EFFECTS OF SALINOMYCIN ON CELL VIABILITY, MIGRATION, INVASION AND INCREASING TAMOXIFEN SENSITIVITY IN ANTI-ESTROGEN RESISTANT BREAST CANCER CELLS

Mr. Suwisit Manmuan

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ผลของยาซาลิโนมัยซินต่อการรอดชีวิต,การเคลื่อนที่,การลุกลามและการเพิ่มความไวต่อยาทาม็อกซิเฟ่นในเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจน

นายสุวิศิษฎ์ แม้นเหมือน

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สุวิศิษฎ์ แม้นเหมือน : ผลของยาซาลิโนมัยซินต่อการรอดชีวิต,การเคลื่อนที่,การลุกลามและการเพิ่มความไวต่อยาทาม็อกซิเฟ่นในเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจน (EFFECTS OF SALINOMYCIN ON CELL VIABILITY, MIGRATION, INVASION AND INCREASING TAMOXIFEN SENSITIVITY IN ANTI-ESTROGEN RESISTANT BREAST CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: วรรณรัศมี เกตุชาติ, 136 หน้า.

ยาทาม็อกซิเฟ่นเป็นยาตัวแรกที่นิยมใช้ในการรักษาผู้ป่วยมะเร็งเต้านมวัยหลังหมดประจำเดือนที่มีการแสดงออกของตัวรับเอสโตรเจน อย่างไรก็ตามประมาณ 50% ของผู้ป่วยมะเร็งชนิดนี้มักเกิดปัญหาการดื้อต่อยาทาม็อกซิเฟ่น ซึ่งการดื้อต่อยาทาม็อกซิเฟ่นนำไปสู่การลุกลาม, การเกิดมะเร็งซ้ำและการแพร่กระจายของมะเร็งไปยังอวัยวะต่างๆ ยาซาลิโนมัยซินเป็นยาต้านจุลชีพที่นิยมใช้กันมากในโรคทางปศุสัตว์ จากการศึกษาก่อนหน้านี้พบว่ายาซาลิโนมัยซินมีความสามารถในการยับยั้งเซลล์มะเร็งหลายชนิด ดังนั้นในการศึกษานี้จึงตรวจสอบผลของยาซาลิโนมัยซินในการยับยั้งการรอดชีวิต, การเคลื่อนที่, การลุกลาม, การเพิ่มความไวต่อยาทาม็อกซิเฟ่น และศึกษากลไกการออกฤทธิ์ในระดับโมเลกุลของยาซาลิโนมัยซินในการยับยั้งการลุกลามผ่านทางการลดการแสดงออกของ matrix metalloproteinase 9 (MMP9) และการเพิ่มความไวต่อยาทาม็อกซิเฟ่นผ่านทางการเปลี่ยนแปลงการแสดงออกของยีนเป้าหมายของตัวรับเอสโตรเจนและยีนที่เกี่ยวข้องกับการดื้อต่อยาทาม็อกซิเฟ่น ฤทธิ์ต้านมะเร็งของยาของซาลิโนมัยซินถูกตรวจสอบโดยเทคนิค MTT cell viability assay ผลการศึกษาพบว่ายาซาลิโนมัยซินมีฤทธิ์ต้านมะเร็งต่อเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจนภายหลังจากได้รับยานาน 24, 48 และ 72 ชั่วโมง เมื่อนำยาชนิดนี้มาศึกษาต่อไปเกี่ยวกับผลการออกฤทธิ์ร่วมกับยาทาม็อกซิเฟ่นในการยับยั้งการรอดชีวิตของเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจนโดยใช้เทคนิค tamoxifen response assay พบว่ายาซาลิโนมัยซินสามารถลดการดื้อต่อยาทาม็อกซิเฟ่นในเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจนและสามารถเพิ่มประสิทธิภาพของยาทาม็อกซิเฟ่นในการยับยั้งเซลล์มะเร็งเต้านมปกติ นอกจากนี้ยาซาลิโนมัยซินยังสามารถยับยั้งการเคลื่อนที่และการลุกลามในเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจนจากการศึกษาโดยใช้เทคนิค scratch assay และ matrigel invasion assay ส่วนผลของยาซาลิโนมัยซินต่อการแสดงออกของ MMP9, ยีนเป้าหมายของตัวรับเอสโตรเจนและยีนที่เกี่ยวข้องกับการดื้อต่อยาทาม็อกซิเฟ่นถูกตรวจสอบด้วยเทคนิค RT-PCR ผลการศึกษาพบว่ายาซาลิโนมัยซินสามารถลดการแสดงออกของ MMP9 อย่างมีนัยสำคัญ ซึ่งเป็นเอนไซม์สำคัญที่ส่งเสริมการเคลื่อนที่และการลุกลามของมะเร็ง รวมถึงยาซาลิโนมัยซินสามารถลดการแสดงออกของ NCOA3 ที่ทำหน้าที่เป็น co-activator ในกระบวนการถอดรหัสยีนเป้าหมายของตัวรับเอสโตรเจน นอกจากนี้ยังสามารถลดการแสดงออกของ cyclin D1 และ c-myc ซึ่งทำหน้าที่เป็น positive regulator ในวัฏจักรเซลล์และเพิ่มจำนวนเซลล์ และยาซาลิโนมัยซินสามารถเพิ่มการแสดงออกของ p21 ที่ทำหน้าที่เป็น negative regulator ในวัฏจักรเซลล์ การศึกษาในครั้งนี้แสดงให้เห็นถึงยาซาลิโนมัยซินอาจจะเป็นยาชนิดใหม่ที่ใช้สำหรับการรักษาหรือใช้ร่วมกับยาทาม็อกซิเฟ่นในการเพิ่มประสิทธิภาพของการรักษาในผู้ป่วยมะเร็งเต้านมชนิดที่ดื้อต่อยาต้านฮอร์โมนเอสโตรเจน

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SUWISIT MANMUAN: EFFECTS OF SALINOMYCIN ON CELL VIABILITY, MIGRATION, INVASION AND INCREASING TAMOXIFEN SENSITIVITY IN ANTI-ESTROGEN RESISTANT BREAST CANCER CELLS. ADVISOR: WANNARASMI KETCHART, M.D.,Ph.D., 136 pp.

Tamoxifen is the first line adjuvant treatment for ER-positive breast cancer in post-menopausal patients. However, approximately 50% of advance stage ER-positive breast cancer patients developed tamoxifen resistance. Resistance to tamoxifen leads to more invasive cancer phenotypes, tumor recurrence, and distant metastasis. Salinomycin is carboxylic polyether ionophore that has been widely used as an antibiotic in poultry diseases. Recent studies demonstrated that salinomycin was able to inhibit various types of cancer cells and cancer stem cells. Therefore, the present study is to investigate the effects of salinomycin in inhibiting cell viability, migration, invasion, and increasing tamoxifen sensitivity and determine molecular mechanism of salinomycin to inhibit cancer cell invasion through matrix metalloproteinase 9 (MMP9) expression and increase tamoxifen sensitivity through altering the expression of ER-target genes and genes involved in tamoxifen resistance in anti-estrogen resistant breast cancer cells. The anti-cancer activity of salinomycin was investigated by MTT cell viability assay. Salinomycin demonstrated anti-cancer effects on the anti-estrogen resistant breast cancer cells after 24, 48 and 72 hours of exposure. This drug was further evaluated for the synergistic effects with tamoxifen on the inhibition of cell viability by tamoxifen response assay. Salinomycin can reverse tamoxifen resistance in the anti-estrogen resistant breast cancer cells and increases the efficacy of tamoxifen on the inhibition of wild-type ER-positive breast cancer cells. Moreover, salinomycin inhibited cell migration and invasion in the anti-estrogen resistant breast cancer cells in scratch assay and matrigel invasion assay. The effect of salinomycin on the mRNA expression of MMP9, ER-target genes, and genes involved in tamoxifen resistance was investigated by RT-PCR. Salinomycin significantly down-regulated the expression of MMP9, which is the main enzymes that promote cancer migration and invasion. In addition, salinomycin down-regulated NCOA3 which functions as a co-activator in ER-target genes transcription. Furthermore, salinomycin profoundly down-regulated cyclin D1 and c-myc which play an important role as positive regulators in cell cycle and cell proliferation. In addition, salinomycin up-regulated the expression of p21 which functions as a negative regulator in cell cycle. These findings suggested that salinomycin is potential to be a promising agent for novel treatment or can be used as a novel combination therapy with tamoxifen for increasing the efficacy of treatment in anti-estrogen resistant breast cancer patients.

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

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

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µl	microliter
^{0}C	degree Celsius
4-OHT	4-hydroxytamoxifen
ABC	ATP-binding cassette
AF-1	activation function 1
AF-2	activation function 2
AIB1	amplified in breast cancer 1
AIs	aromatase inhibitors
ALDH1	aldehyde dehydrogenase 1
ATCC	American Type Cell Culture
CDK	cyclin-depentdent kinases
cDNA	complementary DNA
CK 5/6	cytokeratin 5/6
CO_2	carbon dioxide
CSCs	cancer stem cells
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DOX	doxorubicin
ECM	extracellular matrix
EGFR	endothelial growth factor receptor
EMT	epithelial mesenchymal transition

ER	estrogen receptor
EREs	estrogen response element
ERK	extracellular signaling regulated kinases
ETO	etoposide
FBS	fetal bovine serum
FDA	food and drug administration
H	hour
HCl	hydrochloric acid
HDACs	histone deacetylase
HER2	human epidermal growth factor receptor 2
Hsp90	heat shock protein 90
JNK	c-Jun N-terminal kinase
LBD	ligand binding domain
M	molar (molar per liter)
Ml	milliliter(s)
MMP	matrix metalloproteinase
mRNA	messenger RNA
NaCl	sodium chloride
NAG-1	NSAID-activated gene
NCOA3	nuclear receptor coactivator-3
NcoR	nuclear receptor co-repressor
NSL	nuclear localization signal

PBS	phosphate buffer saline solution
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
P-gp	P-glycoprotein
pH	the negative logarithm of hydrogen ion concentration
PR	progesterone receptor
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpm	revolution per minute
RT	reverse transcription
S.E.M	standard error of mean
Sal	salinomycin
SERD	selective estrogen receptor downregulator
SERMs	selective estrogen receptor modulators
SRC-3	steroid receptor coactivator-3
TIMPs	tissue inhibitors of MMPs

CHAPTER I INTRODUCTION

1. Background and Rationale

Breast cancer is generally a kind of cancer that occurs in women and remains the cause of mortality in Europe and North America **[1]**. Approximately 70% of breast cancer expresses estrogen receptor which is the main target of action of estrogen that can be treated by anti-estrogen **[2]**. Tamoxifen is a member of Selective Estrogen Receptor Modulators (SERMs), used as the first line therapy to block the action of estrogen in ER-positive breast cancer patients particularly in post-menopausal patients. However, half of ER-positive breast cancer patients in advance stage do not respond to ER-antagonist agents and are classified as acquired resistance or intrinsic insensitivity to tamoxifen **[3]**. Fulvestrant is clinically categorized in a group of Selective Estrogen Receptor Down regulator (SERD), used as an alternative therapy for tamoxifen resistant breast cancer and advanced metastatic breast cancer in postmenopausal women. It acts as a pure antagonist to hormone-sensitive breast cancer **[4]**.Therefore, it is more potent and effective than tamoxifin. However, acquired resistance to fulvestrant ultimately appears in the majority of breast cancer patients after prolonged fulvestrant therapy **[5]**.

As mentioned, there are few drug options for patients who become resistant to tamoxifen. Therefore, it is important to discover the new therapeutic targets to decrease and eliminate anti-estrogen resistance in order to prevent tumor recurrence and metastasis. The development of new agents for anti-estrogen resistant breast cancer is challenging. Gupta et al. study identified salinomycin by a high throughput screening method and demonstrated that it was able to effectively inhibit breast

tumor stem cells more than paclitaxel and also inhibit breast cancer cell seeding, cell growth, and metastasis in cancer xenograft nude mice **[6]**. Salinomycin is a monocarboxylic polyether ionophore extracted from *Streptomyces albus* that has been widely used as veterinary drug to eliminate gram-positive bacteria, protozoa, and parasites in poultry diseases. It is also generally fed to bovine animals to enhance nutrient absorption and used as growth promoter in ruminant animals **[7]**. It works in normal transport system of alkali ions in both mitochondrial and cytoplasmic membrane. More recently, salinomycin has been able to inhibit multidrug resistant cancer cells and also function as P-glycoprotein inhibitor in cancer stem cell **[8]**. Moreover, salinomycin has been shown to inhibit cell viability, colony growth, cell migration, and cell invasion of human non-small cell lung cancer cell lines through inducing the expression of the pro-apoptotic protein NAG-1 **[9]**. Recent studies reported that salinomycin induced apoptosis by increasing oxidative stress level via generating intracellular ROS production, leading to disturbance of mitochondrial membrane potential and later releasing of cytochrome c to the cytosol in human prostate cancer cell lines **[10]**. Salinomycin also induced apoptosis through the accumulation of reactive oxygen species and up-regulated pro-apoptotic related genes in cisplatin-resistant colorectal cancer cells **[11]**. However, the effect of salinomycin on anti-estrogen resistant breast cancer cells has not been clarified.

The existence of breast cancer stem cells is one of major mechanisms of tamoxifen resistance that leads to tumor recurrence, more invasive cancer phenotype, and metastasis **[12]**. Since distant metastasis is the main cause of death in breast cancer patients, development of new treatment agents to reduce migration and invasion via down-regulation of matrix metalloproteinase 9 (*MMP9*), which is the

main critical molecule assisting cancer cells to invade surrounding tissue and extracellular matrix during metastatic process, results in the reduction of tumor recurrence and metastasis **[13]**. These findings will provide basic molecular mechanism of potential therapeutic agent that can inhibit anti-estrogen resistant breast cancer cells. Moreover, this study will offer further extension of salinomycin use and evaluation of anti-cancer efficacy of salinomycin on animal tumor models and anti-estrogen resistant breast cancer patients. Therefore, these results will be the preliminary data for clinical study of salinomycin and co-treatment between salinomycin and tamoxifen for anti-estrogen resistant breast cancer patients.

2. Objectives of the study

1. To investigate the effects of salinomycin on cell viability, migration, invasion, and increasing tamoxifen sensitivity in anti-estrogen resistant breast cancer cells.
2. To investigate the effects of salinomycin on mRNA expression of matrix metalloproteinase 9, ER-target genes, and genes involved in tamoxifen resistance in anti-estrogen resistant breast cancer cells.

3. Hypothesis

1. Salinomycin has an inhibitory potential on cell viability, migration, invasion, and increasing tamoxifen sensitivity in anti-estrogen resistant breast cancer cells.
2. Salinomycin has an inhibitory effect on mRNA expression of matrix metalloproteinase 9 and alters ER-target genes and genes involved in tamoxifen resistance in anti-estrogen resistant breast cancer cells.

4. Research design

Experimental research

5. Keywords

Salinomycin, breast cancer, tamoxifen, anti-estrogen resistant, metastasis

CHAPTER II LITERATURE REVIEWS

1. Breast Cancer

Breast cancer is the most general kind of tumor occuring almost totally in women **[14]**. 226,870 women were diagnosed with new breast cancer cases and 39,510 women were dead from breast cancer in 2012 **[15]**. A malignant breast tumor is a group of cancer cells transformed from normal cells and proliferated in breast tissues. Moreover, cancer cells are able to invade into surrounding tissues and spread to other parts and distant organs of the body to grow and form new tumors in secondary distant organs via vascular and lymphatic systems. Most breast cancers usually arise from the cells that line the lobules and ducts of breast. Malignant cells that start off in the lobules and ducts are known as *lobular carcinoma* and *ductal carcinoma*, respectively. The risk factors of breast cancer include life time exposure to estrogen, gender, aging, family history of breast cancer, personal history of breast cancer, nationality, and genetic risk factors such as mutation in *BRCA1* and *BRCA2* genes **[16]**. Breast cancer is classified into 4 major types by their receptor expression : ER positives (2 subgroups : luminal A and luminal B), Basal-like, Her2-enriched, and Normal-like.

The classification depends upon molecular markers including the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6 (CK 5/6), or endothelial growth factor receptor (EGFR) **[17]**. This study focused on ER-positive breast cancer type. Luminal A type is the highest incidence which appears in approximately 70% of breast cancer patients. It has the expression of estrogen receptor without HER2 receptor, and its growth is

stimulated by estrogen **[18]**. The characterization of luminal A subtype is the high expression of ER, lower proliferation rate, less aggressive cancer behavior and higher response to hormonal therapy. Conversely, the luminal B subtype is the lower expression of ER, higher proliferation rate, higher aggressive, and lower response to hormonal therapy **[19]**.

2. Role of estrogen in breast cancer

Estrogen is cyclopentanophenanthrene compounds and steroid hormone of female whose precursor for synthesis is cholesterol. The ovary and adrenal gland are the main sources to produce androstenedione that is the substrate for estrogen biosynthesis. Androstenedione is converted by aromatase to estrone in adipose tissues and skins. Estrone is further converted to estradiol or estrogen in peripheral tissues such as adipose tissues by aromatase enzyme **(Figure 1) [20]**. The most potent and predominant intracellular estrogen in the body is 17β-estradiol. However, other kinds of estrogen that contain estrone (E1) and estriol (E3) are also present at lower level **[21]**. Estrogen plays critical roles in regulation of the physiological functions in the human body, modulation of bone density, and regulation of the ovary during menstrual cycle and also functions in controlling of cell growth and proliferation of mammary glands via Estrogen receptor (ER) **[22]**. Estrogen functions as signaling molecules through the bloodstream and interacting with cells in a variety of target tissues. The main targets of estrogen are breast and uterus. Recently, the study has reported the involvement of estrogen with the tumorigenesis of different types of cancer including endometrial cancer, ovarian cancer, breast cancer, prostate cancer, lung cancer, and colon cancer **[23, 24]**.

Moreover, the higher levels of estrogen were correlated with the increased risk of ER-positive breast cancer in postmenopausal women **[25]**.

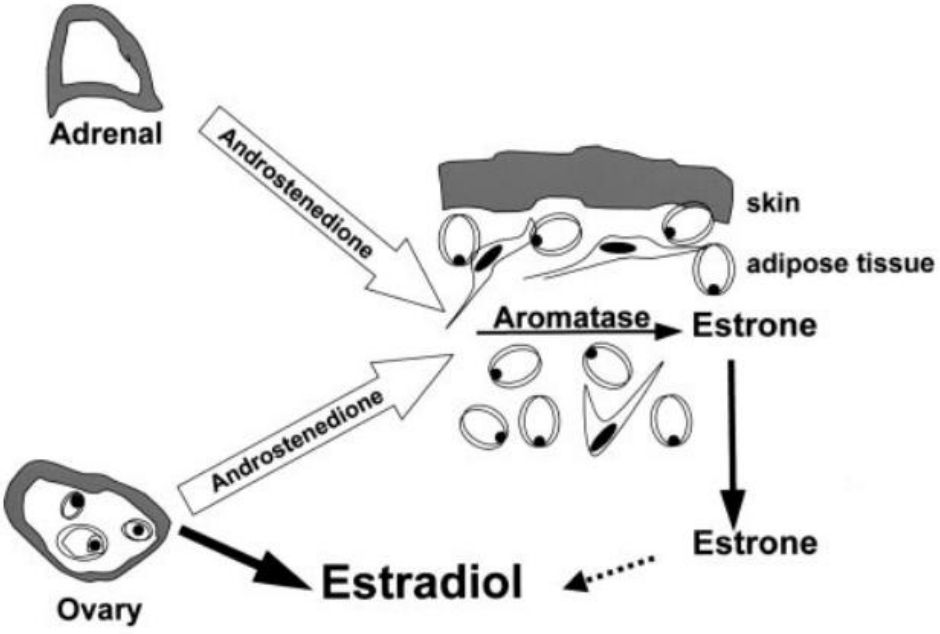


Figure 1 : Estradiol synthetic process **[20]**.

3. Estrogen receptor (ER)

ER is an intracellular nuclear receptor that serves as a transcription factor. There are 2 types of ER, ERα and ERβ. ERα and ERβ were transcribed from *ESR1* and *ESR2* genes and generated by separate genes located on chromosome 6 and 14 respectively. ERα is a receptor that expresses in pituitary gland, uterus, and mammary gland. ERβ is a receptor that expresses in prostate, ovary (granulosa cell), lung, and bone. Both receptors express in normal breast tissues, only ERα involves in breast cancer development and tumorigenesis. The function of ERβ is still unclear. However, previous studies demonstrated ERβ serving as prognostic marker that the higher expression of ERβ was observed in pre-invasive mammary tumors in tamoxifen resistant breast cancer patients and correlated with decreased breast

cancer progression and also increased survival rate of breast cancer patients **[26, 27]**. In addition, *in vivo* study described the expression of ERβ contributing to cell proliferation since ERβ inhibited cell proliferation by decreasing *c-myc*, *cyclin D1*, and *cyclin A* gene and inducing the expression of *p21* and *p27* gene that leads to a cell cycle arrest in G2 phase of cell cycle. These observations support that ERβ is a tumor suppressor in terms of breast cancer prevention and have opposite effects on breast cancer cell proliferation and tumor formation **[28, 29]**.

The structural domains of both receptors are composed of six domains named A/B, C, D, E, and F domain, The N-terminal domain or A/B domain composed of the activation function 1 (AF-1). This region is responsible for transcriptional activity of its receptor in the absence of ligand binding or ligand independent manner. The activity of A/B domain is depending on the phosphorylation level of ER from non-genomic signaling pathways. The C domain or DNA binding domain plays important role in receptor dimerization after the binding of specific DNA sequences with the receptor. The D domain is a flexible hinge and nuclear localization signal (NLS) of receptor. This region has plays an important role in dimerization of receptor and binding with heat shock protein 90 (Hsp90) chaperon molecule. The E domain is known as ligand binding domain (LBD) containing second nuclear localization signal and activation function (AF-2) which is responsible for the activation of ER in ligand-dependent binding. The F domain is a final region that locates at the C-terminal. This domain is not essential for transcriptional activity of ER. However, it plays an important role in modulating both AF-1 and AF-2 region. The transcriptional activity of ER depends on specificity of ligand binding **(Figure 2) [3]**.

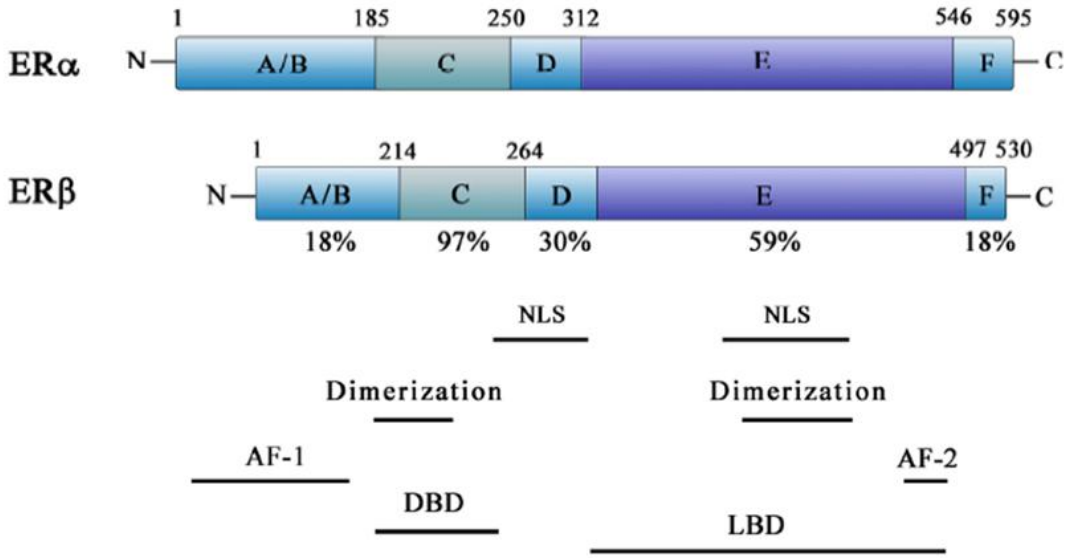


Figure 2 : Functional domain of Human ER**α** and ER**β** **[3]**.

Estrogen plays important roles in tumorigenesis of breast cancer. Estrogen mediates its mitogenic effects via classical pathway or genomic signaling pathway and non-classical pathway or non-genomic signaling pathway. Both pathways play crucial roles in the control of cell differentiation, apoptosis, invasion, and angiogenesis of breast cancer **[30]**. Genomic pathway is initiated by estrogen binding to ER in the outside part of nucleus. This binding stimulates a conformational change of the receptor, leading to receptor phosphorylation and dissociation from Heat shock protein 90 (Hsp90) chaperon molecule. The receptors are dimerized and translocated to inside the nucleus. The ligand-ER complex acts as a transcription factor and binds directly to estrogen response elements (EREs) region on ER-target gene promoters or binds to ER-target gene promoters via binding with other transcription factors at DNA responsive sites. This binding causes the recruitment of co-regulatory proteins such as co-activators or co-repressors. Moreover, ER is able to bind with DNA sequence in promoter region of ER-target genes by protein-protein interaction and other

transcription factors including specificity protein-1 (SP-1) and activator protein-1 (AP-1). The co-activator complexes (COA) induce or modulate gene transcription such as gene encoding growth factor (GFs) and receptor tyrosine kinases (RTKs). On the other hand, the activation of non-genomic pathway arises from ER which is localized outside the nucleus and activates growth factor receptor signaling including the Src, PI3K/AKT, Ras, and MAPK pathways. Moreover, the signaling from the microenvironment includes fibroblasts, endothelial cells, immune system cells, structural components of extracellular matrix (ECM), hypoxic condition, acidity, and other soluble factors such as growth factors and cytokines activated stress-related pathways including the p38 and c-Jun N-terminal kinase (JNK). Moreover, members of integrin family can further modulate components of the transcriptional activity of ER target genes by phosphorylation of ER and co-regulators. However, both genomic and non-genomic pathways lead to the up-regulation of ER target genes, regulating cell proliferation, survival, and invasion of breast cancer **(Figure 3) [30]**.

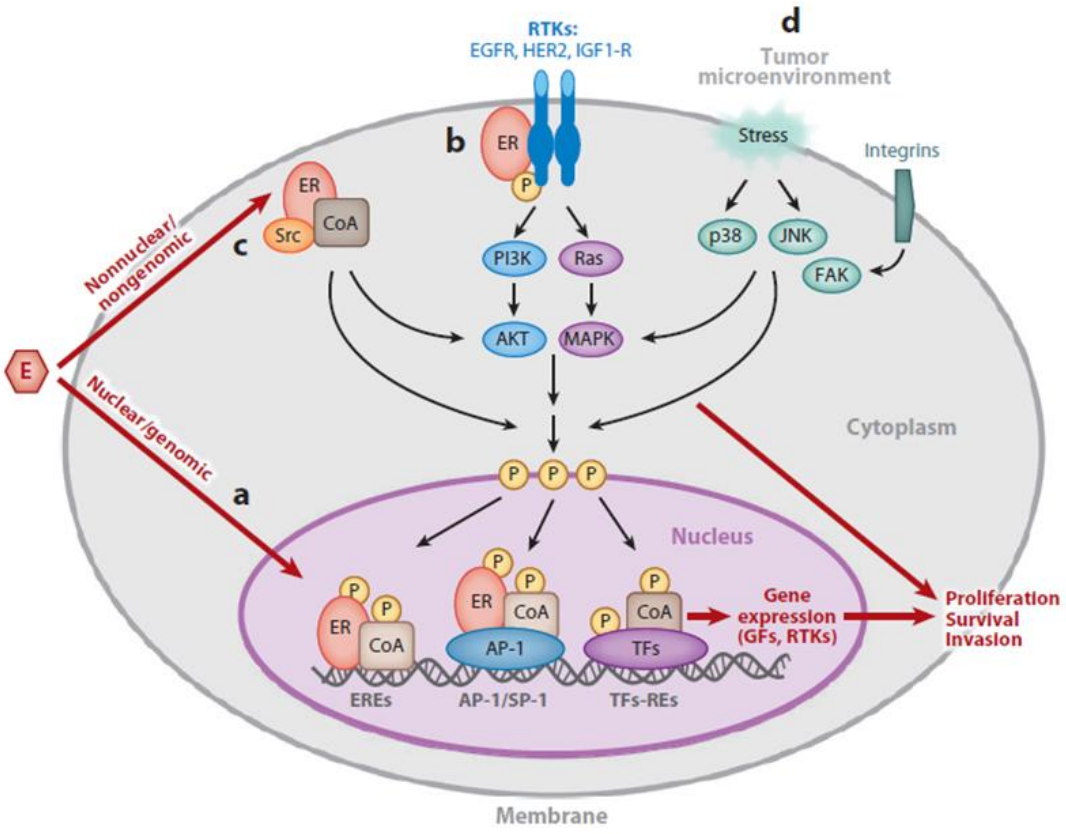


Figure 3 : Mechanism of estrogen action in breast cancer **[30]**.

4. Therapeautic approaches to breast cancer

Currently, breast cancer treatments are considered on the basis the history of women with breast cancer, stage of breast cancer, ages, and hormone receptor status from immunohistochemistry on paraffin-embedded tissues. Particularly, the estrogen receptor and progesterone receptor statuses are important factors used to define breast cancer therapy **[31]**. Current therapy of breast cancer includes surgery, radiation therapy, chemotherapy, targeted therapy, and hormonal therapy. The surgery is the principle approach for breast cancer treatment. However, hormonal therapy is adjuvant therapy for the treatment of hormone-dependent breast cancer to decrease tumor size before and/or after surgery and prevent tumor recurrence.

Hormonal therapy aims to inhibit estrogen biosynthesis or block the action of estrogen at target tissues using selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs), or aromatase inhibitor (AIs)

4.1 SERMs : SERMs is the most common drug group used in ER-positive breast cancer treatment. Tamoxifen, raloxifene, toremifene, and arzoxifene are known as SERMs, a group of synthetic chemical agents whose structures correlated with estrogen and they can bind to ER to modulate ER signaling in target tissue **(Figure 4).** The molecular mechanism of action of SERMs are occured by interacting with ligand binding domain (AF-2) of the ER which cause a conformational change in the AF-2 domain leading to prevent co-activators binding to ER and blocking transcriptional activity of the ER-target genes **[32]**. Tamoxifen is the first line agent used to block mitogenic effects of estrogen at all stages of breast cancer, particularly in pre- and post-menopausal patients. It was approved by the US Food and Drug Administration in 1978, Tamoxifen citrate is the first generation of SERMs. Tamoxifen has the antagonistic effect to ER in breast tissue but acts as partial agonist to bone, cardiovascular system, and uterus. It has been used in pre- and post-menopausal patients for adjuvant and neoadjuvant treatments of ER-positive breast cancer patients to reduce the incidence of breast cancer recurrence after surgery. The primary side effects of tamoxifen are hot flashes, blood clots, thromboembolic disease, and endometrial cancer **[33]**. Tamoxifen increases the risk of endometrial cancer, due to a partial agonist effect in the endometrium. However, the incident rate is very low **[34]**.

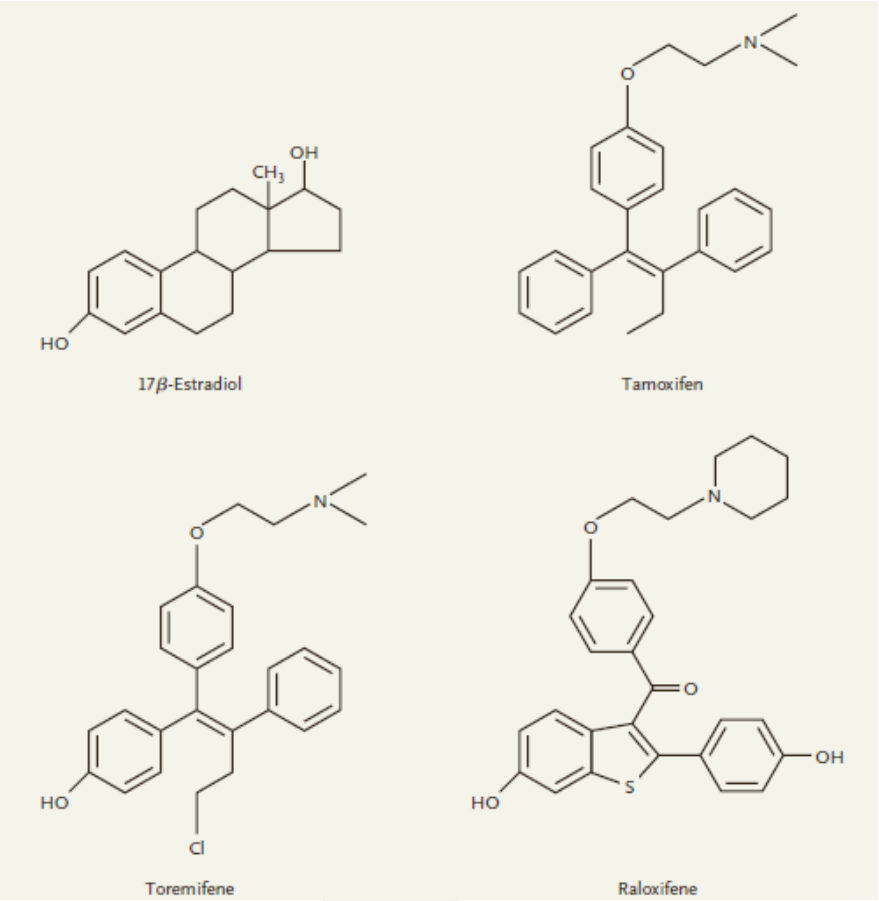


Figure 4 : Chemical structures of 17β-Estradiol, Tamoxifen, Toremifene, and Raloxifene **[35]**.

After tamoxifen entries to the body, tamoxifen is metabolized from prodrug to be active metabolites through phase I and II reactions of biotransformation process. The liver is the main organ for tamoxifen catalyzed, mediated by cytochrome-P450 enzymes; tamoxifen is converted to 4-hydroxytamoxifen and N-desmethyltamoxifen by CYP2D6 and CYP3A4/5 enzymes, respectively. Afterwards, the primary metabolites are converted to the more potent endoxifen through CYP3A4/5 and CYP2D6 enzymes **(Figure 5)**. Endoxifen is a phenolic metabolite. This metabolite has an anti-cancer effect and is responsible for inhibiting agonistic effects of estrogen more effective than tamoxifen **[36]**.

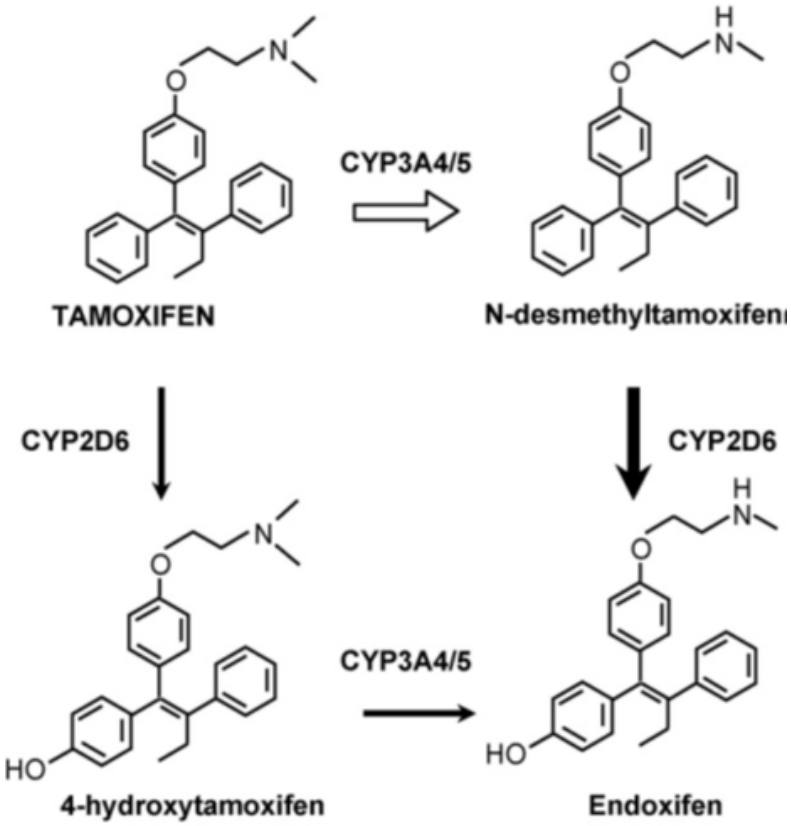


Figure 5 : Mechanism of tamoxifen metabolism **[36]**.

4.2. SERDs : Fulvestrant is the prototype of SERDs. Fulvestrant is an alternative therapy for tamoxifen resistant breast cancer and advance metastatic breast cancer in post-menopausal women. It is a pure antagonist and has no estrogenic effect to the hormone-sensitive breast cancer. Thus, it is able to completely disrupt ER receptor and block ER dimerization. This effect leads to ER degradation and the inhibition of estrogen signaling via ER down-regulation **(Figure 6)**. Fulvestrant also obstructs the activity of ER target genes associated with breast cancer progression, invasion, metastasis, and angiogenesis. Therefore, it serves as an alternative drug for tamoxifen resistant patient **[32, 37]**. However, the effectiveness

of this drug is decreased by the acquired resistance or does not response to fulvestrant therapy in most of ER-positive breast cancer patients **[38]**.

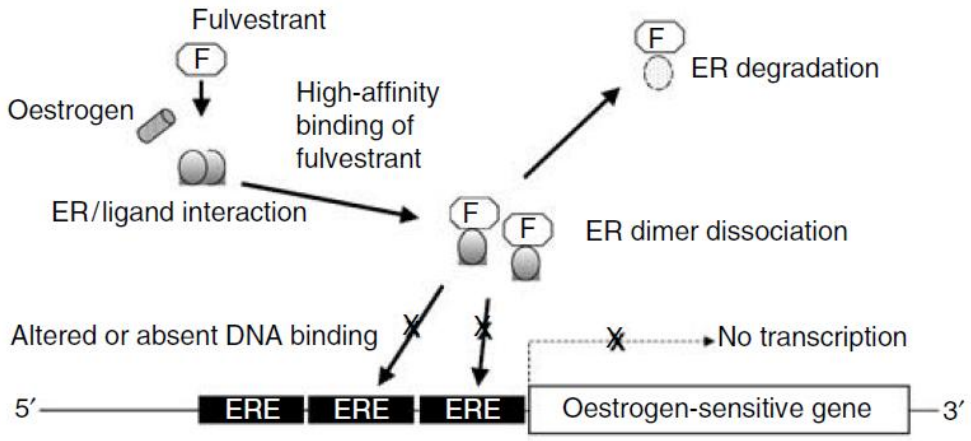


Figure 6 : Mechanism of action of fulvestrant

F = fulvestrant ; ER = estrogen receptor ; ERE = estrogen response element **[39]**.

4.3. AIs : AIs are the inhibitors of aromatase cytochrome P450 or estrogen synthetase **(Figure 7)**. This enzyme is essential for the final step of estrogen biosynthesis in order to convert androgen to estrogen. This drug group is divided into type I (steroidal) inhibitor and type II (non-steroidal) inhibitor by their chemical structures. Currently, available AIs are exemestane, anastrozole, and letrozole. Clinical trials have shown that aromatase inhibitors were more effective and had greater efficacy than tamoxifen in post-menopausal women with early or advance ER-positive breast cancer. This drug cannot be used in pre-menopausal women with breast cancer because of adverse effects from the inhibition of estrogen synthesis in the body **[40, 41]**. However, acquisition of resistance to aromatase therapy is eventually occurred in breast cancer patients **[42]**.

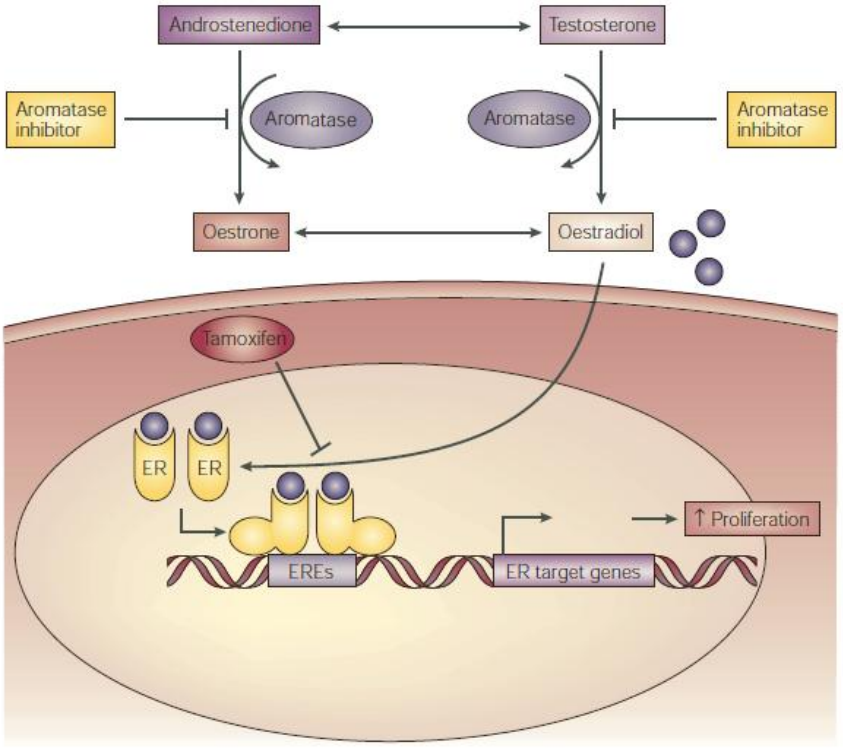


Figure 7 : Mechanisms of action of aromatase inhibitor and tamoxifen **[40]**.

5. Mechanisms of tamoxifen resistance in breast cancer

Tamoxifen resistance is still a problem limiting the potency of breast cancer treatment in ER-positive breast cancer patients **[43]**. Resistance to hormonal therapy can be categorized into two major forms, de novo resistance and acquired resistance. The de novo resistance or intrinsic resistance occurs in breast cancer patients who are primarily unresponsive to tamoxifen. Acquired resistance occurs in breast cancer patients who initially responded to hormonal therapy, and developed resistance since failing to respond to hormonal treatment after prolonged hormonal therapy in half of breast cancer patients **[44, 45]**. Mechanisms underlying tamoxifen resistance are described as following **(Figure 8)**:

5.1 The alteration of tamoxifen bioavailability

The general mechanism of drug resistance is decreasing concentration of drug inside cells. The p-glycoprotein (P-gp) is the efflux protein, which is the member of the ABC transporter superfamily. It also functions as a membrane pump P-gp to decrease drug influx or increase drug efflux from cells. However, the mechanism of modifying tamoxifen accumulation is not well understood since it might not be related only to P-gp **[46]**. Tamoxifen is a prodrug that requires metabolism process to generate active compounds. These processes need CYP2D6 and CYP3A4 metabolizing enzyme in the liver. Both enzymes play a critical role in converting tamoxifen to be 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyltamoxifen. Both isoforms have agonist activity to ER and anti-cancer activity more than tamoxifen. Thus, CYP2D6 and CYP3A4 polymorphisms are correlated with the alteration of tamoxifen metabolites **[3]**.

5.2 Cancer stem like cells

Cancer stem cell is a group of cancer cells which have capability to self-renewal, proliferate and transform to be cancer cells. Breast cancer stem cells are resistant to hormonal therapy that causes tumor recurrence and metastasis. The expression of ATP-binding cassette (ABC) drug pump such as p-glycoprotein/MDR1 increased in cancer stem cells. Cancer stem cells increase the activity of aldehyde dehydrogenase 1 (ALDH1) that functionally involved to proliferation and self-protection in cancer stem cells. They also activate Wnt-β catenin signaling, Hedgehog and Notch signaling pathways **[47]**. Moreover, previous study demonstrated that tamoxifen-resistant breast cancer cells had cancer stem-like cell properties by increasing mRNA levels of SOX-2, Oct-4, and CD133 when compared to

wild-type cells. As mentioned before, this mechanism is one of the main causes of tumor recurrence and failure of hormonal treatment **[48]**.

5.3 Loss of ERα expression

ERα is the main predictive marker of the response to hormonal therapy. Thus, the loss of ERα expression is the main mechanism of intrinsic resistance to tamoxifen treatment. Moreover, the absence of ERα expression related to epigenetic modification. Epigenetic change caused the methylation at CpG islands of the ER promoter and histone deacetylation, resulting in transcriptional inactivation of the ER target genes and was responsible for acquired resistance to hormonal therapy because approximately 17%–28% of breast cancer patients with acquired resistance do not express ERα. In addition, There are other mechanisms which cause the loss of ER expression, such as hypoxic condition, highly expression of EGFR or HER2, MAPKs hyperactivation, and p53 mutation **[49, 50]**.

5.4 Alteration of ERβ expression

The role of ERβ in tamoxifen resistance has not been elucidated. However, the alteration of ERβ expression also involves and plays a crucial role in the mechanism correlated with tamoxifen resistance **[51]**. The study reporting higher expression of ERβ was observed in tamoxifen-resistant breast cancer patients when compared to tamoxifen-sensitive tumors **[52]**. In contrast, another report demonstrating lower level of ERβ protein was observed in pre-invasive mammary tumors when compared to benign tumors. These findings suggested that ERβ may have a protective effect opposed to the effect of estrogen **[53]**. Thus, the role of ERβ in tamoxifen resistance is still not understood well.

5.5 Alteration of co-regulatory proteins.

Co-regulatory proteins, which are co-activator and co-repressor, play pivotal roles in the control of transcriptional activity of ER target genes. Therefore, the alteration of these proteins contributed to tamoxifen resistance. The amplified in breast cancer 1 (AIB1) is an important ER co-activator. It functions in regulating transcriptional activity of ER. It is highly expressed in more than fifty percent of breast tumors **[54]**. It was previously reported that the expression of *AIB1* was higher in human breast tissues and AIB1 increased estrogenic agonist activity of tamoxifen which associated to tamoxifen resistance **[46]**. Additionally, clinical studies showed that the high expression of *AIB1* was correlated with the worse prognosis in breast cancer patients **[55]**. On the other hand, the nuclear receptor co-repressor (NcoR) is an important co-repressor to repress transcription activity of ER target genes. It has been considered as a predictive marker to foretell the response of tamoxifen treatment. Interestingly, clinical studies have significantly demonstrated association between lower expression of NcoR with shorter regress-free survival in invasive ER**α** positive breast tumors from postmenopausal breast cancer patients who recieved tamoxifen therapy after surgery **[56]**.

5.6 Alteration of growth factor signaling

The alteration of growth factor signaling and downstream signaling molecules can induce cancer growth either in concert with genomic signaling or non-genomic signaling and leads to tamoxifen resistance, such as the activation and high expression of tyrosine kinase receptor e.g. epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), PI3K/AKT signaling pathway, stress signaling pathway, and insulin-like growth factor **[3]**. The activation of these pathways can

cause the induction of ER activity and increases the level of co-regulatory proteins and other downstream signaling molecules such as cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors which play roles in controlling cell cycles and increasing cell survival and proliferation of breast cancer cells**.** In addition, the clinical data demonstrated a relationship between ER, HER-2, p38, and ERK (extracellular signaling regulated kinases) in the tamoxifen-resistant specimens which may involved to tamoxifen resistance. These findings suggested that crosstalk between ER and growth factor signaling act as an adaptive mechanism to bypass the cytotoxic effects of tamoxifen **[57]**.

5.7 Alteration of cell cycle signaling molecule

The positive regulators and negative regulators play important roles in cell cycle progression. Thus, the up-regulation of positive regulators and down-regulation of negative regulators contribute to tamoxifen resistance. Especially, cyclin D1 is the main protein to regulate G_1–S phase transition in the progression of cell cycles. This protein is elevated at mRNA and protein levels up to 50% of breast cancer tumors **[58]**. Recent evidence suggested that cyclin D1 associated with breast cancer tumorigenesis since cyclin D1 exerts its oncogenic effects in ER signaling via inducing the elevation of ER-mediated transcription through a CDK-independent mechanism **[59]**. Moreover, cyclin D1 is one of ER-target genes. Therefore, overexpression of cyclin D1 contributes to the response to tamoxifen therapy. In addition, high expression of *c-myc* which is a transcription factor, functions in regulation of p21 that plays a role as a negative regulator in cell cycle progression. *In vitro* study demonstrated that the over-expression of *c-myc* decreased the level of *p21* expression in human breast cancer cells **[60]**.

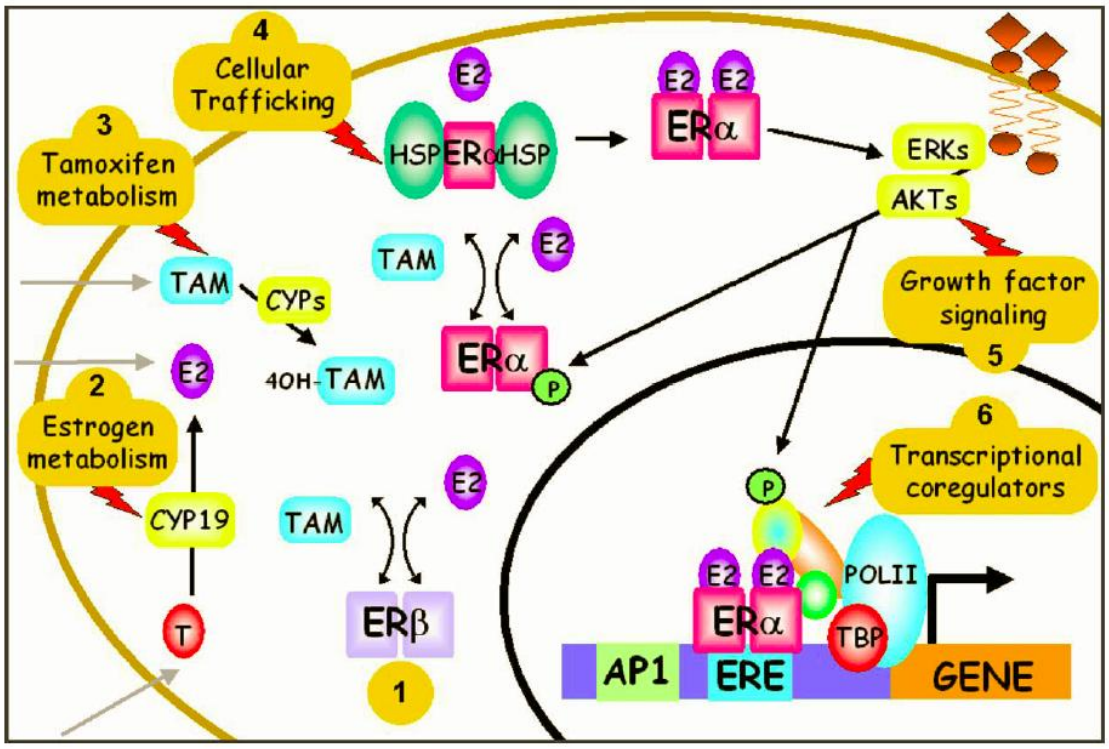


Figure 8 : Mechanisms of tamoxifen resistance in breast cancer **[61]**.

6. Critical factors involved in tamoxifen resistance

6.1 Nuclear receptor coactivator 3

Nuclear receptor coactivator-3 (NCOA3), Amplified in breast cancer 1 (AIB1) or steroid receptor coactivator-3 (SRC-3) is a member of the p160 nuclear receptor co-activator family that has been amplified and overexpressed in approximately 64 % of breast cancer tumors **[62]**. It is an important co-activator and plays significant roles in tumor progression and tumorigenesis of breast cancer. NCOA3 interacts with ER and assembles to be co-activator complex, which recruits histone deacetylases (HDACs) , turns on chromatin remodeling through histone modification and facilitates RNA polymerases II, leading to activating estrogen dependent transcription **(Figure 9) [63]**. Moreover, NCOA3 can bind to signaling molecules mediated cell proliferation and migration such as human epidermal growth factor receptor 2 (HER2). In transgenic mouse model (AIB1-tg), NCOA3 is an ER co-activator and exhibits oncogenic properties when highly expressed in the transgenic mouse model. The mice developed mammary hyperplasia, mammary hypertrophy, and malignant mammary tumors **[64]**. Clinical study demonstrated that the expression of *NCOA3* was correlated with ER**α** positive tumors since *NCOA3* overexpression was observed in 105 unselected tumor specimens of primary breast cancer patients and the expression was higher when compared to normal mammary epithelium in 64 percent of primary tumors from postmenopausal patients **[54]**. It was previously reported that the higher expression of *NCOA3* was correlated with tamoxifen resistance and shortened survival of breast cancer patients **[65]**.

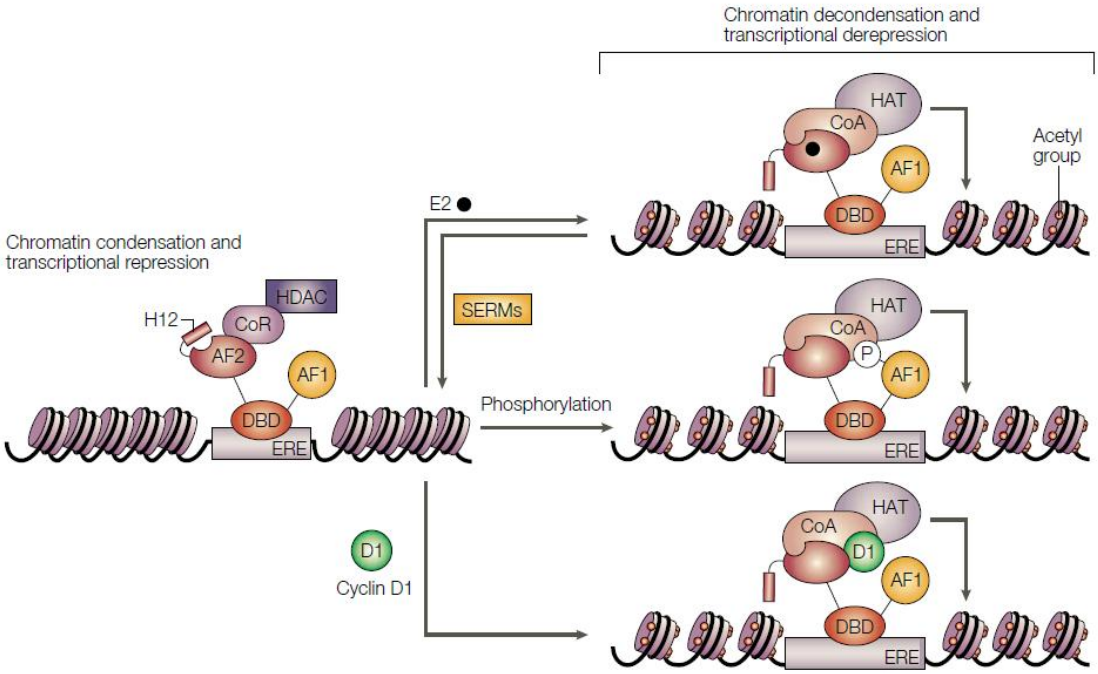


Figure 9 : Roles of co-activators (CoA) in estrogen-receptor activation **[66]**.

6.2 Cyclin D1

Cyclin D1 is the major protein regulator during G_1/S phase transition and initiates mitosis in cell cycle progression **(Figure 10) [67]**. Cyclin D is the protein encoded from *CCND1* (*PRAD1*) gene located on chromosome 11q13 **[68]**. Cyclin D1 is the predominant protein associated with breast cancer tumorigenesis since dysregulation of cyclin D1 activity often causes various types of cancer **[69]**. The regulation in cell cycle of cyclin D1 starts from binding to cyclin dependent kinase 4 (CDK4) or CDK6 to induce the phosphorylation of the retinoblastoma protein (Rb). The amplification and overexpression of *cyclin D1* were found in 50% of human breast cancer tumors correlated with poor prognosis **[70]**. Recent evidence suggested a critical role of cyclin D1 in the development of ER-positive breast cancers and also

contributed to tamoxifen resistance. The overexpression of *cyclin D1* was found in 8.7% of the breast tumors and was involved with increased risk of breast cancer recurrence **[71, 72]**. Moreover, the expression of cyclin D1 correlated strongly with ER status in breast cancer **[73]**.

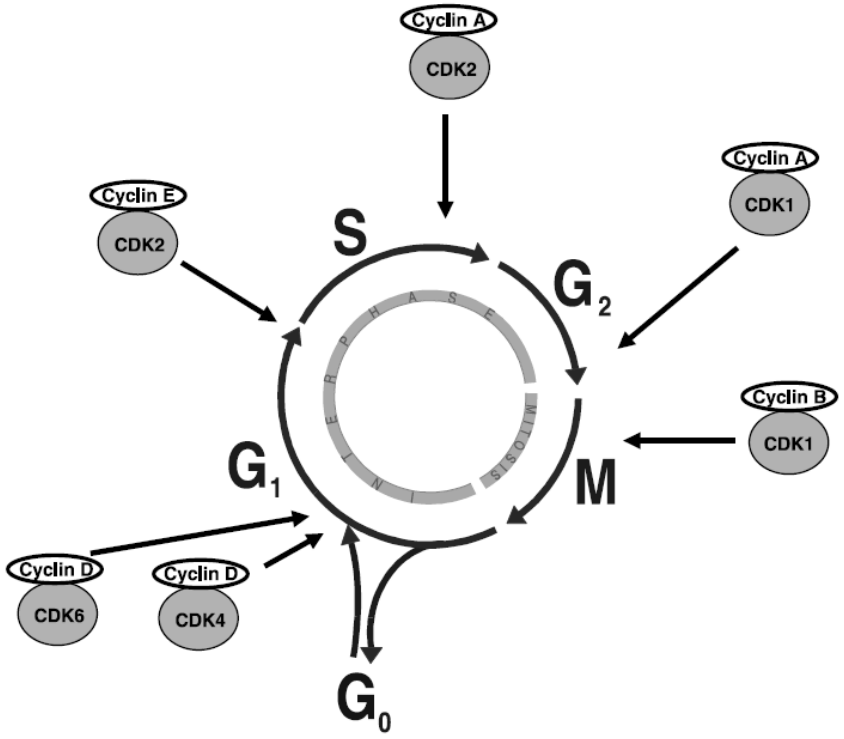


Figure 10 : Stages of the cell cycle and the activity of regulatory cyclin/CDK complex **[74]**.

6.3 C-myc

C-myc is a nuclear transcription factor, a product of c-myc proto-oncogene located on human chromosome 8. It is transcribed to three major proteins including c-myc1, c-myc2, and c-mycs **(Figure 11)**. The c-myc2 is the main form of three c-myc proteins known as "c-myc" overexpressed in approximately 20-30% of breast cancer patients. It plays roles in regulating cell proliferation by driving the cell in G_0 phase into cell cycle, inhibiting cell differentiation and inducing program cell death.

Moreover, c-myc can interact with ER and modulate estrogen dependent signaling. The function of these proteins is started by the activation of C-myc target genes as positive regulators in cell cycle including *cyclins D1*, *D2*, *A* and *E*, cdk4, cdc25A, *e2f1, e2f2*, and *B* **[75]**. The alteration and amplification of *c-myc* expression is frequently observed in various types of tumors including breast cancer, lung cancer, and rare cases of colon cancer. Recent study showed that the de-regulation of *c-myc* expression induced genomic instability as evaluated by the rate of development of aneuploidy and gene amplification in cancer cells **[76]**. The amplification and de-regulation of *c-myc* oncogene occurred in 15–20% of breast cancer patients and related to poor prognosis **[77, 78]**. In addition, the overexpression of *c-myc* gene leaded to the alteration of chromatin structure, tumor aggression, apoptosis, cell adhesion, and angiogenesis and associated with poor clinical outcome **[79]**. Clinical study reported that overexpression of *c-myc* gene involved with the transformation from carcinoma *in situ* to invasive carcinoma and was primarily responsible for being the predictive marker of disease-free survival in tamoxifen treatment patients **[80]**.

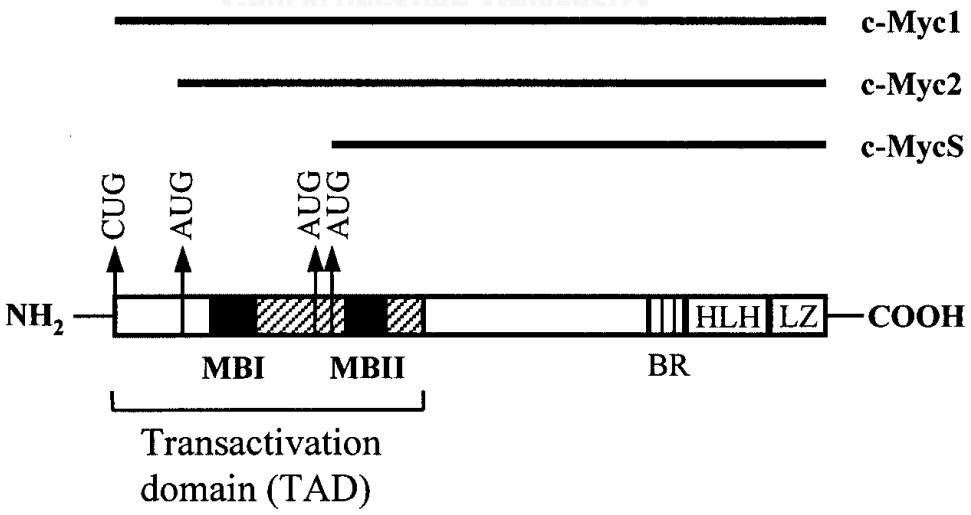


Figure 11 : Schematic diagram of the C-myc family proteins **[75]**.

6.4 P21

P21 is a negative regulator of cell cycle **(Figure 12)**, also called WAF-1 encoded from human *CDKN1A* gene and classified to be a member of the Cip/Kip family of CDK inhibitor proteins which include p21, p27, and p57. These proteins compose a conserved region of sequence at the NH_2 terminus for the suppression of cyclin/CDK complexes **[81]**. Cyclin-CDK complexes play a crucial role during cell cycle progression at G1 phase through activated downstream targets by phosphorylation. The p21 has a tumor suppressor activity in inducing cell cycle arrest, cell differentiation, and cell senescence. This protein mediates its biological activities by binding to CDK and inhibiting the phosphorylation of retinoblastoma (Rb) protein by cyclin-CDK complexes including cyclin A-CDK2, cyclin E-CDK2, cyclin D1-CDK4, and cyclin D2-CDK4 complexes **[82]**. The p21 plays an essential role in arresting cell growth after DNA damage to prevent the development of tumor cells and cell cycle progression to S phase. P21 is composed of a COOH terminal binding site for proliferating cell nuclear antigen (PCNA) and competes with DNA-polymerase to bind to PCNA that contributes to DNA synthesis, resulting in the inhibition of DNA synthesis **[83]**. Previous study showed the loss of *p21* expression occurring in most of breast cancer tumors from a patient with tamoxifen-stimulated breast cancer and contribute to be the predominant mechanism of acquired resistant of tamoxifen **[84]**. Furthermore, the loss of p21 inhibitory protein leaded to the hyperphosphorylation of estrogen receptor-α at serine 118 and 305, causing the recruitment of co-activator, increasing expression of ER-regulated genes, and leading to tamoxifen resistance **[85, 86]**.

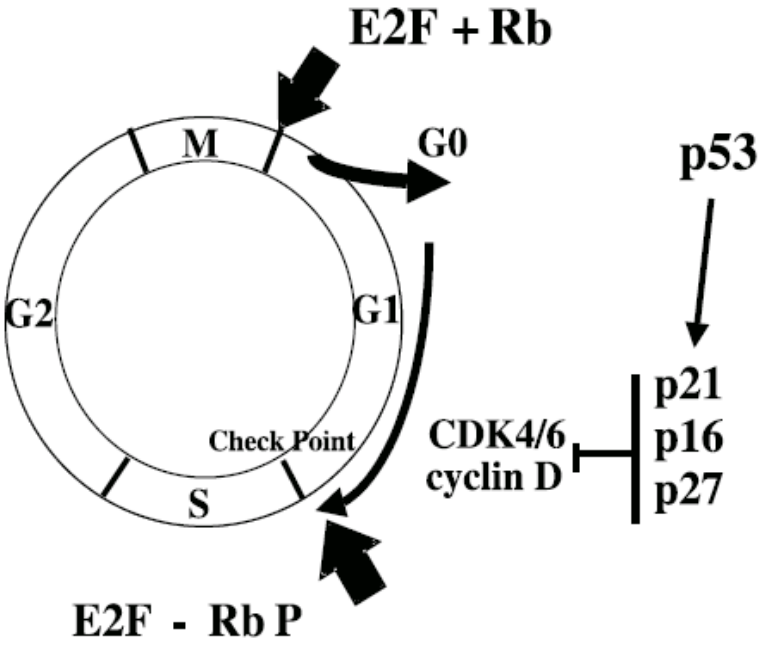


Figure 12 : Role of negative cell cycle regulatory proteins in cell cycle **[87]**.

Table 1 : Alteration of genes related to tamoxifen resistance **[88]**.

Genes related to tamoxifen resistance	Expression in tamoxifen resistance	Mechanism of tamoxifen resistance
NCOA3	↑	Up-regulation of co-activator in ER transcription
Cyclin D1	↑	Increased expression of positive regulator of cell cycle
C-myc	↑	Increased expression of positive regulator of cell cycle
P21	↓	Reduced expression of negative regulator of cell cycle

7. Metastatic process

Metastasis is the main cause of death in breast cancer patients. This process starts from cancer cells disseminating from primary tumors and invading to neighboring tissues by destroying surrounding extracellular matrix (ECM), basement membrane, cell-cell junction, and cell-matrix junction and activates signaling pathways involved with the cytoskeleton control in cancer cells. Afterwards, the cancer cells intravasate into the blood vessel and spread to lymph nodes, then extravasate from blood circulation and lymphatic system through penetrating basement membranes and endothelial walls and reach to secondary organs. Then, cancer cells arrest and colonize at a distant organ and accommodate to survive in the foreign microenvironment **(Figure 13)** **[89, 90]**.

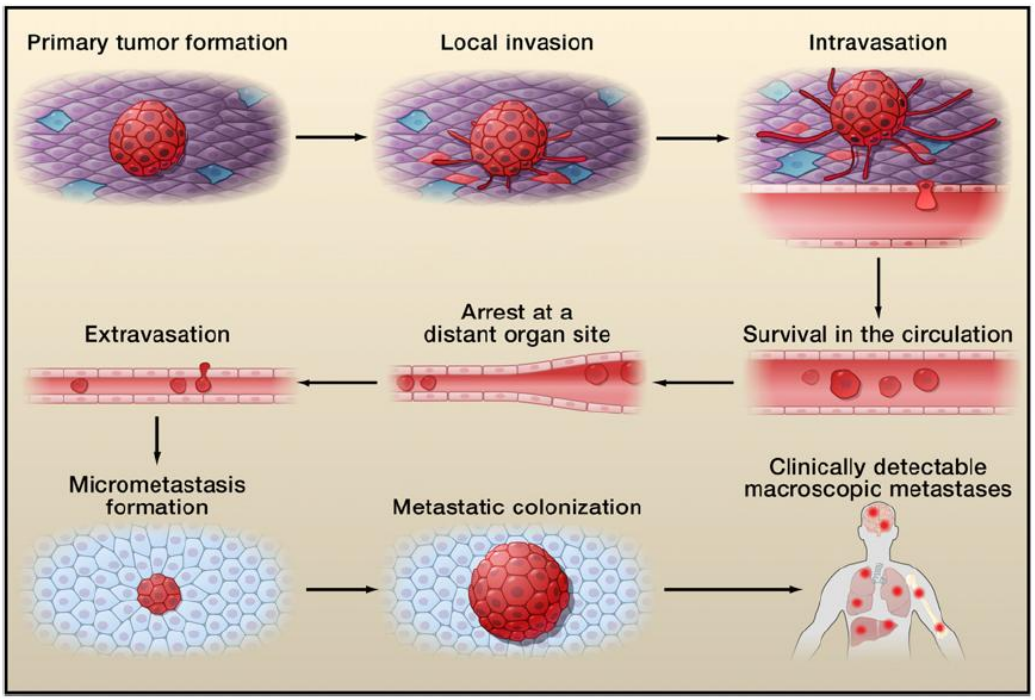


Figure 13 : The invasion and metastatic process of cancer **[91]**.

Matrix metalloproteinase (MMP) is a large family of zinc-dependent endopeptidase. This family shares specific functional domains and structural components. The structure of MMP is composed of the signaling peptide (pre) domain functioning as the conductor of MMP to move into rough endoplasmic reticulum during the synthesis. The propeptide domain plays a role in maintaining the latency of MMPs. The catalytic domain houses a highly conserved Zn^{2+} serving as a binding region of MMP. The hemopexin-like-C-terminal domain (PEX) is a terminal region of its structure and links to the catalytic domain via a hinge region **(Figure 14)**. MMP is regularly secreted by a variety of pro-inflammatory cells and connective tissues such as endothelial cells, fibroblasts, macrophages, osteoblasts, lymphocytes, and neutrophils. The MMP expresses as zymogen that requires proteolytic enzymes including serine proteases, furin, and plasmin to create their active forms. In normal condition, the proteolytic activity of MMP is regulated by various tissue inhibitors of MMPs (TIMPs). In pathological condition, the balance between MMP and TIMPs is altered toward increasing the MMP activity that causes tissue degradation. MMP is highly expressed in various types of tumors including breast cancer **[92, 93]**. MMP plays crucial roles in promoting tumor invasion, angiogenesis, and metastasis by degrading surrounding tissues and releasing growth factors and cytokines inside the ECM and many components of extracellular matrix (ECM) including collagen, elastin, proteoglycan, vitronectin, laminin, and fibronectin. This degradation helps cell migration and spreading of cancer cells **[94]**. Moreover, MMP relates to cancer development and progression through the promotion of cancer cell proliferation by regulating signaling pathways which control cell growth, proliferation, migration, release of growth factors and angiogenesis **[95]**. Matrix metalloproteinase 9 (MMP-9)

is a type-IV collagenase, also known as 92-kDa gelatinase B, which is the major component in basement membrane. MMP-9 involves in cancer invasion and metastasis via degradation of collagen type IV and gelatin which are the main components of ECM. It also functions as a cancer marker and is associated with tumor aggressiveness **[96]**. The high expression of *MMP9* was found in cancer of breast, prostate, ovarian, pancreas, colorectal, bladder, brain, lung, and melanoma **[97]**. Previous study demonstrated that elevation of MMP9 expression in serum and tissue of breast cancer patients was associated with a poor prognosis. Clinical study also demonstrated that *MMP9* gene was correlated with lymph node metastasis **[98, 99]**.

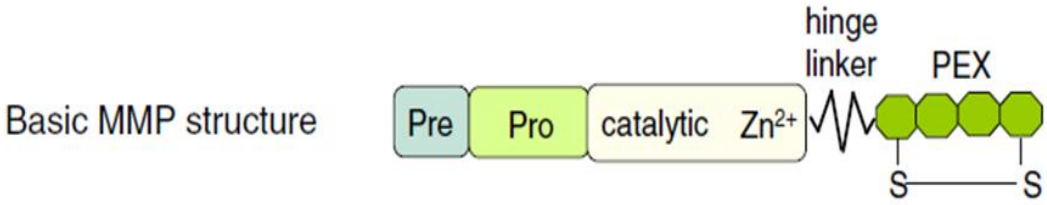


Figure 14 : The structure of the MMP family members **[97]**.

8. Salinomycin

Salinomycin is a monocarboxylic polyether ionophore antibiotic extracted from the cultured supernatant of *Streptomyces albus* **[100]**. It has a mass of 751 dalton and $C_{42}H_{70}O_{11}$ as a molecular formula **[101]**. The structure of salinomycin is composed of pentacyclic molecule with a unique tricyclic spiroketal ring and an unsaturated six-membered ring **(Figure 15) [102]**. It has an effective antimicrobial activity against gram positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus flavus*, *Sarcina lutea*, and *Mycobacterium spp.* Moreover,

salinomycin has an ability to kill some kinds of parasites e.g. *Plasmodium falciparum* and *Eimeria spp* **[47]**. It interferes normal transport system of mitochondria and cytoplasm in prokaryotes and eukaryotes via increasing K^+ efflux and disrupting Na^+/K^+ ion transport across phospholipid bilayer membranes that lead to the disturbance of intracellular pH and cause cell death **[103]**. In addition, it has been widely used as an antibiotic in veterinary medicine for poultry diseases for more than 30 years and used as a growth promoter agent in ruminant animals **[104]**.

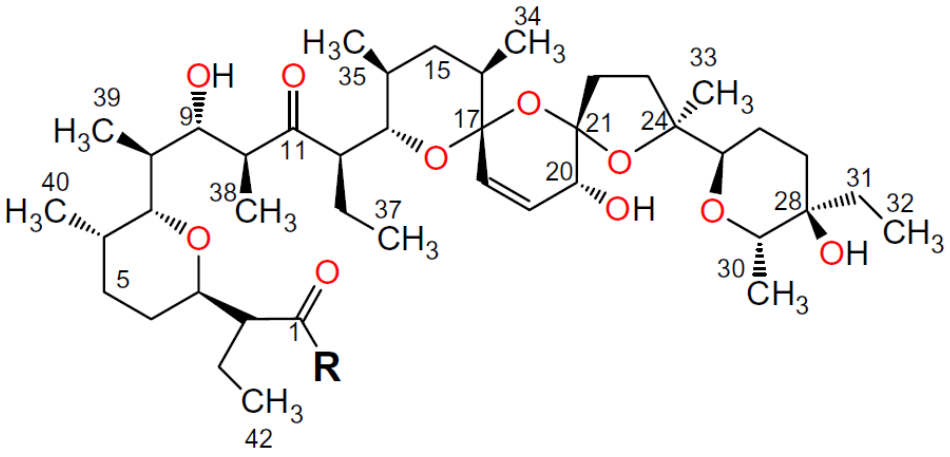


Figure 15 : Structural formulation of salinomycin **[105]**.

Conventional chemotherapeutic drugs can kill a bulk of cancers. However, chemotherapy cannot kill all of cancer stem cells which possess chemo-resistance property **[106]**. Cancer stem cells are associated with chemotherapeutic drug resistance and radiotherapy resistance that lead to the failure of cancer treatment **[107]**. Therefore, the goal of cancer treatment should be the eradication of both cancer cells and cancer stem cells. Thus, the discovery of new therapeutic agents as well as novel therapeutic strategy which has a capability of eliminating cancer stem

cells is still required due to the limitation of current drug options. Recent study reported that salinomycin selectively inhibited human breast cancer stem cells 100-folds more than paclitaxel, a common standard therapeutic drug in breast cancer treatment. In addition, salinomycin was able to inhibit tumor seeding, growth, and cancer metastasis in mice and reduce the expression of cancer stem cells (CSCs)-associated genes from global gene expression analysis after salinomycin treatment compared to paclitaxel treatment. These findings suggested that salinomycin was able to selectively eliminate human breast cancer stem cells **[6].** The recent study revealed that salinomycin had an anti-cancer efficacy to kill various types of cancer including colorectal cancer **[108, 109]**, lung cancer **[110]**, gastric cancer **[111]**, pancreatic cancer **[112]**, hepatocellular carcinoma **[113]**, ovarian cancer **[114]**, prostate cancer **[115]**, and osteosarcoma **[116]**. Other studies demonstrated that salinomycin was able to induce massive apoptosis and overcome chemotherapeutic drug resistance via the reduction of the expression of ATP-binding cassette (ABC) transporters such as p-glycoprotein, a transmembrane protein, responsible for anticancer-drug efflux, to reduce drug concentration in human leukemic stem cell-like KG-1a cells **[117]**. Salinomycin also functions as a p-glycoprotein inhibitor and inhibits doxorubicin (DOX)- or etoposide (ETO)-treated cancer cells through increasing DNA damage and decreasing p21 protein expression **[118, 119]**. Recent report demonstrated that salinomycin was essential in order to deplete cancer stem cells including activation of apoptosis and cell death, inhibition of ABC transporters, inhibition of the Wnt/β-Catenin signaling pathway, inhibition of oxidative phosphorylation in mitochondria, and interference with transmembrane K^+ channel **[47]**.

Recently, the anti-cancer activity of salinomycin has been elucidated that salinomycin decreased cell viability in the human non-small cell lung cancer cell lines, LNM35 and A549 by the activation of a caspase 3/7 cell death pathway and also inhibited colony growth, cell migration, cell invasion mediated by the induction of the expression of the pro-apoptotic protein NAG-1 that contributed to the inhibition of lung cancer cell invasion. These findings suggested that salinomycin has an ability to kill lung cancer cells, supporting the role of salinomycin to eradicate both cancer stem cells and lung cancer **[9]**. Previous study demonstrated that salinomycin reduced the level of fibronectin expression associated with epithelial-mesenchymal transition (EMT) process, induced apoptosis, and inhibited cell proliferation, migration, invasion, and tumorigenesis in endometrial cancer cell lines (Hec-1 and RK12V-SP cells). These findings suggested that salinomycin can be an effective anti-cancer drug for endometrial cancer treatment **[120]**. Moreover, salinomycin inhibited cell growth and migration of pancreatic cancer cells and decreased the proportion of $CD133^{+}$ pancreatic cancer stem cell subpopulation. Further study showed that salinomycin significantly up-regulated E-cadherin expression that serves as an epithelial marker which decreased in epithelial/mesenchymal transition (EMT) and significantly down-regulated the expression of Bcl-2 and proliferating cell nuclear antigen (PCNA). In addition, salinomycin inhibited the expression of Wnt/β-catenin signaling-related proteins including β-catenin and p-GSK-3b that leaded to inhibiting cancer cell proliferation and metastasis. This suggested a possible future use of salinomycin in the pancreatic cancer treatment **[112]**.

The combination therapy is a good promising approach for cancer treatment since it can enhance the efficacy of current cancer therapy and delay cancer resistance. Recent study revealed that salinomycin combined with gemcitabine significantly induced apoptosis in $CD133^{+}$ and $CD133^{-}$ pancreatic cancer cells. Consistently, *in vivo* study demonstrated that salinomycin combined with gemcitabine was able to decrease tumor sizes in mice more effectively than individual agents. This result suggested that salinomycin could be a potential therapeutic agent for novel combination therapy to improve the efficacy of gemcitabine to deplete human pancreatic cancer cells **[7]**. Moreover, salinomycin exhibited anti-cancer activity to inhibit cell growth, arrest cell cycle, and induce apoptosis by inducing the apoptotic signaling pathway through the activation of caspase 3/7 activity, increasing PARP cleavage, and inducing senescence of breast cancer cells through hyperacetylation of histone H3, H4 and upregulation of p21 expression. In addition, salinomycin was able to potentiate the anti-proliferative activity of 4-Hydroxytamoxifen and frondoside A in MCF-7 and MDA-MB-231 breast cancer cells respectively. This data indicated that salinomycin could be a promising alternative strategy for breast cancer treatment and provided a new insight to understand the molecular mechanism of action of salinomycin **[121]**.

In summary, all findings from the mentioned studies suggested that salinomycin was potential to be a promising agent for novel therapeutic targets and novel combination therapy since salinomycin has anti-cancer activity to eradicate cancer stem cells and human cancer cells, overcome apoptotic resistant cancer cells, and sensitize cancer cells to conventional chemotherapeutic drugs. All the aforementioned studies provide a background knowledge for molecular mechanism

and lead to the further study of the anti-cancer efficacy, toxicity, and safety of salinomycin in animal tumor models and cancer patients in the future that may contribute to the development of salinomycin-based combination therapy to improve the efficacy of cancer treatment.

CHAPTER III MATERIALS AND METHODS

1. Materials

1.1 Salinomycin and 4-hydroxytamoxifen

Salinomycin and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma-Aldrich and dissolved in DMSO at a stock concentration of 20 mM and 10 mM, respectively.

1.2 Human breast cancer cell lines

Human breast cancer cell lines MCF-7 were obtained from ATCC. MCF-7/LCC2 and MCF-7/LCC 9 were kindly provided by Dr. Robert Clarke from Georgetown University Medical Center, Washington, DC, USA. The MCF-7/LCC2 and MCF-7/LCC9 cells are tamoxifen and tamoxifen/fulvestrant-resistant cell lines. All Human breast cancer cells line were maintained in Eagle's minimum essential medium (MEM) containing phenol red and supplemented with 5% of heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/ml of streptomycin in a humidified 37°C incubator containing 5% CO_2.

1.3 Chemicals and reagents

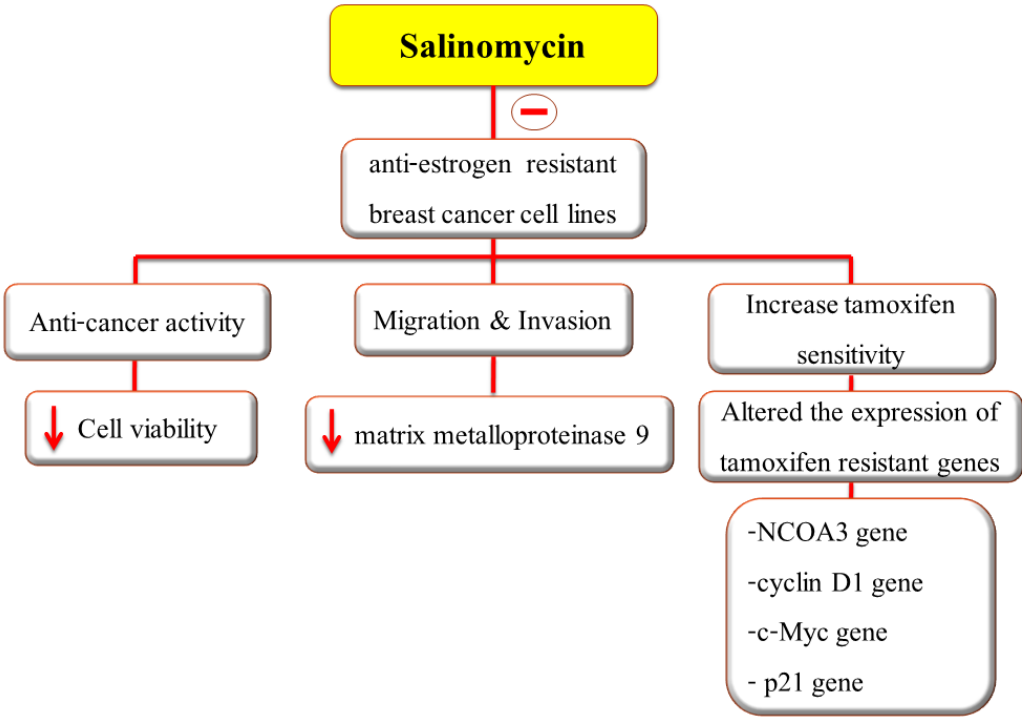
The following chemicals and reagents were used in this study; Methylthiazolyldiphenyl-tetrazolium bromide (Sigma, USA), Dimethyl sulfloxide (DMSO) (Sigma, USA), Fetal bovine serum (Gibco, USA), penicillin/streptomycin (Hyclone, USA), 0.4% Trypan blue dye, dulbecco's modified eagle's medium (DMEM) (Gibco, USA), Charcoal strip fetal bovine

serum (Gibco, USA), phenol red-free IMEM (Gibco, USA), Non-essential amino acids (Gibco, USA), Insulin, Human recombinant, Zinc solution (Gibco, USA), TRiZol reagent (Invitrogen, UK), dietyl pyrocarbonate (DEPC) (Molekula, UK), ImProm-II™ Reverse Transcription system (Promega, USA), primer (Bio Basic, Canada), Taq polymerase (Invitrogen, USA), crystal violet, Matrigel invasion chambers (Corning USA), BD matrigel matrix (Biosciences, USA), absolute ethanol (Merck, Germany), Minimum Essential Medium (MEM) Powder (Gibco, USA), Fungizone® Antimycotic (Gibco, USA), 0.25% Trypsin-EDTA (1X), Phenol Red (Gibco, USA)

1.4 Equipments and Instruments

The followings equipments and instruments were used in this study; Autopipette (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge machine (Hettich, USA), light microscope (Nikon, USA), 96–well plate (Corning, USA), spectrophotometer (Shimadzu, Japan), T-25 Tissue Culture flasks (Corning, USA), hemocytometer (Brand, Germany), ELISA microplate reader (Labsystemsmultiskan, USA), 6–well plate (Corning, USA), gel electrophoresis (Bio-Rad, USA), PCR thermocycler machine (Eppendorf, USA), vortex mixer (Scientific industries, USA), 24–well plate (Corning, USA), analytical balances (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hiclave TM, HVE-50, Hirayama, Japan), incubator (Thermo, USA)

2. Conceptual Framework



3. Methods

3.1 Effect of salinomycin on cell viability in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells

Methylthiazolyldiphenyl–tetrazolium bromide assay (MTT assay) was used to determine the effect of salinomycin on cell viability in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells. Each condition was performed in triplicate and repeated in 3 independent experiments (n=3) as following;

1. Cell were seeded at a density of 5000 cells/well into a 96 well plate and incubated at 37°C in 5% CO_2 atmosphere for 24 hours.
2. The cells were treated for 24 hours with 10, 20, 30, 40, 50 uM of salinomycin and cells were treated for 48 and 72 hours with 2.5, 5, 10, 15, and 20 μM of salinomycin in triplicate.

3. After indicated time periods of treatment, 10 µl of 5 mg/ml MTT in PBS solution was added to each well.
4. Cell were incubated at 37°C in 5% CO_2 atmosphere for 4 hours.
5. Media was removed and 100 µl DMSO was added in each well in order to solubilize formazan crystals.
6. The effect of salinomycin on cell viability was determined using microplate reader at a wavelength of 570 nanometer. The percentage of cell viability of the treated cells in each condition was compared to the vehicle control and was calculated by the following equation ;

$$\% \text{ Cell viability} = \left(\frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}}\right) \times 100\%$$

The percentage of cell viability was calculated using the following equation: ($OD_{sample}/OD_{control}$) × 100%. To calculate inhibition concentration (IC50), series of dose-response data were obtained from MTT cell viability assay as % cell viability (y) and doses of salinomycin (x). The data was plotted as x-y axis to generate the straight line. IC_{50} value was estimated using the fitted line by graphpad prism software.

3.2 Effect of salinomycin on increasing sensitivity of tamoxifen and increasing tamoxifen sensitivity in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells

Tamoxifen response assay was used to determine the effect of salinomycin on increasing sensitivity to tamoxifen and increasing tamoxifen sensitivity in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells. Each

condition was performed in triplicate and repeated in 3 independent experiments (n=3) as following;

Before experiment, cells were weaned estrogenic effect in FBS serum. All cell lines were cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 5% FBS for 2 days and then all cell lines were cultured in phenol red-free IMEM contained with 5% charcoal strip FBS serum for 2 days before seeded cell in 96-well plate.

1. Cells were seeded at a density of 5,000 cells/well in 96-well plates in phenol red-free IMEM supplemented with 5% charcoal strip FBS serum for 24 hours.
2. Cells were incubated with combination of 0.5, 1.5, and 2.5 μM of salinomycin and 0.1, 0.5, 1, 2,5, 5, 7.5, and 10 μM of 4-OHT for 72 hours.
3. After indicated time period of treatment, Cell were incubated with phenol red-free IMEM and 4-hydroxytamoxifen for 96 hours.
4. Cell survival were measured using MTT cell viability assay.

3.3 Effect of salinomycin on the inhibition of cell migration in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells

Scratch assay was used to screen the effect of salinomycin on the inhibition of cell migration in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells. Each condition was performed in duplicate and repeated in 2 independent experiments (n=2) as follow;

1. Cells were seeded at a density of 100,000 cells/well in a 6-well plate and allowed to form a confluent monolayer until 100% confluence.
2. The monolayer were scratched with a yellow plastic pipette tip of 1 mm diameter.
3. Afterwards, the six-well plate were washed with serum-free media to remove floating cells and photographed at time 0.
4. Cells were incubated at 37ºC in MEM medium containing 5% FBS in the presence of the concentrations of 2.5 μM salinomycin. While control cells were treated with 0.1% DMSO.
5. Cells were then photographed at time 0, 6, 24, and 30 hours with an inverted microscope (objective 5x) to measured the width of the wound.
6. The percent of open wound area were expressed as the mean ± SEM at time 0, 6, 24, and 30 hours after wound.
7. The results were analyzed by Tscratch software.

3.4 Effect of salinomycin on the inhibition of cell invasion in MCF-7/LCC9 cells

Matrigel invasion assay was used to determine the effect of salinomycin on inhibiting cell invasion in MCF-7/LCC9 cells. Because the scratch assay is an experiment to first screen anti-migration effect of salinomycin. The result may explain the effect on inhibiting cell proliferation but not only on cell migration. Therefore, matrigel invasion assay was performed to confirm anti-invasive effect of salinomycin on MCF-7/LCC9 cells.

1. Matrigel was diluted to 1:30 with serum free media and added 40 µl to upper chamber of invasion chamber which was inserted in 24 well plate.
2. Matrigel was dry in the hood for 4 hours and kept at room temperature overnight.
3. Matrigel was rehydrated with 100 µl of serum free media for 1 hours.
4. Cells were trypsinized and wash with serum free media twice.
5. MEM media was added with 5% heat inactivated FBS as a chemo-attractant 500 µl/well to the lower chamber of system.
6. Cells were seeded at a density of 50,000 cells/well into the upper chambers of the system which contains 0.1% DMSO, or 2.5 and 5 µM salinomycin.
7. Cells were incubated at 37°C in 5% CO_2 atmosphere for 72 hours.
8. Media was removed from both upper and lower chamber.
9. Upper and lower chambers were washed with 1XPBS twice.
10. Cells which migrated through the matrigel were fixed with 500 µl of 4% formaldehyde at room temperature for 15 minutes.
11. Formaldehyde was removed and chambers were washed with 1X PBS twice gently.
12. Non-penetrating cells were removed from the upper surface of the transwell with a cotton swab.
13. Upper chambers were stained with 300 µl of crystal violet for 30 minutes.
14. Upper chambers were washed with 1X PBS 4 times until the bottom of the chamber was clear.

15. Cells were counted in 5 random fields per well under a microscope. The percentage proportional invasiveness was calculated by the following equation;

Proportional invasiveness (%) = number of cells migrate in salinomycin treated cells/number of cell migrates in 0.1% DMSO treated cells x 100%

3.5 Effect of salinomycin on the expression of matrix metalloproteinase 9, ER-target genes, and genes involved in tamoxifen resistance in MCF-7/LCC2 and MCF-7/LCC9 cells

The effect of salinomycin on MCF-7/LCC2 and MCF-7/LCC9 at molecular mechanism was investigated by RT-PCR to evaluate the mRNA expression of gene involved in invasion and metastatic process including *MMP9* gene, ER-target genes, and genes involved in tamoxifen resistance including *NCOA3*, *cyclin D1*, *c-myc*, and *p21* genes. The assay was performed in 3 independent experiments (n=3) as the following procedures.

3.5.1 Isolation of total RNA

1. MCF-7/LCC2 and MCF-7/LCC9 cells were added in the complete MEM media at a density of 1×10^6 cells/ml in each well of 24-well plate.

2. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours.

3. Cells were treated with 10, 20 μM salinomycin for 48 hours in CO_2 incubator.

4. The supernatant was removed and cells were homogenized with 500 µl of Trizol® reagent by passing cells up and down through a pipette.

5. Lysate cells were transfered to a 1.5 ml microcentrifuge tube and incubated at room temperature for 5 minutes.

6. 200 µl of chloroform was added, vigorously shake the tube by vortex for 15 seconds and incubated at room temperature for 2-3 minutes.

7. Tubes were centrifuged at 12,000 rpm 4°C for 15 minutes and the aqueous phase was transfered to a fresh tube.

8. 500 µl of isopropyl alcohol was added to each tube and incubated at -20^{0}C for 60 minutes.

9. Tubes were centrifuge at 12,000 rpm at 4°C for 10 minutes.

10. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol and mixed by vortex.

11. The RNA pellet was separated by centrifugation 7,500 rpm at 4° C for 5 minutes.

12. The supernatant was removed and RNA pellet was air-dry for 5-10 minutes.

13. The RNA pellet was dissolved in 10 µl of DEPC-treated water, incubated at 55-60 °C for 10 minutes.

14. The total RNA samples were stored at -80°C for complementary DNA (cDNA) production.

15. The amount of RNA in the sample was determined by nanodrop spectrophotometer at 260°C. The total RNA samples should have optical density ratio; OD_{260}:OD_{280}> 1.8.

3.5.2 Preparation of complementary DNA (cDNA) by reverse transcription polymerase chain reaction

1. Total RNA of each sample was mixed with nuclease free water and 1 µl oligo dT15 primer for a final volume of 5 µl per tube in 0.2 ml PCR tube.

2. Tubes were heated at 70° C for 5 minutes and immediately placed on ice for 5 minutes until preparation of master mix was finished.

3. Prepare transcription mixture solution was prepared containing nuclease free water, imProm-ll™ 5x reaction buffer, $MgCl_2$ 25 mM, dNTP mix 10 mM, RNA ribonuclease inhibitor, and imProm-ll™ reverse transcriptase in 1.5 ml microcentrifuge tube on ice.

Component	Volume (1x) (µl)
Nuclease free water	6.9
imProm-II™ 5x reaction buffer	4
$MgCl_2$ 25 mM (final conc. 2 mM)	1.6
dNTP mix 10 mM (final conc. 0.5 mM)	1
Recombinant RNA ribonuclease inhibitor	0.5
imProm-II™ reverse transcriptase	1
total	15

1. 15 µl of mixture solution was added into each tube for a final volume of 20 µl per tube.
2. The PCR tube was centrifuged to maintained the final volume of reaction tube.
3. Place the PCR reaction tube in the PCR thermocycle machine to generated cDNA by using the following conditions; 25°C for 5 minutes, then 42°C for 1 hour and 30 minutes, and finally 70°C for 15 minutes.
4. The cDNA samples were stored at -20°C for using as the template to determine gene expression.

3.5.3 Determine mRNA expression of matrix metalloproteinase 9, ER-target genes, and genes involved in tamoxifen resistance

cDNA to PCR product

PCR	**1X (µl)**
Nuclease free water (ddH_2O)	19.05
$MgCl_2$ 50 µM	0.75
10x PCR buffer	2.5
dNTP 10 mM	0.5
Primer forward 10 µM	0.5
Primer reverse 10 µM	0.5
Tag polymerase 5 U/µl	0.2
cDNA	1
total	25

1. PCR was performed by the following conditions; 94°C for 2 min followed by 35 cycles of 30 seconds for denaturation at 94 °C, 30 seconds for annealing at appropriate melting temperature (Tm) of the primers, 1 minutes for extension at 72°C, and finally 10 minutes for extension at 72°C.
2. 3 µl of DNA ladder was added at the first well of agarose gel of each row.
3. 4 µl of PCR product was mixed with 2 µl of 6X loading dye.
4. The mixture of PCR product of each sample was added to the well of 1.5% agarose gel.
5. The PCR products were analyzed by electrophoresis in 1.5 % agarose gel at 100 V,3.0 A, 300 W for 35 minutes.
6. The agarose gel was stained with 0.5 µg/ml of ethidium bromide in 1xTBE buffer for 15 minutes and destained in 1XTBE buffer for 30 minutes.
7. The density of PCR product was determined their densities by gel documentation and image lab™ software which express the densities of the PCR products as % of internal control gene (*GAPDH*).

Table 2 : Primers for RT-PCR experiment and their annealing temperatures.

Gene	Primer sequences	Tm°C	PCR product (bp)
NCOA3	Forward : 5'-AGC-CAT-CAG-TGA-AGG-TGT-GG-3' Reverse : 5'-ACT-TGT-GCA-AAA-TCC-GGT-GC-3'	57.6	482
Cyclin D1	Forward : 5'-TTC-GCT-TTC-TCC-TGA-CCG-AC-3' Reverse : 5'-TGC-TTC-AAG-AAG-CGC-AGA-GA-3'	58.2	335
C-myc	Forward : 5'-GCT-TCT-CTG-AAA-GGC-TCT-CCT-3' Reverse : 5'-CCA-TTC-CCG-TTT-TCC-CTC-TG-3'	56.2	295
p-21	Forward : 5'-TGG-CTA-TGT-CGG-TGA-AGC-TC-3' Reverse : 5'-AAG-GGG-TGG-TTT-GTC-TGC-AT-3'	56.2	312
MMP-9	Forward : 5'-ACA-CCT-CTG-CCC-TCA-CCA-T-3' Reverse : 5'-TCG-ACT-CTC-CAC-GCA-TCT-CT-3'	58.2	210
GAPDH	Forward : 5'-GAG-AAG-GCT-GGG-GCT-CAT-TT-3' Reverse : 5'-AGT-GAT-GGC-ATG-GAC-TGT-GG-3'	57.6	231

4. Statistical analysis

The data was performed at least three experiments (n=3), Data presented as means ± SEM from three independent experiments determined by one-way ANOVA with Tukey's Honestly significant Difference (HSD) post hoc test. Student t test was used to compared difference between control group and treated group in the RT-PCR experiment. The *p*-value less than 0.05 were considered as statistically significant.

CHAPTER IV RESULTS

1. The effect of salinomycin on the inhibition of cell viability in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells

To investigate the anti-cancer activity of salinomycin on the inhibition of cell viability of MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells were determined using MTT cell viability assay. Cells were treated with 10, 20, 30, 40, and 50 μM of salinomycin for 24 hours and cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin for 48, 72 hours. Complete MEM media and 0.1, 10 μM 4-OHT were used as the negative control and positive control in this experiment. The result demonstrated that salinomycin had the anti-cancer activity on MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells by decreasing cell viability of MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells in a concentration- and time- dependent manner at 24, 48, and 72 hours. The IC_{50} values of salinomycin in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells at 24 hours were 24.25 ± 2.060 μM **(Figure 16)**, 24.17 ± 1.645 μM **(Figure 17)**, and 19.74 ± 5.496 μM **(Figure 18)**, respectively. The IC_{50} values of salinomycin at 48 hours were 20.41 ± 1.634 μM **(Figure 19)**, 18.15 ± 0.454 μM **(Figure 20)**, and 15.77 ± 1.165 μM **(Figure 21)**, respectively. The IC_{50} values of salinomycin in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells at 72 hours were 7.63 ± 0.300 μM **(Figure 22)**, 9.23 ± 0.434 μM **(Figure 23)**, and 6.56 ± 0.192 μM **(Figure 24)**, respectively. Moreover, these results indicated that anti-estrogen resistant breast cancer cells were relatively sensitive to salinomycin when compared to wild type cells.

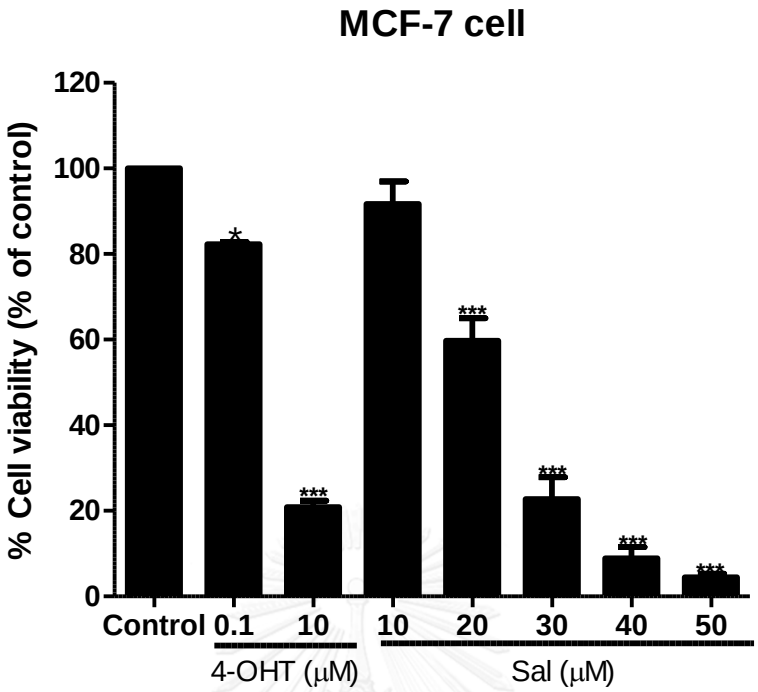


Figure 16 : The inhibitory effect of salinomycin on MCF-7 cells for 24 hours. Cells were treated with 10, 20, 30, 40, and 50 μM of salinomycin (Sal) for 24 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

*$p < 0.05$ denotes statistically significant difference from negative control

***$p < 0.001$ denotes statistically significant difference from negative control

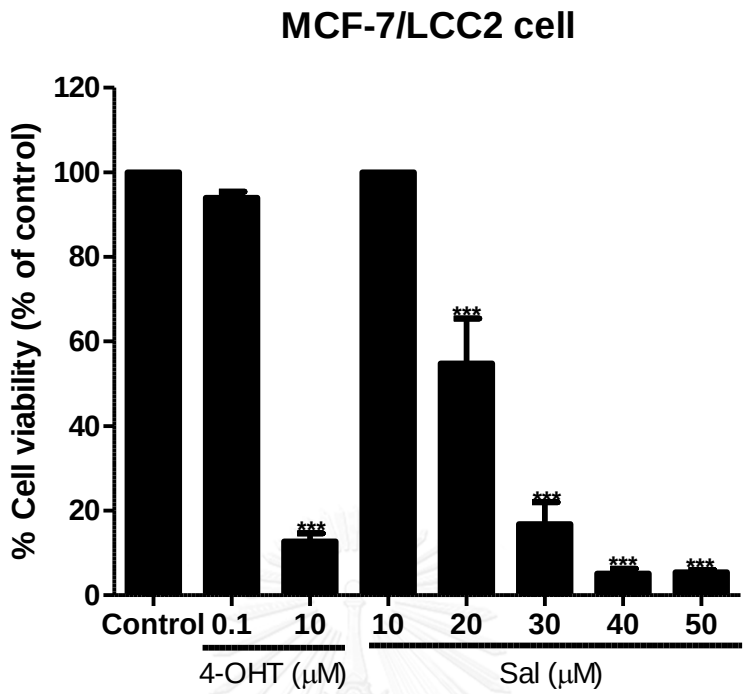


Figure 17 : The inhibitory effect of salinomycin on MCF-7/LCC2 cells for 24 hours. Cells were treated with 10, 20, 30, 40, and 50 μM of salinomycin (Sal) for 24 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

***$p < 0.001$ denotes statistically significant difference from negative control

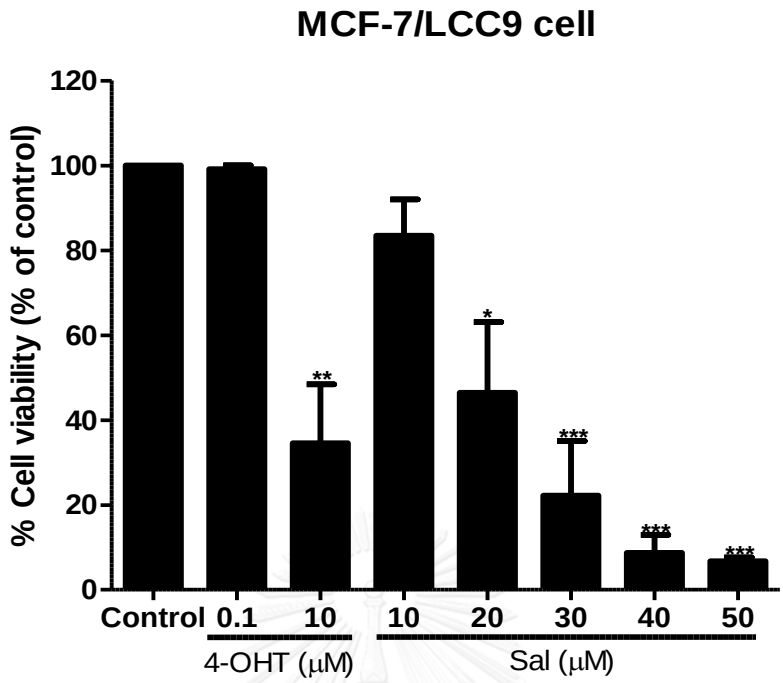


Figure 18 : The inhibitory effect of salinomycin on MCF-7/LCC9 cells for 24 hours. Cells were treated with 10, 20, 30, 40, and 50 μM of salinomycin (Sal) for 24 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

*$p < 0.05$ denotes statistically significant difference from negative control

**$p < 0.01$ denotes statistically significant difference from negative control

***$p < 0.001$ denotes statistically significant difference from negative control

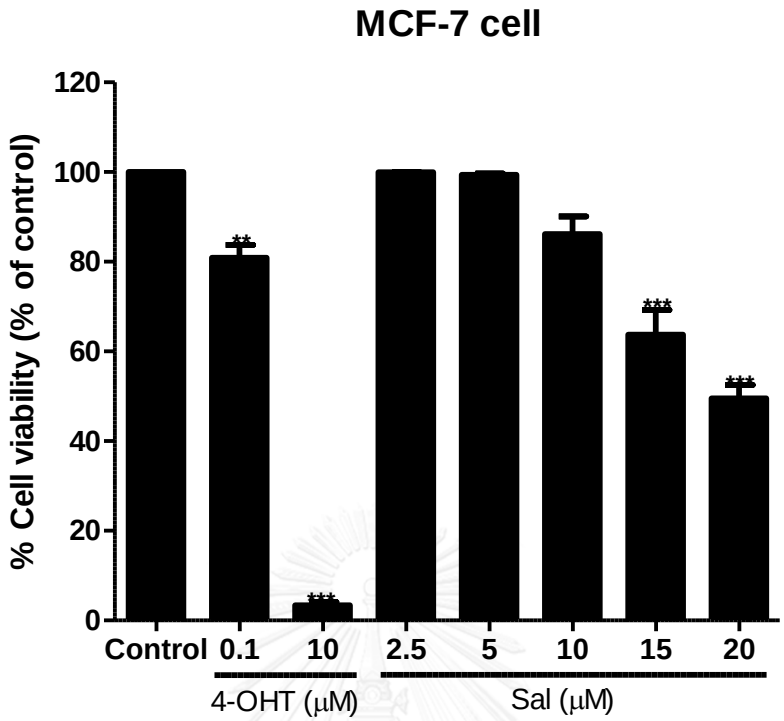


Figure 19 : The inhibitory effect of salinomycin on MCF-7 cells for 48 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 48 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

***p*< 0.01 denotes statistically significant difference from negative control

****p* < 0.001 denotes statistically significant difference from negative control

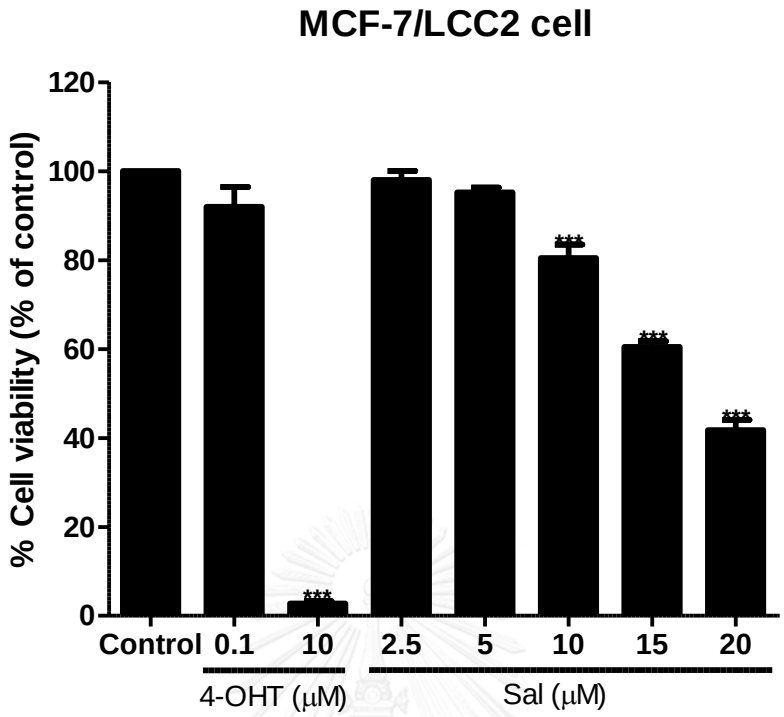


Figure 20 : The inhibitory effect of salinomycin on MCF-7/LCC2 cells for 48 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 48 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

***$p < 0.001$ denotes statistically significant difference from negative control

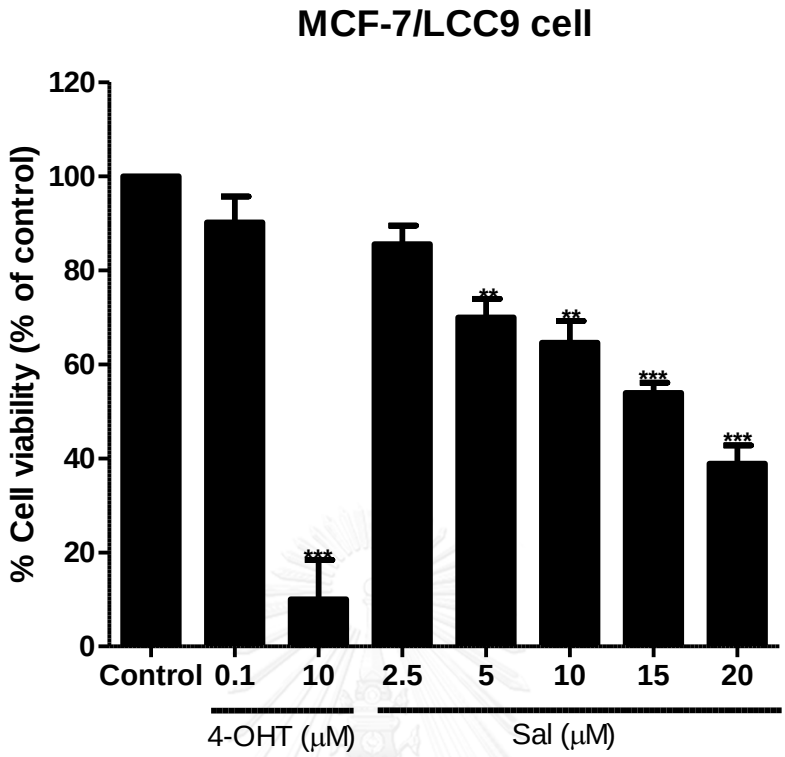


Figure 21 : The inhibitory effect of salinomycin on MCF-7/LCC9 cells for 48 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 48 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

**$p < 0.01$ denotes statistically significant difference from negative control

***$p < 0.001$ denotes statistically significant difference from negative control

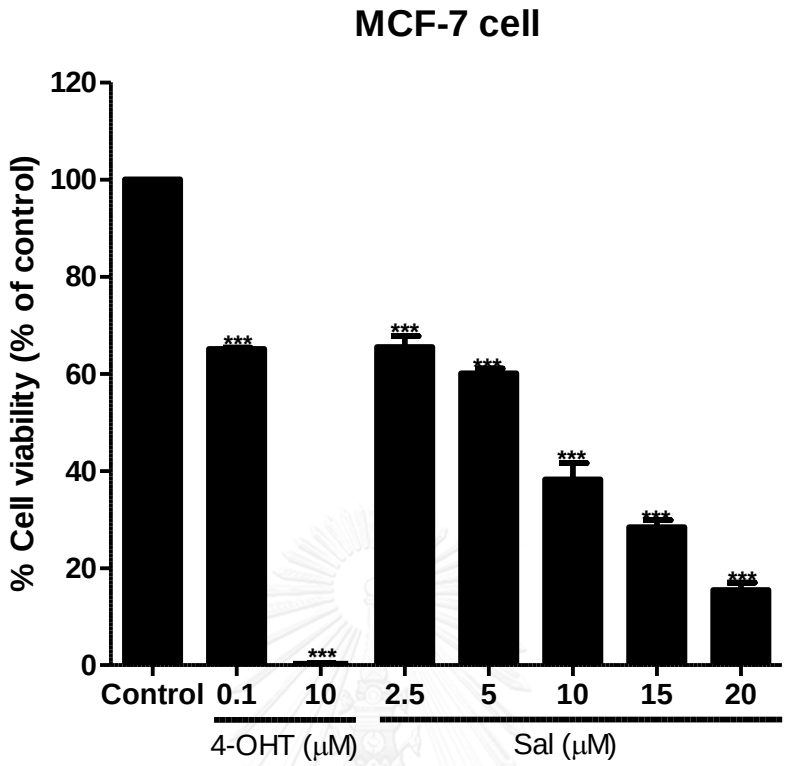


Figure 22 : The inhibitory effect of salinomycin on MCF-7 cells for 72 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 72 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

***$p < 0.001$ denotes statistically significant difference from negative control

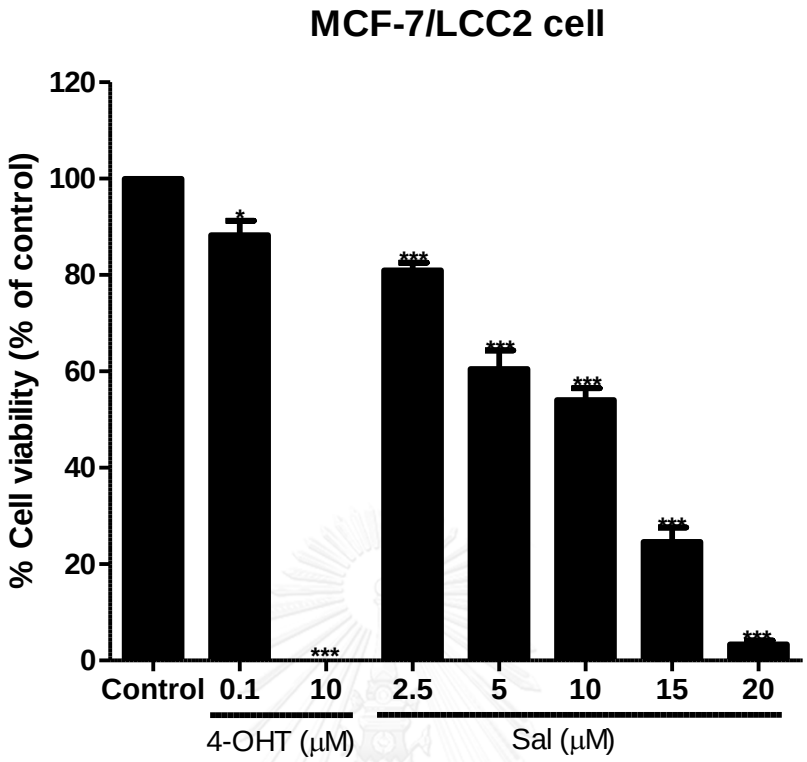


Figure 23 : The inhibitory effect of salinomycin on MCF-7/LCC2 cells for 72 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 72 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

*p< 0.05 denotes statistically significant difference from negative control

***p < 0.001 denotes statistically significant difference from negative control

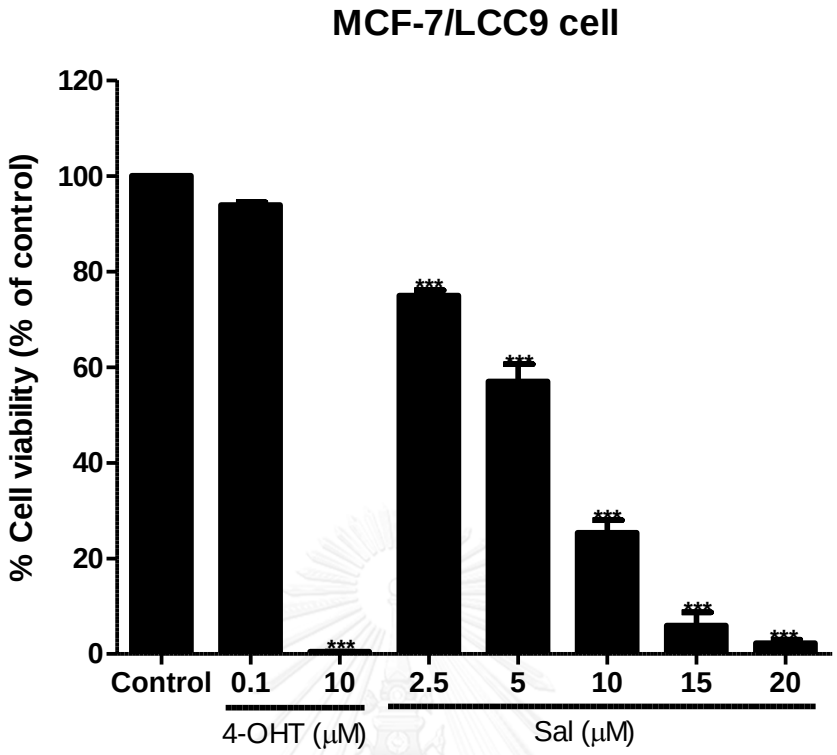


Figure 24 : The inhibitory effect of salinomycin on MCF-7/LCC9 cells for 72 hours. Cells were treated with 2.5, 5, 10, 15, and 20 μM of salinomycin (Sal) for 72 hours. The effect of salinomycin on cell viability was determined by MTT cell viability assay. The data was presented as means ± SEM from three independent experiments.

***$p < 0.001$ denotes statistically significant difference from negative control

2. The synergistic effect of salinomycin on the inhibition of cell viability with tamoxifen in MCF-7 cells and increasing tamoxifen sensitivity in MCF-7/LCC2 and MCF-7/LCC9 cells

The synergistic effect of salinomycin on the inhibition of cell viability with tamoxifen in MCF-7 cells and increasing tamoxifen sensitivity in MCF-7/LCC2 and MCF-7/LCC9 cells was investigated using tamoxifen response assay. The result showed dose response survival curve when compared between the range of 4-OHT concentrations from 0.1, 0.5, 1, 2.5, 5, 7.5, 10 μM, and 4-OHT combined with 0.5, 1.5, or 2.5 μM of salinomycin. The result demonstrated that salinomycin was able to increase sensitivity of tamoxifen in MCF-7 cells when compared to 4-OHT treatment alone **(Figure 25)**. The result indicated that salinomycin enhanced the anti-cancer effect of tamoxifen by decreasing their IC_{50} values. Moreover, salinomycin induced a significant increase in sensitivity of 4-OHT treatment in MCF-7/LCC2 **(Figure 26)** and MCF7/LCC9 cells **(Figure 27)**. The IC_{50} value of tamoxifen was decreased from 3.04 ± 0.648 to 1.48 ± 0.431 μM for the combination of tamoxifen with 0.5 μM salinomycin in MCF-7 cell. Moreover, The IC_{50} value of tamoxifen decreased from 3.88 ± 0.264 to 1.51 ± 0.742 μM and 4.38 ± 0.307 to 2.72 ± 0.171 μM for the combination of tamoxifen with 0.5 μM of salinomycin in MCF-7/LCC2 cells and MCF-7/LCC9 cells, respectively **(Table 3)**. This finding suggested anti-cancer activity of salinomycin and synergistic effect of salinomycin and tamoxifen on MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells.

Table 3 : Represent the IC_{50} values either 4-hydroxytamoxyfen (4-OHT) alone or combination with 0.5 μM salinomycin in MCF-7 and anti-estrogen resistant breast cancer cell lines.

Type of cell lines	**4-hydroxytamoxifen (4-OHT) alone**	**4-OHT combined with 0.5 μM salinomycin**	***p*-value**
MCF-7	IC_{50} = 3.04 ± 0.648	IC_{50} =1.48 ± 0.431	0.022
MCF-7/LCC2	IC_{50} =3.88 ± 0.264	IC_{50} =1.51 ± 0.742	0.067
MCF-7/LCC9	IC_{50} =4.38 ± 0.307	IC_{50} =2.72 ± 0.171	0.027

p-value less than 0.05 denotes statistically significant difference from 4-OHT treatment alone

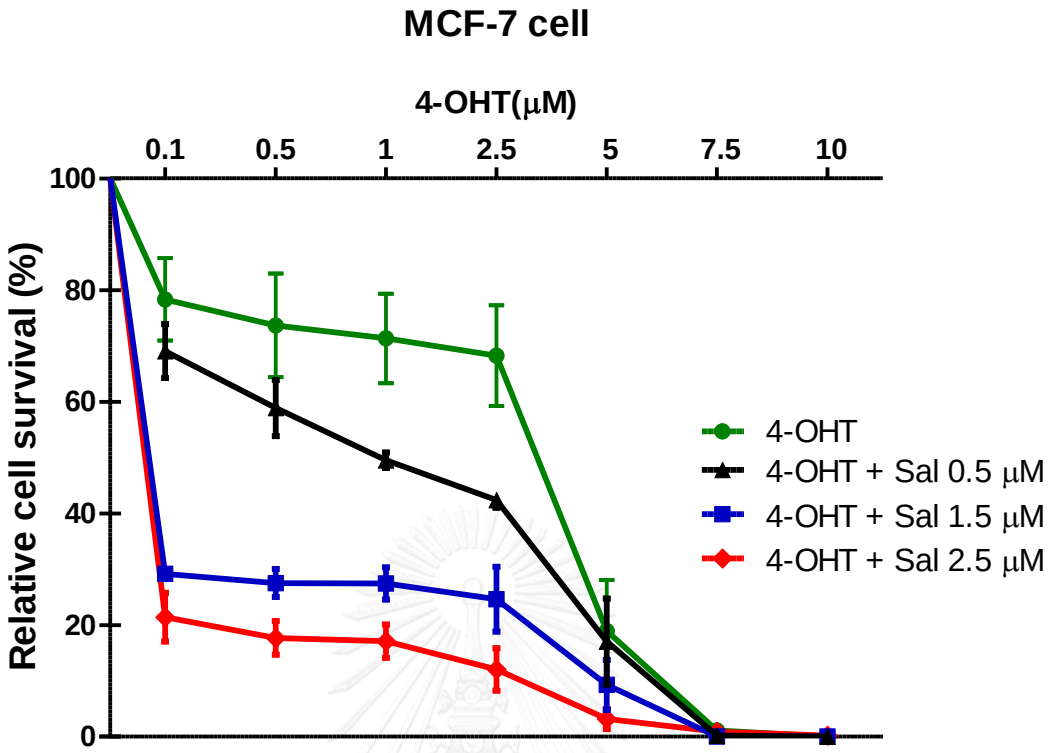


Figure 25 : The effect of salinomycin on the inhibition of cell viability and increasing tamoxifen sensitivity in MCF-7 cells. Cell were treated with combination of increasing concentrations of 4-OHT and 0.5, 1.5, or 2.5 μM of salinomycin (Sal) for 3 days and then treated with 4-OHT alone for the next 4 days. The data was presented as means ± SEM from three independent experiments.

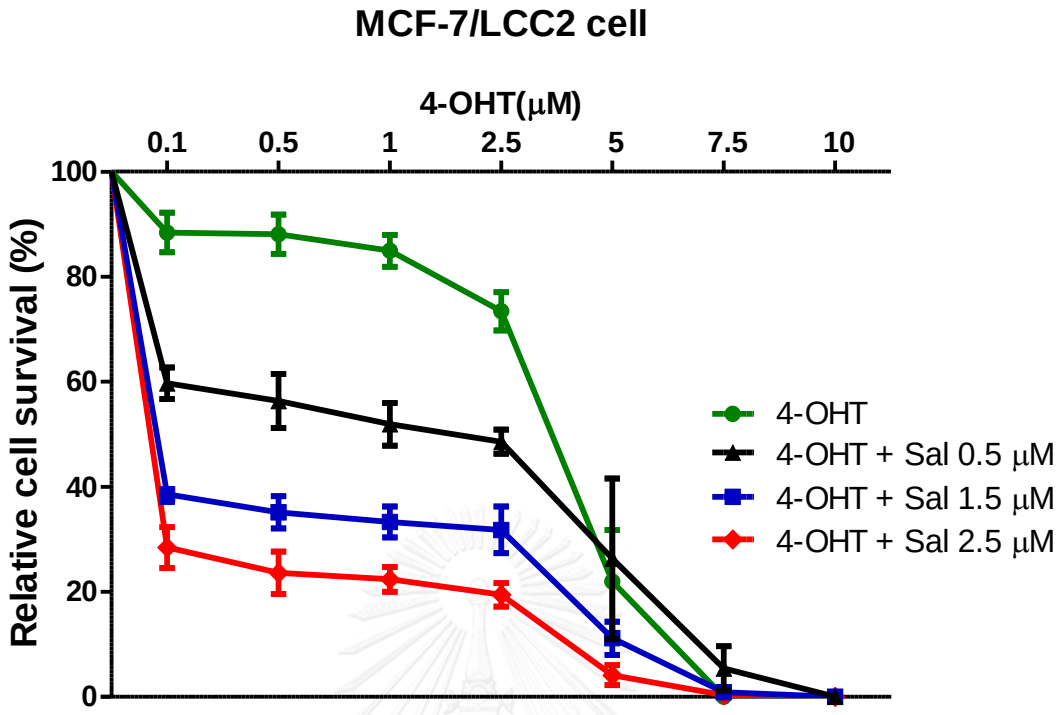


Figure 26 : The effect of salinomycin on the inhibition of cell viability and increasing tamoxifen sensitivity in MCF-7/LCC2 cells. Cell were treated with combination of increasing concentrations of 4-OHT and 0.5, 1.5, or 2.5 μM of salinomycin (Sal) for 3 days and then treated with 4-OHT alone for the next 4 days. The data was presented as means ± SEM from three independent experiments.

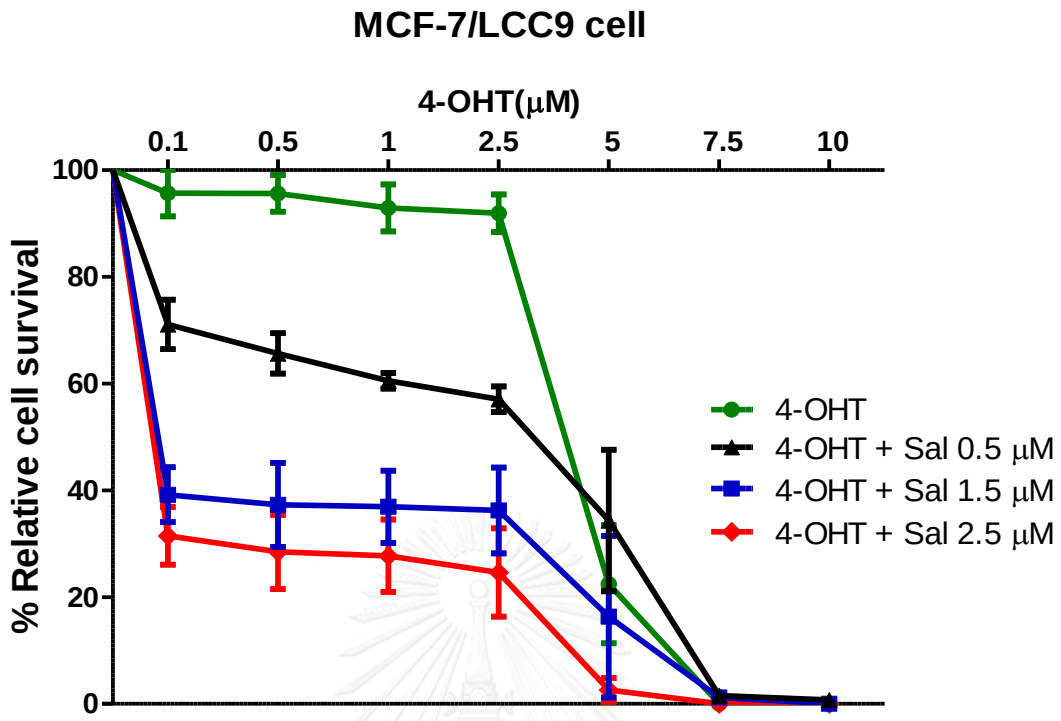


Figure 27 : The effect of salinomycin on the inhibition of cell viability and increasing tamoxifen sensitivity in MCF-7/LCC9 cells. Cell were treated with combination of increasing concentrations of 4-OHT and 0.5, 1.5, or 2.5 μM of salinomycin (Sal) for 3 days and then treated with 4-OHT alone for the next 4 days. The data was presented as means ± SEM from three independent experiments.

3. The effect of salinomycin on the inhibition of cell migration in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells

The effect of salinomycin on the inhibition of cell migration in MCF-7, MCF-7/LCC2, and MCF-7/LCC9 cells was investigated using wound scratch assay. Cells were exposed to 2.5 μM salinomycin and wound confluence was monitored at 0, 6, 24, and 30 hours. The result indicated that salinomycin reduced cellular migration of MCF-7 cells in a time-dependent manner. Moreover, salinomycin significantly greater inhibited on cell migration in MCF-7 cells ($p< 0.001$) after 24 and 30 hours of wound scratch **(Figure 28)**. Similarly, salinomycin was able to inhibit cell migration in MCF-7/LCC2 cells ($p< 0.05$) after 24 and 30 hours of wound scratch **(Figure 29)**. In addition, salinomycin inhibited cell migration in MCF-7/LCC9 cells ($p< 0.05$) after 30 hours of wound scratch **(Figure 30)**.

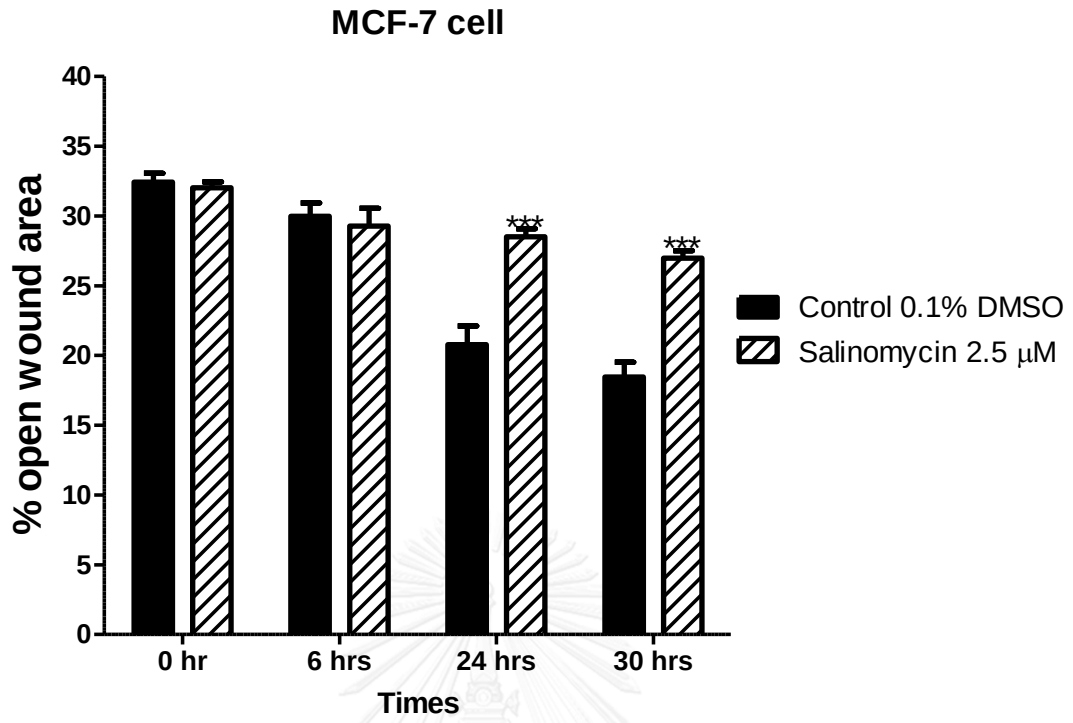


Figure 28 : The effect of salinomycin on the inhibition of cell migration in MCF-7 cells. Wounds were introduced in MCF-7 cells. The confluent mono-layers were cultured in the presence or absence (control) of salinomycin (2.5 μM). The width of the wound was photographed with an inverted microscope (objective 5x). Data was expressed as the mean ± SEM of the difference between the measurements at 0, 6, 24, and 30 hours after wound scratch from two independent experiments. The percentage of open wound area was analyzed by Tscratch software.

***p< 0.001 denotes statistically significant difference from negative control

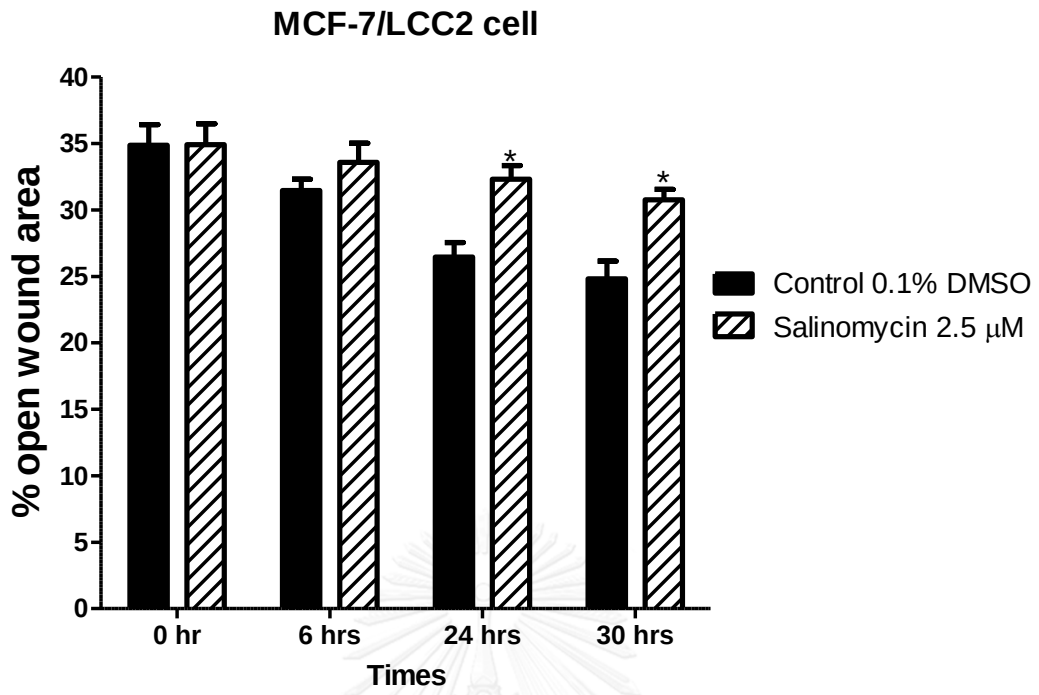


Figure 29 : The effect of salinomycin on the inhibition of cell migration in MCF-7/LCC2 cells. Wounds were introduced in MCF-7/LCC2 cells. The confluent mono-layers were cultured in the presence or absence (control) of salinomycin (2.5 μM). The width of the wound was photographed with an inverted microscope (objective 5x). Data was expressed as the mean ± SEM of the difference between the measurements at 0, 6, 24, and 30 hours after wound scratch from two independent experiments. The percentage of open wound area was analyzed by Tscratch software.

*p< 0.05 denotes statistically significant difference from negative control

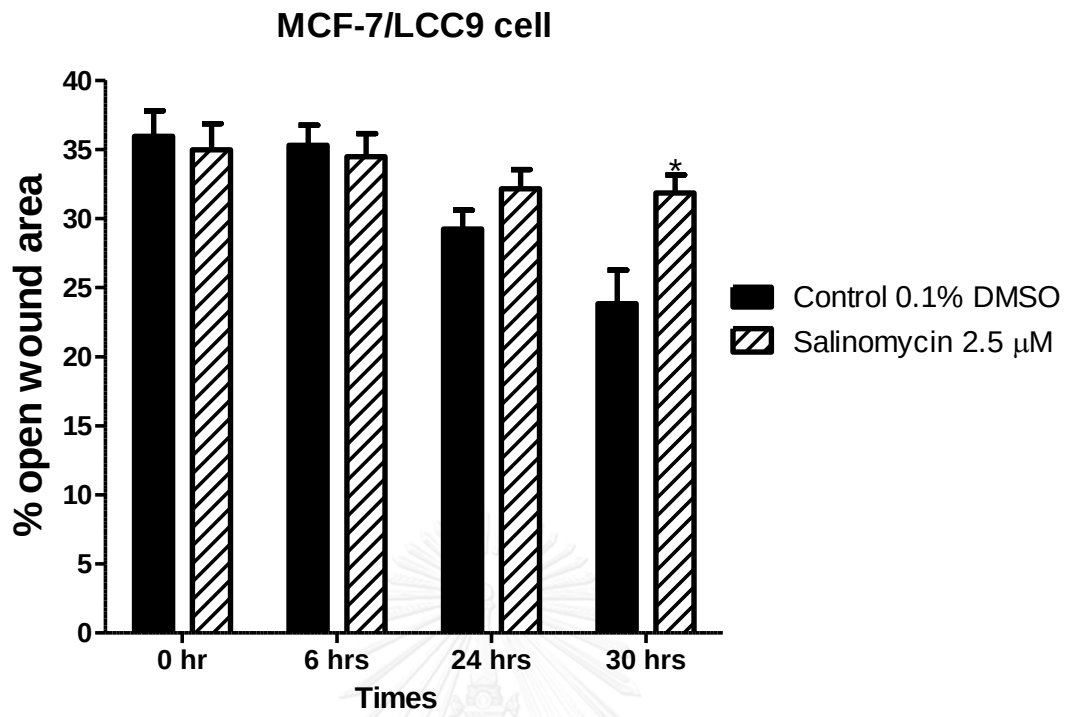


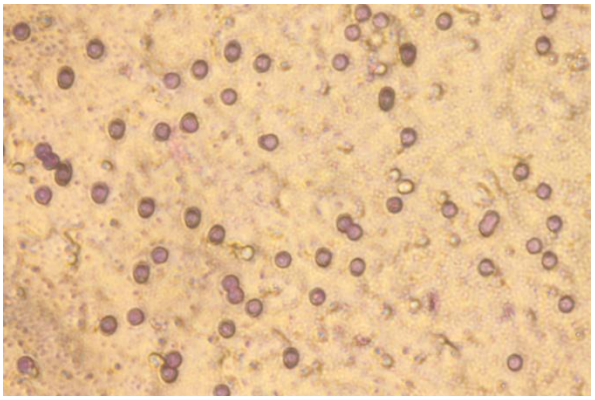
Figure 30 : The effect of salinomycin on the inhibition of cell migration in MCF-7/LCC9 cells. Wounds were introduced in MCF-7/LCC9 cells. The confluent mono-layers were cultured in the presence or absence (control) of salinomycin (2.5 μM). The width of the wound was photographed with an inverted microscope (objective 5x). Data was expressed as the mean ± SEM of the difference between the measurements at 0, 6, 24, and 30 hours after wound scratch from two independent experiments. The percentage of open wound area was analyzed by Tscratch software.

*p< 0.05 denotes statistically significant difference from negative control

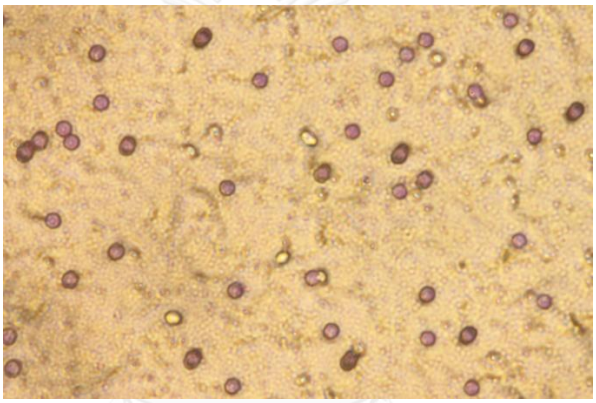
4. The effect of salinomycin on the inhibition of cell invasion in MCF-7/LCC9 cells

Because cell invasion plays a crucial role in tumor invasion and metastasis, the ability of salinomycin to reduce cell invasion was determined using matrigel invasion assay. MCF-7/LCC9 cells were treated for 72 hours with 2.5 and 5 μM of salinomycin in matrigel-coated invasion chamber. Cells which invaded matrigel were fixed with 4% formaldehyde and stained with crystal violet. Numbers of cells invading through chamber assay with coating with matrigel were counted in 5 random fields under a microscope. The results demonstrated that salinomycin at 2.5 μM ($p< 0.001$) and 5 μM ($p< 0.001$) significantly inhibited cell invasion through decreased numbers of migrated cell and percent proportional invasiveness in MCF-7/LCC9 cells in a concentration dependent manner after 72 hours of treatment **(Figure 31, 32)**. This result demonstrated that salinomycin can strongly inhibit cell invasion *in vitro*.

A.



B.



C.

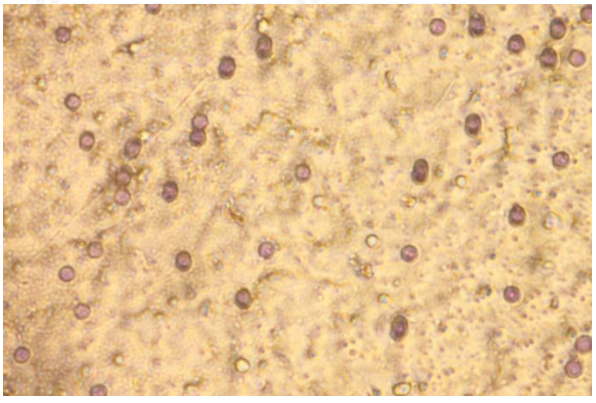


Figure 31 : The effect of salinomycin on cell invasion in MCF/LCC9 cells. A : control (0.1% DMSO) ; B. Sal 2.5 μM ; C. Sal 5 μM. Quantification of numbers of cells migrated from invasion chamber assay in MCF-7/LCC9 cells. The images are representative from three independent experiments using a magnification of 100X.

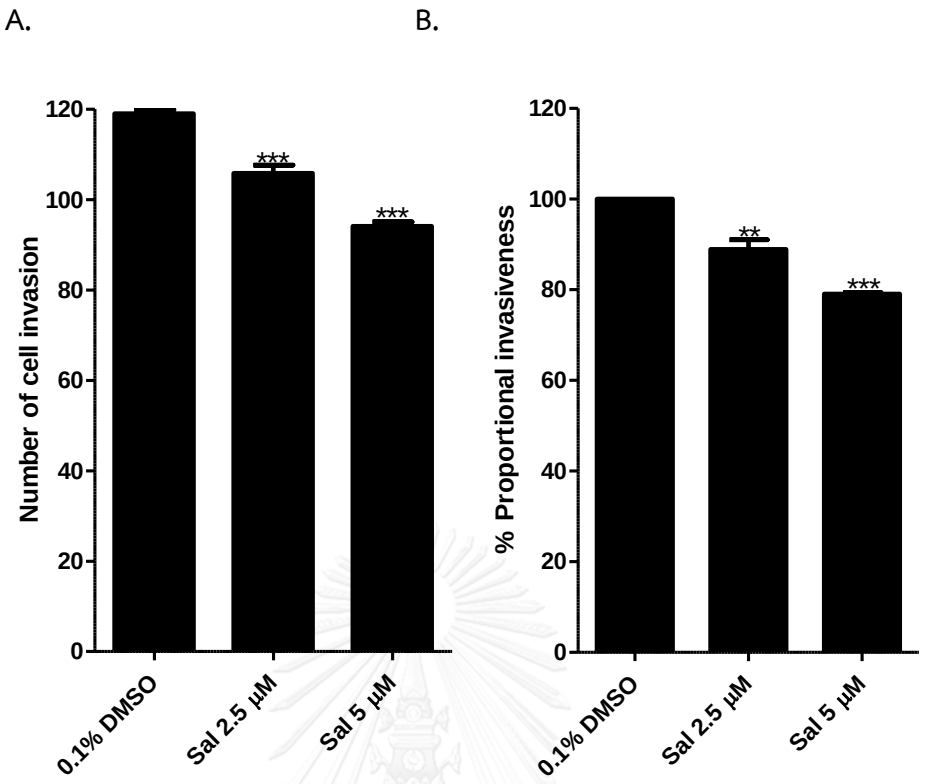


Figure 32 : The effect of salinomycin on cell invasion in MCF/LCC9 cells. MCF-7/LCC9 cells were incubated on transwell chambers in the presence of 0.1% DMSO, Sal 2.5 µM, and Sal 5 µM for 72 hours. Migrated cells were fixed with 4% formaldehyde and stained with crystal violet. Migrated cells were counted in 5 random fields using microscrope. The data was presented as mean ± SEM of three independent experiments (n=3).

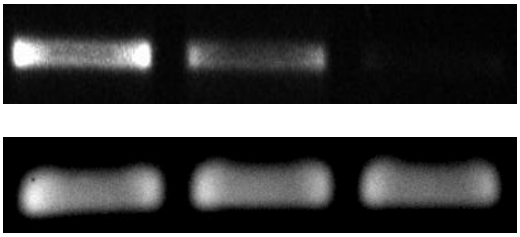
***p*< 0.01 denotes statistically significant difference from control (0.1% DMSO)

****p*< 0.001 denotes statistically significant difference from control (0.1% DMSO)

5. The effect of salinomycin on the expression of matrix metalloproteinase 9, ER-target genes, and genes involved in tamoxifen resistance in MCF-7/LCC2 and MCF-7/LCC9 cells

To examine molecular mechanisms underlying salinomycin reducing invasion and restoring tamoxifen sensitivity in anti-estrogen resistant breast cancer cells : MCF-7/LCC2 and MCF-7/LCC9 cells, the effect of salinomycin on the expression of matrix metalloproteinase 9, ER-target genes, and gene involved in tamoxifen resistance was investigated by RT-PCR. Cells were treated with 10 and 20 μM salinomycin for 48 hours and determined mRNA expression of *MMP9*, ER-target genes, and genes involved in tamoxifen resistance genes including *NCOA3*, *Cyclin D1*, *C-myc*, and *p21* genes. The result showed that salinomycin significantly down-regulated the mRNA expression of *MMP9* in a dose-dependent manner in both MCF-7/LCC2 and MCF-7/LCC9 cells **(Figure 33, 38)**. Moreover, 20 μM of salinomycin significantly decreased the expression of *NCOA3* in MCF-7/LCC2 and MCF-7/LCC9 cells, respectively **(Figure 34, 39)**. Furthermore, 20 μM of salinomycin diminished *cyclin D1* **(Figure 36, 41)** and *c-myc* **(Figure 37, 42)** expression in both MCF-7/LCC2 and MCF-7/LCC9 cells. In addition, the expression of *p21* was up-regulated in both MCF-7/LCC2 and MCF-7/LCC9 cells **(Figure 35, 40)**.

A.



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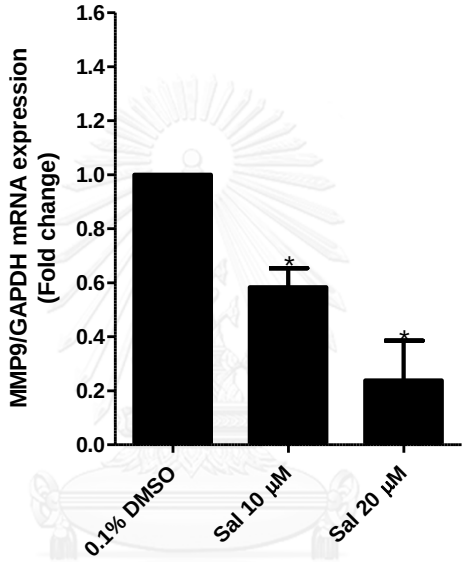
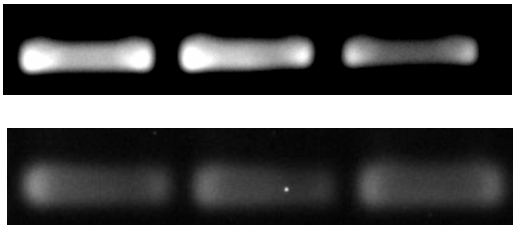


Figure 33 : The effect of salinomycin on the mRNA expression of *MMP9* in MCF-7/LCC2 cells. Cell were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *MMP9* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

* $p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

A.



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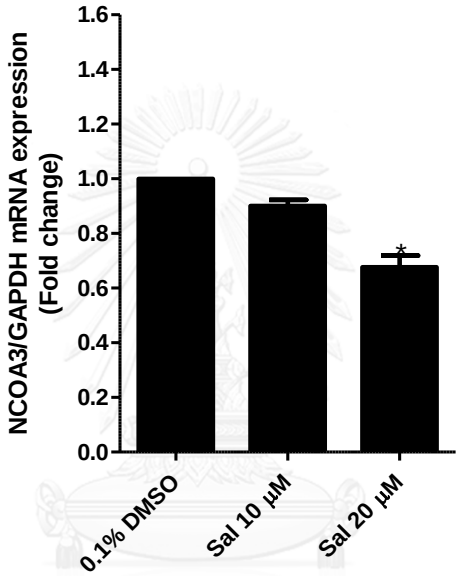
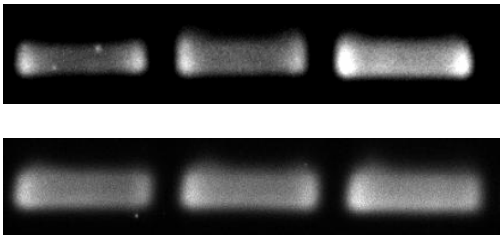


Figure 34 : The effect of salinomycin on the mRNA expression of *NCOA3* in MCF-7/LCC2 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *NCOA3* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

* $p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

A.



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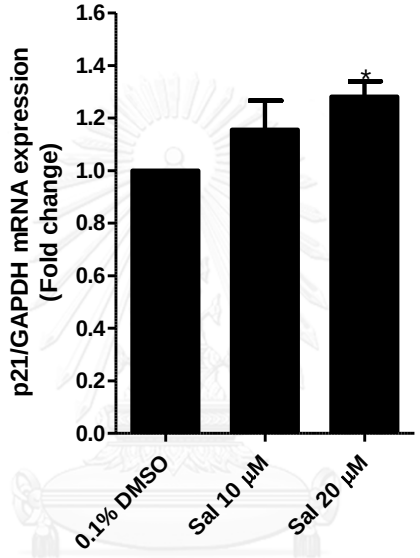
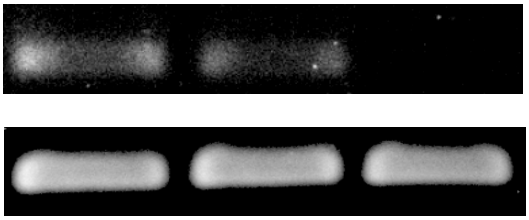


Figure 35 : The effect of salinomycin on the mRNA expression of *p21* in MCF-7/LCC2 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *p21* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

* $p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

A.



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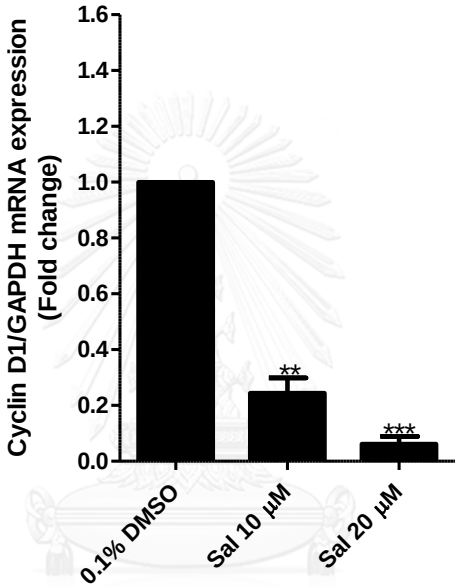
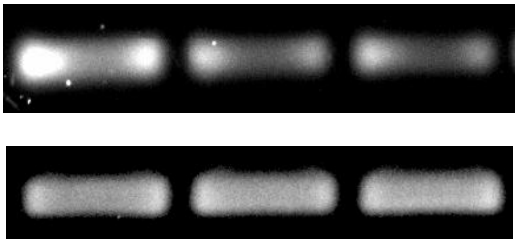


Figure 36 : The effect of salinomycin on the mRNA expression of *cyclin D* in MCF-7/LCC2 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *cyclin D1* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

* $p< 0.05$ denotes statistically significant difference from 0.1% DMSO.

A.



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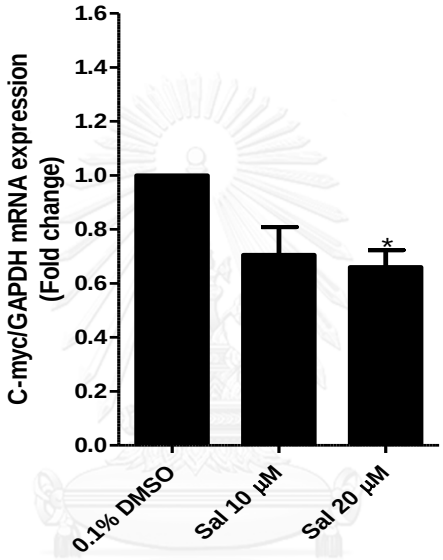


Figure 37 : The effect of salinomycin on the mRNA expression of *c-myc* in MCF-7/LCC2 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *c-myc* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

*p< 0.05 denotes statistically significant difference from 0.1% DMSO.

A.

B.

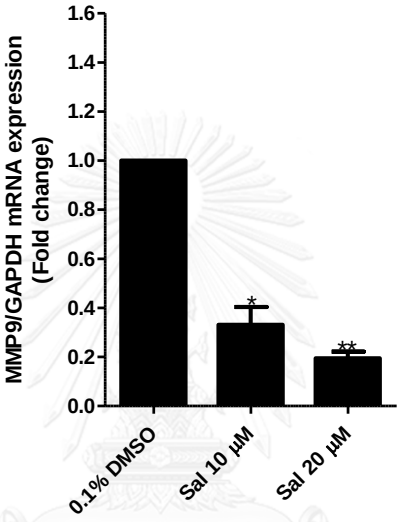
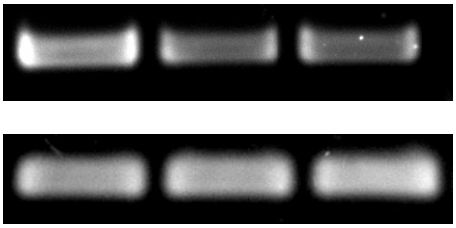


Figure 38 : The effect of salinomycin on the mRNA expression of *MMP9* in MCF-7/LCC9 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *MMP9* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

* $p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

** $p < 0.01$ denotes statistically significant difference from 0.1% DMSO.

A.



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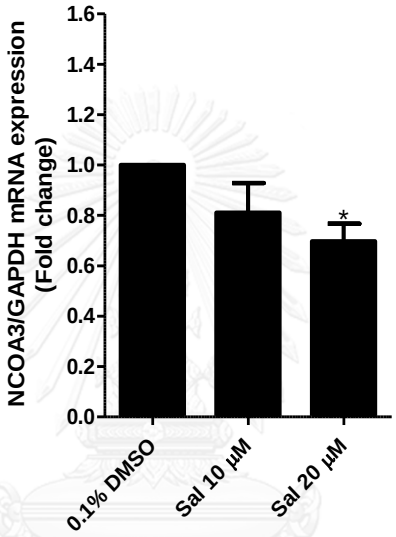
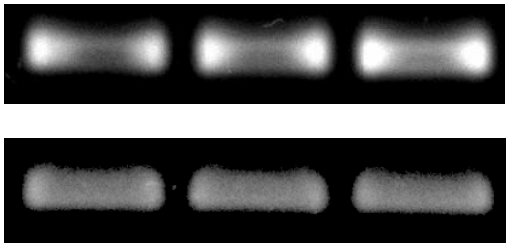


Figure 39 : The effect of salinomycin on the mRNA expression of *NCOA3* in MCF-7/LCC9 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *NCOA3* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

*$p < 0.05$ denotes statistically significant difference from 0.1% DMSO

A.



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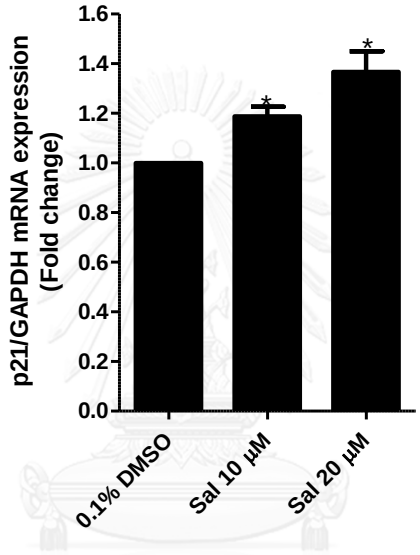
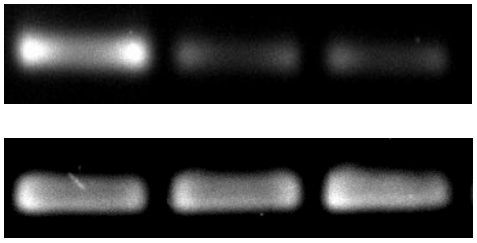


Figure 40 : The effect of salinomycin on the mRNA expression of *p21* in MCF-7/LCC9 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *p21* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

*$p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

A.



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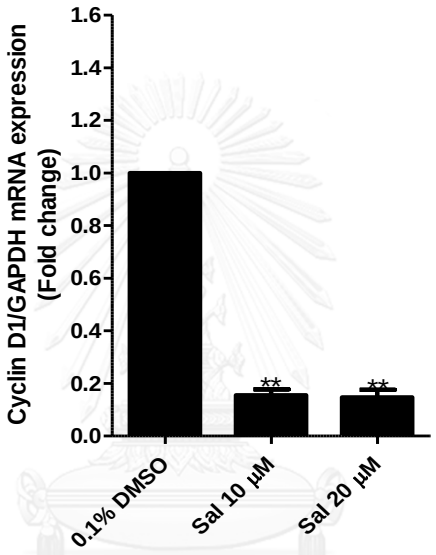
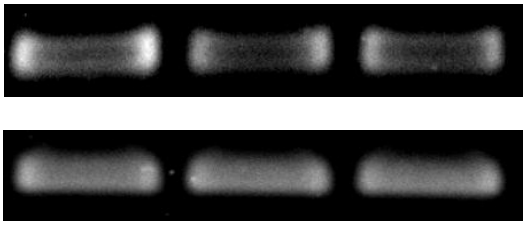


Figure 41 : The effect of salinomycin on the mRNA expression of *cyclin D1* in MCF-7/LCC9 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *cyclin D1* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

** $p < 0.01$ denotes statistically significant difference from 0.1% DMSO

A.



B.

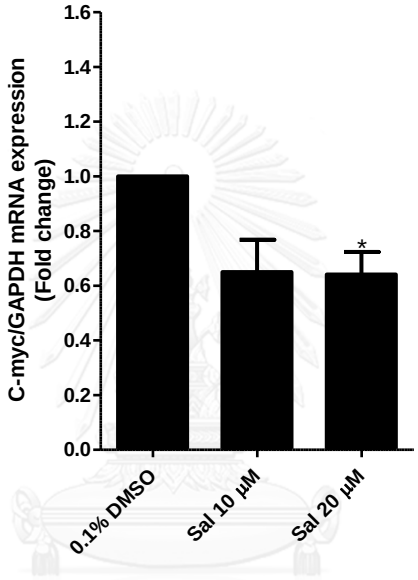


Figure 42 : The effect of salinomycin on the mRNA expression of *c-myc* in MCF-7/LCC9 cells. Cells were treated with 10 and 20 µM of salinomycin for 48 hours. The total RNA was extracted from treated cells and reverse transcribed to amplify with specific primer by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A). A representative PCR products of *c-myc* from control and salinomycin treated cells. (B). Densitometric analysis of PCR products relative to *GAPDH* represented as Relative Quantitation. The data was presented as mean ± SEM of three independent experiments (n=3).

*$p < 0.05$ denotes statistically significant difference from 0.1% DMSO.

CHAPTER V DISCUSSION AND CONCLUSION

This study focused on the investigation of inhibitory effects of salinomycin on cell viability, migration, invasion, and increasing tamoxifen sensitivity via down-regulation of *MMP9* expression, ER-target genes, and genes involved in tamoxifen resistance. Salinomycin is an antibiotic extracted from the cultured supernatant of *Streptomyces albus*. Salinomycin is used as an antimicrobial and anticoccidial drug in poultry diseases and helps improve nutrient absorption in cattle and swine **[122]**. Recent study reported that salinomycin exhibited anti-cancer activity against multi-drug resistance in various types of cancer. However, mechanisms of salinomycin on anti-estrogen resistant breast cancer have not been elucidated. The result from these study demonstrated that salinomycin had an anti-cancer effect on anti-estrogen resistant breast cancer cells: MCF-7/LCC2 and MCF-7/LCC9 cells determined by MTT cell viability assay. Salinomycin inhibited cell viability in a concentration- and time-dependent manner of MCF-7/LCC2 cell line with its IC_{50} values which were 24.17, 18.15, and 9.23 μM at 24, 48, and 72 hours, respectively and IC_{50} values of MCF7/LCC9 cell which were 19.74, 15.77, 6.56 μM at 24, 48, and 72 hours, respectively. In addition, anti-estrogen resistant breast cancer cells were more sensitive to salinomycin than MCF-7 wild type cells. Therefore, the results demonstrated that salinomycin is a potent anti-cancer compound against anti-estrogen resistant breast cancer cells.

Tamoxifen has been used as the standard treatment for all stages of ER-positive breast cancer. Approximately, 50% of breast cancer patients developed resistance to tamoxifen **[3]**. Thus, the development of new emerging treatment and

novel combination therapy for ER-positive breast cancer to increase efficacy of tamoxifen or decrease resistance of hormonal therapy is necessary. Previous studies reported that salinomycin improved the efficacy of gemcitabine to eradicate pancreatic cancer cells which exhibited stem cell-like features. Moreover, co-treatment between gemcitabine and salinomycin suppressed colony formation *in vitro* and completely suppressed tumor growth more effectively than individual treatment against pancreatic cancer **[7]**. Consistently, salinomycin sentitized docetaxel-, paclitaxel-, visblastine-, colchicine- treated cancer cell lines to anti-mitotic drugs with very low concentration of salinomycin. The data indicated that salinomycin was able to improve efficacy of chemotherapy for cancer patients **[123]**. Tamoxifen response assay was used to investigate the synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability and increasing tamoxifen sensitivity in the anti-estrogen resistant breast cancer cells. The results demonstrated that salinomycin was able to combine with tamoxifen treatment since salinomycin concurrently increased tamoxifen sensitivity in the anti-estrogen resistant breast cancer cell lines and increased tamoxifen efficacy in MCF-7 wild type cells.

The principle steps of tumor metastasis include cell migration, degradation of extracellular matrix, and cell invasion. The metastasis of cancer cells is the main problem that limits the efficacy of cancer treatment and leads to the cause of death in majority of cancer patients **[89, 90]**. Thus, the development of new treatment regimen to reduce cell migration and invasion is important for improving cancer therapy. The effect of salinomycin on migration and invasion on human endometrial cancer stem-like cells was previously investigated. Salinomycin decreased the level of fibronectin as well as inhibited cell proliferation, migration, and invasion in human

endometrial cancer stem-like cells **[120]**. This study found that salinomycin significantly inhibited cell migration in time dependent manner in MCF-7/LCC2 and MCF-7/LCC9 cells in scratch assay. Salinomycin has the similar effect in significantly inhibiting cell migration of MCF-7 wild type cells after treatment with salinomycin for 24 and 30 hours. Previous study showed that salinomycin inhibited cell growth and migration in prostate cancer cell via decreased expression of prostate cancer oncogenes and induction of oxidative stress **[115]**. To confirm anti-migratory effect of salinomycin in anti-estrogen resistant breast cancer cells, the result from scratch assay may also explain the effect on cell proliferation. Therefore, matrigel invasion assay was performed to confirm anti-invasive effect of salinomycin on anti-estrogen resistant breast cancer cells. The result confirmed that salinomycin had anti-invasive effect in the anti-estrogen resistant breast cancer cells since salinomycin significantly decreased numbers of cell invasion and percentage of proportional invasiveness in concentration dependent manner in MCF-7/LCC9 cell after treatment with salinomycin for 72 hours. This study suggested that salinomycin was able to be an anti-invasive agent for anti-estrogen resistant breast cancer patients.

To better understand the molecular mechanisms involved in salinomycin inhibiting cell viability, migration, invasion, and increasing tamoxifen sensitivity in anti-estrogen resistant breast cancer cells, the effect of salinomycin on the mRNA expression of *MMP9*, ER-target genes, and genes involved in tamoxifen resistance including *NCOA3*, *Cyclin D1*, *C-myc*, and *P21* was also investigated by RT-PCR. MMP9 is the main critical molecule enhancing tumor migration and invasion by degrading basement membrane structures and collagen type IV which is the composition of ECM. MMP9 is mainly expressed in the cytoplasm of both tumor and stromal cells

[98]. The overexpression of *MMP9* was associated with a poor prognosis in many types of cancer. The effect of salinomycin on the mRNA expression of *MMP9* was investigated by RT-PCR. Salinomycin significantly down-regulated mRNA expression of *MMP9* in the anti-estrogen resistant breast cancer cells, resulting in the inhibition of cell migration and invasion in anti-estrogen resistant breast cancer cells and finally inhibiting metastasis.

NCOA3 is nuclear receptor co-activator, functioning as a prognostic marker that plays the major role in the tumorigenesis of breast cancer and also promotes breast cancer growth, cell invasion, and tamoxifen resistance. NCOA3 can interact with ligand-ER complex to activate transcription of ER-target genes involved in cell proliferation, survival, and migration of cancer **[63]**. NCOA3 amplification and overexpression were found in breast cancer that enhanced mitogenic effects of estrogen that stimulated cell proliferation and related to high histological grade, poor prognosis, and tamoxifen resistance **[62, 124]**. The effect of salinomycin on increasing tamoxifen sensitivity through *NCOA3* was investigated. The result demonstrated that salinomycin significantly down-regulated the mRNA expression of *NCOA3* in the anti-estrogen resistant breast cancer cells. This finding suggested that salinomycin was able to increase tamoxifen sensitivity via decreasing mRNA expression of *NCOA3* in the anti-estrogen resistant breast cancer cells.

The alteration of downstream signaling molecules plays critical roles in cell cycle progression and regulation of cell proliferation that can promote tamoxifen resistance. Cyclin D1 is the central protein controlling G_1/S phase in the cell cycle. The activity of cyclin D1 begins from binding to CDKs 4, 6 and leads to phosphorylate Rb protein, resulting in activation of numerous genes which are responsible in S

phase progression **[69]**. The high expression of *cyclin D1* was observed in one third of breast cancer patients. The amplification of *cyclin D1* (*CCND1*) gene was found in tumors in a major randomized cohort of ER-positive breast cancer in postmenopausal patients with hormonal therapy that linked to an aggressive disease and was either associated with increased risk of breast cancer recurrence or related to tamoxifen resistance **[71]**. This study demonstrated that salinomycin significantly decreased mRNA expression of *cyclin D1* in anti-estrogen resistant breast cancer cells. Moreover, salinomycin significantly inhibited the mRNA expression of *c-myc.* C-myc is the transcription factor which is responsible for promoting cell proliferation by activating quiescent cells into cell cycle. Moreover, c-myc binds to ER to modulate ER-signaling and is highly expressed in approximately 20-30% of breast cancer patients **[125]**. The elevated expression of *c-myc* alone was adequate to induce breast cancer cell growth in the presence of pure antagonist and was able to confer anti-estrogen resistance in human breast cancer cells. Consistently, *c-myc* was strongly expressed in biopsies of metastatic lesion of breast cancer patients who had received adjuvant hormonal therapy **[126, 127]**. Thus, c-myc can serve as a predictive marker of effectiveness of tamoxifen therapy and associates with tamoxifen resistance **[80]**. Therefore, the down-regulated expression of *c-myc* may lead to a decrease in tamoxifen resistance. In addition, salinomycin also induced mRNA expression of *p21* gene. P21 is a cyclin-dependent kinase inhibitor (CKI) that functions as a negative regulator in G1/S phase of cell cycle. P21 mediates its effects through binding to CDK2 and CDK1 and inhibiting the kinase activity of the CDK2 and CDK1, resulting in cell growth arrest. It also plays a crucial role in inhibiting cell cycle progression, modulating DNA repair process, and inducing gene correlated with senescence **[83]**.

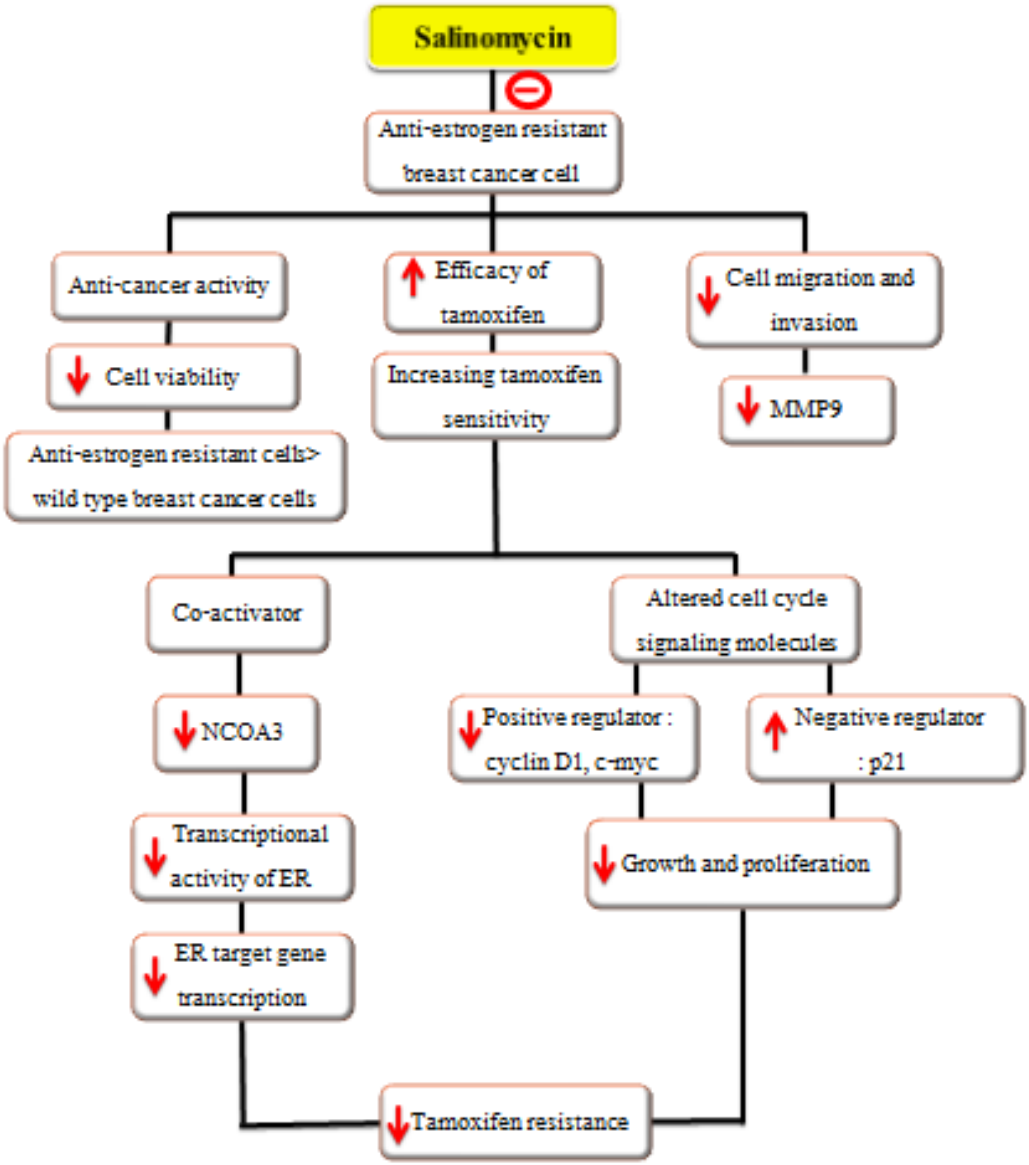
The loss of *p21* expression was highly observed in a high percentage of human breast cancers and was a predominant mechanism of acquired tamoxifen resistance which associated with poor response to tamoxifen **[84, 128]**. This finding suggested that salinomycin inhibited cell viability and increased tamoxifen sensitivity through down-regulation of expression of cell cycle signaling molecules involving growth maintenance and positive regulator of cell cycle and also up-regulation of the negative regulator of cell cycle. It is possible that salinomycin works as a cell cycle specific agent to trigger cell cycle arrest because the cell cycle arrest was correlated with the increase of p21 expression and decreased the expression of *cyclin D1* and *c-myc* in cell cycle. The effect of salinomycin on the expression of cell cycle regulators should be further elucidated for precisely explaining their effect on cell cycle arrest.

In summary, salinomycin demonstrated anti-cancer activity to inhibit cell viability in anti-estrogen resistant breast cancer cells. It also increased tamoxifen efficacy in MCF-7 wild type cells and salinomycin can increase tamoxifen sensitivity through down-regulation of *NCOA3* expression since it is one of critical ER-coregulators in concert with other ER-target genes including *cyclin D1*, *c-myc* and up-regulation of *p21* which involves in cell cycle. Thus, salinomycin acts on both inhibition of cell viability and decreasing important mediators in ER-target gene transcription. However, the effect of salinomycin in the combination with tamoxifen extremely decreased IC_{50} and increased tamoxifen sensitivity. This might not be explained by decreasing only one co-activator and mediators in this study. It is possible that other co-activators or co-repressors are also altered by salinomycin which further study will be required for better understanding of molecular mechanism of salinomycin action. Furthermore, this finding suggested that

salinomycin can significantly inhibit cell migration and invasion by down-regulation of *MMP9* expression in the anti-estrogen resistant breast cancer cells. It is possible that salinomycin inhibits not only *MMP9* expression but also other factors which involved with metastasis in the anti-estrogen resistant breast cancer cells. To clarify this issue, the anti-cancer activity of salinomycin is further investigated in the future.

This finding provides basic molecular mechanism of salinomycin that can inhibit anti-estrogen resistant breast cancer cells. Salinomycin can be a promising agent for novel therapeutic targets and novel combination with tamoxifen for improving the efficacy of the treatment in the anti-estrogen resistant breast cancer patients. The data from this study also provides benefits for further extension of anti-estrogen resistant study. Moreover, this study offers further studies of salinomycin to evaluate anti-cancer efficacy of salinomycin in animal tumor models and anti-estrogen resistant breast cancer patient.

Conclusion


Salinomycin
Anti-estrogen resistant breast cancer cell
Anti-cancer activity
Cell viability
Anti-estrogen resistant cells> wild type breast cancer cells
Efficacy of tamoxifen
Increasing tamoxifen sensitivity
Cell migration and invasion
MMP9
Co-activator
NCOA3
Transcriptional activity of ER
ER target gene transcription
Altered cell cycle signaling molecules
Positive regulator : cyclin D1, c-myc
Negative regulator : p21
Growth and proliferation
Tamoxifen resistance

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APPENDIX A

Buffers and Reagents

1. Incomplete MEM medium

- MEM medium powder	9.5 g
- $NaHCO_3$	3.75 g
- ddH_2O	900 ml

Adjust pH to 7.3 with 1M HCl

Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 membrane filter

2. Complete MEM medium 100 ml

- MEM medium stock	94 ml
- Inactivated Fetal Bovine Serum	5 ml
- Penicillin/Streptomycin	1 ml

3. Complete IMEM medium 100 ml

- Phenol red-free IMEM medium	93.9 ml
- Charcoal dextran-treated FBS	5 ml
- Non-essential amino acid	1 ml
- Insulin 10^{-6} M	100 µl

4. 10x Phosphate Buffered Saline (PBS) 1 liter

- NaCl	80.65 g
- KCl	2 g
- KH_2PO_4	2 g
- Na_2HPO_4	11.5 g
- ddH_2O	900 ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and sterilized by autoclaving

5. EDTA 0.5 M pH 8.0 100 ml

- EDTA	18.612 g
- ddH_2O	80 ml

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 ml and sterilized by autoclaving

6. 5xTBE Buffered 1 liter

- Tris base	54 g
- Boric acid	27.5 g
- EDTA 0.5 M pH 8.0	20 ml

Add ddH_2O to 1000 ml and sterilized by autoclaving

APPENDIX B

Appendix B-1 : Effect of salinomycin on the inhibition of cell viability in MCF-7 cells at 24 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	81.334	82.377	83.152	82.290	82.29 ± 0.527
4-OHT 10 μM	18.367	20.879	23.421	20.890	20.89 ±1.459
Salinomycin 10 μM	98.308	81.292	95.522	91.710	91.71 ±5.269
Salinomycin 20 μM	64.111	49.147	65.844	59.700	59.70 ±5.300
Salinomycin 30 μM	24.440	13.127	30.654	22.740	22.74 ±5.131
Salinomycin 40 μM	10.154	3.979	12.687	8.940	8.940 ±2.586
Salinomycin 50 μM	5.475	2.687	5.1665	4.443	4.443 ± 0.882

Appendix B-2 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC2 cells at 24 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	92.689	96.887	92.351	93.980	93.98 ±1.459
4-OHT 10 μM	15.969	9.188	12.908	12.690	12.69 ±1.961
Salinomycin 10 μM	100.000	100.000	100.000	100.000	100.000 ± 0.000
Salinomycin 20 μM	70.707	34.776	58.964	54.820	54.82 ±10.580
Salinomycin 30 μM	25.974	8.049	16.335	16.790	16.79 ±5.180
Salinomycin 40 μM	7.407	4.100	3.825	5.111	5.111 ±1.151
Salinomycin 50 μM	5.051	6.530	4.622	5.401	5.401 ± 0.578

Appendix B-3 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC9 cells at 24 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	100.000	97.470	100.000	99.160	99.16 ± 0.843
4-OHT 10 μM	61.820	16.866	25.083	34.590	34.59 ±13.820
Salinomycin 10 μM	113.383	71.469	78.974	83.480	83.48 ±8.539
Salinomycin 20 μM	78.768	23.472	37.086	46.440	46.44 ±16.630
Salinomycin 30 μM	47.592	6.254	12.914	22.250	22.25 ±12.810
Salinomycin 40 μM	17.167	4.920	4.139	8.742	8.742 ±4.219
Salinomycin 50 μM	8.193	6.817	5.298	6.769	6.769 ± 0.836

Appendix B-4 : Effect of salinomycin on the inhibition of cell viability in MCF-7 cells at 48 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	76.406	79.871	86.342	80.873	80.873 ± 2.912
4-OHT 10 μM	1.888	3.789	4.430	3.369	3.369 ± 0.763
Salinomycin 2.5 μM	99.823	100.000	100.000	99.941	99.941 ± 0.059
Salinomycin 5 μM	99.096	99.120	100.000	99.405	99.405 ± 0.297
Salinomycin 10 μM	79.827	85.149	93.471	86.149	86.149 ± 3.970
Salinomycin 15 μM	61.974	55.277	74.084	63.780	63.778 ± 5.503
Salinomycin 20 μM	49.037	44.452	55.030	49.506	49.506 ± 3.063

Appendix B-5 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC2 cells at 48 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	100.000	84.756	91.257	92.004	92.004 ± 4.416
4-OHT 10 μM	2.459	2.041	3.783	2.761	2.761 ± 0.525
Salinomycin 2.5 μM	94.042	100.000	100.000	98.014	98.014 ± 1.986
Salinomycin 5 μM	93.108	95.585	96.931	95.208	95.208 ± 1.120
Salinomycin 10 μM	74.659	81.758	84.952	80.456	80.456 ± 3.042
Salinomycin 15 μM	60.445	58.267	62.757	60.490	60.490 ± 1.296
Salinomycin 20 μM	45.154	37.526	42.707	41.796	41.796 ± 2.249

Appendix B-6 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC9 cells at 48 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	100.000	89.576	80.919	90.165	90.165 ± 5.516
4-OHT 10 μM	1.779	1.565	26.776	10.040	10.040 ± 8.368
Salinomycin 2.5 μM	82.410	80.947	93.336	85.564	85.564 ± 3.909
Salinomycin 5 μM	72.039	62.390	75.519	69.983	69.983 ± 3.927
Salinomycin 10 μM	73.818	60.443	59.457	64.573	64.573 ± 4.631
Salinomycin 15 μM	55.313	49.675	56.744	53.911	53.911 ± 2.158
Salinomycin 20 μM	39.197	31.997	45.451	38.882	38.882 ± 3.887

Appendix B-7 : Effect of salinomycin on the inhibition of cell viability in MCF-7 cells at 72 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	65.637	64.708	65.052	65.132	65.132 ± 0.271
4-OHT 10 μM	0.088	0.523	0.266	0.292	0.292 ± 0.126
Salinomycin 2.5 μM	69.121	61.429	66.010	65.520	65.520 ± 2.234
Salinomycin 5 μM	58.142	61.006	61.185	60.111	60.111 ± 0.986
Salinomycin 10 μM	31.514	41.851	41.370	38.245	38.245 ± 3.368
Salinomycin 15 μM	26.288	27.847	31.205	28.446	28.446 ± 1.451
Salinomycin 20 μM	13.938	14.064	18.520	15.508	15.508 ± 1.507

Appendix B-8 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC2 cells at 72 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	85.501	85.156	94.215	88.291	88.291 ± 2.964
4-OHT 10 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000
Salinomycin 2.5 μM	77.917	83.495	81.461	80.958	80.958 ± 1.630
Salinomycin 5 μM	57.387	55.996	68.200	60.528	60.528 ± 3.857
Salinomycin 10 μM	50.076	53.780	58.390	54.082	54.082 ± 2.405
Salinomycin 15 μM	18.733	28.081	27.097	24.637	24.637 ± 2.966
Salinomycin 20 μM	3.016	4.874	2.097	3.329	3.329 ± 0.817

Appendix B-9 : Effect of salinomycin on the inhibition of cell viability in MCF-7/LCC9 cells at 72 hours.

Test Compounds	The percentage of cell viability				Mean ± S.E.M
	1	2	3	Mean	
MEM media	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	94.760	92.670	94.251	93.894	93.894 ± 0.629
4-OHT 10 μM	0.419	0.479	0.530	0.476	0.476 ± 0.032
Salinomycin 2.5 μM	72.906	75.642	76.425	74.991	74.991 ± 1.067
Salinomycin 5 μM	59.349	61.763	49.899	57.004	57.004 ± 3.620
Salinomycin 10 μM	28.048	20.013	27.937	25.333	25.333 ± 2.660
Salinomycin 15 μM	11.434	4.055	2.421	5.970	5.970 ± 2.772
Salinomycin 20 μM	3.720	1.713	1.437	2.290	2.290 ± 0.720

Appendix B-10 : Effect of tamoxifen on the inhibition of cell viability in MCF-7 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	86.812	63.615	84.533	78.320	78.320 ± 7.382
4-OHT 0.5 μM	81.760	55.161	84.078	73.666	73.666 ± 9.277
4-OHT 1 μM	77.881	55.375	80.755	71.337	71.337 ± 8.024
4-OHT 2.5 μM	76.998	50.168	77.585	68.250	68.250± 9.043
4-OHT 5 μM	0.811	26.126	29.859	18.932	18.932 ± 9.124
4-OHT 7.5 μM	0.000	3.063	0.276	1.113	1.113 ± 0.978
4-OHT 10 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-11 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 0.5 μM	71.830	59.637	75.673	69.047	69.047 ± 4.834
4-OHT 0.5 μM + Sal 0.5 μM	58.917	50.101	67.564	58.861	58.861 ± 5.041
4-OHT 1 μM + Sal 0.5 μM	48.379	47.856	52.225	49.486	49.486 ± 1.378
4-OHT 2.5 μM + Sal 0.5 μM	41.257	40.767	45.053	42.359	42.359 ± 1.354
4-OHT 5 μM + Sal 0.5 μM	2.142	21.140	27.869	17.051	17.051 ± 7.703
4-OHT 7.5 μM + Sal 0.5 μM	0.463	0.000	0.176	0.213	0.213 ± 0.135
4-OHT 10 μM + Sal 0.5 μM	0.029	0.000	0.000	0.010	0.001 ± 0.001

Appendix B-12 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 1.5 μM	29.039	27.321	31.148	29.169	29.169 ± 1.107
4-OHT 0.5 μM + Sal 1.5 μM	22.554	29.541	30.474	27.523	27.523 ± 2.499
4-OHT 1 μM + Sal 1.5 μM	21.772	30.802	29.742	27.439	27.439 ± 2.849
4-OHT 2.5 μM + Sal 1.5 μM	13.144	32.114	28.630	24.629	24.629 ± 5.830
4-OHT 5 μM + Sal 1.5 μM	0.405	14.531	12.939	9.292	9.292 ± 4.467
4-OHT 7.5 μM + Sal 1.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000
4-OHT 10 μM + Sal 1.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-13 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 2.5 μM	27.360	12.928	23.959	21.415	21.415 ± 4.356
4-OHT 0.5 μM + Sal 2.5 μM	21.540	11.771	19.768	17.693	17.693 ± 3.005
4-OHT 1 μM + Sal 2.5 μM	18.877	11.268	21.119	17.088	17.088 ± 2.981
4-OHT 2.5 μM + Sal 2.5 μM	10.336	6.539	19.245	12.040	12.040 ± 3.765
4-OHT 5 μM + Sal 2.5 μM	0.550	2.314	6.672	3.179	3.179 ± 1.819
4-OHT 7.5 μM + Sal 2.5 μM	0.000	1.509	1.130	0.880	0.880 ± 0.453
4-OHT 10 μM + Sal 2.5 μM	0.000	0.352	0.221	0.191	0.191 ± 0.103

Appendix B-14 : Effect of tamoxifen on the inhibition of cell viability in MCF-7/LCC2 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	90.260	81.124	93.849	88.411	88.411 ± 3.788
4-OHT 0.5 μM	89.532	80.969	93.691	88.064	88.064 ± 3.745
4-OHT 1 μM	89.594	79.264	85.949	84.936	84.936 ± 3.025
4-OHT 2.5 μM	79.376	74.031	66.842	73.416	73.416 ± 3.631
4-OHT 5 μM	40.895	7.907	17.095	21.966	21.966 ± 9.829
4-OHT 7.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000
4-OHT 10 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-15 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC2 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 0.5 μM	58.772	65.314	55.089	59.725	59.725 ± 2.990
4-OHT 0.5 μM + Sal 0.5 μM	56.712	65.052	47.283	56.349	56.349 ± 5.133
4-OHT 1 μM + Sal 0.5 μM	49.490	59.873	46.382	51.915	51.915 ± 4.079
4-OHT 2.5 μM + Sal 0.5 μM	47.659	52.954	45.152	48.588	48.588 ± 2.299
4-OHT 5 μM + Sal 0.5 μM	3.143	55.180	20.624	26.316	26.316 ± 15.289
4-OHT 7.5 μM + Sal 0.5 μM	0.000	13.706	2.582	5.429	5.429 ± 4.205
4-OHT 10 μM + Sal 0.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-16 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC2 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 1.5 μM	41.436	37.136	37.097	38.556	38.556 ± 1.440
4-OHT 0.5 μM + Sal 1.5 μM	39.334	29.180	36.844	35.120	35.120 ± 3.055
4-OHT 1 μM + Sal 1.5 μM	36.046	27.469	36.381	33.299	33.299 ± 2.916
4-OHT 2.5 μM + Sal 1.5 μM	34.860	23.056	37.476	31.797	31.797 ± 4.435
4-OHT 5 μM + Sal 1.5 μM	5.078	15.521	12.871	11.157	11.157 ± 3.134
4-OHT 7.5 μM + Sal 1.5 μM	0.000	2.642	0.000	0.881	0.881 ± 0.881
4-OHT 10 μM + Sal 1.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-17 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC2 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 2.5 μM	36.212	23.994	25.132	28.446	28.446 ± 3.897
4-OHT 0.5 μM + Sal 2.5 μM	31.238	17.461	22.140	23.613	23.613 ± 4.045
4-OHT 1 μM + Sal 2.5 μM	26.660	18.573	21.898	22.377	22.377 ± 2.347
4-OHT 2.5 μM + Sal 2.5 μM	21.582	14.950	21.772	19.434	19.434 ± 2.243
4-OHT 5 μM + Sal 2.5 μM	1.353	7.760	3.286	4.133	4.133 ± 1.898
4-OHT 7.5 μM + Sal 2.5 μM	0.000	0.770	0.169	0.313	0.313 ± 0.234
4-OHT 10 μM + Sal 2.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-18 : Effect of tamoxifen on the inhibition of cell viability in MCF-7/LCC9 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM	100.000	87.046	100.000	95.682	95.682 ± 4.318
4-OHT 0.5 μM	100.000	88.828	98.126	95.651	95.651 ± 3.454
4-OHT 1 μM	100.000	84.921	93.873	92.931	92.931 ± 4.378
4-OHT 2.5 μM	93.922	85.058	96.828	91.936	91.936 ± 3.540
4-OHT 5 μM	25.909	1.782	39.596	22.429	22.429 ± 11.054
4-OHT 7.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000
4-OHT 10 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-19 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC9 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 0.5 μM	73.943	62.012	77.290	71.082	71.082 ± 4.640
4-OHT 0.5 μM + Sal 0.5 μM	73.011	63.385	60.600	65.665	65.665 ± 3.760
4-OHT 1 μM + Sal 0.5 μM	58.313	63.256	60.024	60.531	60.531 ± 1.450
4-OHT 2.5 μM + Sal 0.5 μM	52.851	61.240	57.123	57.071	57.071 ± 2.422
4-OHT 5 μM + Sal 0.5 μM	7.977	49.764	45.341	34.361	34.361 ± 13.253
4-OHT 7.5 μM + Sal 0.5 μM	0.000	3.539	1.212	1.584	1.584 ± 1.040
4-OHT 10 μM + Sal 0.5 μM	0.000	2.124	0.000	0.708	0.708 ± 0.708

Appendix B-20 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC9 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 1.5 μM	38.955	30.398	48.223	39.192	39.192 ± 5.147
4-OHT 0.5 μM + Sal 1.5 μM	38.435	23.132	50.283	37.284	37.284 ± 7.859
4-OHT 1 μM + Sal 1.5 μM	37.850	24.743	48.190	36.928	36.928 ± 6.784
4-OHT 2.5 μM + Sal 1.5 μM	40.559	20.631	47.536	36.242	36.242 ± 8.061
4-OHT 5 μM + Sal 1.5 μM	1.192	1.165	46.620	16.326	16.326 ± 15.147
4-OHT 7.5 μM + Sal 1.5 μM	0.000	0.000	3.554	1.185	1.185 ± 1.185
4-OHT 10 μM + Sal 1.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-21 : Synergistic effect of salinomycin and tamoxifen on the inhibition of cell viability in MCF-7/LCC9 cells.

Test Compounds	The percentage of cell survival				Mean ± S.E.M
	1	2	3	Mean	
0.1% DMSO	100.000	100.000	100.000	100.000	100.000 ± 0.000
4-OHT 0.1 μM + Sal 2.5 μM	37.845	20.768	35.920	31.511	31.511 ± 5.400
4-OHT 0.5 μM + Sal 2.5 μM	35.589	14.565	35.235	28.463	28.463 ±6.950
4-OHT 1 μM + Sal 2.5 μM	34.430	14.256	34.587	27.758	27.758 ± 6.751
4-OHT 2.5 μM + Sal 2.5 μM	25.063	10.075	38.755	24.631	24.631 ± 8.282
4-OHT 5 μM + Sal 2.5 μM	0.689	0.137	7.016	2.614	2.614 ± 2.207
4-OHT 7.5 μM + Sal 2.5 μM	0.000	0.137	0.000	0.0457	0.0457 ± 0.0457
4-OHT 10 μM + Sal 2.5 μM	0.000	0.000	0.000	0.000	0.000 ± 0.000

Appendix B-22 : Effect of salinomycin on cell migration in MCF-7 cells after 0, 6, 24, and 30 hours exposure.

Test compound : 0.1 % DMSO	**% open wound area**			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	29.66	30.38	20.36	16.78
	32.15	30.06	23.93	18.38
	31.02	28.60	22.80	15.87
	29.13	29.42	17.88	19.82
	33.70	31.71	18.04	20.69
N2	33.38	33.60	17.58	11.76
	31.36	30.33	16.71	24.85
	34.34	25.15	27.06	18.55
	34.52	34.77	27.40	18.32
	34.95	25.80	15.97	19.47
Mean	32.42	29.98	20.77	18.45
Mean ± SEM	32.42 ± 0.655	29.98 ± 0.957	20.77 ± 1.347	18.45 ± 1.070

Appendix B-23 : Effect of salinomycin on cell migration in MCF-7 cells after 0, 6, 24, and 30 hours exposure.

Test compound : Salinomycin 2.5 μM	**% open wound area**			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	32.15	32.92	32.39	26.57
	31.91	27.53	26.47	24.46
	33.31	24.41	29.19	29.50
	32.98	36.47	28.60	27.04
	31.85	22.32	26.02	26.39
N2	31.23	28.35	29.61	29.83
	29.27	29.31	27.62	26.57
	32.68	29.49	29.55	27.65
	33.93	32.32	28.38	26.80
	30.89	29.60	27.21	25.02
Mean	32.02	29.27	28.50	26.98
Mean ± SEM	32.02 ± 0.424	29.27 ± 1.290	28.50 ± 0.584	26.98 ± 0.536

Appendix B-24 : Effect of salinomycin on cell migration in MCF-7/LCC2 cells after 0, 6, 24, and 30 hours exposure.

Test compound : 0.1 % DMSO	**% open wound area**			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	30.13	27.78	23.75	21.07
	28.77	31.13	24.77	22.27
	28.80	29.20	23.58	20.31
	33.18	27.09	23.29	19.18
	31.02	32.36	21.62	22.44
N2	40.72	34.17	31.16	30.03
	37.54	32.14	29.16	27.20
	39.69	34.34	28.20	26.89
	38.98	31.50	30.81	27.88
	39.83	34.86	28.30	30.88
Mean	34.87	31.46	26.46	24.82
Mean ± SEM	34.87 ± 1.564	31.46 ± 0.859	26.46 ± 1.089	24.82 ± 1.340

Appendix B-25 : Effect of salinomycin on cell migration in MCF-7/LCC2 cells after 0, 6, 24, and 30 hours exposure.

Test compound : Salinomycin 2.5 μM	**% open wound area**			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	30.19	30.33	27.95	29.92
	31.49	30.23	30.06	27.41
	30.38	27.82	27.55	29.58
	30.99	27.49	30.47	27.38
	28.43	31.24	30.93	29.31
N2	38.70	38.15	35.25	33.47
	39.17	37.62	35.61	34.48
	39.92	37.98	35.78	32.87
	38.94	37.30	35.54	31.78
	41.00	37.72	33.98	31.53
Mean	34.92	33.59	32.31	30.77
Mean ± SEM	34.92 ± 1.573	33.59 ± 1.434	32.31 ± 1.037	30.77 ± 0.775

Appendix B-26 : Effect of salinomycin on cell migration in MCF-7/LCC9 cells after 0, 6, 24, and 30 hours exposure.

Test compound : 0.1 % DMSO	% open wound area			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	29.29	30.97	24.90	15.42
	30.10	31.75	25.92	20.74
	31.95	29.84	27.66	15.32
	32.08	31.67	23.54	15.94
	29.35	30.41	24.69	16.35
N2	42.51	39.66	34.70	29.95
	41.17	39.43	33.81	31.04
	42.37	39.68	32.58	30.73
	40.52	40.47	33.04	31.05
	40.27	39.06	31.67	31.90
Mean	35.96	35.29	29.25	23.84
Mean ± SEM	35.96 ± 1.838	35.29 ± 1.470	29.25 ± 1.365	23.84 ± 2.416

Appendix B-27 : Effect of salinomycin on cell migration in MCF-7/LCC9 cells after 0, 6, 24, and 30 hours exposure.

Test compound : Salinomycin 2.5 μM	**% open wound area**			
	0 hr	**6 hrs**	**24 hrs**	**30 hrs**
N1	30.74	30.96	29.72	27.11
	28.72	29.43	28.49	27.39
	28.96	30.14	25.57	27.20
	29.18	28.49	28.80	29.27
	29.18	28.57	27.82	29.40
N2	41.01	39.13	36.32	34.15
	40.70	38.94	36.36	36.22
	39.83	40.05	36.53	36.85
	41.20	39.73	35.81	36.14
	40.22	39.26	36.11	34.66
Mean	34.97	34.47	32.15	31.84
Mean ± SEM	34.97 ± 1.884	34.47 ± 1.668	32.15 ± 1.398	31.84 ± 1.301

Appendix B-28 : Effect of salinomycin on cell invasion in MCF-7/LCC9 cells after 72 hours treatment.

N1	Number of cell migration		
Test compound	0.1% DMSO	Sal 2.5 μM	Sal 5 μM
	120	103	98
	120	102	93
	117	104	98
	117	103	87
	129	103	104
Mean ± SEM	120.6 ± 2.205	103 ± 0.3162	96 ± 2.846

N2	Number of cell migration		
Test compound	0.1% DMSO	Sal 2.5 μM	Sal 5 μM
	117	105	98
	120	107	97
	118	104	88
	125	105	91
	113	105	91
Mean ± SEM	118.6 ± 1.965	105.2 ± 0.4899	93 ± 1.924

Appendix B-29 : Effect of salinomycin on cell invasion in MCF-7/LCC9 cells after 72 hours treatment.

N3	Number of cell migration		
Test compound	0.1% DMSO	Sal 2.5 μM	Sal 5 μM
	122	113	95
	116	106	83
	116	108	100
	117	113	91
	118	106	97
Mean ± SEM	117.8 ± 1.114	109.2 ± 1.594	93.2 ± 2.939

Appendix B-30 : Effect of salinomycin on mRNA expression of *MMP9*, ER target genes, and genes involved with tamoxifen resistance in MCF-7/LCC2 cells.

Test compound	*MMP9/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.4849	0.7197	0.5458	0.5835 ± 0.07035
Sal 20 μM	0.0907	0.5328	0.0909	0.2381 ± 0.1473

Test compound	*NCOA3/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.9145	0.8569	0.9297	0.9004 ± 0.02217
Sal 20 μM	0.6980	0.5950	0.7367	0.6766 ± 0.04229

Test compound	*P21/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.9351	1.2489	1.2828	1.156 ± 0.1107
Sal 20 μM	1.1693	1.3696	1.3053	1.281 ± 0.05904

Appendix B-31 : Effect of salinomycin on mRNA expression of ER target genes and genes involved with tamoxifen resistance in MCF-7/LCC2 cells.

Test compound	*Cyclin D1/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.2901	0.3059	0.1347	0.2436 ± 0.05462
Sal 20 μM	0.00690	0.10010	0.07646	0.06115 ± 0.02797

Test compound	*C-myc/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.6013	0.6045	0.9122	0.7060 ± 0.1031
Sal 20 μM	0.6425	0.5623	0.7765	0.6604 ± 0.06248

Appendix B-32 : Effect of salinomycin on mRNA expression of *MMP*, ER target genes, and genes involved with tamoxifen resistance in MCF-7/LCC9 cells.

Test compound	*MMP9/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.3958	0.4109	0.1878	0.3315 ± 0.07198
Sal 20 μM	0.2197	0.2226	0.1421	0.1948 ± 0.02636

Test compound	*NCOA3/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.5896	0.9942	0.8479	0.8106 ± 0.1183
Sal 20 μM	0.5757	0.8170	0.7003	0.6977 ± 0.06967

Test compound	*P21/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	1.1238	1.1806	1.2576	1.187 ± 0.03877
Sal 20 μM	1.2580	1.3130	1.5301	1.367 ± 0.08306

Appendix B-33 : Effect of salinomycin on mRNA expression of ER target genes and genes involved with tamoxifen resistance in MCF-7/LCC9 cells.

Test compound	*Cyclin D1/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.1386	0.1262	0.2003	0.1550 ± 0.02291
Sal 20 μM	0.1023	0.1379	0.2003	0.1468 ± 0.02864

Test compound	*C-myc/GAPDH* mRNA expression			Mean ± SEM
	1	2	3	
0.1% DMSO	1.0000	1.0000	1.0000	1.0000 ± 0.000
Sal 10 μM	0.4143	0.7772	0.7580	0.6498 ± 0.1179
Sal 20 μM	0.4759	0.7290	0.7189	0.6413 ± 0.08273

VITA

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Publication

1. Manmuan S, Ketchart W. (2014) Effect of Salinomycin on Inhibits Cell Proliferation and Restores Tamoxifen Sensitivity in the Tamoxifen Resistant Breast Cancer Cells. Proceedings of the 36th Pharmacological and Therapeutic Society of Thailand meeting. Thai Journal of Pharmacology, Vol.36, Suppl.1, 2014, 117-120.

Poster Presentation

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