated apoptosis (164). Furthermore,

in vitro and *in vivo* study of ACA in multiple myeloma cells showed that ACA provided growth inhibition via apoptotic induction through the repression of anti-apoptotic proteins' expression, activation of caspase-8, and inhibition of NFκB activation (156). ACA can induce the activities of caspase 3, 9, and 8 and induced GO-G1 phase cancer cell arrest in myeloma cells (156). This suggested that ACA-induced apoptosis in myeloma cells mediates both mitochondrial- and Fas-dependent signalling (156). Moreover, ACA can inhibit the phosphorylation of amino acid serine and promote the degradation of IκBα (156). The study in RPMI8226 cells showed that the nuclear expression of NFκB was blocked by ACA, resulting in the accumulation of cytosolic NFκB (156). The mode of ACA action through the inhibition of nuclear expression of NFκB was consistent in RPMI8226-grafted NOD/SCID mice and the result from RPMI8226 cell line (156). Also, the result has shown that ACA has significantly decreased tumour weight in ACA treatment group compared to control group, ultimately all these information has led to confirm that ACA possessed the inhibitory effect on NFκB and induced apoptosis in myeloma cells both *in vitro* and *in vivo* studies (156). Further investigation of In and colleagues in 2005 showed that the mechanism of ACA on apoptosis induction in RPMI8226 was related to the up-regulation of both TNF-related apoptosis-inducing ligand/Apo2 ligand (TRAIL/Apo2L) (168).

ACA treatment in oral squamous cell carcinoma (SCC) showed the antiproliferation, apoptosis induction and antimigration without causing toxicity to HMEC cell (169). Combination of ACA with cisplatin showed a synergistic cytotoxic effect indicated by using combination index studies (169). ACA alone also suppression of IKK/β activation consequently inhibited the constitutive activation of

NFκB (169). Human oral tumour mice xenografts studies revealed that ACA alone was as effective as in combination with cisplatin in decreasing tumour volume with minimal bodyweight loss and further potentiated cisplatin outcomes when used in combination (169). The effects of ACA also associated with a down-regulation of NFκB regulated genes (FasL and Bim), covering pro-inflammatory (NFκB and COX-2) and proliferative (cyclin D1) biomarkers in tumour tissue (169).

Another study of ACA demonstrated that ACA had a time- and dose-dependent cytotoxicity to both MCF7 and MDA-MB-231 at the concentration tested at 10 - 50 μM (170). ACA showed a dominant mode of action through the suppression of RANKL-induced NFκB activation in a time- and concentration-dependent manner and blocked the osteoclastogenesis induced by MCF7, multiple myeloma MM1 cells, and head and neck squamous cell carcinoma LICR-LON-HN5 cells (171). This results showed that ACA might have a beneficial action for osteoporosis and cancer-related bone deterioration (171). ACA was also reported the antimigration/invasion and antimetastasis effect in the concentration vary from 5-40 μM from the results of *in vitro* matrigel invasion assay and skeletal metastasis in breast cancer mouse model (172). ACA had cytotoxic effects and antimigration and mechanistically worked through the suppression of constitutive and interleukin-6-inducible STAT3 activation and decreased the accumulation of nuclear STAT3 as well as DNA-binding activity of STAT3 (172). Moreover, ACA treated cells had a higher amount of Src homology region 2 domain-containing phosphatase 1 (SHP-1) and inhibited STAT3 signalling resulting in the repression of STAT3 regulated genes such as matrix metalloproteinase (MMP)-2 and -9 which responsible for cell migration and invasion (172). This led to the conclusion that ACA inhibited cancer cell migration via SHP-1/STAT3/MMPs

signalling pathway (172). In mouse macrophages (RAW 264.7) cells using LPS to induce cyclooxygenase (COX)-2 mRNA expression, the activation of MAPK, p38, ERK1/2, JNK1/2 and AKT, and the degradation of the IκB-α protein and nuclear translocation of NFκB p65 subunit revealed that ACA can abrogate ERK1/2 and JNK1/2 as well as the activation of AP-1, NFκB, and CREB transcription factors (173).

The dominant published mechanism of ACA was the inhibition of NFκB activation which caused by many ligands and substances such as TNF, IL-1β, PMA, LPS, H_2O_2, and doxorubicin (171). Regarding the inhibition of NFκB activation, ACA did not interfere with the DNA binding activity of NFκB (171). Instead, ACA inhibited the translocation of p65 subunit by blocking the activation of IκBα kinase, IκBα phosphorylation, IκBα degradation, and p65 phosphorylation (171). ACA also inhibited NFκB-dependent gene expression stimulated by TNF, TNFR1, TNFR-associated death domain protein, TNFR-associated factor-2, and IκBα kinase, but not that stimulated by p65 (171). ACA suppressed the expression of TNF-induced NFκB-mediated proliferative genes (cyclin D1 and c-Myc), antiapoptotic genes (survivin, inhibitor of apoptosis protein-1 (IAP1), IAP2, X-chromosome-linked IAP, Bcl-2, Bcl-xL, Bfl-1/A1, and FLIP), and metastatic genes (COX-2, ICAM-1, VEGF, and MMP-9) (171). Therefore, ACA exhibited the ability to induce apoptosis and inhibit invasion via inhibition of NFκB activation and NFκB-targeted genes involved in cell proliferation, metastasis, and apoptosis (171). ACA suppressed VEGF-induced proliferation, migration, adhesion and tubulogenesis of HUVECs in a dose-dependent manner (174). In Matrigel plugs vasculature formation assay, ACA inhibited VEGF-induced microvessel growing from aortic rings and suppressed new vessel formation.

In vivo study confirmed the mechanism of inhibition of VEGF by ACA through blocking the activation of VEGF-mediated Src kinase, FAK and Rho family of small guanosine triphosphatases (GTPases) (Rac1 and Cdc42, but not RhoA) (174). In human prostate cancer cells (PC-3), the treatment of ACA at 5-50 μM decreased cell viability and abolition of angiogenic factor by interfering with dual Src/FAK kinases. In a PC-3 xenograft model, ACA (6 mg/kg/day) significantly inhibited the level of Src, CD31, VEGF and Ki-67 as well as tumour weight and volume (174). ACA inhibited glioblastoma cell proliferation as a result of promoting apoptotic cell death by enhancing the activities of caspase 3 (175). ACA notably diminished the migration of glioblastoma cells by lowering their adhesive functions (175). Additionally, ACA increased the protein expression of the pro-survival signalling cytokines, IL-6 and IL-1α which compensated pro-survival response in addition to pro-apoptotic ACA-induced caspase 3 response (175).

Ohnishi and team (1996) performed the xanthine oxidase inhibitor effect of ACA on 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis in male F344 rats (176). The result showed that ACA administration group significantly reduced tongue polyamine levels (35-40% inhibition) (176). These results evidenced that ACA inhibited rat oral carcinogenesis, and such inhibition might be associated with suppression of cell proliferation in the oral mucosa by the xanthine oxidase inhibitor (176). In 1997, Tanaka and colleagues performed *in vivo* investigation of the effects of ACA to inhibit the development of azoxymethane-induced colon carcinogenesis (177). They reported that ACA suppressed cell proliferation in the colonic mucosa and its induction of glutathione S-transferase (GST) and quinone oxidoreductase 1 (NQO1) (177). Further *in vitro* study of ACA in rat intestinal-epithelial cells (IEC6)

showed that ACA-induced glutathione S-transferase (GST) and NAD (P)H: quinone oxidoreductase 1 (NQO1) activities, increased intracellular glutathione (GSH) level, and upregulated intranuclear Nrf2 and cytosolic p21 (178). Impliedly, that activation of phase II enzymes via Nrf2 related to p21 is a possible mechanism of ACA to halt the advance of carcinogenesis (178). In Ehrlich ascites tumour cells, ACA showed the decrease in the intracellular GSH levels, suggesting that ACA-induced reduction of the cellular GSH levels can lead to growth arrest of the cancer cell (179).

ACA can also modulate the expression of miRNAs in cervix carcinoma cells (Ca Ski, HeLa); therefore, tumour suppressor miRNAs miR-138, miR-210 and miR-744 were upregulated (180). In 2013, there was a study reported that ACA significantly inhibited the growth of human head and neck squamous cell carcinoma cell line HN4 and induced cell apoptosis (181). Additional studies indicated that ACA (17.70 μM) downregulated the expression of miR-23a in HN4 cells which associated with phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a target of miR-23a (181). In human non-small cell lung cancer (NSCLC), ACA showed cytotoxicity and induced formation of cytoplasmic vacuoles (182). Moreover, ACA has been shown its action on the induction of pro-autophagy pathway through Beclin-1-independent pathway in NSCLC (182). In cervical cancer, it has been stated that ACA targeted decapentaplegic homolog 4 (SMAD4) resulting in down-regulation of miR-210 expression, which commonly overexpressed in cervical cancer (183).

ACA has been reported to inhibit the proliferation of a broad range of cancer types in both *in vitro* and *in vivo* studies, including bile duct cancer, oesophageal cancer, large intestinal cancer, oral cancer and hepatocellular carcinoma (176, 177, 184-186). ACA induced apoptosis in myeloma cells through the inhibition of NFκB-

related functions and also inhibited the phosphorylation of inhibitor of nuclear factor kappa B kinase subunit alpha/beta (IKKα/β) leading to the suppression of NFκB activation in human squamous carcinoma (HSC-4) cells (169, 187). Furthermore, the effect of ACA on myeloma cell can induce apoptosis as well as inhibited ERK MAPK signalling pathway (188). Also, ACA has been reported to repress tumour volume and weight, Proto-oncogene tyrosine-protein kinase (Src), Cluster of differentiation 31 (CD31), proliferative marker Ki-67, and blocking the activation of Vascular endothelial growth factor (VEGF)-mediated Src kinase *in vivo* (174). In MCF7 and MDA-MB-231 cell lines, ACA has revealed to be apoptotic induction agent via c-Jun N-terminal kinase 1/2 (JNK1/2) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) signallings (170, 173).

1.3.3 Proposed mechanism of action of ACA on endocrine resistant breast cancers

Crosstalk between the NFκB pathway and endocrine therapy resistance in breast cancers has been established (189). Also, it has been shown that MCF7/LCC9 cells (ER-positive, oestrogen-independent, and anti-oestrogen (tamoxifen and fulvestrant) cross-resistant) and MCF7/RR cells (ER-positive, oestrogen-independent for growth, tamoxifen-resistant and fulvestrant-sensitive) have higher p65 basal levels compared to MCF7 cell (ER-positive and anti-hormone sensitive) (190). Increased expression of p65 in endocrine-resistant breast cancer cells may be involved in promoting cancer cell growth (190). Also, the enhancement of NFκB and AP-1 transcriptional activities were reported in endocrine-resistant breast cancer cells (191). Treatment of the endocrine-resistant breast cancer cells with pathenolide (NFκB inhibitor), significantly decreased the transcriptional activity of NFκB regulated genes compared to MCF7 cells (190). This evidence shows that inhibiting NFκB pathway results in the inhibition of proliferation and NFκB transcriptional activity in both of the resistant cell lines, compared to MCF7 control cells.

ACA, as a potent NFκB inhibitor through inhibition of nuclear translocation of the p65 subunit and its effects on the repression of AKT and ERK1/2 activity (171, 173, 192) could inhibit endocrine-resistant breast cancer cell growth, and other related effects.

1.3.4 Safety profile of ACA

ACA has been studied the safety both *in vitro* and *in vivo* as revealed in the study of Awang and partners (2010) showed that ACA at the concentration range up to 80 μM had no adverse cytotoxic effects on normal dermal microvascular endothelium (HMEC cells) (163). Also, in human oral tumour xenografts, studies in mice revealed that ACA alone was as effective in reducing tumour volume without any toxicity (169). In 2000, Moffatt *et al.* demonstrated that ACA had no cytotoxic effect on normal rat hepatocytes at increasing concentration up to 40 μM (166). Furthermore, ACA (3 mg/kg) had been shown no toxic effects *in vivo* in a nonobese diabetic/SCID mice leukaemia model (164).

1.4 The zebrafish model

Danio rerio is a vertebrate and a non-mammalian specie that commonly used in developmental process and human diseases including, gastrointestinal disorders, brain disorders, muscle disease, and cancers (193-197). Zebrafish xerograft model is increasingly used in cancer research as a screening model, particularly with tumour proliferation, angiogenesis, metastasis, and antineoplastic drug screens (198). Around 70% of all human disease genes are functional homolog with zebrafish specie (199). The advantages of zebrafish for using as an animal experiment are its transparency of embryos and embryonic development (200). Therefore, it can significantly aid imaging and protein/cell marker tracking to observed biological and disease processes (200). In addition, it gives high number of offspring for each breeding under laboratory conditions, and allowing high confidence in statistical analysis (201, 202). Also, zebrafish models are easy-manipulated method and allow real-time visualisation of tumour mass and volume (203). Zebrafish xenotransplantation is the superior transplantation model in avoiding the issue of graft rejection compared to mouse models (203, 204). In mouse models, the aspect to avoid graft rejection is to use the genetically modified mouse by immunosuppression of T, B, and natural killer cells, NOD/SCID mouse (205). Unlike in zebrafish, their innate and adaptive immunity are not fully functioned until 21 days of life (204). At this stage, their T and B cells enter thymus and processing the immune maturation (204). The absence of the immune system until 3 to 4 days post fertilisation (dpf) giving the benefit of unnecessary usage of immunosuppressants in zebrafish engrafted humanised tumours (206). Thus, this model is favourable and convenient in xenotransplantation experiments (206).

Zebrafish is well-established for various cancer studies such as breast cancer, prostate cancer, liver cancer, glioblastoma, and melanoma (207-210). It has been used for studying the tumour microenvironment of tumour-induced angiogenesis and tumour neovascularisation in tumour growth and metastatic stage (211). In breast cancer, it has been suggested that zebrafish has possessed the oestrogen-responsive genes and signalling pathways (212). Furthermore, zebrafish has highly conserved of oestrogen-responsiveness, which responsible for cell proliferation, DNA damage, and cell death (212). Therefore, this model can potentially be used in oestrogen-dependent research (212). Additionally, zebrafish model has been used in triple negative breast cancer (TNBC) type to investigate epithelial-mesenchymal transition (EMT) through β1 integrin mediated transforming growth factor-β (TGF-β) pathway (213). Also, zebrafish has been used in TNBC xenograft metastatic model and micro-metastatic formation through the CXCR4-CXCL12 signalling axis to project the new pharmacological therapy for TNBC (214, 215).

With all striking genetic information of zebrafish models, it promotes the utilisation of zebrafish in the physiopathology of human diseases (216, 217). Also, this model benefits for the studying of promised therapeutic compounds in terms of efficacy, safety, and mechanism of action in various classes of disease researches (218).

1.5 Molecular Docking Simulation

The molecular docking approach was a computational tool, which commonly used for target identification (219). This approach can identify protein targets (e.g. receptors, ligands) of the pharmacologically active substances (219, 220). It can be used as a high throughput screening for structured-based lead optimisation in the drug discovery process (220, 221). There are several processes to perform molecular docking approach (222). The processes are composed of two main methods, which are the preparation of ligand and receptor (222). The preparation of ligand method includes generation, optimisation, and analysis of ligand's 3D structure (222). In addition, the Protein Data Bank (PDB) is the primary source for the 3D structure of proteins, protein fragments, nucleic acids, and protein-ligand complexes (222). This well-established source is used for analysing the proteins' structure or comparing any protein structure (222). The receptor preparation is performed by using the rigorous target protein conveying a single conformation of the receptor (222).

This docking technique is also used for predicting and matching the ligand to the protein binding site (223). It is noted that the protein-ligand binding interaction is a key for molecular signal transduction and biological mechanism of action (223, 224). One of the most significant points is the versatility of molecules involved in the complex molecule (225). These molecules include both the target macromolecule (typically proteins) and the ligand (225). Typically, the rationale for drug development is to evaluate the compound capable of inhibiting the functioned protein responsible for diseases progression (225). On the one hand, is to fill the gap to find the organic compound work as a selective inhibitor (226). From the aforementioned, this approach is very useful to support the results of molecular experiments (227).

1.6 Research question

- Does ACA inhibit proliferation, migration/invasion, and angiogenesis in endocrine-resistant breast cancer cells?

- Does ACA inhibit the expression or activity of HER2, NCOA3 and PI3K/AKT or ERK1/2 and suppress NF-κB targeted genes involved in endocrine-resistant breast cancer cells?

- Does ACA inhibit proliferation of endocrine-resistant breast cancer cells in zebrafish xenograft model?

1.7 Objectives of the study

ACA is a promising compound, prominently for its anticancer attributes through inhibition of NF-kB activation; it may implement a new option in breast cancer therapeutics. This study aims to investigate the effects of ACA on hormonal-sensitive breast cancer (MCF7) and hormonal-resistant breast cancer cells (MCF7/LCC2 and MCF7/LCC9) to unpack the signalling pathways that are affected by ACA. Therefore, this study focuses primarily on the effects of ACA on the inhibition of NF-kB targeted genes which involved in breast cancer cell proliferation (Cyclin D1; CCND1, MYC proto-oncogene; c-Myc), invasion (C-X-C chemokine receptor type 4; CXCR4, urokinase plasminogen activator; uPA), angiogenesis (VEGF, Basic fibroblast growth factor 2; FGF2). Subsequently, the *in vivo* antiproliferative activity of ACA in MCF7/LCC9 grafted zebrafish xenograft model. Additionally, the signalling mechanisms of ACA via the growth factor receptors including HER2 and its downstream molecules such as MAPK/ERK1/2 and PI3K/AKT. Also, the effect of ACA on the abolishment of NCOA3 transcriptional

activity and protein expression since NCOA3 the important co-activator of ER and is phosphorylated by ERK1/2. Therefore, the expression of NCOA3 protein could be altered by the effect of ACA.

1.8 Hypothesis

- ACA exhibits its antiproliferation effects through inhibition of HER2, NCOA3, AKT, and ERK1/2 which are key molecules involved in endocrine-resistant breast cancer cells.

- ACA inhibits the mRNA expression of NF-κB targeted genes which are reported to be overexpressed in endocrine-resistant breast cancer namely, proliferative factors (*CCND1*, *C-myc*), invasive factors (*uPA*, *CXCR4*), angiogenic factors (*VEGF*, *FGF2*).

- ACA inhibits proliferation of endocrine-resistant breast cancer in zebrafish xenograft model.

1.9 Experimental design

The experiment is divided into four parts: Isolation and purification, *in vitro* anticancer activities, *in vivo* zebrafish model, and molecular docking.

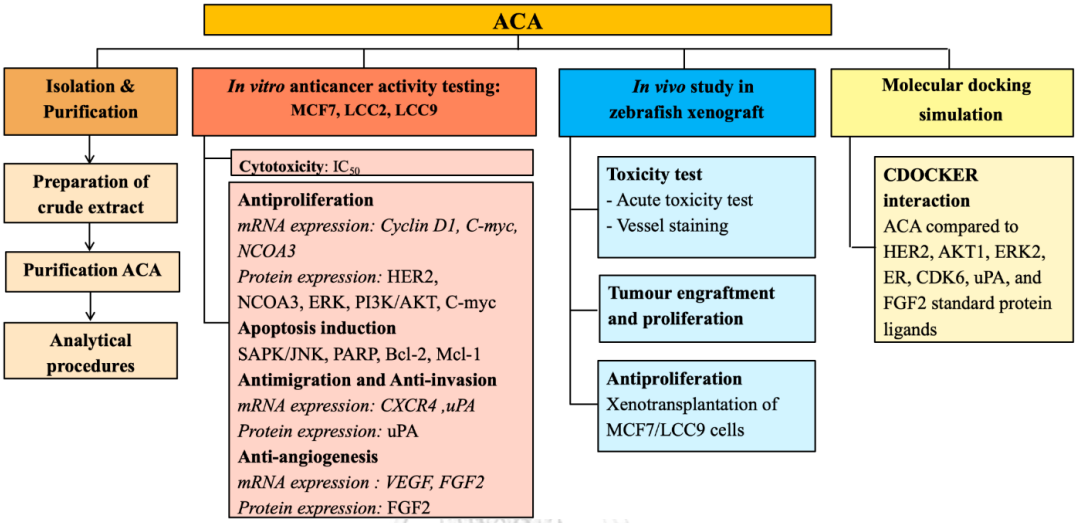


Figure 1.8.1. Flow chart of experimental design. There are 4 main sections of the experiment. Firstly, isolation and purification which divides into 3 subsections namely, extraction, purification, and structural elucidation. Secondly, *in vitro* anticancer activities which are divided into antiproliferation, apoptosis induction, anti-invasion, and anti-angiogenesis. Thirdly, *in vivo* zebrafish model which consists of toxicity test, tumour engraftment and proliferation, and antiproliferation. Finally, molecular docking.

CHAPTER 2 RESEARCH METHODOLOGY

2.1 Cell lines and its characteristics

The human ER positive breast cancer (Michigan Cancer Foundation-7; MCF7) (American Type Culture Collection (ATCC®) HTB-22™) cell line was purchased from ATCC, USA. The tamoxifen-resistant breast cancer (MCF7/ Lombardi Cancer Center 2; LCC2) and tamoxifen/fulvestrant-resistant breast cancer (MCF7/LCC9) cell lines were obtained from Dr. Robert Clarke (Georgetown University Medical center, Washington DC, USA).

The MCF7/LCC2 and MCF7/LCC9 cells are sublines of the ovarian-dependent human breast cancer cell line MCF7 (228). These cell lines were established after isolation from one of three small, slowly-proliferating MCF7 tumours in vivo in ovariectomised-athymic nude mice (MIII) after 6 months of inoculation (229). The initial MCF7/MIII line was further passaged in ovariectomised nude mice and re-established in vitro as the continuous cell line MCF7/LCC1 (ovarian-independent) (230). The baseline level of oestrogen receptor was equivalent in MCF7/MIII, MCF7/LCC1, and MCF7 (230). The basal level of oestrogen-regulated pS2 mRNA was evaluated and shown to be higher in MCF7/MIII and MCF7/LCC1 compared to the parental MCF7 cell (230). The ovarian-independent breast cancer cells (MCF7/MIII and MCF7/LCC1) showed higher sensitivity to oestrogen, which was associated with elevation of the expression of progesterone receptor (229).

The oestrogen-independent MCF7/LCC2 (tamoxifen-resistant) cell line was derived from the oestrogen-responsive MCF7/LCC1 after continuous exposure to 10-

6M of 4-hydroxytamoxifen for 5 years (231). MCF7/LCC2 is still sensitive to the inhibitor, ICI 182,780 (fulvestrant) similar to MCF7 cells (231). MCF7/LCC2 cell line was reported to have expression of basal progesterone mRNA level comparable to parental MCF7 cell (232). The MCF7/LCC9 cell line was derived from MCF7/LCC1 after continuous exposure to ICI 182,780 (fulvestrant), and showed cross-resistance to tamoxifen, although these cells have never been exposed to the drug (228). MCF7/LCC9 cell line was reported to have higher baseline mRNA level of progesterone receptor compared to parental MCF7 cell (228).

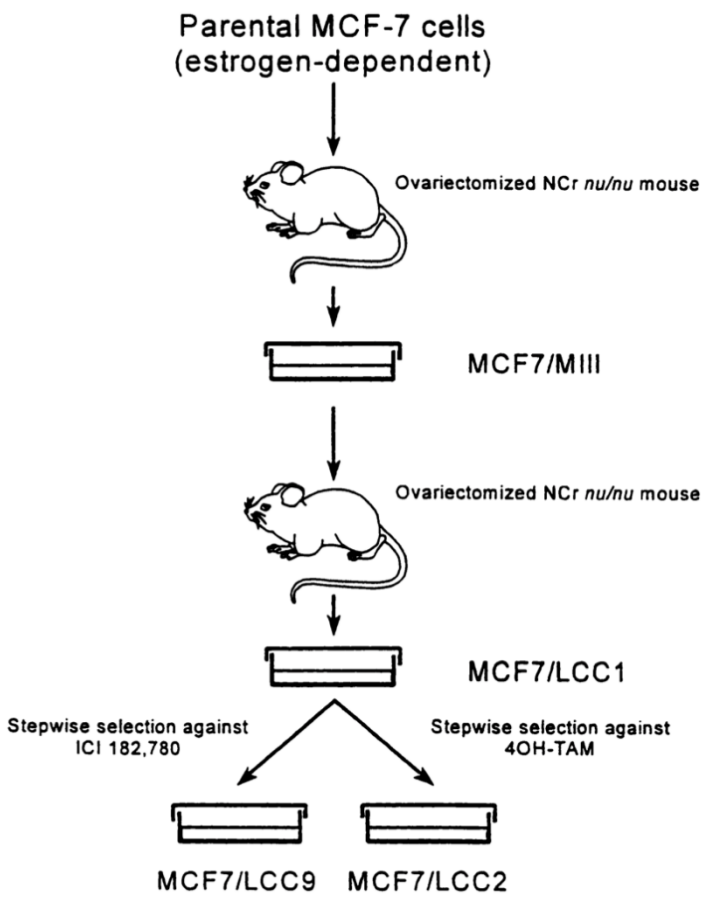


Figure 2.1.1. The derivation of MCF7 sublines MCF7/LCC2 and MCF7/LCC9 (228)

In this study MCF7/LCC2 and MCF7/LCC9 were selected for use as representatives of the most common oestrogen receptor-positive breast cancer cells which have developed resistance to hormonal therapy post-treatment, and hence relevant to endocrine-resistant breast cancer patients. In our lab, routine confirmation of the resistance of cell lines was performed using real-time qPCR to determine the expression of genes involved in endocrine-resistant breast cancer such as ESR1, NCOA3, and HER2.

2.2 Materials

0.4% Trypan blue dye (Sigma, USA)

1’ Acetoxychavicol acetate (ACA) isolated from fresh Alpinia galanga rhizomes

4-Hydroxytamoxifen (Sigma, USA)

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma, USA)

96–well, 24–well, 6–well plates (Corning, USA)

Acetone (Sigma, USA)

Alkaline phosphatase (AP) buffer (Sigma, USA)

Autopipette (Gilson, USA)

BD matrigel matrix (Biosciences, USA)

Biohazard laminar flow hood (ESSCO, USA)

CellTracker™ CM-DiI Dye (Thermo Fisher, US)

Centrifuge (Hettich, USA)

Charcoal strip fetal bovine serum (Gibco, USA)

Crystal violet (Sigma, USA)

Deuterochloroform ($CDCL_3$) (Sigma, USA)

Diethyl pyrocarbonate (DEPC) (Molekula, UK)

Dimethyl sulfloxide (DMSO) (Sigma, USA)

Dulbecco's modified eagle's medium (DMEM) (Gibco, USA)

Ethyl acetate (Sigma, USA)

Fetal bovine serum (FBS) (Gibthai)

Forceps

Fresh *Alpinia galanga* (L.) Willd rhizomes

Fourier Transform Infrared (FTIR) spectrometer (PerkinElmer, USA)

Gel electrophoresis (Bio-Rad, USA)

Hemocytometer (Brand, Germany)

Hexane (Sigma, USA)

ImProm-IITM Reverse Transcription system (Promega, USA)

Improved MEM (Gibco, USA)

Insulin, Human recombinant (Gibco, USA)

Light microscope (Nikon, USA)

Matrigel invasion chambers (Corning USA)

Micromass LCT mass spectrometer (Waters, USA)

Minimum Essential Medium (Gibco, USA)

Methanol (MeOH) (Sigma, USA)

Methylthiazolyldiphenyl- tetrazolium bromide (MTT) (Sigma, USA)

NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer with Wi-Fi (Thermo Fisher, US)

Nitro blue tetrazolium (NBT) (Sigma, USA)

Non-essential amino acids (NEAA) (Gibco, USA)

Nuclear Magnetic Resonance (NMR) spectrometer AV500 (Bruker, USA)

Penicillin/streptomycin (Hyclone, USA)

Phenylthiourea (PTU) (Sigma, USA)

Phosphate-Buffered Saline/Tween-RNAse (PBST-RNAse) (Sigma, USA)

Polysorbate 20 (Tween 20) (Sigma, USA)

Potassium chloride (KCl) (Sigma, USA)

Primers (Bio Basic, Canada)

Promega GoTaq™ qPCR Master Mix (Promega, UK)

Promega ImProm-II™ Reverse Transcription (Promega, UK)

Spectrophotometer (Shimadzu, Japan)

Sodium chloride (NaCl) (Sigma, USA)

T-25 and T-75 tissue Culture flasks (Corning, USA)

Taq polymerase (Vivantis, USA)

Tetramethylsilane (TMS) (Sigma, USA)

Thermocycler machine (Eppendorf, USA)

TLC silica gel 60 F_{254} (Merck, USA)

Tricaine methanesulfonate (MS222) (Sigma, USA)

TRiZol reagent (Invitrogen, UK)

UV-Vis Spectrophotometer, UV-1800 (Shimadzu, Japan)

Vortex mixer (Scientific industries, USA)

Zinc solution (Gibco, USA)

Zebrafish apparatus

- Eppendorf FemtoJet 4i (Eppendorf, UK)
- G-100/100L Thin-walled glass capillary (Narishige, Japan)

- Handmade hair-inserted glass capillary loop
- Leica stereo microscope S9i (Leica, UK)
- Nikon microscope camera (model DS-Ri2) (Nikon, US)
- Nikon stereo microscope SMZ18 (Nikon, USA)
- P-1000 Micropipette puller (Sutter Instrument Company, USA)
- Pneumatic Microinjector IM300 (Narishige, Japan)
- Zebrafish Housing (ITS-Z) (IWAKI Aquatic, USA); additional information can be found in **Appendix D**

2.3 Methods

2.3.1 Preparation of crude extract and purification of 1'-acetoxychavicol acetate (ACA)

The fresh *A. galanga* (L.) Willd rhizomes were collected locally in Bangkok, Thailand (November 2017). The plant was identified by Dr. Thanapat Songsak (Department of Pharmacognosy, College of Pharmacy, Rangsit University, Thailand). A voucher specimen number was CP-Ag-29 and the specimen was placed in the College of Pharmacy, Rangsit University, Thailand.

Figure 2.3.1.1. The rhizome of greater galangal. The figure shows fresh rhizomes of *A. galangal.*

The fresh *A. galanga* rhizomes (10.0 kg) were chopped to increase the surface area for performing cold extraction using hexane as an extraction solvent. The crude extract in hexane was prepared from raw material by cold percolation for 5 minutes. The process of extraction was repeated three times with hexane. The hexane extracts were combined and concentrated under vacuum at 40 °C and pooled together to

obtain 7.2 g of brown oily substance (crude hexane extract). After that crude hexane extract was initial screened by performing spot thin layer chromatography (spot TLC) with the condition of Hexane 8 : Ethyl acetate 2 was performed and followed by UV detection using UV-Vis Spectrophotometer SHIMADZU, UV-1800 of spot TLC of ACA at the wavelength 254 nm.

After that crude hexane extract (7.2 g) was chromatographed on silica gel glass column. This was eluted with stepwise gradient of hexane-acetone. The fraction (hexane 85 : acetone 15) was collected separately and concentrated in a vacuum at 40 °C. The yield of pure 1' acetoxychavicol acetate (ACA) after process through column chromatography was 6.5 g and will be used for further analysis and for the study of anticancer activities.

2.3.2 Analytical procedures

To identify the bioactive compound in the column chromatographic fraction (hexane 85 : acetone 15). This fraction was responsible for cytotoxic activity and was further subjected to characterisation of bioactive compound ACA (233, 234).

Proton nuclear magnetic resonance (^{1}H NMR) and carbon thirteen nuclear magnetic resonance (^{13}C NMR) experiments were carried out on a Bruker AV500 NMR spectrometer, operating at 500 MHz for hydrogen and carbon and then recorded in $CDCl_3$ with tetramethylsilane as an internal standard. Electrospray ionization (ESI)/Time-of-Flight (TOF) mass spectra were obtained from a Micromass LCT mass spectrometer.

2.3.3 Maintenance of MCF7, MCF7/LCC2, MCF7/LCC9, and PCS201-010 cell lines

ER positive (MCF7), tamoxifen-resistant (MCF7/LCC2), and tamoxifen/fulvestrant-resistant (MCF7/LCC9) cell lines were cultured on 25-cm^2 flasks with MEM (minimum essential media) medium with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin, and incubated at 37°C in humidified air and 5% CO_2. While the normal neonatal fibroblast (PCS201-010) cell line was culture in DMEM (Dulbecco's Modified Eagle Medium) high glucose and supplement with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. Culture medium was changed every 48 hours to assure that essential nutrition was available to the cells. For further experiments, MCF7, MCF7/LCC2 and MCF7/LCC9 cell lines were sub-cultured at 85-100% confluency by using trypsin-EDTA (Ethylenediaminetetraacetic acid) mixture to detach cells from the coated plate surface and seeded into 25-cm^2 flasks or onto 96 well plates (5000 cells/well) for *in vitro* study.

2.3.4 *In vitro* studies

2.3.4.1 Viability assay

The cytotoxic activity of 1’ acetoxychavicol acetate in MCF7, MCF7/LCC2, and MCF7/LCC9 cells was assessed by the (methylthiazolyldiphenyl–tetrazolium bromide assay) MTT assay.

Cell viability/cytotoxicity was determined by a modified MTT assay as previously described (235) Cells (5×10^5 cells/mL) were seeded into each well of a 96-well plate and incubated for 24 hours. The cells were exposed to ACA at 0 - 60 μM for MCF7, MCF7/LCC2, and MCF7/LCC9 cells. While the PCS201-010 cells were treated with ACA for toxicity test at the concentration up to 320 μM. Tamoxifen (0 - 20 μM) was used as a positive control for MCF7 cells. Palbociclib (0 - 20 μM) was used for MCF7/LCC2 and MCF7/LCC9 cells. The negative control/vehicle control used in the study was 0.1%v/v ethanol (0.1% EtOH) in completed medium. In addition, this study was also performed the effects of vehicle control on untreated control (completed medium). All cell lines were treated and incubated with 3-time points; 24 hours, 48 hours, and 72 hours. Following the treatment incubation time, the medium was then replaced by MTT (final concentration 0.5 mg/mL) and incubated for a further 4 hours at 37°C. The purple formazan crystals were dissolved in DMSO. The relative number of viable cells was assessed by measuring the absorbance of the formazan product at 570 nm with a microplate reader (BioTek Synnergy, USA). After the initial exploratory experiments, the MTT assay was performed in triplicates for each cell line with ACA concentrations ranging from 0 - 60 μM for the determination of IC_{50}. All the work was repeated in three independent experiments.

The calculation of the percentage cell viability as shown below:

$$\frac{\text{Mean absorbance obtained from treated cells}}{\text{Mean absorbance obtained from untreated cells}} \times 100$$

The half inhibition concentration (IC_{50}) from 48 hours incubation and diluted for other two concentrations as twofold dilution (IC_{25} and $IC_{12.5}$) will be used for further studies.

In the *in vitro* viability test, there were two positive controls have been used for the study. 4OH-Tam was used as a positive control for MCF7 cell while palbociclib was used for MCF7/LCC2 and MCF7/LCC9 as these two cell lines were resistant to hormonal treatment in which palbociclib was recommended for clinically used as combination with endocrine therapy in ER positive advanced breast cancers (236).

ACA could have different potency for each cell line. Therefore, the concentration used for further studies was very critical in terms of the accuracy and precision of the data. In all gene and protein analysis studies, all cell lines were incubated with ACA for 24 hours. However, the IC_{50} at 48 hours of ACA treatment will be used for gene and protein assays. This is because the IC_{50} value at 24 hours incubation was higher concentration compared to IC_{50} at 48 hours incubation. Thus, the higher concentration will drastically cause cell death which could affect and alter the expression and function of genes and proteins. Therefore, IC_{50} values at 48 hours incubation were the selected concentration for the equally efficacious effect of ACA for each cell line and for not causing a significant cell death interfering the results of experiments.

2.3.4.2 Invasion assay

The inhibitory effect of 1’ acetoxychavicol acetate on cell migration and invasion in endocrine-resistant breast cancer cells was investigated by the matrigel invasion assay

MCF7/LCC2 and MCF7/LCC9 cell lines were cultured on 24-well plate at 37°C in humidified air and 5% CO_2 for 24 hours. Following this incubation period, the medium was removed and replaced with fresh, containing ACA at three non-toxic concentrations. Medium with 0.1% EtOH was used as a negative control. Cells were incubated for 48 hours before coating the invasion chamber with matrigel and incubating for another 24 hours before performing further experiments. After the incubation of matrigel, the cells were harvested using Trypsin/EDTA and plated 1×10^5 cells/mL for 150 μL of cells suspension into the upper chamber (final cell density 50,000 cells in 150 μL). Then the cells were treated with 7.5 and 15 μM ACA in MCF7/LCC2 cells and 10 and 20 μM ACA in MCF7/LCC9 cells then incubate for 24 hours. 0.1% EtOH was used as a negative control. After the incubation, non-invaded cells were scraped off from the upper chamber with a cotton swab and fixed the invasive cells with 4% formaldehyde and stained with crystal violet dye. Invasive cells were counted for 25 random fields under an inverted microscope and analysed the proportion invasiveness.

The proportion invasiveness was calculated from the formula below;

$$\text{Proportion invasiveness (\%)} = \frac{\text{number of invaded cells in treated group}}{\text{number of invaded cells in control group}} \times 100\%$$

2.3.4.3 Gene expression analysis

The inhibitory effects of 1’ acetoxychavicol acetate on the expression of genes and proteins involved in cell proliferation, migration, invasion, angiogenesis, and tamoxifen resistance were studied by quantitative real-time-PCR.

MCF7/LCC2 and MCF7/LCC9 cell lines (50,000 cell/well) were cultured on a 6-well plate at 37°C in humidified air and 5% CO_2 for 24 hours. After that medium was removed and added 1’ acetoxychavicol acetate at the concentration of IC_{50}, IC_{25}, and $IC_{12.5}$. Ethanol at 0.1% was used as a negative control then incubated for 24 hours. RNA was extracted from the cells after complete incubation using Trizol reagent. The cells were rinsed using PBS and lysed with 1 mL of Trizol reagent and scraped off the adherent cells. Then, the Trizol added cells were transferred into 0.2 mL tubes and added 200 µL of chloroform (0.2 mL of chloroform/1 mL Trizol) and vortexed the sample vigorously for 15 seconds and incubated 3 minutes at room temperature. After that, the samples were centrifuged at 12,000 g, 4 degree Celsius for 15 minutes. After centrifugation, the samples were separated into lower pink, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The samples in upper aqueous phase were transferred carefully into new 0.2 mL tubes as the RNA remained mainly in the aqueous phase. The samples were then going through precipitation steps. The samples were mixed with 500 µL of isopropyl alcohol (0.5 mL of isopropyl alcohol/1 mL Trizol) and incubated the samples at room temperature for 10 minutes. The samples were centrifuged at 12,000 g, 4 degree Celsius for 10 minutes. After that, the supernatants were removed, and the RNA pellets were washed using 1 mL of 75% ethanol/DEPC-treated water and centrifuged 7,500 g at 4 degree Celsius for 5 minutes.

Before performing real-time qPCR, the RNA was measured the concentration via spectrophotometry using NanoDrop spectrophotometer. The RNA was air-dried for 10 minutes and diluted the RNA with 20 μL of DEPC-treated water then measured absorbance at 260 nm and 280 nm. After that, the RNA was converted to complementary DNA (cDNA) using ImProm-II reverse transcription system.

The real-time qPCR using SYBR green was performed using cDNA as a template with primers (**Table 2.3.1.3.1)** specific for Cyclin D1 (*CCND1*), *NCOA3*, MYC proto-oncogene (*c-Myc*), *CXCR4*, *uPA*, *VEGF*, and *FGF2* under the following conditions: amplification at 95°C for 15 seconds, extension at 60°C for 30 seconds and denaturation at 72°C for 30 seconds for 40 cycles followed by a melt curve stage (95°C for 15 seconds, 60°C for 1 minutes, 95°C for 15 seconds), and holding stage (50°C for 2 minutes, 95°C for 2 minutes) for one cycle.

Table 2.3.4.3.1. Primer sets and sequence for studied genes

Primer set	**Primer**	**Sequence**
Cyclin D1	Forward	5’-GGATGCTGGAGGTCTGCGAGGAAC-3’
	Reverse	5’-GAGAGGAAGCGTGTGAGGCGGTAG-3’
CXCR4	Forward	5’-AATGTAGTAAGGCAGCCAACAG-3’
	Reverse	5’-CTTCTACCCCAATGACTTGTGG-3’
NCOA3	Forward	5’-GGTAGGCGGCATGAGTATGTC-3’
	Reverse	5’-TGTTACTGGAACCCCCATACCT-3’
VEGF	Forward	5'-GAGATGAGCT TCCTACAGCAC-3'
	Reverse	5'-TCACCGCCTCGGCTTGTCACAT-3'
FGF2	Forward	5’-GAGAAGAGCGACCCACACG-3’
	Reverse	5’-GGCACACACTCCCTTGATAGA-3’
uPA	Forward	5’-AAATGCTGTGTGCTGCTGAC-3’
	Reverse	5’- AGGCCATTCTCTTCCTTGGT-3’
c-Myc	Forward	5’- AAAGGCCCCCAAGGTAGTTA-3’
	Reverse	5’- GCACAAGAGTTCCGTAGCTG-3’

2.3.4.4 Protein expression analysis: Western blotting analysis

Whole-cell extracts were prepared by lysing the cells with RIPA buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 8), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor and phosphatase inhibitor cocktails (Sigma- Aldrich). The cell extracts were separated by 7.5% SDS-PAGE or 17% 7.5% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Samples were incubated in blocking buffer [0.1%

Tween 20 and 5% nonfat milk powder in Tris-buffered saline (TBS)] for 1 h at room temperature. Afterward, the membrane was incubated with primary antibody in blocking buffer overnight at 4 ℃ before being washed twice with TBST (0.1% Tween in TBS) and incubated with the appropriate secondary antibody at the concentration of 1:3000 in blocking buffer for 1 h at room temperature. The blot was developed using ECL Western blotting substrate (Millipore) and analysed using a luminescent image analyzer (C-DiGit Blot Scanner, US). The primary antibodies used were the following;

- rabbit anti-NCOA3 antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-HER2 antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-ERK 1/2 (phospho-Thr202/Tyr204) antibody 1:1000 dilution (Abcam)
- rabbit Anti-AKT1 (phospho S473) antibody 1:1000 dilution (Abcam)
- rabbit anti-c-Myc antibody 1:1000 dilution (Cell signaling Technology)
- mouse anti- uPA antibody 1:1000 dilution (Abcam)
- mouse anti-FGF2 antibody 1:1000 dilution (Merck Millipore)
- mouse anti-BCL2 antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-MCL1 antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-PARP antibody 1:1000 dilution (Cell signaling Technology)

- rabbit anti-cleaved-PARP antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-p-SAPK/JNK antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-SAPK/JNK antibody 1:1000 dilution (Cell signaling Technology)
- rabbit anti-GADPH antibody 1:2000 dilution (Sigma-Aldrich)
- anti-rabbit IgG, HRP-linked antibody 1:2000 dilution (Cell signaling Technology)
- anti-mouse IgG, HRP-linked antibody 1:2000 dilution (Cell signaling Technology)

2.3.5 *In vivo* studies

2.3.5.1 Acute toxicity test

The zebrafish embryo toxicity test was performed according to the published Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TG236) for a Fish Embryo Toxicity (FET) test (237). For each experiment 20 fertilised eggs at the beginning of the epiboly stage (0.50 hours) were used. The selected eggs were exposed to 1,000 µL of 10, 20, 40, 80, and 100 µM ACA in 0.1% EtOH in E3/PTU medium. The samples were incubated at 28°C for 24, 48, 72, and 96 hours post fertilisation (hpf) and embryonic development was observed with Leica microscope after 24, 48, 72, and 96 hpf. The safety concentration from this experiment will be used for further *in vivo* antiproliferation of ACA. The information from this toxicity tests were used as a supportive data for the safety concentration for further *in vivo* experiment.

2.3.5.2 Zebrafish vessel staining

Dehydration of zebrafish

The zebrafish embryos were dechorionated and fixed at 72 and 96 hpf with 4% Paraformaldehyde (PFA) - store at 4^{o} C overnight. After that fixed embryos were rinsed with Phosphate-Buffered Saline/Tween-RNAse (PBST-RNAse) free for 5 mins twice by using a shaker. The embryos were then dehydrated with methanol (MeOH) in a 25% increase series. The dehydration steps starting with 25% MeOH/75% PBST-RNAase free for 3 minutes, 50% MeOH/50% PBST-RNAase free for 3 minutes, 75% MeOH/25% PBST-RNAse free for 3 minutes, and 100% MeOH for 3 minutes twice. After finish the dehydration process the embryos were kept in in 100% MeOH and stored at -20^{o} C for future vessel staining experiment as it can last for 3 months.

Rehydration of zebrafish

Before using the dehydrated embryos for performing vessel staining, the embryos were required to perform the rehydration by reverse steps of dehydration process. In the rehydration process the embryos were performed without the use of shaker.

Staining (alkaline phosphatase activity), RT

The embryos were rinsed with alkaline phosphatase (AP) buffer/0.1%Tween for 10 mins twice. Freshly prepare 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) staining mix (substrate of AP) by using NBT stock solution 1 µL, BCIP stock solution 3.5 µL and adjust with AP buffer/0.1%Tween to get 1 ml. Then the embryos were moved from 1.5 ml tube into 24 well-plate and removed AP buffer/0.1%Tween. Added 300 µL of NBT/BCIP staining mix into the embryos and incubated in the dark for 60 minutes and observed

every 15 minutes under microscope. After 60 minutes of incubation, PBST-RNAse free was added to stop the staining reaction after that images of the vessels were taken. The optimisation of staining time was validated before performing the experiment (additional information can be found in **Appendix E**). The results from this test will be taken into consideration further *in vivo* study of ACA.

2.3.5.3 Antiproliferation

The early-stage embryos (48 hpf) of *Danio Rerio* (Tubingen AB) were used in *in vivo* observation of growth changes in CM-Dil-labeled MCF7/LCC9 cell transplantation, and were immune insufficient to allow the growth of human cells. Before the injection process, zebrafish embryos needed to be selected for a particular stage followed by dechorionation. Then the embryos were anaesthetised using 0.4% tricaine and mounted on the agar for the nanoinjection. ACA was treated into zebrafish medium at 1 day post injection (dpi). The MCF7/LCC9 cell growth in zebrafish embryos was observed at 1 day post injection and 3 days post injection (238). CM-Dil-labeled MCF7/LCC9 cells injected in the yolk of zebrafish could be observed by fluorescence microscopy 1 dpi, and retained fluorescent-labeled MCF7/LCC9 cell for 3 days, providing an sufficient range for ACA screening (239). Quantification of tumour area (n = 23/group) was performed using image acquisition (LAS Va.12), quantitative image software.

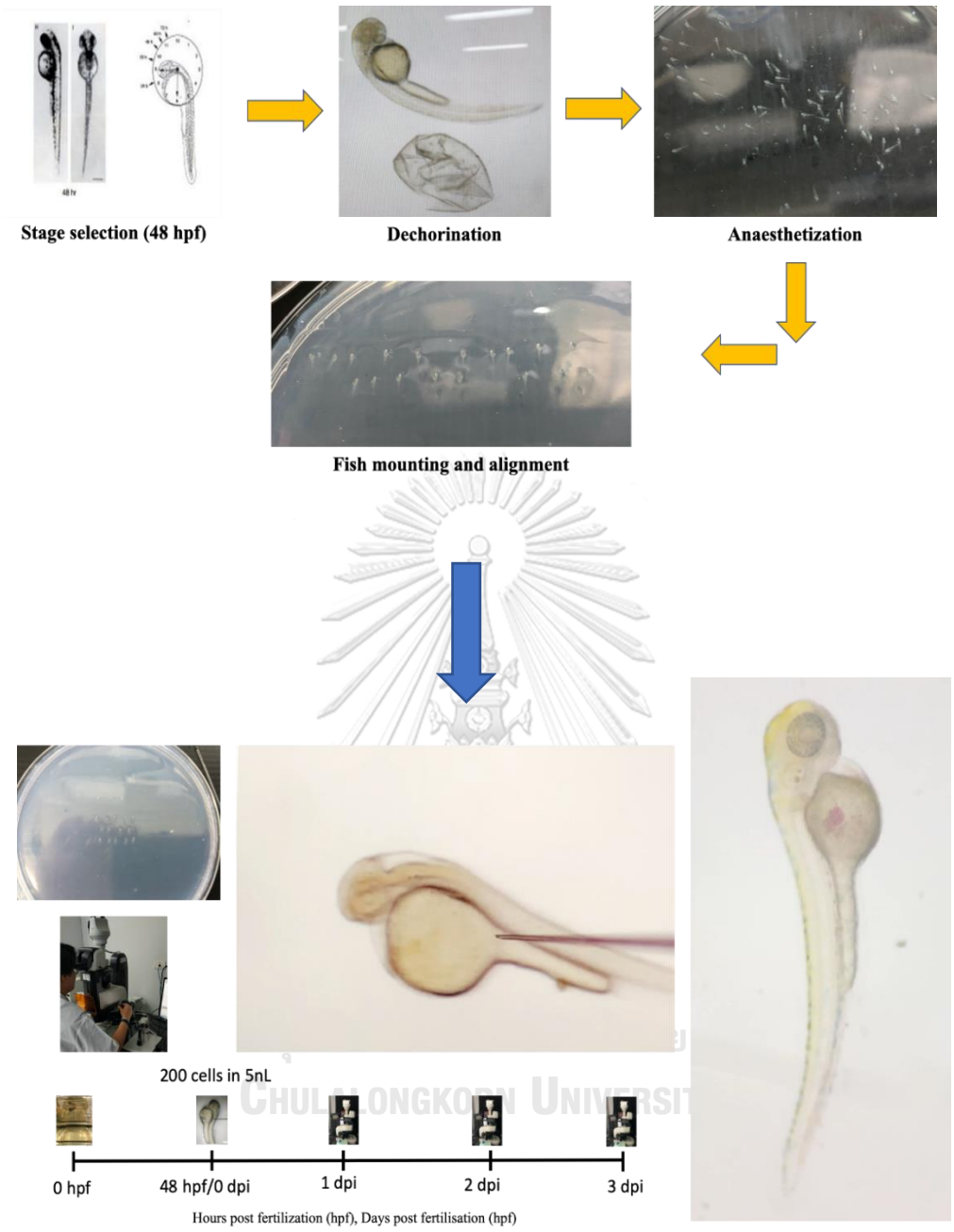


Figure 2.3.5.3.1. The processes of nanoinjection of MCF7/LCC9 labeled CM-dil into the yolk sac of zebrafish. The zebrafish embryos at 48 hpf were dechorinated and anaesthetized before the injection. The tumour areas of the injected zebrafishes were observed the tumour area everyday under fluorescent microscope for 3 days.

2.3.6 Molecular docking simulation

The molecular docking simulation was performed by Dr. Panupong Mahalapbutr. The crystal structures of HER2 (PDB ID: 3RCD (240)), AKT1 (PDB ID: 4GV1 (241)), ERK2 (PDB ID: 5NHJ (242)), ER (PDB ID: 3ERT (243)), uPA (PDB ID: 5YC7 (244)), CDK6 (PDB ID: 1XO2 (245)), and FGF2 (PDB ID: 1FQ9 (246)) were obtained from Protein Data Bank. The three-dimensional (3D) structure of all the studied ligands were built and fully optimised by the HF/6-31(d) level of theory using Gaussian09 program (247). The protein-ligand complexes were generated using CDOCKER module implemented in Accelrys Discovery Studio 2.5[Accelrys Inc.] (248) with 100 independent docking runs. Note that the co-crystalised inhibitors were defined as docking centre with a spherical radius of 15 Å.

2.3.7 Data and Statistical Analysis

Results were represented as mean and standard deviation. Data was analysed and graphed with GraphPad Prism, version 6.0. The *in vivo* study, G power programme was used for sample size calculation and tumour area and SIV length determination was analysed using image acquisition (LAS Va.12), quantitative image software. Significant differences between the mean values within the group were determined by using a Student's t test or one-way analysis of variance (ANOVA) followed by a Tukey's test for further comparison. The level of significant was set up at $p \leq 0.05$.

2.3.8 Ethical consideration

The human cell lines used in this study were reviewed and exempted by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB Number: 616/60). The animal ethic for zebrafish study was reviewed by Siriraj Animal Care and Use Committee (SiACUC), Faculty of Medicine, Siriraj Hospital (SI-ACUP 006/2559).

CHAPTER 3 STRUCTURAL ELUCIDATION OF ACA

3.1 Results: Structural elucidation of 1'-acetoxychavicol acetate

The purity of crude hexane extract was initially checked by spot thin layer chromatography which was comparable with the crude working standard of galangal extract (**Figure 3.1.1).** After the initial check with spot TLC, the crude hexane extract was further purified through column chromatography. Subsequently, the chromatographic fractions of the compound were evaluated the purity by using ^{1}H-NMR, ^{13}C -NMR, and mass spectroscopy and compared the spectra with published purified ACA.

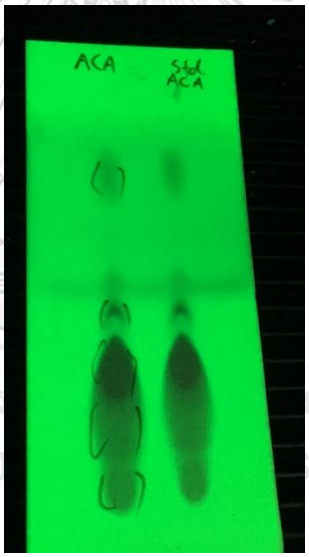


Figure 3.1.1. Spot thin layer chromatography of crude hexane extract. The figure showed crude hexane extract of ACA from the experiment (left lane) and the crude working standard (right lane)

The typical chemical shift values of organic compound in part per million (ppm) relative to tetramethylsilane was showed for emphasising proton in functional groups, which bolster the interpretation of the chemical shift values of ACA in ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum was interpreted into four mains information. Firstly, the number of groups of signals there were in the spectrum. Secondly, types of proton indicated as the chemical shift of each group. Thirdly, the number of protons of each group indicated as an integration which was the area under the peaks that proportioned with proton numbers. Finally, the coupling patterns which represented proton numbers of adjacent carbon that caused the signals to be split into "n+1" lines.

The ^{1}H NMR data (**Figure 3.1.2 - 3.1.3, Table 3.1.1)** showed 14 protons in the molecule. Two symmetrical doublets at 7.38 and 7.08 ppm were four protons on a para-substituted benzene ring. The proton resonances at 5.99 (1H, *m*), 5.38 (1H, *dd*, *J* = 16.2, 1.2 Hz), 6.23 (1H, *d*, *J* = 6.0 Hz), correspond to protons on 2’ and 3’ – olefinic carbons, respectively. The signal at 5.23 (1H, *d*, *J* = 10.2, 1.2 Hz) correspond to proton 1’ carbon. Two methyl signals of two acetyl groups were clearly due to the presence of two singlet signal at 2.27 (3H, *s*), 2.08 (3H, *s*).

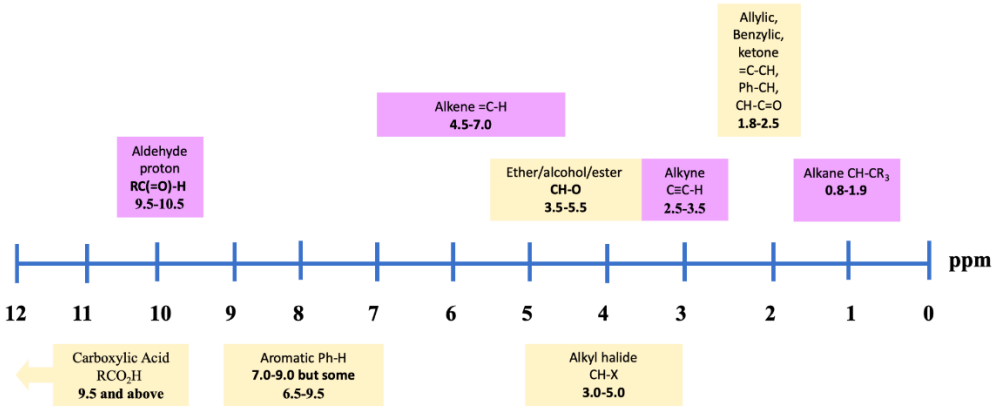


Figure 3.1.2. The typical chemical shift values of organic compounds (249). The diagram shows the chemical shift values of organic compounds in parts per million (ppm) relative to tetramethylsilane.

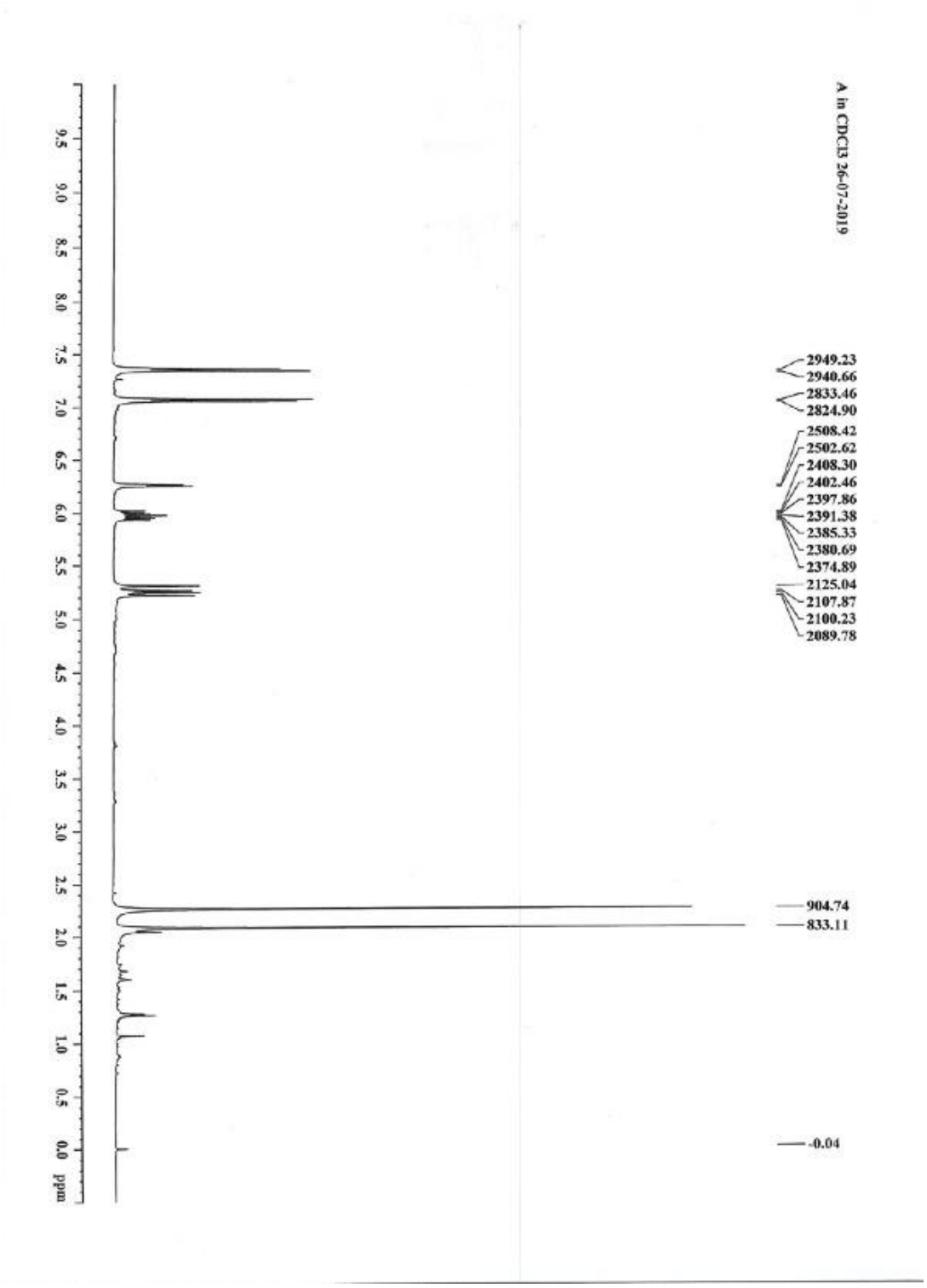


Figure 3.1.3. ^{1}H-NMR spectrum of ACA

The ^{13}C NMR **(Figure 3.1.4, Table 3.1.1)** displayed the ^{13}C signals. Among these, two methyl signals were clear due to the presence of chemical shift value of 20.8 and 20.9 ppm. Two carbon signals at 169.1 and 169.6 ppm were typically from carboxyl groups. Two parts of carbon signal have chemical shifts at 128.2 and 135.8 ppm, and two quaternary carbon signals at 136.2 and 150.4 confirming a para substituted benzene ring. The downfield sp2 carbons at δc 150.2 (C-1) indicated the presence of oxygen group attached to an aromatic carbon. The carbon signal with the chemical shift of 121.5 and 116.8 ppm should be assigned as sp2 carbons at 2' and 3', respectively. The downfield sp3 carbons at δc 75.3 (1'-C) indicated the presence of oxygen that attached to a carbon. Combination of the fragments mentioned above led to assignment of structure of compound ACA. Therefore compound A was identified as (1'S)-1'-acetoxychavichol acetate (250).

The high-resolution mass spectrum **(Figure 3.1.5)** showed a molecular ion peak [M+Na] at m/z 257.0786 corresponding to a molecular formula of $C_{13}H_{14}O_4$+Na. The identification of ACA structure was confirmed by the NMR and mass spectra with published articles of Azuma *et al*. (251). The structure of 1'-acetoxychavicol acetate and its appearance were showed in **Figure 3.1.6** (additional information can be found in **Appendix A**).

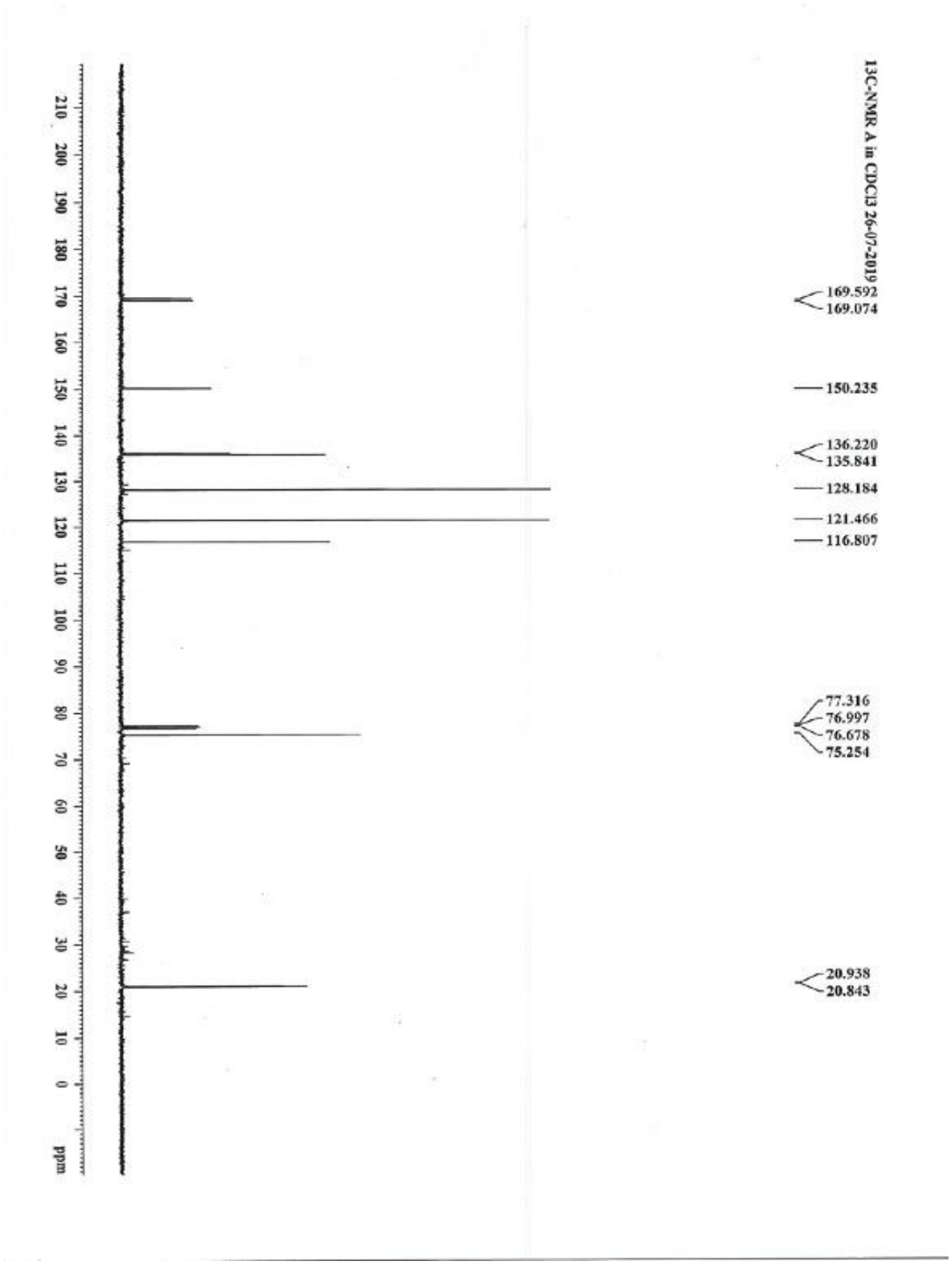


Figure 3.1.4. ^{13}C-NMR spectrum of ACA

Table 3.1.1. The ^{1}H and ^{13}C NMR spectra of 1’ acetoxychavichol acetate

Position	**1’ Acetoxychavichol acetate**	
	***δ*c, mult.[a]**	***δ*H, mult., *J* in Hz**
1	150.2, *s*	
2	128.2, *d*	7.08 *d*, (8.5)
3	135.8, *d*	7.38 *d*, (8.4)
4	136.2, *s*	
5	135.8, *d*	7.38 *d*, (8.4)
6	128.2, *d*	7.08 *d*, (8.5)
1’	75.3, *d*	5.23, *dd*, (10.2, 1.2)
2’	116.8, *d*	5.99, *m*
3’a	121.5, *d*	5.38, *dd*, (16.2, 1.2)
3’b		6.23, *d*, (6.0)
1’-COO	169.1, *s*	
-CH3	20.9, *q*	2.08, *s*
1-COO	169.6, *s*	
-CH3	20.8, *q*	2.27, *s*

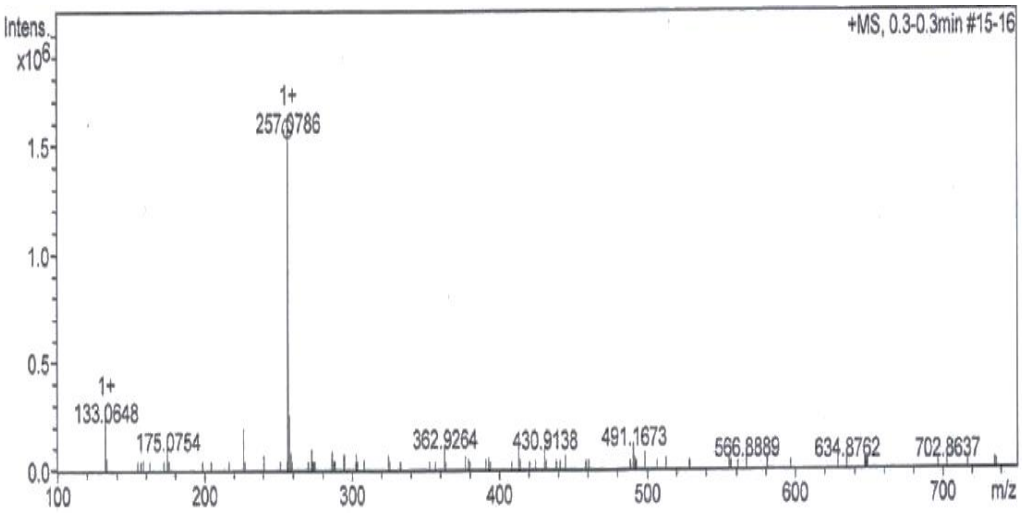
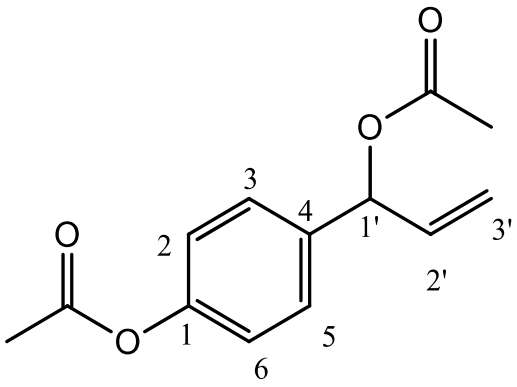


Figure 3.1.5. Mass spectrum of ACA. The figure shows a molecular ion peak [M+Na] at m/z 257.0786 corresponding to a molecular formula of $C_{13}H_{14}O_4$+Na.



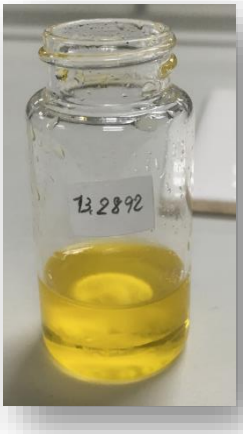


Figure 3.1.6. Structure of 1’-acetoxychavicol acetate and the yellow oil of purified 1’-acetoxychavicol acetate

3.2 Discussion

Purified ACA is a natural product that exerts anticancer effects by inducing apoptosis, inhibiting angiogenesis and metastasis (172, 174). This study performed multidisciplinary processes of crude extraction, purification, and identification. Plant identification was essential as the source and period to collect the plant associated with the quality of plant and the number of significant compounds as well as the species of this genus was similar to each other (233). Also, the parts of the plant (e.g. leaves, pseudostem, roots, rhizomes, and aerial parts) are the critical factor as different parts of the plant can be extracted different plant-derived compounds in terms of the amount and type of major compounds (233). Besides, appropriate solvents and extraction techniques are also crucial for the extraction of different parts of the plant (252). The organic solvents that commonly used for plant-derived compounds extraction are methanol, ethanol, ethyl acetate, hexane, dichloromethane, aqueous, chloroform, and petroleum ether (233).

In this study, hexane was used as a solvent for cold extraction as it was nonpolar and effective for oil extraction. In this step, the rhizomes were chopped and ground using mortar into tiny pieces for increasing the surface area of contacting with the solvent to bolster a more efficient extraction. The crude hexane extract of ACA was performed spotted on thin layer chromatography and compared with crude extract of ACA from Chulabhorn Reseasrch Institute, Thailand. Then the crude extract was passed through column chromatography to isolate the purified fraction of ACA. The purified ACA was analysed by multidisciplinary spectroscopy to identify proton numbers and positions, as well as carbon groups in the structure. High-performance mass spectroscopy was performed to investigate the molecular weight of the purified

ACA. The ^{1}H-NMR and ^{13}C-NMR spectra for ACA from this study were identical to the ^{1}H-NMR and ^{13}C-NMR spectra obtained from the working standard of Chulabhorn Research Institute, as well as spectra published by Azuma et al and Seo et al (160, 250, 251). Also, the high- performance mass spectroscopy spectrum demonstrated the molecular weight of purified ACA from this study as identical to the molecular weight of published ACA (253). Therefore, the recorded spectroscopy data confirmed that the pure compound was identical to 1'- acetoxychavicol acetate, using one-dimensional ^{1}H-NMR and ^{13}C-NMR spectroscopy followed by mass spectroscopy, compared to published ACA (250, 251, 253).

Identification of the chemical structure is essential as it is associated with the biological activity of such compound. Murakami and colleagues reported the SAR of ACA that the acetoxyl group at the para position and the acetoxyl group at 1' carbon position were compulsory for cytotoxic activity (254). This implied that the functional group at 1' position and the phenolic hydroxyl group needed to be acetylated. Also, the double bond at 2' carbon was essential for the activity (254). Different substitution groups of the compulsory groups affected the pharmacological activities of the compounds, for example, without the acetoxyl group at the para position and the acetoxyl group at 1' carbon position as well as different orientational isomer of phenolic group of ACA can affected cytotoxic activity (254).

ACA isolated in this study was structural identity with published 1'-acetoxychavicol acetate pure compound from the work of Murakami *et al* and Azuma *et al* (251, 254). The results also showed that ACA structure composed of type and orientation of substitution groups, and pharmacophore that was crucial for anticancer,

which were acetoxyl group at the para position and the acetoxyl group at 1’ carbon position as well as phenolic group at the para position. Also, the purity of ACA was more than 97% as shown by the recorded ^{1}H-NMR and ^{13}C-NMR spectra.

CHAPTER 4 *IN VITRO* ACTIVITY OF ACA ON BREAST CANCER CELL LINES

4.1 Results

4.1.1 Cytotoxic activity of ACA on breast cancer cells

Prior performing the cytotoxic effect of ACA treatment on breast cancer cells, the effect of vehicle control (0.1%EtOH in completed medium) on cell viability compared to untreated control (completed medium) was examine in MCF7, MCF7/LCC2, and MCF7/LCC9 cells at 24, 48, and 72 h **(Figure 4.1.1.1)**. The result demonstrated that the vehicle control was not cytotoxic to the cells which can safely be used for dissolving ACA to be used for the further studies.

The percentage viability of MCF7 **(Figure 4.1.1.2a)**, MCF7/LCC2 **(Figure 4.1.1.2b)**, and MCF7/LCC9 **(Figure 4.1.1.2c)** significantly decreased after treated with ACA at increasing concentration (0-60 μM) in the concentration- and time-dependent manners **(Table 4.1.1.1)**. The bright field images of MCF7, MCF7/LCC2, and MCF7/LCC9 after treatment with ACA for 48 hour has shown in **Figure 4.1.1.3**. The studied cell lines were treated for three different incubation time which were 24, 48, and 72 hours. There was a significant difference of IC_{50} value in each cell line. The IC_{50} value of ACA was higher in the endocrine-resistant cell lines (MCF7/LCC2 and MCF7/LCC9) than a wide-type breast cancer cell line (MCF7). Therefore, the concentration used for further experiments needed to be selected based on the concentration acquired from the viability assay. The IC_{50} value of ACA at 48-hour incubation time and its two-fold dilution of each cell line were selected and used for further *in vitro* experiments as the concentrations did not cause major cell death which

interfere the interpretation of the study results as further *in vitro* studies were treated ACA for 24 h treatment.

The positive controls in this study were chosen differently based on the standard treatment and sensitivity to the drug of a particular cell line. MCF7 was a tamoxifen sensitive cell line, and therefore, 4-hydroxy tamoxifen (4OH-TAM) was used as a positive control. MCF7/LCC2 and MCF7/LCC9 were no information reported the sensitivity to drugs; hence, the selection of positive control used for these two cell lines was selected using the information of known resistant-breast cancer cell lines. MCF7/LCC2 and MCF7/LCC9 had molecular characters similar to HER2-overexpressed cell lines such as SKBR3 and AU565 regarding ER, PR and HER2 (228, 255, 256). In addition, there was a report stated that the cyclin-dependent kinase inhibitor, palbociclib (Pal) was effective for metastatic breast cancer (45, 257-259). Therefore, Pal was selected and used as a positive control for MCF7/LCC2 and MCF7/LCC9.

Tamoxifen resistant test was also performed in MCF7, MCF7/LCC2 and MCF7/LCC9. This experiment aimed to confirm that the resistant cell lines had higher IC_{50} value compared to a wild-type MCF7. The result suggested that MCF7/LCC2 and MCF7/LCC9 was resistant to tamoxifen compared to MCF7 **(Figure 4.1.1.4)**.

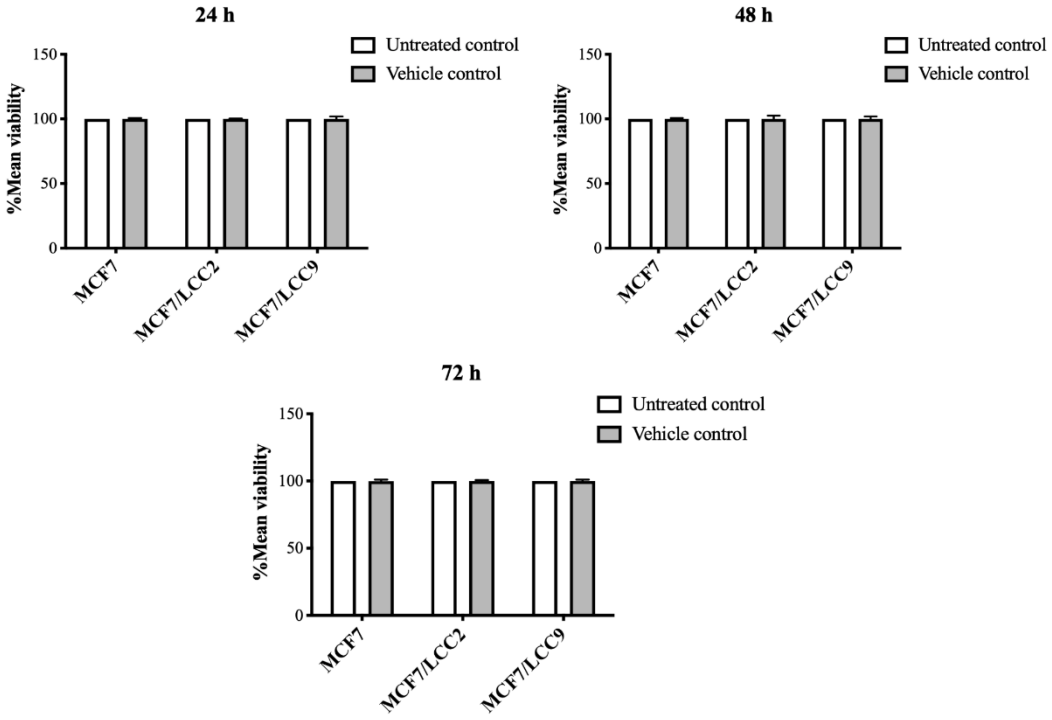


Figure 4.1.1.1. The effects of vehicle control on viability of untreated controls. The viability assay compared the effect of complete medium (untreated control) and 0.1%EtOH in complete medium (vehicle control) on MCF7, MCF7/LCC2, and MCF7/LCC9 cells at 24, 48, and 72 h. Data are represented as mean ± SEM of three independent experiments (n = 3) and each experiment was performed in triplicate.

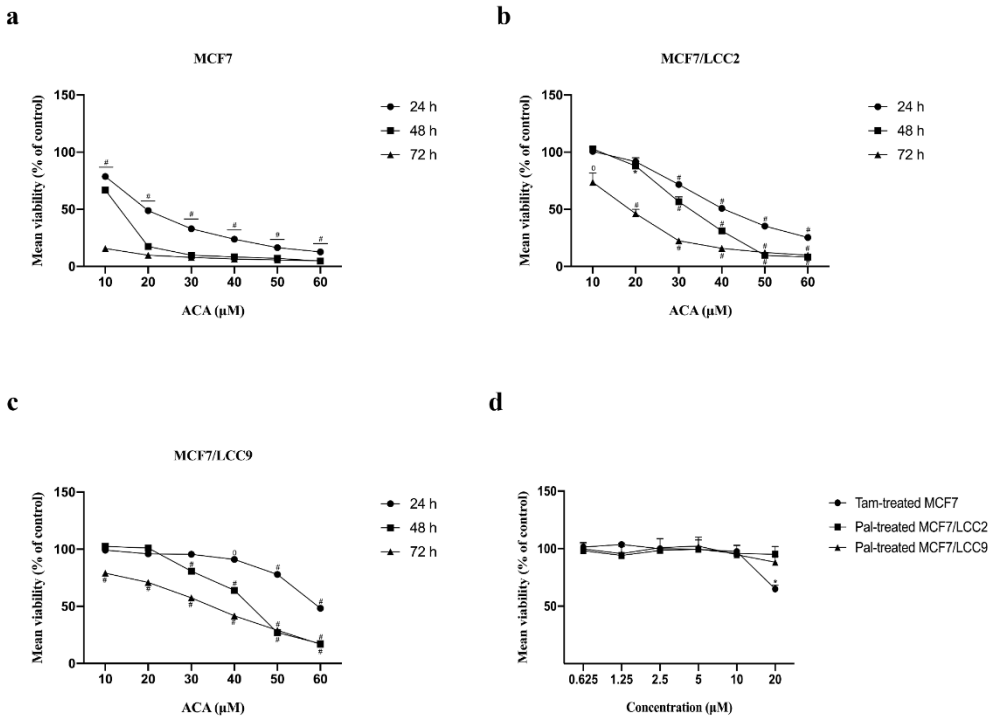


Figure 4.1.1.2. The viability assay of ACA. The percentage viability of MCF7 **(a)**, MCF7/LCC2 **(b)**, and MCF7/LCC9 cells **(c)** after treated with ACA at increasing concentration for 24, 48, and 72 h compared with vehicle control. The cell lines were treated with the positive controls for 48 h. A positive control used for MCF7 cell was 4-hydroxy tamoxifen (4OH-TAM) while palbociclib (Pal) was used for MCF7/LCC2 and MCF7/LCC9 **(d)**. 0.1%EtOH was used as a vehicle control for ACA and 4OH-TAM treatment, while 0.2%DMSO was used as a vehicle control for Pal treatment. Data are represented as mean ± SEM of three independent experiments (n = 3) and each experiment was performed in triplicate, $^{*}p \leq 0.05$, $^{0}p \leq 0.01$, $^{\#}p \leq 0.0001$ compared to the control.

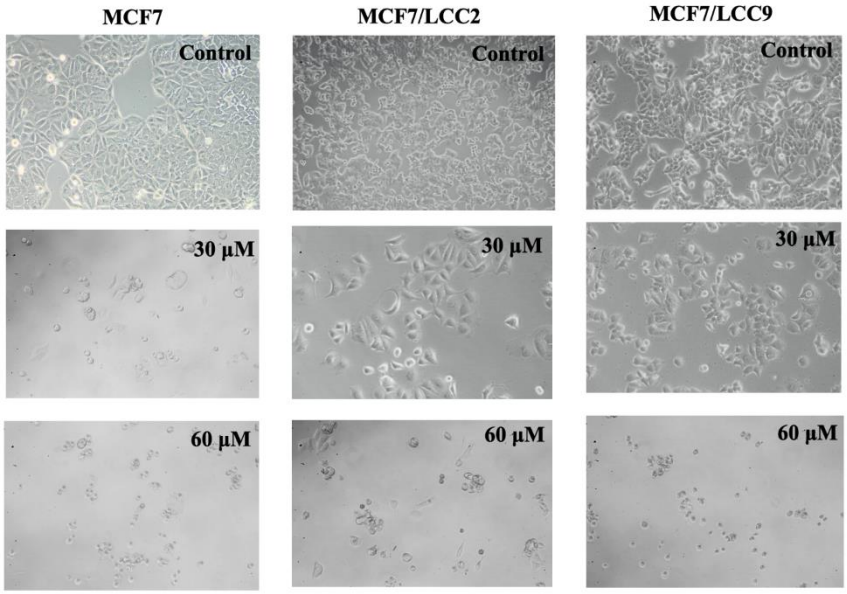


Figure 4.1.1.3. Bright field images of MCF7, MCF7/LCC2, and MCF7/LCC9 after treatment with ACA for 48 h.

Table 4.1.1.1. The comparison of IC_{50} values of ACA on MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines

Cell lines	**IC_{50} (24 h)**	**IC_{50} (48 h)**	**IC_{50} (72 h)**
MCF7	19.54 µM ± 1.24	11.78 µM ± 0.50*	5.01 µM ± 0.46$^{*, **}$
MCF7/LCC2	40.44 µM ± 1.84	31.46 µM ± 1.00*	18.62 µM ± 0.69$^{*, **}$
MCF7/LCC9	59.51 µM ± 2.04	41.38 µM ± 1.80*	34.56 µM ± 1.94$^{*, **}$

IC_{50} Values (µM) of ACA obtained by MTT assay after 24, 48, and 72 h of the treatment against MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines. Values shows as mean IC_{50} ± SEM, n = 3, $^{*}p \leq 0.05$ and $^{**}p \leq 0.05$ compared to each particular cell at 24 and 48 hours of incubation time, respectively.

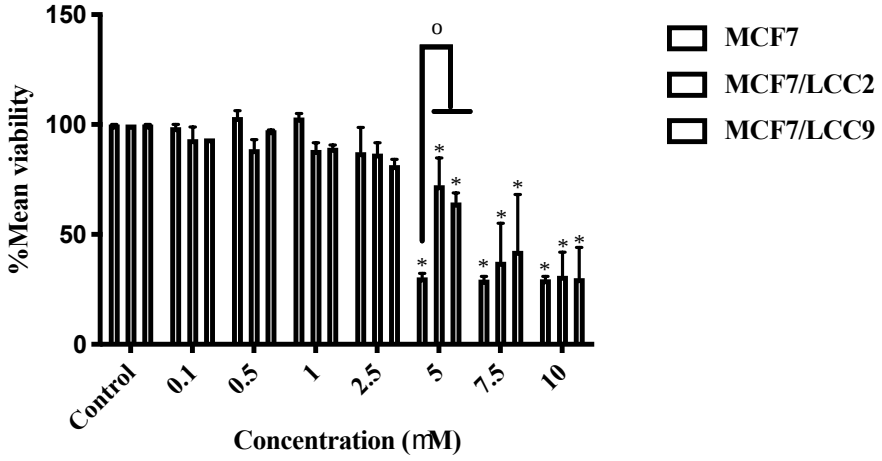


Figure 4.1.1.4. The effect of 4OH-Tam in MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines. Tamoxifen resistance was determined after treating cells with 4OH-Tam for 120 hours using MTT assay (mean ± SEM, n = 3). The medium used for this assay was improved MEM (Gibco, USA) supplemented with 2% Charcoal stripped FBS, 1% penicillin streptomycin, and 1% insulin. The IC_{50} values for MCF7, MCF7/LCC2, and MCF7/LCC9 cells were 3.13 μM ± 1.55, 5.71 μM ± 2.70, and 5.98 μM ± 2.19, respectively. 0.1%EtOH was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3) and each experiment was performed in triplicate, $*p < 0.05$ compared to control of each cell line and $^{o}p < 0.05$ compared to MCF7 cell at the same concentration.

4.1.2 The *in vitro* toxicity test of ACA on primary dermal fibroblast cell (PCS201-010)

ACA has treated the cytotoxicity with primary dermal fibroblast (PCS201-010) cell for 48 hours, and the result showed that ACA up to 80 μM did not significantly reduce the viability of the PCS201-010 cell **(Figure 4.1.2.1)**. Therefore, the selected concentrations of ACA for the further experiments were selected with regard of both *in vitro* toxicity test from this experiment and the IC_{50} of ACA on the previous *in vitro* viability test on studied breast cancer cell lines

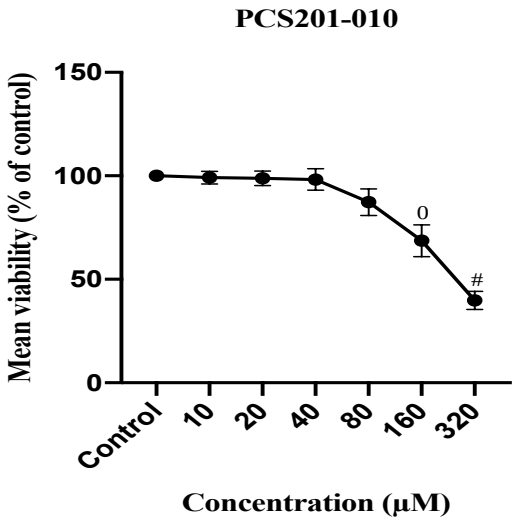


Figure 4.1.2.1. The *in vitro* toxicity test of ACA on PCS201-010. The percentage viability of primary dermal fibroblast (PCS201-010) cells was measured after treatment with ACA at increasing concentration for 48 h. The concentration of ACA up to 80 μM caused no significant cytotoxicity to PCS201-010. 0.1%EtOH was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3) and each experiment was performed in triplicate, $^{0}p \leq 0.01$ and $^{\#}p \leq 0.0001$ compared to the control.

4.1.3 Antiproliferative activity of ACA in endocrine-resistant breast cancer cells

HER2 receptor is a receptor of tyrosine kinase which found to express in breast cancer approximately 20 – 30% (33). Current drugs treatment for breast cancer expressed HER2 receptor are trastuzumab mechanistic work by blocking HER2 receptor and lapatinib wherein blocking the tyrosine kinase domain inside the cytoplasm (260). However, the responsiveness of breast cancer cell is not preferable (261). This study showed the basal level of the studied proteins namely, HER2, NCOA3, AKT, c-Myc, uPA, ERK1/2, and FGF2. The result demonstrated the higher protein expression trend in endocrine-resistant cell lines compared to a wild-type MCF7 cell line **(Figure 4.1.3.1)**. ACA showed the potential to downregulate HER2 receptor in all studied cell line. Albeit, ACA seems to have better effects on HER2 downregulation in tamoxifen-sensitive breast cancer MCF7 cell **(Figure 4.1.3.2a)** than those tamoxifen-resistant breast cancer MCF7/LCC2 **(Figure 4.1.3.2b)** and tamoxifen-fulvestrant resistant breast cancer MCF7/LCC9 cell **(Figure 4.1.3.2c).** These results can be interpreted that ACA has a preferable function on breast cancer resistant cell lines than the tamoxifen-sensitive breast cancer cells. It was confirmed by the statistical analysis from the results wherein ACA significantly reduced the expression of the HER2 protein level much more efficient in MCF7/LCC9 starting at IC_{25} value. While in MCF7/LCC2 and MCF7, ACA can only downregulate HER2 expression at the highest concentration (IC_{50} value) used in the study.

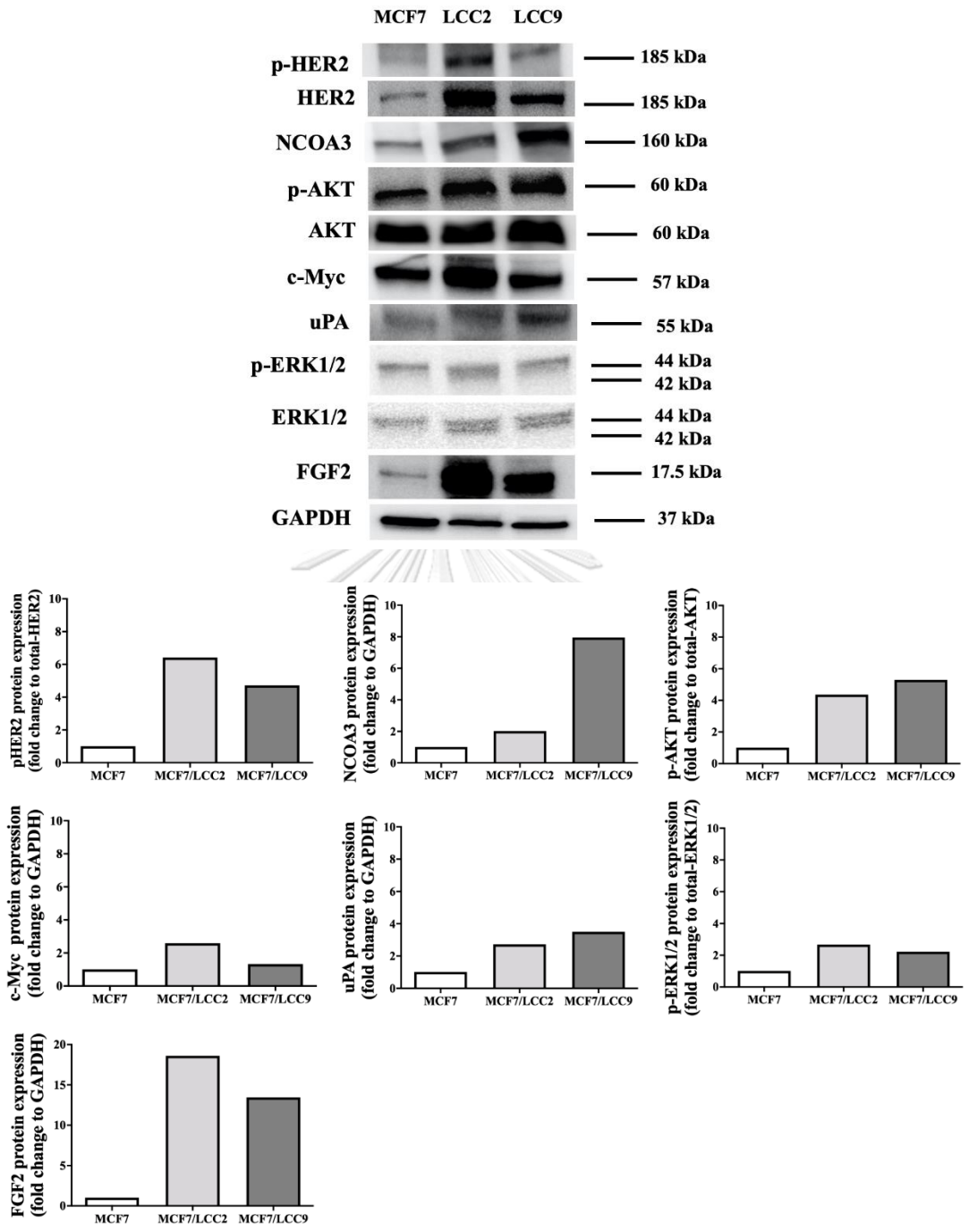


Figure 4.1.3.1. Basal level of studied proteins in MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines. The protein bands in western blots and bar charts of densitometry, showed the baseline expression levels of 7 studied proteins namely, HER2, NCOA3, AKT, c-Myc, uPA, ERK1/2, and FGF2 in MCF7, MCF7/LCC2, and MCF7/LCC9.

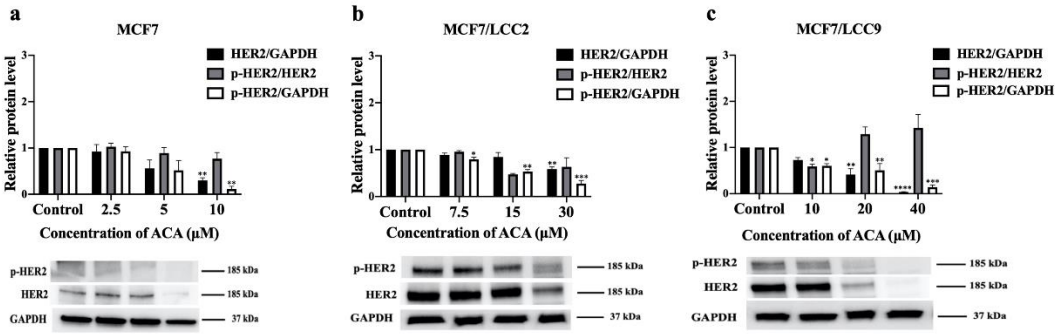


Figure 4.1.3.2. The effects of ACA on pHER2 and HER2 protein expressions on MCF7, MCF7/LCC2 and MCF7/LCC9 cell lines. The bar chart shows the expression level of pHER2 quantified with GAPDH, pHER quantified with total-HER2, and total form of HER2 quantified with GAPDH. ACA downregulated HER2 protein expression in ER positive tamoxifen sensitive MCF7 cell **(a)** and endocrine-resistant MCF7/LCC2 **(b)** and MCF7/LCC9 **(c)** cell lines at increasing concentrations after 24 hours of ACA treatment. Data are represented as mean ± SEM of three independent experiments (n = 3), $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ compared to vehicle control.

Signalling cascades through PI3K/AKT and ERK1/2 also play an essential role in breast cancer progression and these molecules are downstream pivotal molecules of HER2 signalling (262). Also, both PI3K/AKT and ERK1/2 were involved in the phosphorylation of NCOA3 which was important in the proliferation and survival of breast cancer cell (39). Hyperactivation and overexpression of ERK1/2 and AKT were found in tamoxifen-resistant breast cancer cells (263). Overexpression and activation of growth factor receptors, such as EGFR, HER2 and IGF1R, drive the proliferation and survival through activation of MAPK and PI3K/AKT signalling pathways in endocrine-resistant breast cancer (37). Overexpression of constitutively active AKT in breast cancer cell lines can induce oestrogen independence and resistance to tamoxifen and fulvestrant (71), while inhibition of PI3K or AKT restores tamoxifen sensitivity (72).

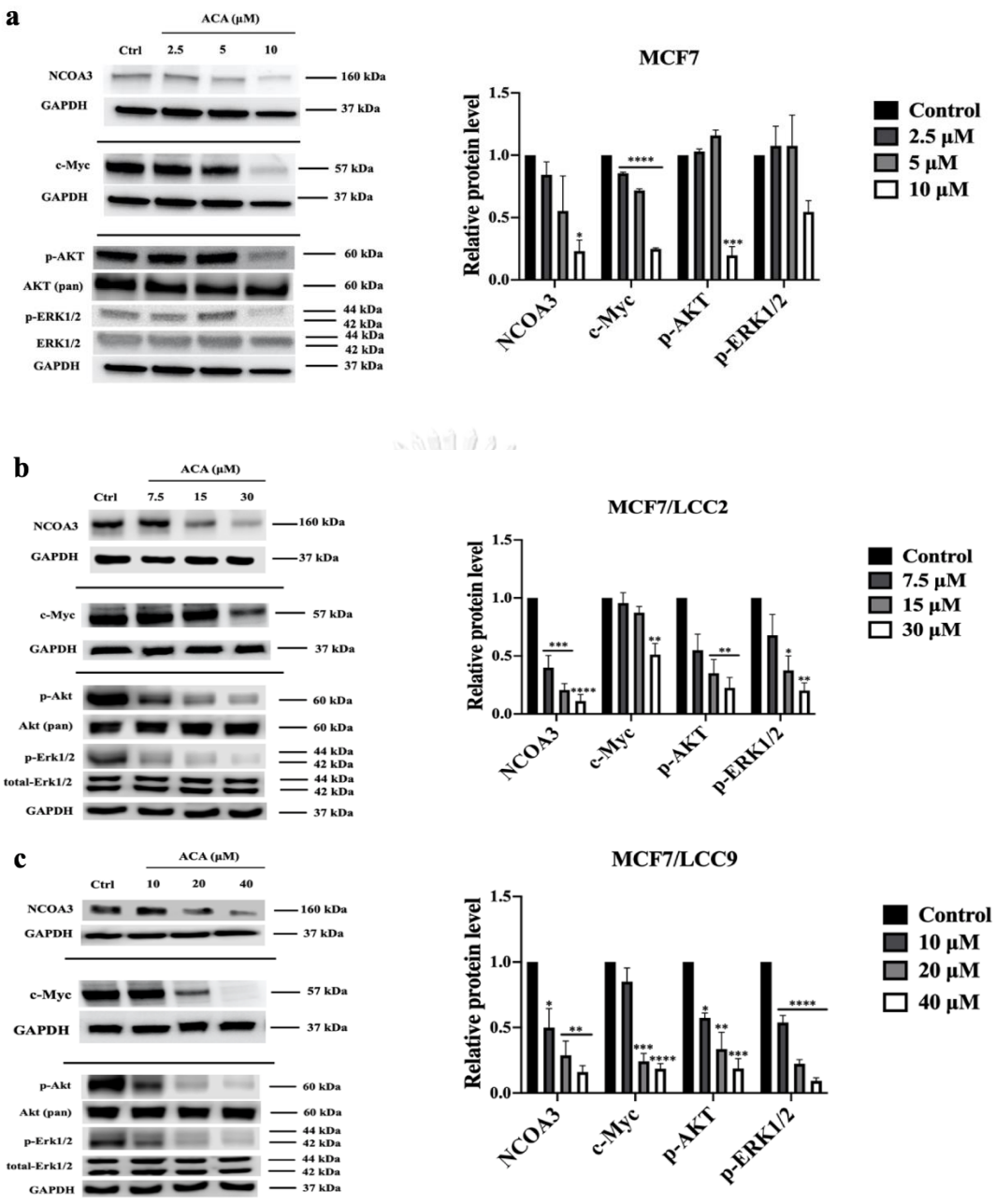
The results of this study demonstrated that ACA showed less effects on the downregulation of pAKT and pERK1/2 on ER positive tamoxifen- sensitive MCF7. As ACA can only inhibit the expression of pAKT at the highest concentration (10 μM) used for the treatment. Yet, there were no significant differences in the expression of pERK1/2 in ACA-treated MCF7 compared to control **(Figure 4.1.3.3a)**. While ACA effect on MCF/LCC2 at the concentration of 7.5 – 30 μM has shown the repression of pERK1/2 and pAKT **(Figure 4.1.3.3b)**. Similarly, ACA at the concentration of 10 – 40 μM has significantly down-regulated pERK1/2 and pAKT in MCF7/LCC9 **(Figure 4.1.3.3c).**

NCOA3 has been revealed to be overexpressed in breast cancers and pivotally involved in oestrogen-mediated cancer cell proliferation (264). In this experiment, NCOA3 mRNA level was significantly reduced in ACA-treated MCF7/LCC2 and

MCF7/LCC9 cells compared to the control. In wild-type ER positive tamoxifen sensitive MCF7 cell, ACA can significantly downregulate NCOA3 protein only at the highest concentration **(Figure 4.1.3.3a)**. The NCOA3 protein expression was also significantly repressed in ACA-treated endocrine-resistant breast cancer MCF7/LCC2 and MCF7/LCC9 cells as a dose-dependent manner **(Figure 4.1.3.3b-c)**. The protein results in MCF7/LCC2 and MCF7/LCC9 cell lines were correlated with the gene results. This study showed that potentiality of ACA to inhibit the expression of NCOA3 was more potent in hormonal-resistant breast cancer cells than hormonal-sensitive cell.

This study highlighted that the effect of ACA on the inhibition of NCOA3 could relate with the abolishment of ERK pathway since NCOA3 is phosphorylated by ERK. Consequently, the transcription of ER-targeted genes is terminated. More importantly, this study suggested that the effects of ACA on breast cancer cells growth functioned through the inhibiting of AKT and ERK1/2 signalling pathways which was essential mechanisms of anti-oestrogen resistant breast cancer.

Correlatedly, the protein analysis of ACA treatment showed that ACA potentially downregulated c-Myc protein in ACA-treated MCF7 (**Figure 4.1.3.3a**), ACA-treated MCF7/LCC2 and MCF7/LCC9 cells (**Figure 4.1.3.3b - c**).


a
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GAPDH
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GAPDH
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MCF7
Relative protein level
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p-ERK1/2
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Ctrl
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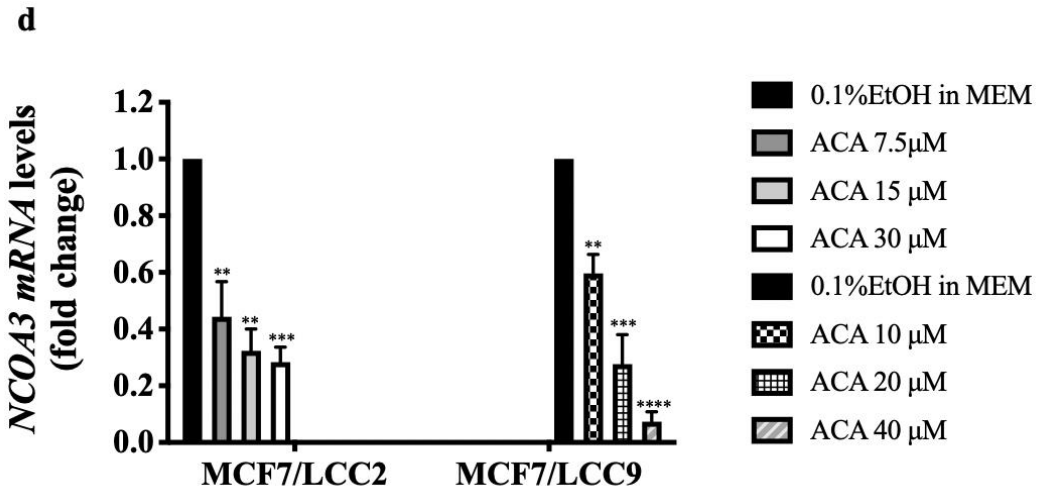

Figure 4.1.3.3. The effect of ACA on NCOA3, c-Myc, pAKT, and pERK1/2 protein expression in MCF7, MCF7/LCC2 and MCF7/LCC9 cell lines and mRNA level of NCOA3 in MCF7/LCC2 and MCF7/LCC9 cell lines. The bar chart represents the expression levels of pAKT relative to the total AKT protein and pERK1/2 relative with the total ERK1/2 protein. NCOA3 and c-Myc protein levels are expressed relative to GAPDH. ACA down-regulated pAKT and pERK1/2, NCOA3, and c-Myc protein expression in MCF7 (a), MCF7/LCC2 (b) and MCF7/LCC9 (c) cell lines at increasing concentrations after treatment for 24 h. NCOA3 mRNA expression (relative to GAPDH expression) in MCF7/LCC2 and MCF7/LCC9 cell lines (d) was also significantly downregulated in ACA-treated groups compared to vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3), $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ compared to vehicle control.

4.1.4 Inhibitory effects of ACA on the expression of NFκB targeted genes involved in cells migration/invasion and angiogenesis in endocrine-resistant breast cancer cells

NFκB plays a significant role in cancer progression , and especially it is a key associated between inflammation and cancer (265). There was also crosstalk between NFκB and ER signalling in endocrine-resistant breast cancer cells (189). The phosphorylation of IKKα can cause by the activation of PI3K signalling, resulting in both ER and NCOA3 increased its transcriptional activity and ultimately increased cell cycle progression (189).

In both ACA-treated MCF7/LCC2 and MCF7/LCC9 cells showed the repression of the expression of NFκB targeted genes including, genes that responsible for cell proliferation; *CCND1* and *c-myc* in all three concentration ($IC_{12.5}$, IC_{25}, and IC_{50}) used in the experiment **(Figure 4.1.4.1a - c)**. For invasive factors; *CXCR4* and *uPA*, ACA showed that it was more effective in MCF7/LCC2 than MCF7/LCC9 cell lines as the concentration than significantly downregulated *CXCR4* and *uPA* was seen at the IC_{25} value **(Figure 4.1.4.1c - d)**. Regarding angiogenic factors, ACA can repress the expression of *VEGF* at the IC_{50} value. On the other hand, the expression of *FGF2* was downregulated by ACA treatment at all three concentration in MCF7/LCC2 and at IC_{25}, and IC_{50} in MCF7/LCC9 **(Figure 4.1.4.1e - f)**. Additionally, ACA can significantly downregulate uPA and FGF2 proteins in MCF7 cell line at the IC_{50} (**Figure 4.1.4.2a**). While, ACA can repress the expression of uPA and FGF2 proteins in MCF7/LCC2 and MCF7/LCC9 cells at the concentration of IC_{25} and IC_{50} (**Figure 4.1.4.2b - c**).

This study revealed that ACA can inhibit the expression of genes involved in survival, metastasis/invasion, and angiogenesis of endocrine-resistant breast cancer cells through the NFκB regulated genes.

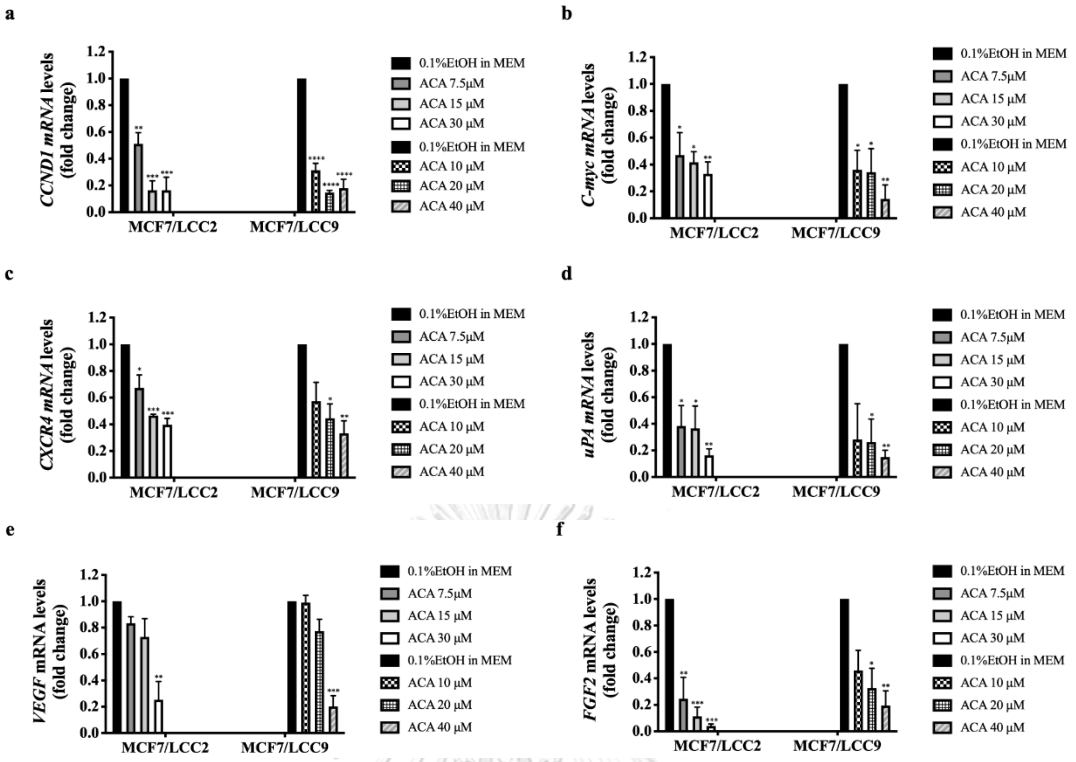

Figure 4.1.4.1. The inhibitory effects of ACA on down-regulation of *CCND1*, *C-myc*, *CXCR4*, *uPA*, *VEGF*, and *FGF2* mRNA expressions in MCF7/LCC2 and MCF7/LCC9 cell lines. The bar chart represents the expression of mRNA levels of studied genes quantified with GAPDH. ACA can significantly down-regulated *CCND1* **(a)**, *C-myc* **(b)**, *CXCR4* **(c)**, *uPA* **(d)**, *VEGF* **(e)**, and *FGF2* **(f)** mRNA expressions (as measured using real-time qPCR) on MCF7/LCC2 and MCF7/LCC9 cell lines at increasing concentrations after the treatment for 24 hours. Data are represented as mean ± SEM of three independent experiments (n = 3), $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$ compared to vehicle control.

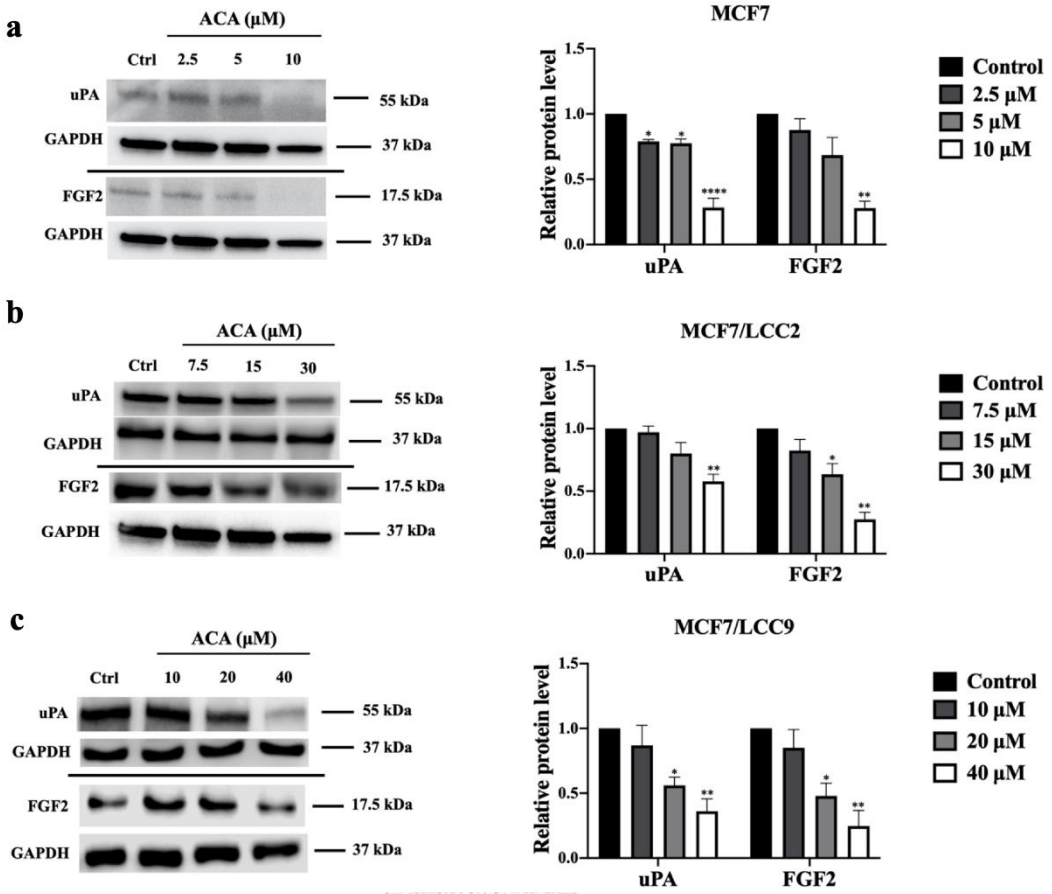

Figure 4.1.4.2. The effects of ACA on uPA and FGF2 protein expressions in MCF7, MCF7/LCC2 and MCF7/LCC9 cell lines. The bar chart represents the expression of uPA and FGF2 protein levels quantified with GAPDH. ACA downregulated the expression of uPA and FGF2 in MCF7 **(a)**, MCF7/LCC2 **(b),** and MCF7/LCC9 **(c)** cell lines at increasing concentrations after the treatment for 24 h with ACA treatment. Data are represented as mean ± SEM of three independent experiments (n = 3), *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$ compared to vehicle control.

4.1.5 Anti-invasive activity of ACA on endocrine-resistant breast cancer cells

The functional assay of ACA on cell invasion was performed using a matrigel invasion assay. The selected cell lines for this essay were MCF7/LCC2 and MCF7/LCC9 as these two cell lines were more aggressive invasion than the wild-type MCF7 cell (255).

This study suggested that ACA significantly inhibited cell invasion in endocrine-resistant breast cancer (**Figure 4.1.5.1a - b)** at non-toxic concentration. This pharmacological invasion assay showed that ACA inhibited invasion of MCF7/LCC2 at 7.5 μM and 15 μM, and in MCF7/LCC9 cell line ACA inhibited cell invasion at 10 μM and 20 μM. This implying that ACA can be a promised substance for inhibition of the cancer cell invasion in aggressive types of breast cancer.

However, *in vivo* study of ACA on antimetastasis and angiogenesis are still required to support ACA as an adjuvant treatment for breast cancer resistant to endocrine treatment in order to augment efficacy and reduce adverse drug reaction.

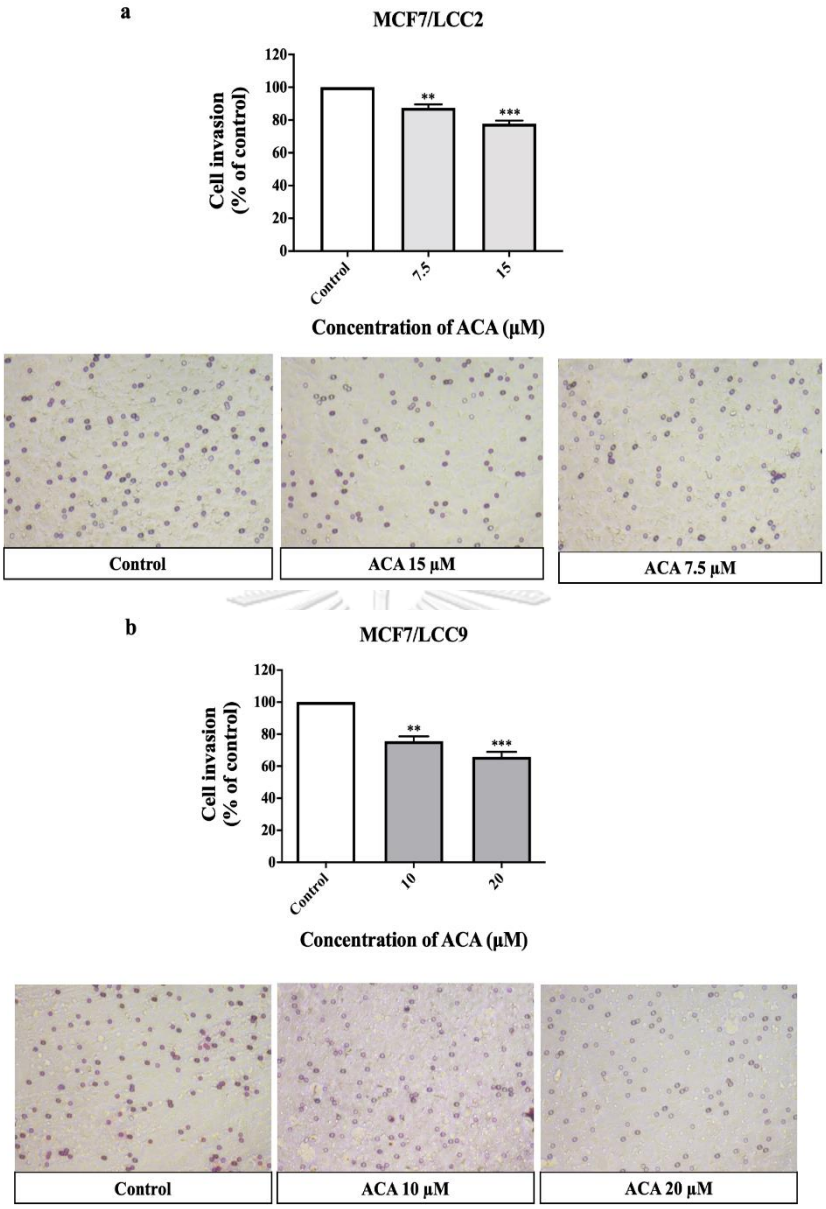

Figure 4.1.5.1. The anti-invasive activity of ACA in MCF7/LCC2 and MCF7/LCC9 cell lines. The invasion assay showed that the relative of cell invasion (%) of MCF7/LCC2 and MCF7/LCC9 **(a, b)** cell lines treated with non-toxic concentration of ACA for 24 hours was significantly lower than the control. 0.1%EtOH was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3), $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ compared to vehicle control.

4.1.6 Shift towards induction of apoptosis

The loss of expression and activation of stress-activated kinases (SAPKs) or c-Jun N-terminal kinases (JNKs) was reported to be involved in various cancers including breast cancer (266). To investigate the induction of apoptosis, protein expression of SAPK/JNK and anti-apoptosis proteins were studied. The three studied cell lines were treated with various concentrations of ACA for 24 hours. Interestingly, while ACA-treated MCF7 showed no significant changes in the expression of neither phospho-SAPK/JNK nor SAPK/JNK compared to non-treated MCF7, ACA treatment of MCF7/LCC2 and MCF7/LCC9 cell lines resulted in upregulation of phospho-SAPK/JNK and SAPK/JNK expression up to tenfold compared to the control. To further evaluate the induction of apoptosis, the expression of Mcl-1 and Bcl-2 were also analysed. ACA exert the ability to downregulate the anti-apoptotic protein Mcl-1 and Bcl-2 in all three studied cell lines. In addition, ACA exerted the apoptotic induction activity via cleavage of PARP and increase the expression of the cleaved form in all studied cells (**Figure 4.1.6.1)**.

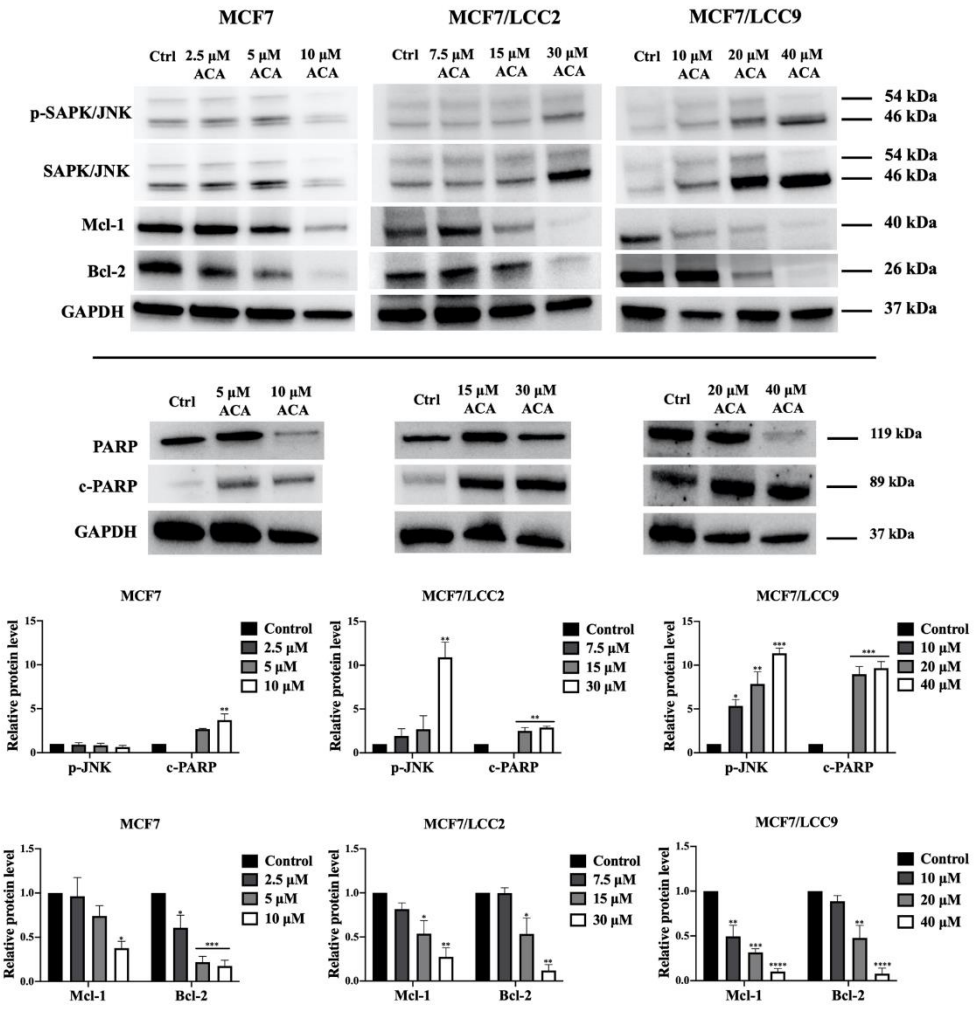

Figure 4.1.6.1. The effects of ACA on apoptotic induction. The bar chart represents the expression of SAPK/JNK, cleaved PARP, Mcl-1, and Bcl-2 protein levels quantified with GAPDH. ACA induced phospho-SAPK/JNK and cleaved PARP protein expression in MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines, while Mcl-1 and Bcl-2 were downregulated in all studied cell lines. The studied cell lines were treated with ACA at increasing concentrations for 24 hours. 0.1%EtOH was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3), *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ compared to untreated control.

4.2 Discussion

In the viability study, ACA exerted the growth inhibition of MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines in 3-time points (24, 48, 72 hours) at increasing concentrations and the IC_{50} values of each particular time point was calculated. From the comparison of IC_{50} value of each incubation time, ACA showed cytotoxic activity as a dose dependent manner. Besides, when compare the IC_{50} value of all three cell lines at 3-time points. The result showed that there was a significant difference between the IC_{50} value of each time point of the same cell line. To be more precise, at 24, 48, and 72 hours of ACA treatment, IC_{50} value of MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines were significantly different. This implied that ACA exhibited cytotoxic activity as a time-dependent manner. Also, the results showed that ACA had different potency for each cell line. In the *in vitro* viability test, there were two positive controls have been used for the study. 4OH-Tam was used as a positive control for MCF7 cell while palbociclib was used for MCF7/LCC2 and MCF7/LCC9. MCF7/LCC2 and MCF7/LCC9 cell lines were resistant to hormonal treatment in which palbociclib was recommended for clinically used as combination with endocrine therapy in ER positive advanced breast cancers (236). The cells were also treated with its particular positive control for 48 hours at the concentration up to 20 μM. The result showed that in MCF7 cell, 4OH-Tam significantly inhibited the growth of MCF7 cell at the concentration of 20 μM which was very high concentration. The resistant test of 4OH-tam was performed using MTT assay, and the result showed that MCF7/LCC2 and MCF7/LCC9 had the IC_{50} value two times higher than the wild-type MCF7. In this resistant test, the incubation time used for 4OH-tam was 120 hours. Tamoxifen generally has cytostatic action rather than

cytotoxic action (267). Tamoxifen was reported to competitively bind to ER and block the molecular signalling of ER that drives the transcriptional activity of ER-regulated genes, thereby promoting breast cancer cell survival (268). Therefore, the appropriate incubation time for cytostatic action of tamoxifen was longer than that for the chemotherapeutic agents that possessed cytotoxic activity to cancer cells which showed pharmacological activity within 24 hours. Palbociclib-treatment of MCF7/LCC2 and MCF7/LCC9, for 48 hours showed that the IC50 values were greater than 20 μM. These results imply that the endocrine-resistant MCF7/LCC2 and MCF7/LCC9 cells were possibly resistant to the CDK4/6 inhibitor palbociclib. This resistance of MCF7/LCC2 and MCF7/LCC9 cells to palbociclib treatment could be explained that these ER-positive cells showed higher baseline level of expression of various molecules involved in survival pathways such as HER, NCOA3, AKT, ERK1/2, and c-Myc. Therefore, treatment with CDK4/6 inhibitor palbociclib alone may not provide effective cytotoxicity to the cells. Additionally, recent evidence has identified that ER positive breast cancers could develop resistance to CDK4/6 inhibitors when used in a long-term treatment, as an adaptive response via the mutation of Rb protein, which in turn alters the growth signalling pathway of CDK4/6, as well as the activation of AKT pathway (236). Also, the long-term treatment with CDK4/6 inhibitors can promote acquired resistant of ER positive breast cancer to CDK4/6 inhibitors (236, 269).

In this thesis, the tamoxifen sensitivity test was performed in MCF7, MCF7/LCC2, and MCF7/LCC9 cells. The results showed that the ER-positive MCF7/LCC2 and MCF7/LCC9 cells had approximately two times higher IC_{50} values compared to a parent MCF7 cell. This result indicated that ER-positive MCF7/LCC2

and MCF7/LCC9 cells were tolerant to tamoxifen treatment. Also, this study confirmed the phenotypes of the all 3 studied cell lines by demonstrating the baseline levels of the proteins (HER2, NCOA3, AKT, ERK1/2, c-Myc, uPA, and FGF2) that are involved in the proliferative-, invasive-, and angiogenic-phenotypes of the resistant cells, compared to the control parental MCF7 cell.

Chemotherapeutic agents used for cancer therapy commonly cause adverse drug reactions to normal cells; for example, doxorubicin-induced cutaneous cytotoxicity (270). Common adverse drug reactions associated with cancer chemotherapy include prolonged QT interval, papulopustular rash, nail disorders and a hand-foot skin reactions (271). In addition, off-target skin reactions have been indicated for several chemotherapeutic drug classes (e.g. alkylating agents, antimetabolites, and antitumor antibiotics) with a broad range of cutaneous manifestations (272-276). Other common cutaneous adverse effects include anagen effluvium, xerosis, thrombophlebitis, generalised pruritus, melanonychia, hand-foot syndrome, and extravasation reactions etc (276, 277). Therefore, the primary dermal fibroblast (PCS201-010) cell line was selected as a model of normal skin cells for the *in vitro* toxicity test of ACA. The *in vitro* toxicity test of ACA was tested using PCS201-010 cell and the result after ACA treatment for 48 hours showed that ACA up to 80 μM was not toxic to the normal cell. Additionally, ACA at 160 μM had approximately 40% cytotoxicity to PCS201-010. This result was to emphasise that ACA was not cytotoxic to the normal connective tissues, which was important in terms of the application of ACA as a future promised adjuvant therapy in the clinic. The toxicity test of ACA on MCF10A cells, a breast epithelial cell line could have been used as an additional control cell line to confirm the safety of ACA on a

nontumourigenic breast cells. MCF10A human mammary epithelial cell is commonly used for studying normal breast cancer function and transformation (278). The phenotype of MCF10A in 3D culture was positive for basal markers and the milk proteins β-casein and α-lactalbumin, which rarely found in normal human breast tissue (278). Therefore, the suitability of using MCF10A as a model for studying normal breast cancer is still questioned (278). Also, culturing MCF10A requires 100 ng/ml cholera toxin to stimulate cell growth (279) , and this toxin is prohibited from entering Thailand. These issues hampered the use of MCF10A as a toxicity control cell line.

Resistance of breast cancer can be develop either in the beginning of the endocrine therapy (intrinsic resistance) and/or during the treatment (acquired resistance) (280). There are several molecular mechanisms of ER-positive breast cancer to develop resistant to endocrine therapy such as the genetic variation of cytochrome P450 isozymes, the upregulation of ER coactivator (e.g. NCOA3), upregulation of cyclins (e.g. cyclin D and E), the overexpression and hyperactivation of PI3K/AKT and ERK1/2, and the activation of NFκB (39, 263, 281). Several studies have revealed that the growth factors and its signallings play pivotal roles in breast cancer resistance (282). The upregulation of HER2 or EGFR was confirmed to affect the expression of ERα in the cell line-based study, and this occurred clinically relevant with the responsiveness of ER-positive breast cancer for endocrine treatment (283, 284). Also, the high expression of NCOA3 was found to contribute to the agonistic action of tamoxifen to ER (285). Therefore, triggering the classical and non-classical genomic pathways of ER signallings therein lied tamoxifen resistance of ER-positive breast cancer (285). One of the important survival pathways was the

PI3K/AKT pathway, the downstream PI3K can be driven by MEK, ERK, and/or JNK (68). This pathway can regulate genes which involved in cell survival and notably crosstalk with ERα activation and signalling (68). Cyclin D1 was reported to promote the resistance of breast cancer via promoting the high proliferation of cancer cell; moreover, amplification of cyclin D1 caused a three-fold higher risk of death in ER-positive breast cancer patient (286). This study has determined the basal level of significant proteins in which related to endocrine-resistant breast cancer. It was confirmed that the resistant cell lines used for the study which were tamoxifen-resistant MCF7/LCC2 cell and tamoxifen/fulvestrant-resistant MCF7/LCC9 expressed higher basal level expression of HER2, NCOA3, AKT, c-Myc, uPA, ERK1/2, and FGF2 proteins compared to the wild-type ER-positive tamoxifen-sensitive MCF7 cell line. MCF7 cell line was characterised molecular subtypes as a luminal A subtype (287). However, there was controversy on the expression HER2, which either HER2 negative or very low-grade expression of HER2 (287-289). In addition, this study suggested that ACA could have a potential to inhibit key signallings, which involved in the resistant cell lines.

ACA has been reported to inhibit cell growth and angiogenesis on various cancer cells. Albeit, the antiproliferative, anti-invasive, and anti-angiogenic effects through HER2 signalling, ER coactivator; NCOA3, and NFκB targeted molecules on hormonal-resistant breast cancer cells have never been reported. The expression of HER2, a significant growth factor receptor for breast cancer in the non-classical genomic pathway, was also determined in this study. This protein analysis on total-HER2 expression after 24 hours of ACA treatment demonstrated that at the concentration below IC_{50}, ACA could not significantly downregulate HER2

expression in MCF7, MCF7/LCC2 cell lines while ACA significantly affected the expression of HER2 in MCF7/LCC9 at the IC_{25} and IC_{50}. In this study, the pHER was also performed. In MCF7 cell line, ACA only at the IC_{50} concentration could downregulate the protein expression of pHER2 (normalised with GADPH). While the expression of pHER2 (normalised with GADPH) in MCF7/LCC2 and MCF7/LCC9 was significantly decreased in all three studied concentrations. However, the result of pHER2 protein expression in all three cell lines when normalised with total-HER2 did not statistically difference compared to untreated control. These results can be implied that the downregulation of pHER2 in ACA-treated groups was due to the influence of total-HER2 downregulation. Hence, ACA effect on HER2 receptor was through the downregulation of HER2 receptor not the activity.

Among three cell lines, the results noticed that ACA had preferable effects on MCF7/LCC9 more than the others. Apart from that, the study of ACA on the expression of pERK1/2 and pAKT was also performed. As the pERK1/2 and pAKT were the downstream molecules of HER2 signalling (262). Therefore, the insight study for these proteins was essential to identify the mechanism of action of ACA on HER2 signalling pathway (262). The results in this study identified that ACA inhibited proliferation through the downregulation of pERK1/2 and pAKT. These finding is consistent with the study of Murakami *et al*, 2005 that ACA showed the potential to inhibit the expression of pERK1/2 and pAKT which performed in RAW264.7 murine macrophages (173). Signalling cascades through ERK1/2 and PI3K/AKT are an essential pathway in breast cancer progression (263). Hyperactivation and overexpression of ERK1/2 and AKT were found in tamoxifen-resistant breast cancer cells (263).

The antiproliferative effects of ACA was also performed via evaluation of gene and protein level by using real-time qPCR and western blotting analysis. The results indicated that ACA affected the expression of NCOA3 in both gene and protein levels. The expression of NCOA3 protein in endocrine-resistant breast cancer cell lines was higher than ER-positive wild-type breast cancer cell. NCOA3 has has been revealed to be overexpressed in breast cancers and pivotally involved in oestrogen-mediated cancer cell proliferation (264). In breast cancer patients, the expression of NCOA3 was determined, and it showed that NCOA3 expression was detected in 60 % of the tumours (57). Also, the analysis of clinical samples indicated that patients with elevated NCOA3 did not respond well with tamoxifen therein lied breast cancer resistance to hormonal therapy (57).

This study suggested that the treatment with ACA at three different concentrations ($IC_{12.5}$, IC_{25}, and IC_{50}) on MCF7/LCC2 and MCF7/LCC9 caused the repression of NCOA3 both gene and protein as a dose-dependent manner as can be seen by the significance of all concentrations used for the treatment in both cell lines represented in gene and protein results. Whereas, in MCF7 ACA can significantly suppress the expression of NCOA3 only at the IC_{50} concentration. Intriguingly, ACA exhibited a better efficiency on endocrine-resistant breast cancer cells than the ER-positive wild-type breast cancer cell. This study revealed that ACA could have a mode of action via the downregulation of NCOA3 in endocrine-resistant breast cancer cells. Hence, ACA could inhibit the transcriptional activity of ER-mediated genes in both classical and non-classical genomic pathways as NCOA3 was a major co-activator of ER signalling pathways.

ACA has been reported to rapidly decrease the nuclear expression of NFκB , but increase the accumulation of cytosol NFκB in RPMI82 cells (37). This indicating that ACA blocks the translocation of NFκB from the cytosol to the nucleus (168). NFκB plays a significant role in cancer initiation and progression (265). ACA has also been reported to inhibit angiogenesis in human prostate cancer through VEGF (174). It can inhibit metastasis through matrix metalloprotease-9 (MMP-9) (171), whereas the effects of ACA on VEGF and FGF2 in endocrine-resistant breast cancer cells have never been reported. NFκB activation was originally associated with the progression of hormone-independent breast cancers (107). NFκB bestowed transcriptionally to induce genes mediating cell proliferation, such as *cyclin D1 (CCND1)* (290-292), *c-myc* (293) as well as genes that responsible for invasion including urokinase-type plasminogen activator *(uPA)* (294-296) and *Chemokine receptor (CXCR4*) (148, 297, 298) and angiogenic factors such as vascular endothelial growth factor (*VEGF*) (299) and basic fibroblast endothelial growth factor (*FGF2*) (300-304).

In this study, we showed that ACA had the potentiality to inhibit the expression of NFκB regulated genes that involved in resistant breast cancer cells. NFκB was activated in the presence of AKT and ERK1/2 (305-307). The effects of ACA on pAKT and pERK1/2 could affect the transcriptional activity of NFκB resulting in anticancer activities of ACA. Therefore, the downregulation of NFκB regulated genes could be observe in this study. More interestingly, with regard to angiogenesis aspect, we pointed out that ACA inhibited not only VEGF but also FGF2, which was a critical angiogenic factor in breast cancer. Giavazzi *et al* have revealed that FGF can function synergistically with VEGF to magnify tumour angiogenesis (308). This study highlighted that ACA targeted both the FGF and

VEGF may be more efficacious in repressing tumour growth and angiogenesis than targeting either factor alone.

Apart from that, ACA shifted up of the pro-apoptotic signal via the upregulation of SAPK/JNK protein expression but not the activity. Its effect predominantly affected hormonal resistant breast cancer cells rather than the wild-type breast cancer. Evading apoptosis and overexpression of anti-apoptotic proteins were found to be the cause of cancer resistance to cancer chemotherapeutics and poor prognosis (309-311). Therefore, we also demonstrated the apoptotic induction activity of ACA was through the anti-apoptotic factors Mcl-1 and Bcl-2. Liew *et al* showed that ACA induced apoptosis via the induction of the mitochondrial pathway (192). Their results supported our study that ACA may have apoptotic induction on hormonal resistant breast cancer cells through mitochondrial pathway.

In conclusion, ACA exhibited anticancer activities through HER2/MAPK/ERK1/2, PI3K/AKT pathways as well as through the inhibition of NCOA3. It can also inhibit the gene expressions, which involved in proliferation and invasion. As of anti-angiogenic activity, ACA potentially repressed both angiogenic factors; VEGF and FGF2. According to apoptotic induction, ACA promoted the expression of pro-apoptotic molecules and repressed of pro-survival molecules.

Future studies could investigate the effects of ACA on reversal of tamoxifen sensitivity in endocrine-resistance breast cancer cells, compared to endocrine-sensitive breast cancer cells. Viability tests, such as MTT or BrDU assays in endocrine-sensitive breast cancer cells could be used to compare the IC50 values of ACA-tamoxifen co-treatment with tamoxifen treatment alone. Also, performing

antiproliferative assays, such as colony forming assays or anchorage-independent growth (soft agar assays) plus additional of gene expression and protein expression analysis of combined ACA-tamoxifen treatment could be compared to tamoxifen treatment alone. This will then give information of the effects of ACA on reversal of tamoxifen sensitivity in endocrine-resistance breast cancer cells. To further explore the effects of ACA on HER2 expression, it would be interesting to study the effects of ACA on HER2 over-expressing cells (e.g. SKBR3, AU565, HCC1008) as endocrine-resistant breast cancer cells showed upregulated of baseline HER2 protein expression. Additionally, the phenotype of endocrine-resistant breast cancer cells (MCF7/LCC2 and MCF7/LCC9 cells) in this study demonstrated the upregulation of molecules such as AKT and ERK1/2 that are downstream of HER2, as well as downregulation of ER protein levels. This could support the rationale for further studies of ACA in HER2 over-expressing subtypes. In addition to the effects of ACA, which targets multiple molecules, the effects of ACA in triple-negative breast cancer cells (e.g. MDA MB231, MDA MB436, HCC70) could be interesting experiments. There are several subtypes of triple-negative breast cancer patients which are reported to have abnormal gene expression profiles. For example, genes for cell-cycle regulation and DNA repair genes (e.g. *MYC, PIK3CA, CDK6, AKT2, FGFR1*) are observed as well as hyperactivation of signalling pathways such as EGFR (312, 313). In addition, ACA has been reported in multiple studies to have effective anticancer effects on MDAMB231 via apoptosis induction (170, 192). Other experiments that will further knowledge of the pharmacological effects of ACA would include different culture methods, such as 3D *in vitro* culture models (e.g. spheroid and organoid 3D culture) that could use to determine its effects within the cell microenvironment. Such

approaches would support further pre-clinical studies of the potential of ACA as a therapy.

CHAPTER 5 *IN VIVO* ACTIVITY OF ACA ON ZEBRAFISH MODEL

5.1 Results

5.1.1 Acute toxicity test of zebrafish embryos

The acute toxicity test of ACA on zebrafish embryos was done as followed the OECD test guideline (TG236). After treated the zebrafish embryos with ACA at increasing concentration (up to 160 μM). The result showed that ACA up to 20 μM did not cause any of four apical indicators (coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tailed-bud from yolk sac, and lack of heart-beat; additional information of four apical indicators can be found in **Appendix C**) to the zebrafish embryos.

The nondetectable heartbeat, tail-bud detachment, and coagulation of zebrafish embryos was initially seen at 48 hours post fertilisation (hpf) of 40 μM ACA treatment. The 50% lethality concentration (LC_{50}) of ACA at 24, 48, 72, and 96 hpf was shown in **Table 5.1.1.1.** and **Figure 5.1.1.1**.

This study also illustrated that the LD_{50} of ACA on zebrafish embryos (at 24, 48, 72, and 96 hours), which indicated that LD_{50} of ACA between 24 and 48 hours (41.20 μM ± 5.03 and 40.47 μM ± 2.87) and 72 and 96 hours (35.45 μM ± 2.20 and 32.16 μM ± 1.54) did not significantly change through these particular exposure time.

Table 5.1.1.1. The LC_{50} of ACA on zebrafish embryos at 24, 48, 72, and 96 hpf

Time (hpf)	**Mean LC_{50} ± SEM**
24	41.20 μM ± 5.03
48	40.47 μM ± 2.87
72	35.45 μM ± 2.20
96	32.16 μM ± 1.54

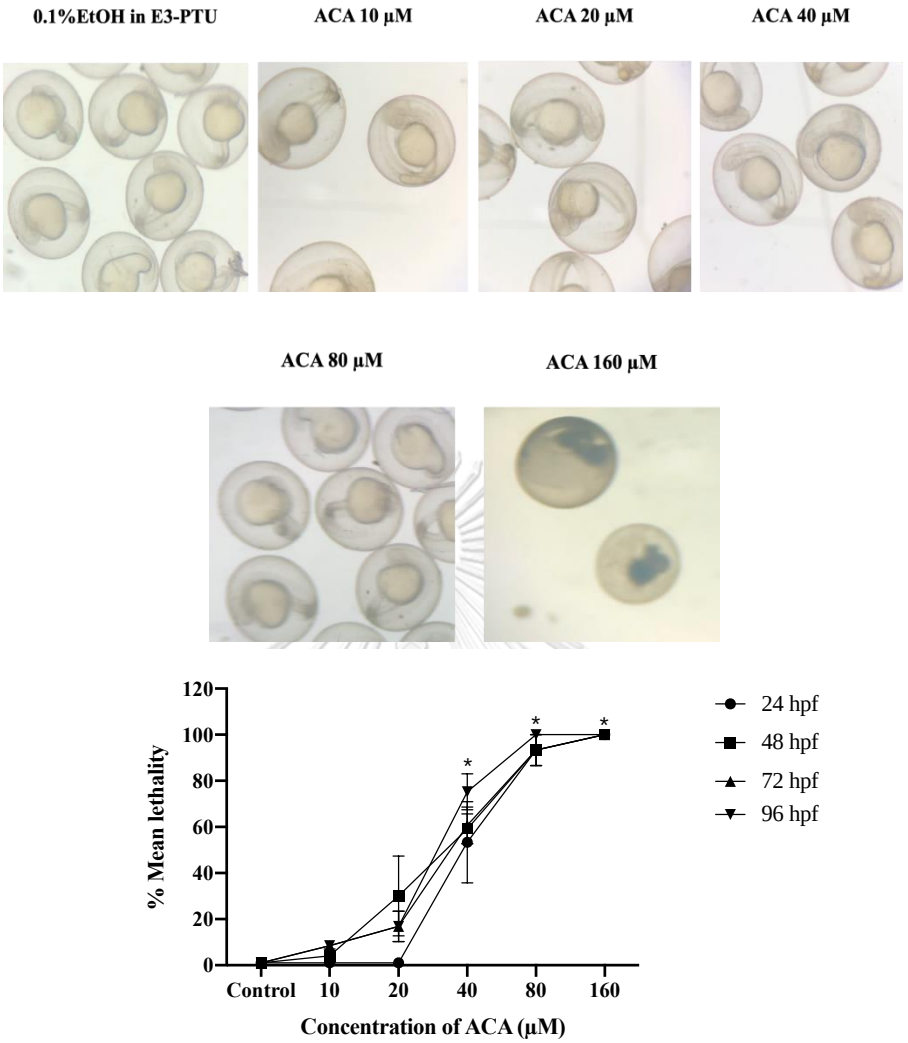

Figure 5.1.1.1. The toxicity of ACA in zebrafish embryo was followed OECD guidelines. Top figures show images of zebrafish embryos at 48 hpf after ACA treatment. Bottom figure shows the 50% lethality concentration (LC_{50}) of ACA at 24, 48, 72, and 96 hpf was 41.20 µM ± 5.03, 40.47 ± 2.87 µM, 35.45 µM ± 2.20, and 32.16 µM ± 1.54 respectively. 0.1%EtOH in E3-PTU was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3), *$P \leq 0.05$ vs. untreated cells (mean ± SEM, n = 3).

5.1.2 Zebrafish vessel staining

In this experiment, ACA was treated to zebrafish embryos at 24 hpf for 48 hours. Then the whole-mount alkaline phosphatase vessel staining was performed. After staining the vessels with alkaline phosphatase, the vessels were easily visualised by using a digital camera and image acquisition (LAS Va.12), publicly available quantitative image software. The results showed that after 48 hours of ACA treatment, the concentration up to 40 μM did not cause the abnormal formation of the subintestinal vessels (SIV) to zebrafish embryos compared to ACA-untreated zebrafish embryos (**Figure 6.2.1)**.

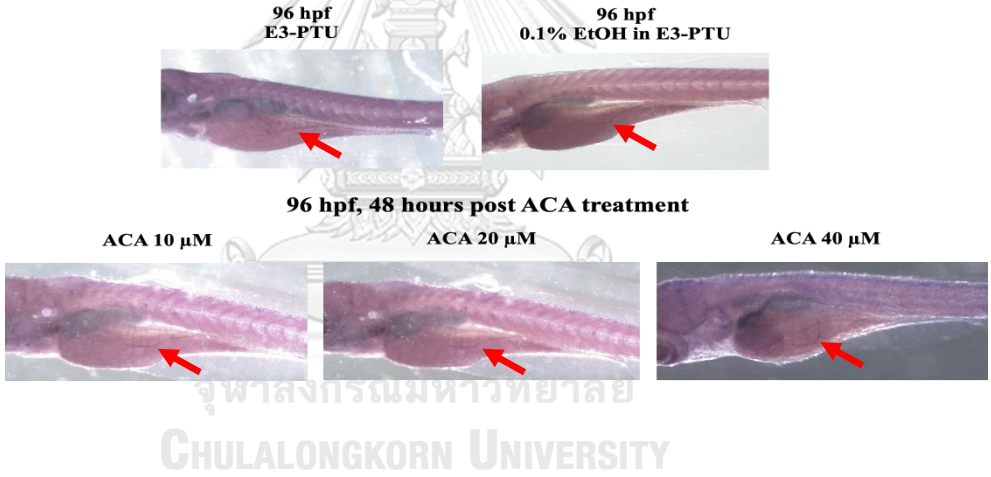

Figure 5.1.2.1. The whole-mount alkaline phosphatase vessel staining. ACA (0-40 μM) did not inhibit normal SIVs development form on the dorsolateral surface of the yolk on both sides of the embryo, as detected using whole-mount alkaline phosphatase vessel staining. Red arrow points indicate the SIVs of the zebrafish that lined behind the yolk of the zebrafish. E3-PTU was used as an untreated control and 0.1%EtOH in E3-PTU was used as a vehicle control.

5.1.3 Tumour engulfment and proliferation

The optimisation of conditions for MCF7/LCC9-grafted zebrafish was performed to optimise three optimal conditions of tumour-grafted zebrafish survival. Firstly, the appropriate number of cancer cells to be injected without causing zebrafish death (314). Secondly, the incubated condition of tumour-engrafted zebrafish (315). Finally, the percentage of tumour engulfment (314).

The result showed that the amount of appropriate cancer cells was 200 cells/5nL. And the optimal conditions for tumour-engrafted zebrafish was 36^{o} Celsius, 5%CO_2 with humidified air. This condition allowed humanised-cancer cell growth without causing zebrafish death. Also, this condition did not cause the aberrant staging development of the zebrafish

Regarding the optimal conditions, the cancer cell can efficiently engraft and proliferate in the zebrafish as shown by the increase of tumour area and fluorescence intensity at 3 days post injection (dpi), which was 1 - 2 times greater than 0 dpi **(Figure 5.1.3.1)**.

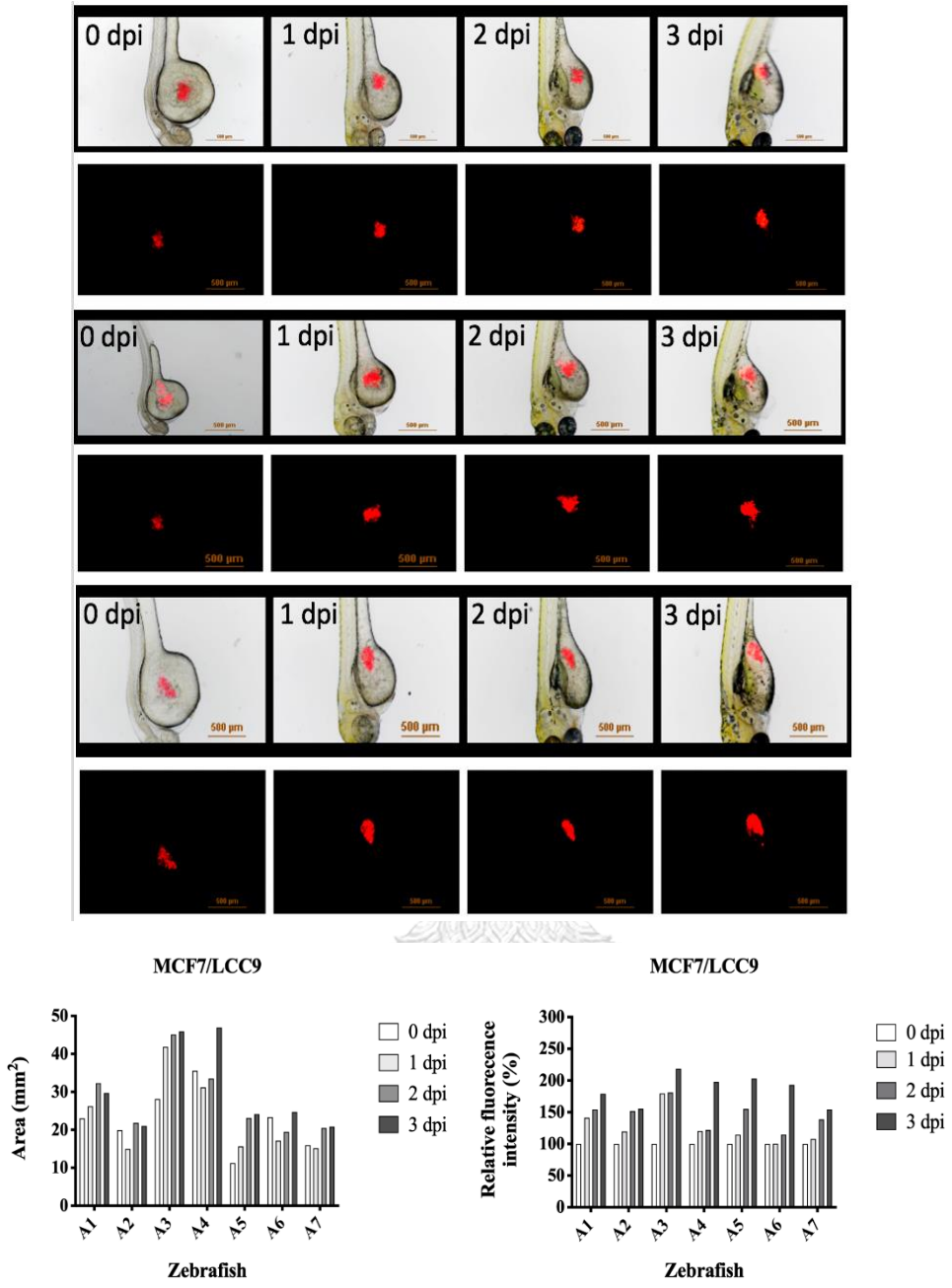

Figure 5.1.3.1. Tumour engulfment and proliferation. The injection of endocrine-resistant breast cancer cell (MCF7/LCC9) into the yolk sac of zebrafish embryos at 48 hpf (0 dpi). The observation of tumour area and fluorescence intensity were measured at 0, 1, 2, and 3 dpi. The fluorescence intensity data were represented as relative values quantified with fluorescence intensity 0 dpi. A1 - A7 represented zebrafish sample number.

5.1.4 The inhibitory effect of ACA on MCF7/LCC9 cells proliferation engrafted zebrafish embryos

Zebrafish has many homolog genes that plays role in crucial signalling pathways involved in cell proliferation, apoptosis, metastasis, angiogenesis of human cancers (316, 317). With this zebrafish xenograft model is the platform for xenotransplantation approach to investigate real-time visualisation of tumour mass (318).

In this experiment, the zebrafish was injected with MCF7/LCC9 cell for 200 cells/5nL at 48 hpf (0 dpi). ACA was treated into the zebrafish medium at 1 dpi. The tumour area was regularly observed at 1 and 3 dpi. The result demonstrated that at 3 dpi (48 hours of ACA treatment), ACA significantly reduced the tumour area in all studied concentrations in the experiments (5, 10, 15 μM). The reduction of tumour area in ACA-treated group was approximately four times lesser than the control group (**Figure 5.1.4.1)**.

This experiment confirmed that ACA exhibited the antiproliferative effect in zebrafish xenograft model.

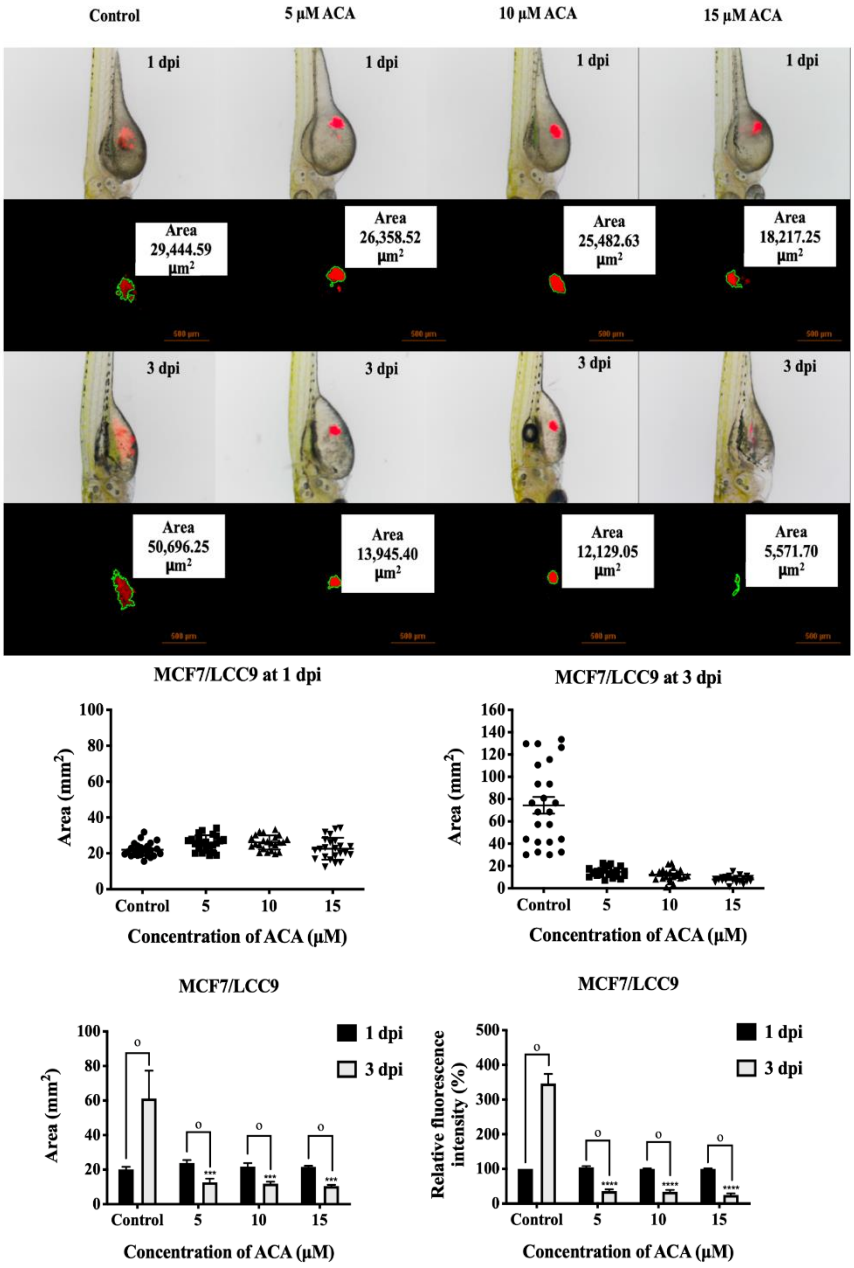

Figure 5.1.4.1. The antiproliferative effect of ACA in zebrafish model. ACA significantly inhibited the tumour area of MCF7/LCC9 cells engrafted zebrafish embryos at 1 and 3 dpi. 0.1%EtOH in E3-PTU was used as a vehicle control. Data are represented as mean ± SEM of three independent experiments (n = 3), ***$P \leq 0.001$ and ****$P \leq 0.0001$ vs. control at 3 dpi, $^{o}P \leq 0.05$ vs. the same concentration of ACA treatment at 1 dpi (mean ± SEM, n = 23/group).

5.2 Discussion

Regrading *in vivo* study of zebrafish, the husbandry and well-being of zebrafish need to be considered because different stages of zebrafish had different living conditions (e.g. types of food, sizes of food, and water level for their survival) (319).

The acute toxicity test showed that ACA concentration used for further *in vivo* experiment was not toxic to the zebrafish. Furthermore, the additional toxicity test, alkaline phosphatase staining, showed that ACA up to 40 μM did not significantly inhibit normal subintestinal vessels (SIVs) development form on the dorsolateral surface of the yolk on both sides of the embryo. Both toxicity assays confirmed that the concentration and treatment time of ACA used in zebrafish experiment did not toxic to zebrafish, regardless of neither causing acute toxicity to zebrafish embryos nor the development of normal SIVs formation. Thus, these experiments ascertained that the concentration of ACA did not interfere with the results from further *in vivo* studies.

Zebrafish (*Danio rerio*) required regular care and appropriate protocol for staging growth (315). Unlike human-derived cancer cells that required human body temperature (37.4 degree Celsius) to proliferate, but the optimum temperature for zebrafish is 28.5 degree Celsius (315). In the *in vivo* MCF7/LCC9-implanted zebrafish experiment, the method was never published before. Therefore, the transplantation of endocrine-resistant breast cancer cells into zebrafish xenograft was needed to evaluate the engraftment efficiency and the optimal condition for both human cell and zebrafish was required to be evaluated. Also, in this study, some mandatory equipment was needed to be manually modified; for instance, the diameter

of the microneedle, injection pressure, and injection time. All those conditions affected the number of injected cancer cells. The microneedle, in particular, was necessary to be nipped for adjusting the inner diametre before performing the nano-injection for a specific cell line. This procedure was done to prevent either cancer cell clogged within the microneedle or the uncontrolled number of cancer cell per injection. Regarding, the injection equipment, it was set the pressure and time for injection as the same condition in every experiment. However, the weight and duration of pushing the paddle for injection could be another significant factor for a variation in the cancer cell numbers. Therefore, it could be a variation in the number of cancer cells from each experiment. Consequently, the optimisation of conditions for MCF7/LCC9-grafted zebrafish need to be validated. Also, the number of cancer cells are significant for engrafted and proliferative capabilities and zebrafish survival (314). Thus, the tumour engraftment and proliferation in zebrafish validation necessitate being done before. Efficient engraftment can also be obtained by transplanting cells into early embryo zebrafish that have not yet developed a functional acquired immune system.

By labelling MCF7/LCC9 with a red fluorescent CM-dil dye, this allowed the cells to be tracked tumour area and fluorescence intensity. The result demonstrated that the tumour area and fluorescence intensity value were directly correlated to each other. Therefore, the proliferation of cancer cells within the zebrafish can be represented by the tumour area. In the antiproliferation experiment, very low concentrations of ACA can significantly inhibit the proliferation of MCF7/LCC9-implanted zebrafish compared to the control.

To conclude, this study suggested that ACA possessed antiproliferative effect in zebrafish xenograft model, which consistently correlated with the results from *in vitro* study on the antiproliferative effects of ACA in breast cancer cell lines.

CHAPTER 6 MOLECULAR DOCKING SIMULATION

6.1 Results: ACA's affinity for protein targets

This research was studied seven target molecules (HER2, AKT, ERK, ER, CDK6, uPA, and FGF2) to perform the docking simulation. Then ACA was determined the interaction energy and compared binding affinity to the standard ligands of a specific target. The studied targets were selected based on its molecular functions that crucially involved in signalling pathways of resistant-breast cancer cells. There were rationales for selecting these eight targets. Firstly, ACA was reported to be a potent NFκB inhibitor (156, 171). Secondly, ACA was reported that it could inhibit AKT and ERK in other cell lines (173, 188). These two molecules were reported to overexpress in resistant-breast cancer cells (67, 71, 72). Thirdly, according to the *in vitro* results from this research, it showed that ACA was able to downregulate AKT, ERK, NFκB mediated genes (uPA, FGF2), NCOA3, and HER2. Fourthly, oestrogen receptor and HER2 were selected because these two receptors were crucial for survival signallings for breast cancer (68, 69). Therefore, this study aimed to explore that ACA could bind to the upstream molecules of NFκB signalling (AKT and ERK), NFκB targeted genes (uPA, FGF2), oestrogen receptor, and HER2 (upstream molecule of MAPK/ERK1/2 pathway). Finally, CDK6 was selected as it was a critical molecule that required to work with cyclin D1, which was significant machinery for cell cycle in breast cancer cells (110-112). This research could not perform to all mediators due to the lack of standard molecules of a particular target to be compared with ACA. Thus, this study was selected these seven studied targets.

The molecular docking results (**Table 6.1.1**) demonstrated that ACA has similar binding affinity for HER2, AKT, ERK, ER, CDK6, uPA, and FGF2 ranging from -21.61 to -38.69 kcal/mol. However, among these seven different targets, the binding affinity of ACA toward uPA (-32.73 kcal/mol) and FGF2 (-21.61 kcal/mol) was in the range of their known inhibitors 4-IBTC (-37.43 kcal/mol) and Sm27 (-29.96 kcal/mol) respectively. Due to hydrophobic structure of ACA, van der Waals (green sphere) were the main interactions underlying protein-ligand complexation for both uPA and FGF2 proteins (**Figure 6.1.1**). On the other hand, some electrostatic contributions (e.g., salt bridge, pi-sulfur, and pi-cation) were involved in the binding of 4-IBTC and Sm27 due to the polar moieties in their chemical structures. Notably, the key binding amino acid residues of ACA toward both uPA (Ser195, Ser214) and FGF2 (Lys119, Lys125, Lys129) as well as the hydrogen bond formation patterns (green dash) were relatively similar to those of the known inhibitors, implying that ACA could be an alternative potential inhibitor to uPA and FGF2.

Table 6.1.1. CDOCKER interaction energy (kcal/mol) of all the studied protein-ligand complexes

Protein targets	Compounds	CDOCKER interaction energy (kcal/mol)
HER2	Lapatinib	-77.62
	1'-acetoxychavicol acetate	-33.66
AKT1	Ipatasertib	-63.44
	1'-acetoxychavicol acetate	-34.92
ERK2	Sorafenib	-53.92
	1'-acetoxychavicol acetate	-34.94
Estrogen receptor	4-hydroxytamoxifen	-65.38
	1'-acetoxychavicol acetate	-35.68
uPA	4-iodobenzo[b]thiophene-2-carboxamidine (4-IBTC)	**-37.43**
	1'-acetoxychavicol acetate	**-32.73**
CDK6	Palbociclib	-57.62
	1'-acetoxychavicol acetate	-38.69
FGF2	Sm27	**-29.96**
	1'-acetoxychavicol acetate	**-21.61**

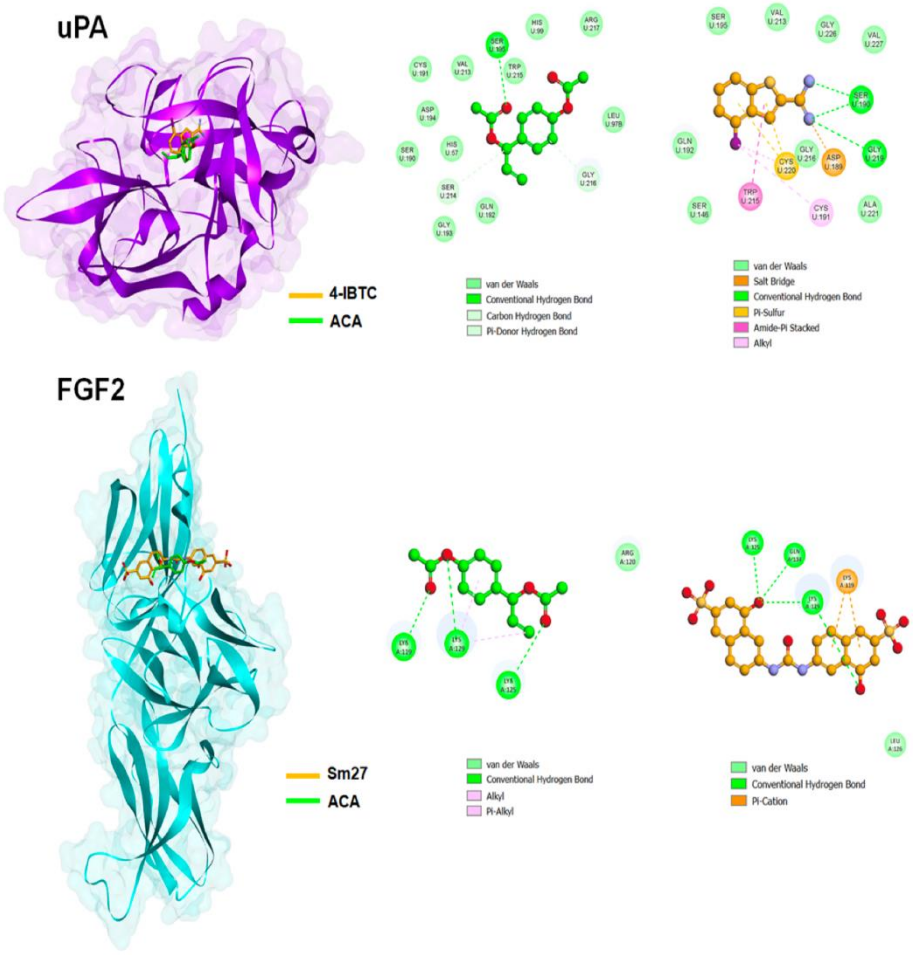

Figure 6.1.1. The 3D superimposed structures obtained from docking as well as the interaction details of all the studied ligands in complex with uPA and FGF2

6.2 Discussion

The molecular docking results was performed by using CDOCKER interaction energy (kcal/mol) of seven studied protein-ligand complexes. Among seven substances, the CDOCKER interaction energy of ACA was close to the interaction energy of 4-IBTC (standard uPA ligand) and Sm27 (standard FGF2 ligand). CDOCKER interaction energy represented an inverse correlation with the binding affinity of protein-ligand complexes wherein, more negative energy value was more binding affinity (320). The lower the binding energy value means that the complex (ligand-protein interaction) is more stable. In addition, a negative value shows that the ligand bound spontaneously without consuming energy, as the negative Gibbs free energy (ΔG) is preferable for better binding affinity. This is because the change in ΔG of the system is negative when the system reaches an equilibrium state at constant pressure and temperature (320, 321). Also, the protein-ligand association extent is determined by the magnitude of the negative ΔG. Therefore, it can be considered that ΔG determines the stability of any given protein-ligand complex (322). The result supported that ACA showed preferable binding interaction to uPA and FGF2 than the other standard molecules. Albeit, the CDOCKER interaction energy of ACA was higher compared with the other five standard molecules; still, ACA could bind with lesser binding affinity to HER2, AKT1, ERK2, oestrogen receptor, and CDK6 compared to its known standard ligands.

To conclude, the docking simulation study suggested that ACA could bind to the molecular targets involved in cancer proliferation, invasion, and angiogenesis, which bolstered the results of ACA effects on the in vitro anticancer activities. However, the binding affinity testing should be confirmed with more direct

approaches such as direct biochemical methods (e.g. affinity chromatography) and quantitative proteomics (e.g. metabolic and chemical labelling). Also, some targets may not be appropriately used this docking simulation method because of the unavailability of published standard ligands to compare with the studied ligand.

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

This study effectively isolated the pure ACA compound from the *A. galangal*; however, the structure of a pure ACA needed to be elucidated. Here in this study, ACA structure was elucidated using multidisciplinary spectroscopy. As of ACA was a known compound. Therefore, more sophisticated methods did not essential.

This study demonstrated that ACA possesses cytotoxicity on both endocrine sensitive ER-positive MCF7 cell line and endocrine-resistant MCF7/LCC2 and MCF7/LCC9 cell lines without causing toxicity to normal fibroblast cell and the cytotoxic effect was more profound in the endocrine resistant cell lines. The mechanisms of ACA regarding its anticancer activities were through the suppression of HER2/MAPK/ERK1/2 and PI3K/AKT pathways. Upon repressed survival pathways, ACA can shift towards the apoptotic pathway through the upregulation of SAPK/JNK and cPARP and downregulation of the antiapoptotic proteins Mcl-1 and Bcl-2. In addition, this study highlighted that ACA could downregulate NCOA3 protein. NCOA3 is the major coactivator of ER, which is an essential coactivator for ER signalling and functions in classical and nonclassical genomic pathways of ER. Consequently, ACA blocked the transcriptional activity of the ER-regulated genes. Apart from that, ACA, as a well-known NFκB inhibitor, can potentially crosstalk by blocking the transcription of NFκB-targeted genes via the inhibition of ERK1/2 and AKT phosphorylation wherein ERK1/2 and AKT activated NFκB activation.

The antiproliferative effect of ACA was established in the endocrine-resistant engrafted zebrafish model. ACA effectively reduced tumour mass in ACA treated

zebrafish/MCF7/LCC9 without adverse effects to the zebrafish embryos. In addition, the acute toxicity experiment reported as the LC_{50} of ACA at 24, 48, 72, and 96 hpf showed that ACA up to 20 μM does not significantly inhibit normal embryo stages development nor the normal formation of subintestinal vessels of the zebrafish. The result from *in vivo* antiproliferation of ACA in zebrafish model supported the result of antiproliferation of ACA in the *in vitro* study parts to demonstrate the function of ACA regarding inhibition of breast cancer cell proliferation.

Furthermore, ACA repressed invasion in endocrine-resistant breast cancer cells by targeting the uPA system confirming via the pharmacological anti-invasion assay. VEGF and FGF2, a potent angiogenic factor, were also inhibited by ACA in both endocrine-sensitive and endocrine-resistant breast cancer cells. Assuredly, the docking analysis results validated the inhibitory effects of ACA on FGF2 and uPA as its binding affinity was comparable to the standard ligands. The gene expression studies and docking experiments imply that ACA can have anti-angiogenic activity. However, more definities experiments are required to confirm this prediction. For example, in vitro anti-angiogenesis of ACA using endothelial cell proliferation assays, endothelial cell migration assays, and endothelial cell differentiation assays would be required. In vivo models could include, for example, zebrafish anti-angiogenesis by visualising the developing vasculature, anti-angiogenesis in mice models (e.g. matrigel plug assay), and chick chorioallantoic membrane (CAM) assays.

This *in vitro* study provided fundamental information of the anticancer activity of ACA in endocrine-resistant breast cancers that could be developed further for future development of ACA into clinical settings. It has also shown for the first time

that ACA can inhibit NCOA3 which is a well-established key protein in ER-positive hormonal-resistant breast cancer, both from in vitro studies and observations in cancer patients. Therefore, the bioactive ACA derived from the edible plant could be a promising compound to be used as adjuvant therapy or combination with tamoxifen for tamoxifen reversal sensitivity in breast cancer patients that develop resistance to tamoxifen, as well as other hormonal treatments. Also, this study showed that ACA possessed activities on the HER2 receptor as well as its downstream survival molecules. Therefore, this novel knowledge can be developed further future studies of ACA on HER2-over-expressing breast cancer cells. The drug development process to develop any new chemical entities into the clinic, needs rigour, that includes defining the mechanism of action and direct target of the substance, pharmacokinetics, pharmacodynamics, adverse drug reactions, drug interactions (drug-drug, herb-drug, and food-drug interactions) as well as the dosage forms, stability and compatibility. In addition, ACA is a natural oily substance, and so could require additional processes such as drug delivery systems to improve the solubility and efficacy as well as reduce off-target toxicity of ACA. Hence, the difficulty of developing ACA as a novel chemotherapeutic agent would require the completion of many studies in order to be approved and established for clinical use.

7.2 Limitation of this study

In the *in vitro* studies of this thesis, the cytotoxic activity of ACA was studied in the endocrine-resistant MCF7/LCC2 and MCF7/LCC9 and the endocrine-sensitive MCF7 breast cancer cells. Tamoxifen was used as the appropriate positive control for MCF7cells (type), but it needed longer treatment time to show its cytostatic activity and there was no specific positive control for the endocrine-resistant cell lines. In addition, the *in vitro* studies were performed in two bases, which were Chulalongkorn University, Thailand and the University of Liverpool, UK. As the aims of the studies at Chulalongkorn University focused primarily in endocrine-resistant cells, the experiments that needed to be performed in the University of Liverpool were involving the endocrine-sensitive MCF7 cells to be able to compare our ACA data on the endocrine resistance to endocrine sensitivity. Albeit, before starting the experiments, the comparison of the IC_{50} values of ACA on each cell line was required. We observed a small variation in the IC_{50} of ACA in the cell lines which could possibly due to the different passage numbers of the cells within the two Universities and their freezing conditions. However, the effect of ACA on MCF7 cells in both bases did not show statistically different IC_{50} values. Hence, the MCF7 cell-based experiments were confidently performed (additional information can be found in **Appendix B**).

This research succeeded in unpacking the effects of ACA on critical molecules involved in survival pathways of aggressive breast cancer cells. However, further investigation is required on identifying the specific mechanism of ACA's action. The use of specific pharmacological inhibitor(s) or a combination of them targeting a particular pathway could possibly identify the dominant mechanism of ACA's action.

Additional studies to support the binding affinity evaluations are required as there are limitations in this docking simulation approach. In this study, ACA binding affinity to seven target proteins was compared with their standard ligands. However, this approach cannot evaluate all targets that may be involved in ACA's action, such as the binding affinity of ACA on p65 subunit. The resistant MCF7/LCC9 cell and ER-positive tamoxifen resistant cells have both been shown to overexpress the p65 subunit of NF-kB, and hence it would have been informative to perform a binding affinity analysis of ACA on p65 subunit. However, this molecular docking simulation could not be performed on the p65 subunit as there is no standard ligand for p65 nuclear localisation inhibitors that have been identified. Consequently, this study could not compare the binding affinity between ACA and the standard ligand of p65 nuclear localisation inhibitors. In addition, other experiments, such as quantitative mass spectroscopy (direct biochemical methods) should be performed in order to demonstrate if ACA binds with p65 subunit, or other target proteins. As the methodology for the MCF7/LCC9 implanted zebrafish experiment was not published before and we had to set and evaluate all the appropriate conditions. It was not feasible to develop an additional MCF7/LCC2 implanted zebrafish.

7.3 Prospective study

The analysis of ACA effects on a breast cancer gene expression panel should be performed in the future to compare the effect of ACA in the endocrine-sensitive and endocrine-resistant breast cancer cells and identify differentially expressed genes for further analysis. In addition, *in vitro* studies on breast cancer cells with combinations of ACA and standard inhibitors (e.g. AKT inhibitor, ERK1/2 inhibitor, anti-HER2, and NFκB inhibitor) and analysis of targeted-gene and protein expressions (e.g. CCND1, C-myc, CXCR4, uPA, VEGF, FGF2) could provide a better insight into the mechanistic actions. ACA experiment on gene silencing (HER2, NCOA3) in breast cancer cells will support more evidence on validating the targets of ACA. By silencing such genes in breast cancer cells, followed by analysing pERK1/2 and pAKT protein expressions will provide the information regarding the targets of ACA. Down-regulating/silencing a gene is not the same as inhibiting a specific region and therefore, multiple methods are needed for confirming specific targets.

Finally, the anti-invasion and anti-angiogenesis of ACA could be investigated in the future both *in vitro* models such as primary breast cancer cells and endothelial cells as well as in *in vivo* models such as zebrafish xenotransplantation and transgenic animals.

7.4 Conclusion

Taken together, our findings demonstrate the anti-cancer activity of the ACA through multiple pathways that directly influence the endocrine resistance of our tested cells, including concomitant pro-apoptotic signalling enhancement and inhibition of pro-survival molecules. Further investigation using specific inhibitors and/or gene silencing could provide a better insight into the mechanism of action of ACA in endocrine resistance.

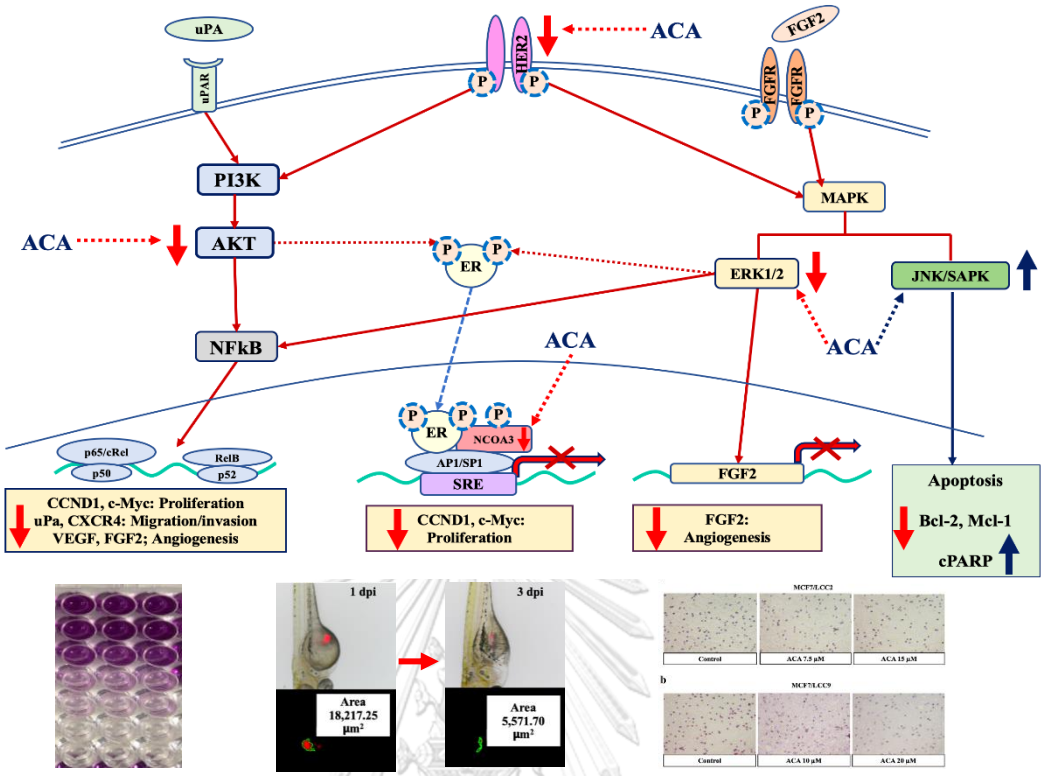

Figure 7.4.1. Proposed mechanism of ACA. The diagram shows the proposed mode of action of ACA which affects multiple molecules namely, HER2, PI3K/AKT, ERK1/2, and NCOA3. The effects lead to the downregulation of targeted molecules which involved in cell proliferation (CCND1, c-Myc), invasion (uPA, CXCR4), and angiogenesis (VEGF, FGF2). This model is based on the results obtained in this thesis.

CHAPTER 8 APPENDICES

Appendix A / Comparison of ^{1}H NMR and mass spectra of ACA

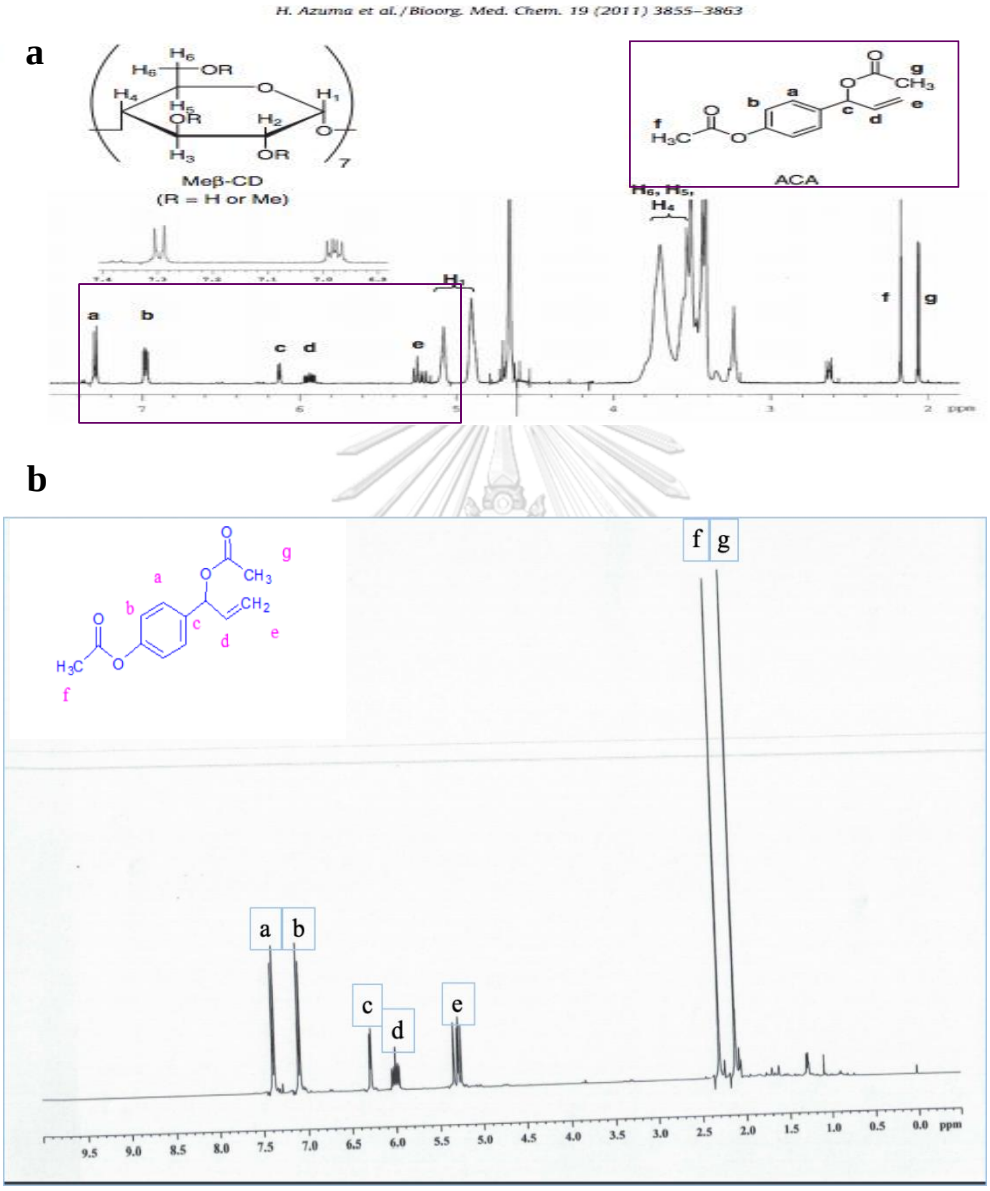

Figure A1. ^{1}H NMR spectrum comparison of ACA. ^{1}H NMR spectrum of ACA from Azuma et al (a) (251) and the ^{1}H NMR spectrum from this study (b)

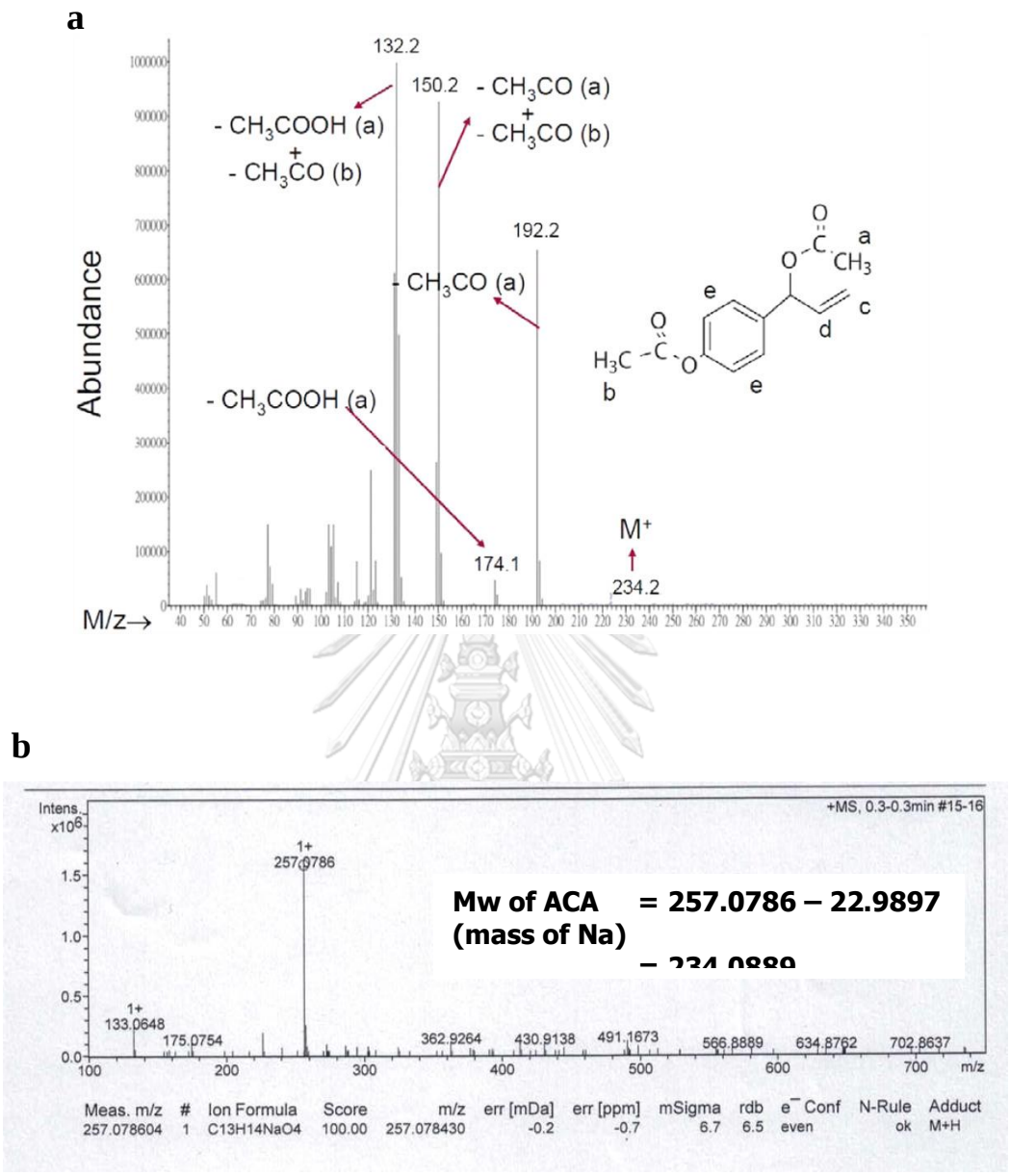

Figure A2. Mass spectrum comparison of ACA. The mass spectrum of ACA from Lin et al (a) (253) and the mass spectrum of this this study (b)

Appendix B / Validation of IC_{50} values of ACA in the University of Liverpool

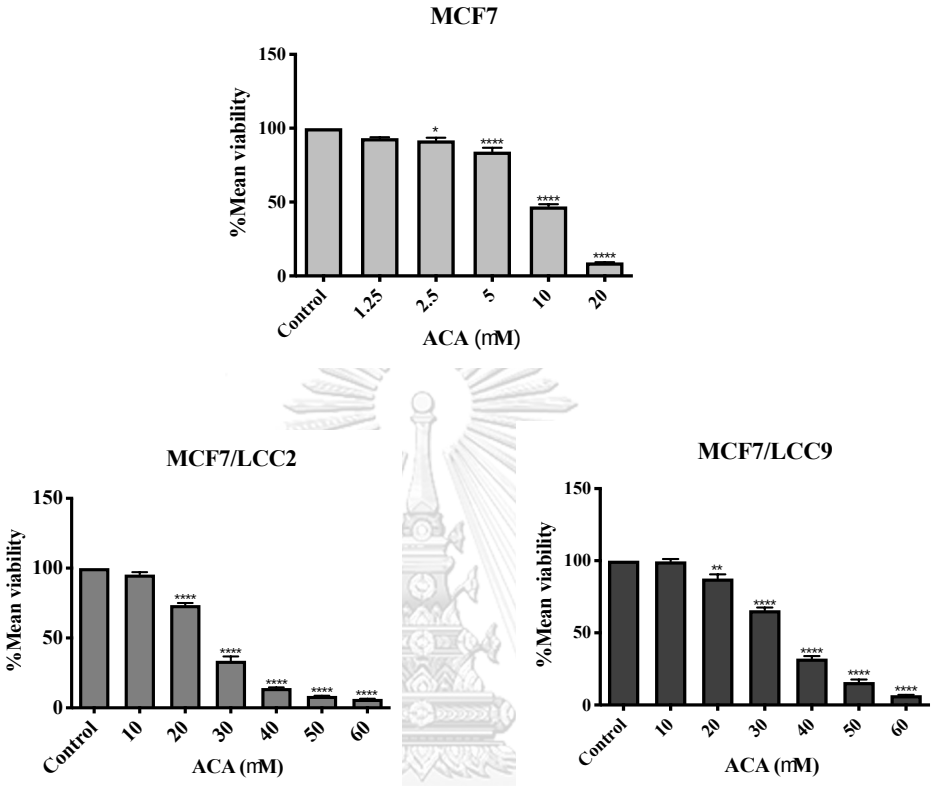

Figure B1. The validation of IC_{50} in MCF7, MCF7/LCC2, and MCF7/LCC9 cell lines. This viability assay was performed in the University of Liverpool. IC_{50} values (μM) obtained by MTT assay after 48 hours of ACA treatment against MCF7, MCF7/LCC2, and MCF7/LCC9. Values shows as mean IC_{50} ± SEM, n = 3, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$compared to control.

Table B1. Comparison of IC_{50} values of ACA (48 h incubation) on 3 cell lines performed at Chulalongkorn University (CU) & University of Liverpool (UoL)

Cell lines	**IC_{50} (µM) - CU**	**IC_{50} (µM) - UoL**
MCF7	11.78 µM ± 0.50	9.61 µM ± 1.09
MCF7/LCC2	31.46 µM ± 1.00	25.80 µM ± 0.88*
MCF7/LCC9	41.38 µM ± 1.80	34.58 µM ± 0.97*

IC_{50} values showed as mean IC_{50} ± SEM, n = 3, $^{*}p \leq 0.05$ compared to IC_{50} values at CU site.

Appendix C / Zebrafish husbandry

Table C1. 60x Stock solution of E3 medium (323)

Chemicals	**Amount (per litre)**
NaCl	17.53 g
KCl	0.76 g
$CaCl_2.2H_2O$	2.94 g
$MgSO_4.7H_2O$	4.93 g

Table C2. Concentration of 60x E3 medium stock solution (323)

Chemicals	**Concentration**
NaCl	300 mM
KCl	10.2 mM
$CaCl_2$	20 mM
$MgSO_4$	20 mM

Preparation of 1x E3 medium (working solution) (323)

Dilute 16.6 mL of 60x E3 medium with 933.4 mL of deionised water and adjust pH to 7.2-7.4 and add 3 mL of 0.01% methylene Blue. After that adjust with deionised water to 1000 mL and store at room temperature.

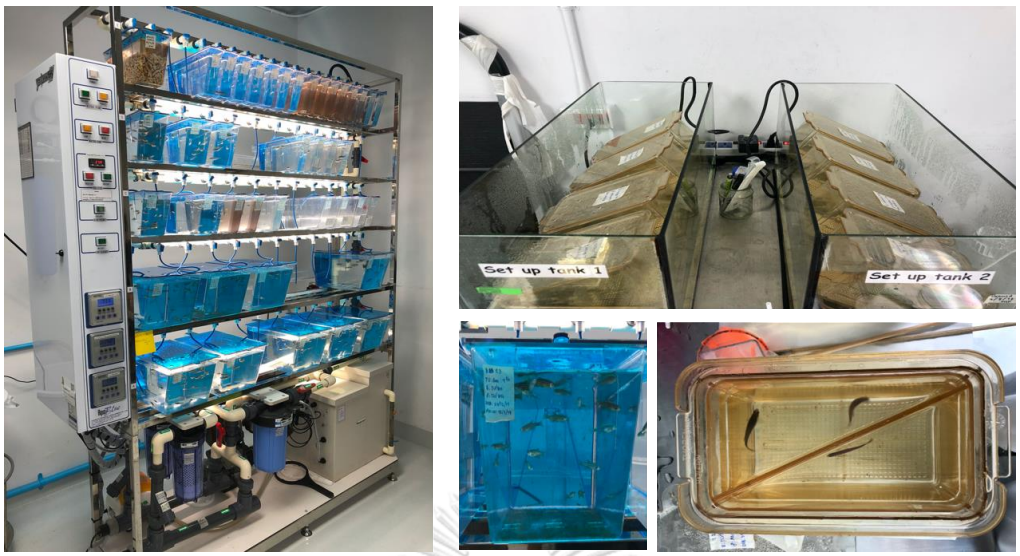

Figure C1. The husbandry of zebrafish and breeding station of zebrafish

Appendix D / Acute toxicity indicators of zebrafish embryo

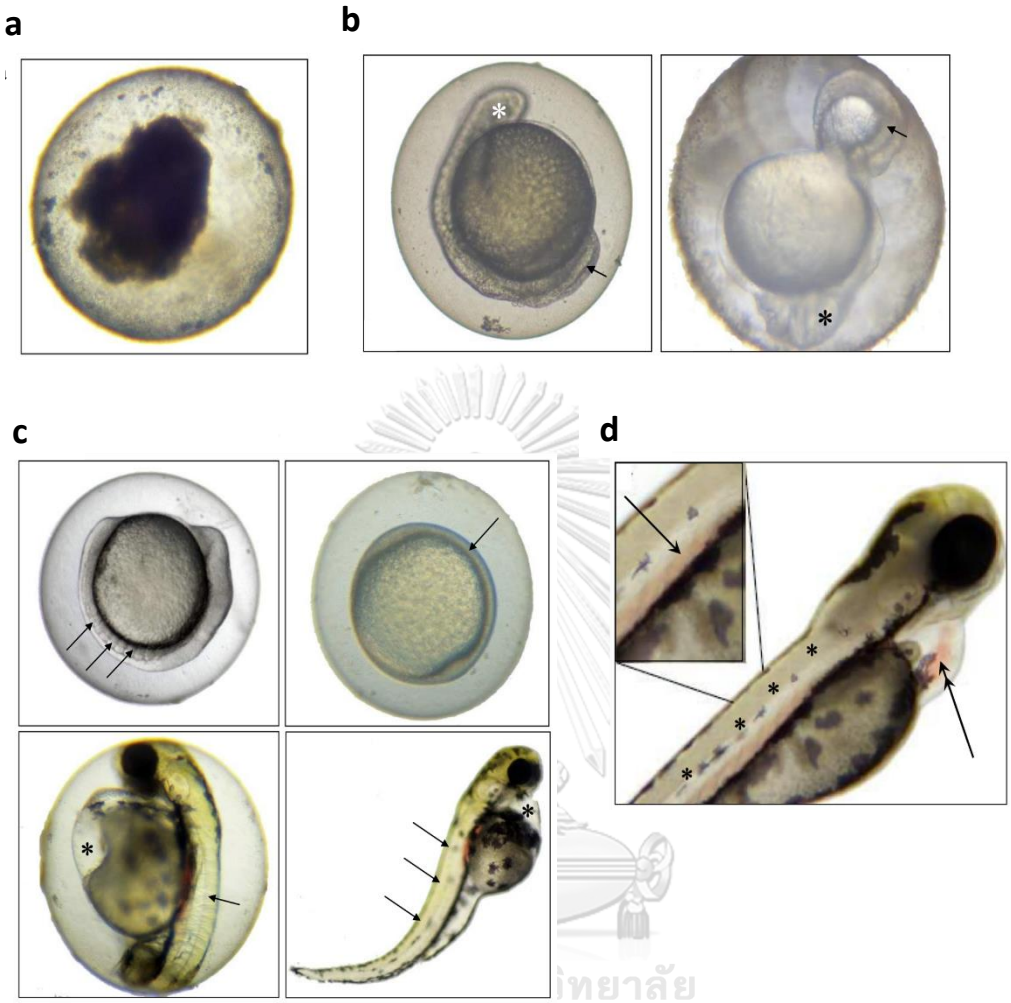

Figure D1. Four atypical indicators of zebrafish embryos acute toxicity as followed OECD test guideline (TG 236) (324)**. (a)** Coagulation of fertilised eggs, **(b)** Lack of detachment of the tailed-bud from yolk sac, **(c)** Lack of somite formation, and **(d)** Lack of heartbeat.

Appendix E / Vessels formation and vessel staining of zebrafish

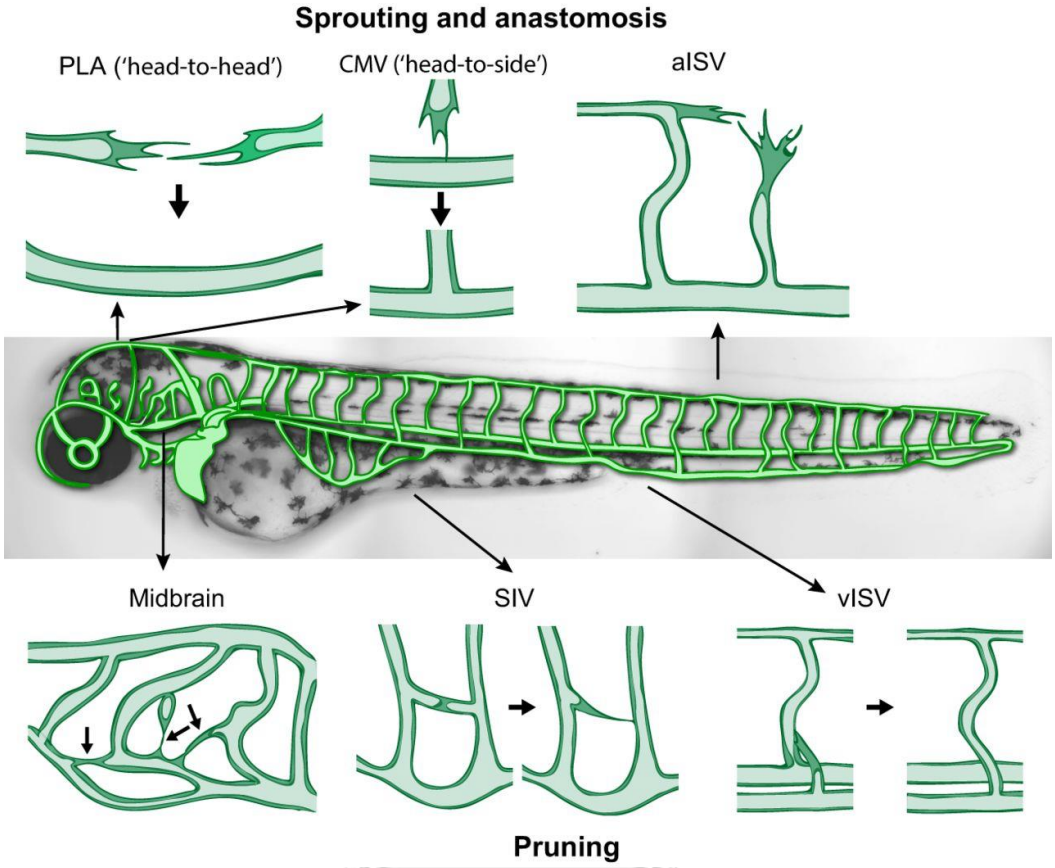

Figure E1. Vascular network formation of zebrafish (325). Palatocerebral artery; PLA, communicating vessel; CMV, nasal ciliary artery; NCA, segmental arteries; aISV, subintestinal vein; SIV, segmental veins; vISV.

Figure E2. Alkaline phosphatase stained-zebrafish and alkaline phosphatase staining reaction at 60 minutes of staining time

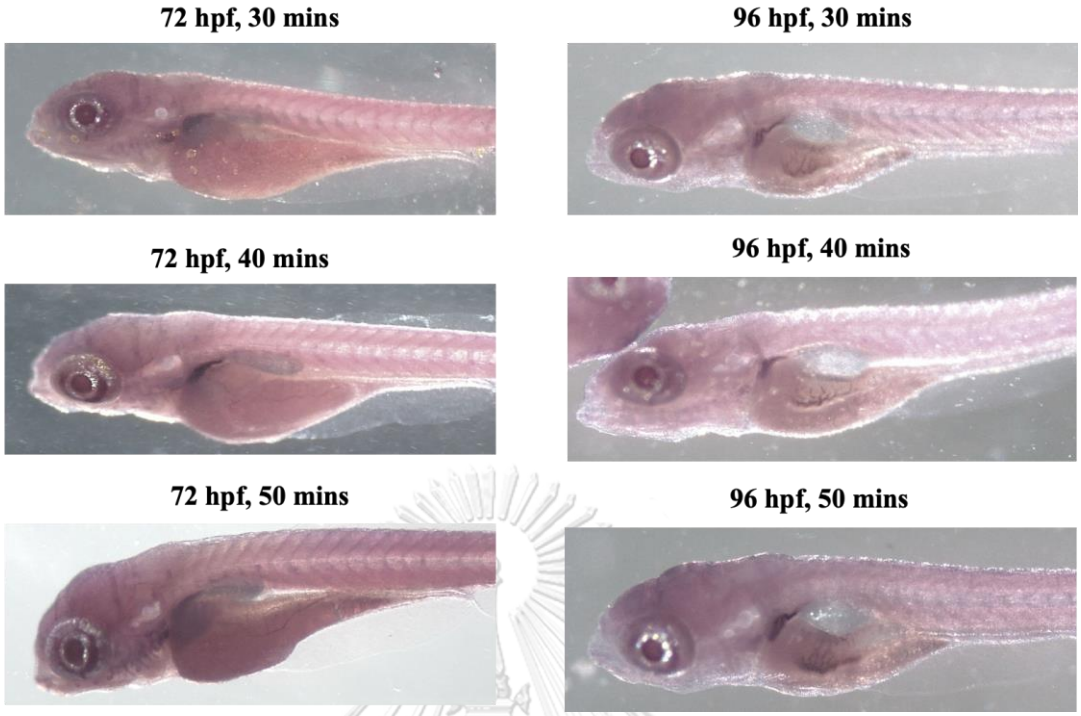

Figure E3. Validation of incubation time for alkaline phosphatase staining in zebrafish at 72 hpf and 90 hpf

Appendix F / Conference Presentations

1) Revision process of Planta Medica journal

"1' Acetoxychavicol acetate from *Alpinia galanga* represses Proliferation, Invasion, and induces Apoptosis via HER-2 signalling in Endocrine-Resistant Breast Cancer cells"

2) 2019 NCRI Cancer Conference (3-5 November 2019), Scottish Event Campus, Glasglow, UK

Poster and silent theater presentations: "Antiproliferation and anti-invasion of 1'-Acetoxychavicol acetate on hormonal-resistant breast cancer cells". **Nalinee Pradubyat**, Athina Giannoudis, Carlo Palmieri, Wannarasmi Ketchart

3) North West Cancer Research Centre (Friday 26th April 2019) - the University of Liverpool Annual Scientific Symposium 2020, Victoria Gallery & Museum, University of Liverpool, UK.

Poster presentation: "1'-acetoxychavicol acetate represses proliferation and invasion and induces apoptosis of endocrine-resistant breast cancer cells" **Nalinee Pradubyat**, Athina Giannoudis, Panupong Mahalapbutr, Chalermchai Mitrpant, Taha Elmitwali, Carlo Palmieri, Wannarasmi Ketchart

Note: The meeting was cancelled due to Covid-19.

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