BREAST CANCER DEVELOPMENT IN RATS UNDER THE INFLUENCE OF PLATELET FACTOR-4 (PF4) AND/ OR RAPAMYCIN

by

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2. **Zaihassni,** Wirdatul-Nur, Damitri TD, Shahidan, Hilda SS, Azman Seeni, Hasnan Jaafar. Effect of Rapamycin and Platelet Factor-4 (PF4) on 1-Methyl-1-Nitrosourea (MNU)-induced Breast cancer. 17TH National Conference on Medical and Health Sciences 2012, 27-28 May 2012, Health Campus, USM.

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LIST OF ABBREVIATIONS

g	- gram
kg	- kilogram
mg	- miligram
ml	- mililiter
mM	- milimolar
M	- molar
ng	- nanogram
µg	- microgram
µl	- microliter
µm	- micrometer
cDNA	- complementary deoxyribonucleic acid
DAB	- diaminobenzidine
DAG	- 1, 2-diacylglycerol
DCIS	- Ductal carcinoma in situ
DEPC	- diethylpyrocarbonate
$dH2_o$	- distilled water
DMBA	- 7,12-dimethylbenz(a)antracene
EDTA	- ethylenediamine tetraacetic acid disodium
ERK	- extracellular signal-regulated protein kinase
FFPE	- formalin fixed paraffin embedded
H_2O_2 -	- Hydrogen peroxide

H&E	- Hematoxylin and Eosin (stain)
HRP	- Horseradish peroxidase
IDC-NOS	- Invasive ductal carcinoma-not otherwise specified
L	- liter
LB	- Lithium Boric acid buffer
MAPK	- mitogen-activated protein kinase
mRNA	- messenger ribonucleic acid
NBF	- Neutral buffered formalin
MNU	- 1-methyl-1-nitrosourea
PF4	- Platelet Factor 4
PI3K	- phosphoinositide 3-kinase
PPAR	- Peroxisome proliferator-activated receptor
qRT-PCR	- quantitative real-time PCR
RNA	- ribonucleic acid
TBS	- Tris Buffer Saline
TDLU	- terminal ductal lobular unit
TEB	- terminal end bud
VEGF	-Vascular endothelial growth factor
ER	- Estrogen Receptor
PR	- Progesterone Receptor
AI	- Aromatase Inhibitor
mTOR	- Mammalian Target of Rapamycin
IDP	- Intraductal proliferations

rhPF4	- Recombinant Platelet Factor 4
HSPG	- Heparan Sulfateproteoglycans
kDa	- Kilo Dalton
RXR	- Retinoid-X Receptor
r.p.m	- rotation perminute
dNTP	- deoxyribonucleotide triphospates
REST-MCS	- Relative Expression Software Tool-Multiple Condition Solver
rRNA	- ribosomal ribonucleic acid
SE	- Standard Error
mTORC1	- Mammalian Target of Rapamycin Complex 1
mTORC2	- Mammalian Target of Rapamycin Complex 2

PERKEMBANGAN KANSER PAYU DARA PADA TIKUS DI BAWAH KESAN PLATLET FAKTOR-4 (PF4) DAN/ ATAU RAPAMAISIN

ABSTRAK

Kanser payu dara merupakan punca utama kematian dalam kalangan wanita di seluruh dunia. Reseptor steroid seperti Estrogen Receptor (ER) dan Progesteron Receptor (PR) memainkan peranan penting dalam perkembangan kanser payu dara. Dalam kajian ini, ER dan PR dipilih sebagai penanda untuk menentukan kehadiran reseptor steroid kerana perkembangan kanser payu dara bergantung kepada hormon steroid seperti yang telah dilaporkan dalam kebanyakan kajian terdahulu. Selain itu, Peroxisome Proliferator Activation Receptor γ (PPARγ), salah satu ahli keluarga dalam kumpulan reseptor hormon nukleus juga ditentukan kerana ianya berperanan dalam pembahagian sel tumor. Rapamaisin, sejenis bahan daripada mikrolid bakteria dan Platelet Faktor-4 (PF4), sejenis kemokin mempunyai ciri-ciri antikanser. Oleh itu, ianya menarik untuk mengkaji kesan Rapamaisin dan PF4 dalam menghalang perkembangan kanser payu dara disebabkan oleh kesan hormon. Dalam kajian ini, kanser payu dara diaruh dengan menggunakan 1-Methyl-1-Nitrosourea (MNU) dengan dos 70mg/ kg berat badan terhadap 80 ekor tikus betina strain Sparague Dawley. Pengekspresan gen dan protein untuk ketiga-tiga reseptor ini ditentukan dengan mengggunakan teknik imunohistokimia dan Real-Time PCR. Hasilnya, semua tumor payu dara merupakan 100% malignan, mempunyai ciri *invasive ductal carcinoma (IDC)* yang kebanyakannya adalah jenis *cribriform, papillary* and *Not Otherwise Specified (NOS*). Perawatan dengan Rapamaisin menunjukkan perencatan perkembangan tumor dan pengurangan keagresifannya. Walaupun pengekspresan ER, PR berlebihan, pengaktifan PPARγ boleh mengaruh pembahagian sel tumor dan

seterusnya menukar fenotip agresif tumor kepada fenotip kurang agresif. Manakala, perawatan dengan PF4 tidak merencat perkembangan tumor dan juga tidak menunjukkan pengkspresan yang signifikan bagi ER, PR dan PPARγ. Pengaktifan kawalaturan bagi ketiga-tiganya dalam perawatan kombinasi menyebabkan tumor berjaya direncat dan keagresifan tumor berjaya disekat. Ianya dijangka bahawa Rapamaisin lebih menunjukkan kesan antikanser berbanding PF4. Oleh itu, kajian ini mencadangkan bahawa Rapamaisin bukanlah penggalak atau sinergi dengan PF4. Kesimpulannya, Rapamaisin berpotensi dalam memainkan peranan sebagai antikanser kerana ia menghalang perkembangan tumor dan mengaruh pembezaan sel tumor melalui pengekspresan yang positif bagi reseptor hormon steroid. Kajian yang lebih mendalam diperlukan bagi mengkaji pengawalaturan jenis isoform bagi ER (ERα dan ERβ) dan PR (PR-A dan PR-B) bagi meningkatkan strategi rawatan kanser payu dara dengan menggunakan antikanser seperti Rapamaisin.

BREAST CANCER DEVELOPMENT IN RATS UNDER THE INFLUENCE OF PLATELET FACTOR-4 (PF4) AND/ OR RAPAMYCIN

ABSTRACT

Breast cancer is a leading cause of morbidity and mortality among women worldwide. Steroid hormone receptors such as Estrogen Receptor (ER) and Progesterone Receptor (PR) play a critical role in breast cancer growth. In this study, ER and PR were selected as markers for steroid receptor determination due to the strong association between breast cancer development and the influence of steroid hormones as demonstrated in many studies. On the other hands, the Peroxisome Proliferator Activation Receptor γ (PPARγ), a family of nuclear hormone receptor was also determined as it was a potential effector for tumour cell differentiation. Rapamycin, a drug from bacteria microlide and Platelet Factor-4 (PF4), a platelet-derived chemokine have anticancer properties. Therefore, it will be interesting to analyse the effect of Rapamycin and PF4 in blocking the growth of breast cancer from responding to hormone stimulation. In this study, invasive mammary carcinoma was induced with 70mg/kg body weight 1-Methyl-1-Nitrosourea (MNU) in 80 young female Sprague Dawley rats. The gene and protein expressions of ER, PR and PPARγ markers were evaluated by using semiquantitative immunohistochemistry analysis and quantitative real-time PCR assay. Findings from the untreated-control group demonstrated that all mammary lesions are 100% malignant, histopathologically characterized with invasive ductal carcinoma (IDC) of three major type ie. cribriform, papillary and Not Otherwise Spesified (NOS). Rapamycin treatment showed significant inhibition of mammary tumour progression as well as reduction of tumour agressiveness. Even though treatment with Rapamycin

significantly overexpressed ER and PR , activation of PPARγ promotes differentiation of tumour cells which lead to a more differentiated mammary tumour and consequently reversing the aggressive phenotype of the lesion. Meanwhile, treatment with PF4 did not regress tumour growth and consequently showed no significant expression of ER, PR and PPARγ. Upregulation of all these three markers in combination treatment lead to significant tumour regression and phenotypically decreased aggressiveness. It was predicted that Rapamycin predominantly showed anticancer effect rather than PF4. Thus, present findings suggested that Rapamycin is neither synergistic nor additive with PF4. It was concluded that Rapamycin is a potent anticancer agent for breast cancer because it halt tumour growth and thus promote tumour cells differentiation through a positive expression of hormone receptors analysis. Further study will be needed to analyse the regulation of ER isoforms (ERα and ERβ) and PR isoforms (PR-A and PR-B) to improve potential therapeutic strategy in breast cancer treatment through anticancer effects of Rapamycin.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Increased incidences of cancer have been noted worldwide. According to The International Agency for Research on Cancer (IARC), a total of 14.1 million new cancer cases were reported in 2013 compared to 12.7 million in 2008. Consequently, the number of cancer related death also increased from 7.6 million cases to 8.2 million cases within the five years (Ferlay J. *et al.*, 2013). Moreover, In the United States, the incidence rate of invasive cancer was ranged from 387 to 509 per 100,000 population in 2009 compared to 380 to 511 per 100,000 population in 2010 (Henley *et al.*, 2014). Among the invasive cancers, breast cancer is commonly related to women and the most prevalence cancer among women. It was the second disease commonly diagnose after lung cancer and also known as silent killer to women (522 000 deaths in 2012). Furthermore, according to GLOBOCAN 2012, a new version of IARC's online database, the global breast cancer risk also rises sharply as the frequently diagnosed cancer among women in 140 of 184 countries worldwide (Ferlay *et al.*, 2013).

In Malaysia, The National Cancer Registry 2007 (NCR) also reported breast cancer as the first most common cancer among population regardless of sex. There were 3, 242 female breast cancer cases diagnosed in 2007 among Malaysians with the highest percentage of occurrence (32.1%) followed by other nine malignancies

such as colorectal (10%), cervix uteri (8%), trachea; bronchus; lung (5.4%), corpus uteri (4.1%), leukemia (3.2%), lymphoma (3.2%), thyroid (3.0%) and stomach (2.8%).

Cancer is malignant neoplasm which is critically dependent on the host for nutrition and blood supply. Some neoplasms require endocrine support such as breast cancer. Estrogen and Progesterone are steroid hormones; play a major role in controlling the progression of breast cancer cells. However, both hormones are activated through the binding to their respective receptors namely Estrogen Receptor (ER) and Progesterone Receptor (PR). It was reported that up to 75% of breast cancers express positivity of ER and/ or PR which is marked as prognosis factor breast cancer therapy. Thus, patients with at least 10% ER positive breast cancer cells in primary tumour effectively responded to endocrine therapy and had better survival after relapse (Yamashita *et al.*, 2006). On the other hand, a member of nuclear hormone receptor superfamily, Proxisome Proliferator Activated Receptor-γ (PPARγ) also tends to become prognosis factor for cancer therapies. Deficient expression of PPARγ can be a determinant to breast cancer carcinogenesis (Mueller *et al.*, 1998; Apostoli *et al.*, 2014). As ER expression increases, the inverse relationship between receptor expression and proliferation becomes dysregulated. Increased ER expression is one of the very earliest changes occurring in the tumorigenic process to malignant transformation of breast cancer cells. Therefore, cancer cells progression also depend on several molecular pathways which dysregulated in cancer cells in order to activate the cell proliferations.

Angiogenesis is defined as the formation of a new vascular network out of pre-existing vessels (Folkman, 1995; Papetti and Herman, 2002). It plays a pivotal role in sustaining tumour growth in cancer through the activation of angiogenic and angiostatic

factors such as vascular endothelial growth factor, basic fibroblast growth factor and platelet-derived growth factor (Boudreau and Myers, 2003). On the other hands, other stimuli can also regulate angiogenesis in direct or indirect manners such as soluble growth factors, membrane-bound proteins, cell-matrix and cell-cell interactions (Papetti and Herman, 2002; Otrock *et al.*, 2007a), hypoxia (Boudreau and Myers, 2003), inflammation and others (Milkiewicz *et al.*, 2006). Nowadays, inhibition of angiogenesis is a kind of strategy for cancer treatment, whereas Platelet factor-4 (PF4) is proven to has anticancer property of angiogenesis inhibition by suppressing endothelial cell proliferation, migration, angiogenesis and metastasis *in vitro* and *in vivo* (Jouan *et al.*, 1999; Struyf *et al.*, 2007; Verpelli *et al.*, 2010).

Moreover, several findings have ruled out that Rapamycin inhibits tumour cell growth by blocking tumour cell proliferation and angiogenesis. It also proven to successfully regress the cancer progression by blocking the cell cycle at S and G1 phase during mitosis and also induced apoptosis in cancer cell (Guba *et al.*, 2002). Rapamycin exerts its activation through a tyrosine/ kinase protein mammalian Target of Rapamycin (mTOR) which is one of the key modulator for cell proliferation (Namba *et al.*, 2006; Sabine *et al.*, 2010).

Nowadays, Tamoxifen and Aromatase Inhibitors become favorable therapies in hormonal positive breast cancer treatments by halting hormonal activities and hormone production for cancer cell progression and survival. It is interesting to discover the anticancer potential of Rapamycin and PF4 toward hormonal activities in breast cancer. In order to determine gene and protein expression of steroid receptors in Rapamycin and/or PF4-treated breast cancer, this study was undergone by using animal model for breast cancer. For in vivo experiments, MNU-induced mammary carcinoma in rats is the model of choice. The MNU induced mammary

tumours are comparable to human due to the site of origin, hormone dependant and characterized with malignant and aggressive histological features like human (Russo and Russo, 2000; Thompson and Singh, 2000).

1.2 Objectives of the study

The general objective of the study is to analyse the expression of steroid growth receptors i.e ER, PR and PPARγ of MNU induced breast cancer under the influences of PF4 and/or Rapamycin in animal model.

1.2.1 Specific objectives

The specific objectives of the study are:

1. To analyse histopathological features of MNU induced tumours treated with Rapamycin and/ or PF4.
2. To analyse protein expression of ER, PR and PPARγ of treated tumours using immunohistochemistry.
3. To analyse gene expression of ER, PR and PPARγ of treated tumours using quantitative Real-Time PCR.
4. To correlate expression of ER, PR and PPARγ with aggressiveness of treated tumours.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

2.1.1 The prevalence of breast cancer worldwide

Breast cancer is a common disease in the worldwide. Looking at the world as a whole, the occurrence rate slightly increases every year and commonest among women (Jemal *et al.*, 2010; Chen *et al.*, 2013a; Henley *et al.*, 2014). Figure 2.1 shows the incidence of breast cancer worldwide based on world age-standardized rate per 100, 000 persons in 2012.

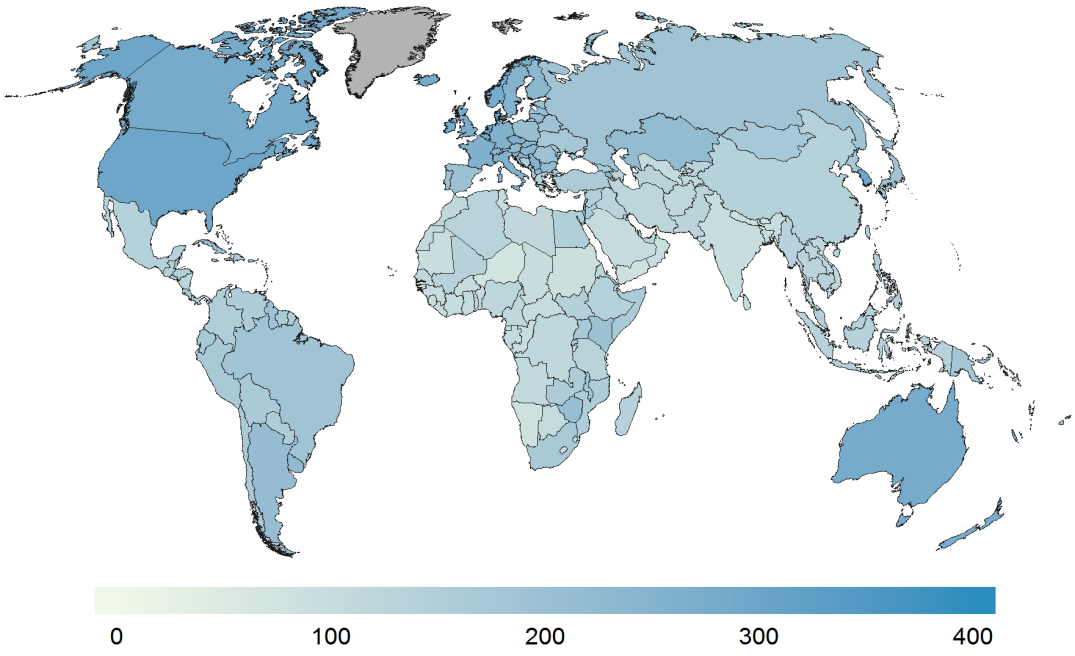

Figure 2.1 Estimated breast cancer incidence worldwide in 2012.
(Source: GLOBACON 2012).

According to GLOBACON 2012, breast cancer (1.7 million cases) is the second common cancer diagnosed after lung cancer (1.8 million cases) from year 2008 to 2012 with a sharp increase for incidence number and mortality rate at 20% and 14% respectively (Bray *et al.*, 2013). Furthermore, Henley and co-workers also reported the invasive malignancies among female in the United State is the breast cancer followed by lung, colorectal, and uterine cancer (Henley *et al.*, 2014).

It was surprising that from year 1990 to 2008, the number of females burdened with breast cancer in European countries is high among female aged below than 35 years old. Moreover, they were also detected with high incidence of ductal carcinomas (Leclere *et al.*, 2013). Ductal carcinoma in situ (DCIS) has became a relatively common malignancy detected upon diagnosis. According to data from International Cancer Screening Network (ICSN), based on age-standardized, detection rates of DCIS varied from 0.41 to 1.38 per 1000 women in Europe, United State and Japan. The rate varies across the countries as a result of different DCIS diagnosis and treatment processes (Ponti *et al.*, 2014).

In Asia, for instance, China cancer registry also stated that breast cancer is one of the top ten common cancers leading to death in age-standardize China population with mortality rate 5.13 per 100, 000 and frequently diagnosed among female (incidence rate 42.55 per 100, 000) in year 2009 (Chen *et al.*, 2013a).

2.1.2 Breast cancer risks in Malaysia

In Malaysia, the exact causes for breast cancer risks are still unknown. However, several risk factors increase the chance of getting this disease were

categorized by uncontrollable and controllable factors. Briefly, the uncontrollable factors are family history and age, whereas the controllable factors are breast feeding, food intake, alcohol intake, smoking habits and physical exercises (Baqutayan *et al.*, 2012)

As shown in Figure 2.2, The National Cancer Registry 2007 has reported that breast cancer (18.1%) is the most frequent diagnosed cancer among the Malaysian compared to colorectal (12.3%), trachea, bronchus, lung (10.2%), nasopharynx (5.2%) ,cervix uteri (4.6%), lymphoma (4.3%), leukemia (4.1%), ovary (3.6%), stomach (3.5%) and liver (3.3%). Moreover, Malaysian females are suffering from breast cancer more than other cancers (Figure 2.3) and it was estimated that one in twenty women in the country developed breast cancer in their lifetime. Among them, Chinese women are commonly diagnosed with this disease followed by Indians and Malay (Baqutayan *et al.*, 2012) as the effect of different culture and lifestyle. Ironically, their overall survival rate was lower than Western, especially Malay women due to delaying for diagnosis and treatment at hospital; after a long period of symptom, presented at the late stage, had larger tumour size and more lymph nodes affected (Ibrahim *et al.*, 2012; Abdullah *et al.*, 2013). Therefore, it is important to promote breast cancer screening in all Malaysian women for early detection and treatment in order to reduce the number of breast cancer fatality and disease burden in Malaysia. Consequently, many breast cancer prevention and control programs are implemented by the government to improve breast health in our country includes encouraging breast self examination in all women, free mammogram screening in government hospital and a RM50 subsidy for every mammogram done in private clinics and hospitals registered with the National Population and Family

Development Board Malaysia (NPFDB) for those who are burdened with high risk of breast cancer (Dahlui *et al.*, 2011).

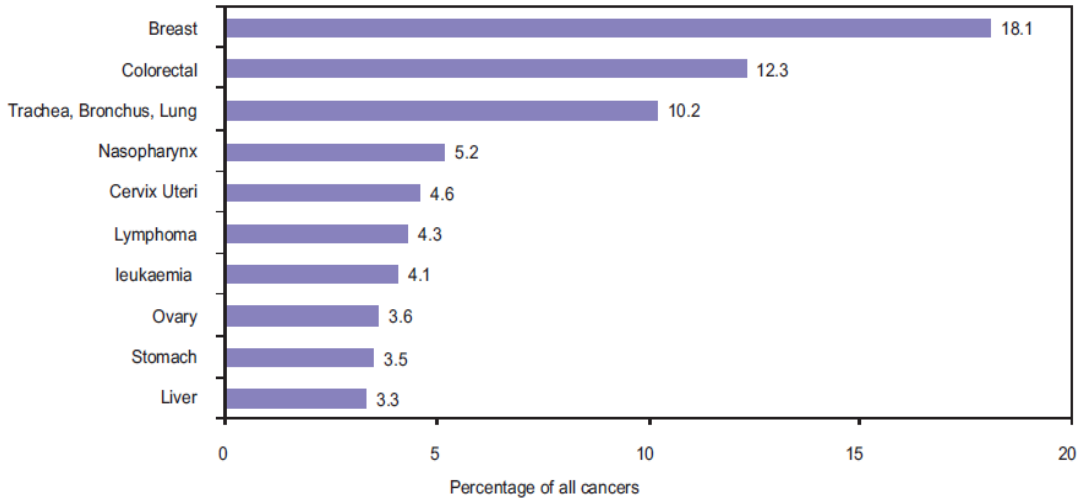

Figure 2.2 The percentage of ten most frequent cancer diagnosed among Malaysian in 2007. (Omar and Ibrahim Tamim, 2011)

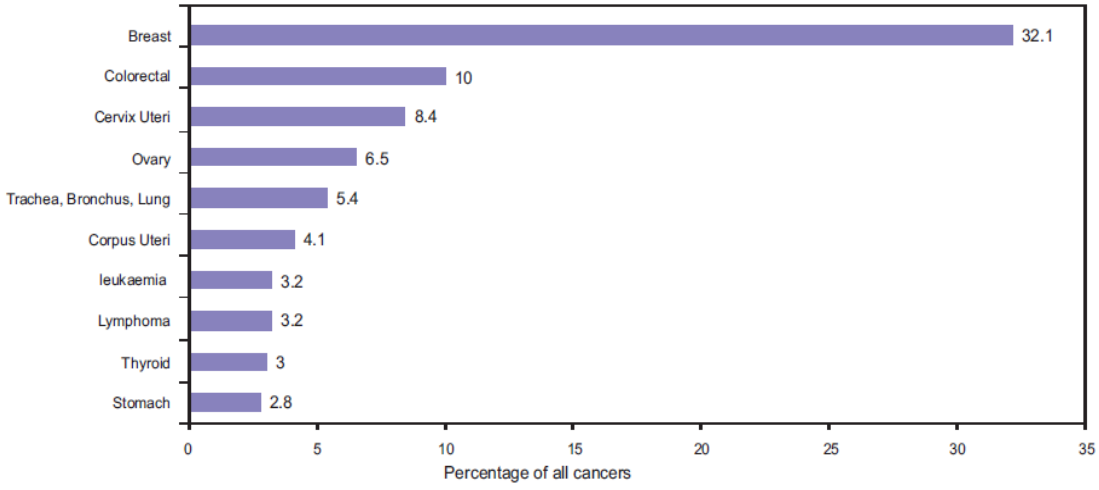

Figure 2.3 Percentage of ten most frequent cancer detected in Malaysian female in 2007 (Omar and Ibrahim Tamim, 2011)

2.2 MNU induced breast cancer animal model

Animal model systems provide invaluable tools in understanding the complexity of multistep carcinogenesis. For decades, the animal model for breast cancer has been used widely in breast cancer prevention and treatment, as its malignancy progression depends on the hormonal status and histopathologically similar to human. Moreover, there are several availability of known initiating agents potentially promotes breast carcinogenesis, such as viral induction, ionizing radiation and chemical carcinogens such as 1-methyl-1-nitrosourea (MNU), 7,12-dimethylbenz[a]anthracene (DMBA), and 2-amino-1-methyl-6-phenylimidazo[4,5 *b*]pyridine (PhIP) (Russo and Russo, 2000; Imaoka *et al.*, 2014).

MNU-induced mammary carcinoma model has been widely used in the investigation of novel breast cancer chemoprevention and therapeutic. Many extensive informations were available, characterizing pathogenesis of tumour growth associated with this compound (Gusterson and Williams, 1981; Thompson and Adlakha, 1991; Thompson and Singh, 2000; Esendagli *et al.*, 2009). MNU induced mammary tumourigensis in a dose-dependent manner as high MNU dosage increased the tumour multiplicity, shortened the latency period and produced more malignant tumour than benign (Thompson and Adlakha, 1991; Thompson *et al.*, 1992). In addition, the carcinomas induced by MNU were ovarian hormone dependence in carcinogenic initiation and tumour progression (Thompson *et al.*, 1998a).

Furthermore, the injection of sexually immature female rats with MNU results in a rapid induction of premalignant and malignant mammary gland lesions. It was proven that the mammary tumour was palpable post 35 days MNU administration with 50 mg MNU/ kg body weight at 21 days of age (Thompson et al.,

1995). The young mammary gland was more susceptible to carcinogenesis. In younger rats, the terminal end buds (TEB) are not well differentiated and frequently mitosis. It is comprised of intermediate cells, myoepithelial cells and have a single lumen with a smooth border. Tumours arise from ductal epithelial cells as in most human breast carcinomas (Thompson and Singh, 2000; Thompson *et al.*, 2000); whereby the early lesions, called intraductal proliferations (IDP) formed after MNU administration (Thompson *et al.*, 1998b). It was the earliest change observed in mammary parenchyma as the dilation of TEB occurs. The epithelial lining thickening up to six layers thick and the cells have a large, round nucleus, prominent nucleolus, and coarse chromatin along the inner leaflet of the nuclear membrane. Recently, in year 2011, Sharma et. al had disclosed the cellular differentiation profile of the MNU-induced rat mammary epithelial cells by quantitative protein expression of the luminal and basal myoepithelial populations (Sharma *et al.*, 2011). The results enhanced rat mammary carcinogenesis model in the study of the role of epithelial cell differentiation in breast cancer.

The epithelial derived mammary neoplasm are predominantly characterized with *in situ* ductal carcinoma (DCIS) and invasive carcinoma of papillary, cribriform and comedo type (Russo and Russo, 2000). Neoplastic transformation of mammary epithelial cells in MNU-induced rats is associated with decrease apoptosis in the lesions (Shilkaitis *et al.*, 2000). Thus, histopathological features of tumour is important because those characteristics have implications for the interpretation of experimental mammary carcinoma in animal.

The pathogenetic characteristics of this experimental model of breast cancer are being defined with the use of molecular techniques, such as an identifiable somatic genetic changes GGA-to-GAA transition in codon 12 of the *Ha-ras* gene is

highly prevalent in MNU-induced rat mammary carcinoma (Lu *et al.*, 1998; Imaoka *et al.*, 2014). The mechanism of initiation of genetic mutation by MNU began when MNU generates O-6-methylguanine, which then base-paired with cytosine and thymidine, leading to a G-to-A transition upon subsequent DNA replication. However, Ha-Ras gene mutation by MNU is inversely proportion to the MNU dosages (Lu *et al.*, 1998; Thompson *et al.*, 2000).

2.3 Anticancer properties of Platelet Factor-4 (PF4)

The CXC-chemokines involve in biological activities such as modulation of inflammation, hemostasis and angiogenesis. Chemokines are cytokines which recruit leukocytes to inflammatory sites, acting as growth factor in tumour development and metastasis and influencing angiogenesis. Platelet Factor 4 (PF4) also known as CXC4 chemokine, a potentially chemokine shown to act as anticancer by inhibiting angiogenesis (Maione *et al.*, 1990; Struyf *et al.*, 2007) and promoting apoptosis (Liang *et al.*, 2013; Din *et al.*, 2014).

PF4 is a product of platelet release reaction, a tetrameric molecule with low molecular weight composed of 70 polypeptide residues. The peptide between amino acids 47 and 70 posses heparin-binding lysine-rich site which play an important role in angiogenesis, especially in the four sites of lysine 61, 62, 65 and 66 which are found on a single α-helix on each PF4 monomer. This heparin binding site located at the carboxyl (COOH) terminal end and positively charged. Meanwhile, the other amino (NH_2) terminal is highly acidic and negatively charged (Deuel *et al.*, 1977; Jouan *et al.*, 1999; Bikfalvi, 2004). Majority of the NH_2 terminal contains three amino acid residues (Glutamine -Leusine- Arginine) named as ELR motif (Belperio et al., 2000). The existence of this motif is also a prediction of pathological features

of CXC chemokine where ELR-positive is potent angiogenesis promoter whilst ELR- negative is potent inhibitor of angiogenesis. The ELR+ CXC chemokines including: CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8. Meanwhile, ELR- CXC chemokines are subdivided into two types: angiostatic ELR- CXC chemokines CXCL4, CXCL4L1 and CXCL14 and Interferon-inducible ELR- CXC chemokines CXCL9, CXCL10, and CXCL11 (Strieter *et al.*, 2004; Keeley *et al.*, 2008). The CXCL4L1, a PF4 variant was successfully inhibited angiogenesis in melanoma and lung carcinoma growth and metastasis in vivo (Struyf *et al.*, 2007). The angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 are mediated by CXCR3 (Struyf *et al.*, 2011). In addition, CXCL4's capacity in inhibiting angiogenesis and promoting immune response had become a tool and target for potential intervention in tumor growth and inflammation with more emphasis on molecular pathways and mechanism of action of PF4 (Kasper and Petersen, 2011).

The mechanism of PF4 inhibition of angiogenesis had been tested as single agents in clinical trials (Hawkins, 1995) whereby the concept of antiangiogenesis therapy was proposed in early 1970s (Folkman and Ingber, 1992). They summarized three strategies for antiangiogenesis; inhibition of release of angiogenic molecules from tumour cells, neutralization of released angiogenic molecule and blocking the vascular endothelial cells from responding to angiogenic stimulation. On the other hand, PF4 blocks the binding of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) to cell surface heparan sulfates which both modulated by heparan sulfateproteoglycans (HSPGs) to their respective receptors during angiogenesis. The heparin binding prevents endothelial cell proliferation, migration and differentiation. In addition, heparin reduces the activation and effects

of matrix metalloproteinases, serine proteases, and heparanases in promoting metastasis (Engelberg, 1999).

PF4 also inhibited cancer cells growth by inducing apoptosis in myeloma cells through a negative regulation of signal-transducer-and-activator-of-transcription-3 (STAT3) in vitro and in vivo (Liang *et al.*, 2013). STAT3 is known to regulate proliferation, apoptosis and angiogenesis in multiple myeloma cells through regulating the expression of its target genes including c-Myc, Bcl-XL, Bcl-2, Survivin and VEGF. This was positively correlated with a study by Din et. al in 2014 who proved that the treatment of PF4 successfully caused overexpression of pro-apoptotic protein Bax and regression of Survivin protein expression in rat mammary carcinoma (Din *et al.*, 2014).

2.4 Rapamycin inhibits cancer cell growth

Rapamycin (sirolimus) is a substance extracted from bacteria macrolide of *Streptomyces hygroscopicus* found in the soil of Easter Island, Rapa Nui. It was originally used as antifungal, antibacterial and an immunosuppresion agent in kidney transplantation. However, recently scientists discovered its anticancer activity, which leads to several test that came into phase II clinical trial as anticancer agent. The chemical structure of Rapamycin (sirolimus) as shown in Figure 2.5.

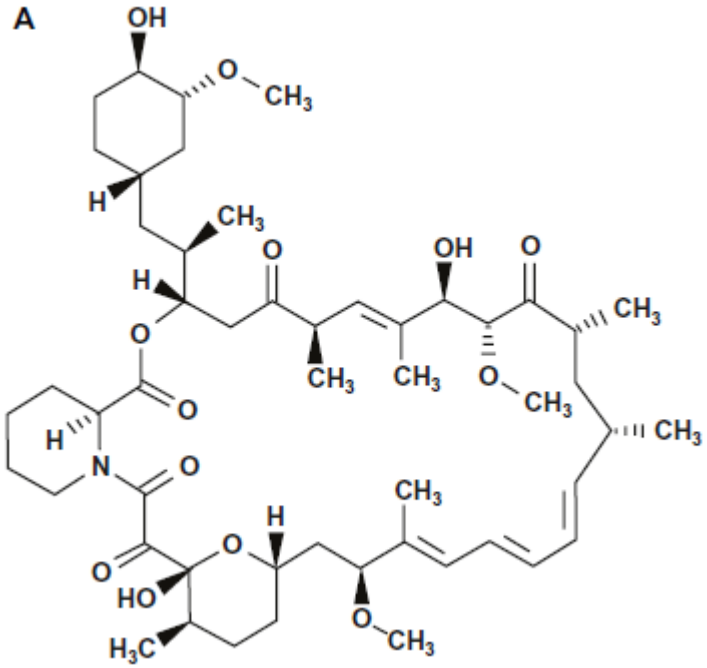

Figure 2.4 The chemical structure of sirolimus (Yardley, 2013)

Several mTOR inhibitors have been developed and evaluated as antitumor therapies, with some distinctive differences in metabolism, formulation, and schedule of administration. Sirolimus is the major metabolite of CCI-779 (a Rapamycin analogue) (Chan, 2004). Another analogues of sirolimus which are demonstrated to have more favorable pharmacokinetics include temsirolimus, everolimus and ridaforolimus. Sirolimus has been approved for renal transplant injection in 1999 and entered phase 2 and 3 in clinical trials. Meanwhile temsirolimus had been approved for advanced renal cell carcinoma in 2007 as well as everolimus, which is also presently approved for renal angiomyolipoma and tuberous sclerosis in 2012. Besides, radifolimus has been entered phase 3 in clinical trial (Yardley, 2013).

Growth inhibition was associated with reduction of cell proliferation and angiogenesis with promotion of apoptosis. Therefore, Rapamycin potentially induced phenotypic transition from invasive spindle, or dome-shaped cells, with exploratory

pseudopodia to noninvasive cuboidal cells that formed cell-to-cell adhesions of renal cancer cells (Luan *et al.*, 2002) and breast cancer cell line proliferation, growth and apoptosis in vitro (Noh *et al.*, 2004; Kasukabe *et al.*, 2005; Chang *et al.*, 2007). Moreover, in vivo treatment with Rapamycin reduced the aggressiveness of invasive renal and bladder cancer cells, inhibited the growth of MCF-7 cells as xenografts and ductal carcinoma in situ malignancy in mouse model (Luan *et al.*, 2002; Kasukabe *et al.*, 2005; Namba *et al.*, 2006).

Mammalian target of rapamycin (mTOR) is a 289-kDa serine/ threonine kinase protein that integrates multiple signals from growth factors and hormones and plays a central role in the control of cell growth, proliferation, and angiogenesis. It was a downstream target of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Activation of PI3K/Akt/mTOR mediates multiple cellular functions to tumour initiation, progression and survival (McAuliffe *et al.*, 2010).

The Rapamycin has affinity to bind to mTOR protein and consequently caused inactivation of mTOR downstream target. Thus, Rapamycin is also recognized as mTOR inhibitor. In preclinical breast cancer models using MCF-7 cells with constitutively active Akt/mTOR that exhibit hormone and chemotherapy resistance, dual inhibiton of PI3K and mTOR inhibitors enhanced the efficacy of selective ER modulator tamoxifen in breast cancer (Chen *et al.*, 2013b). The summary of mechanism of mTOR inhibition in cancer cells is shown in Figure 2.5.

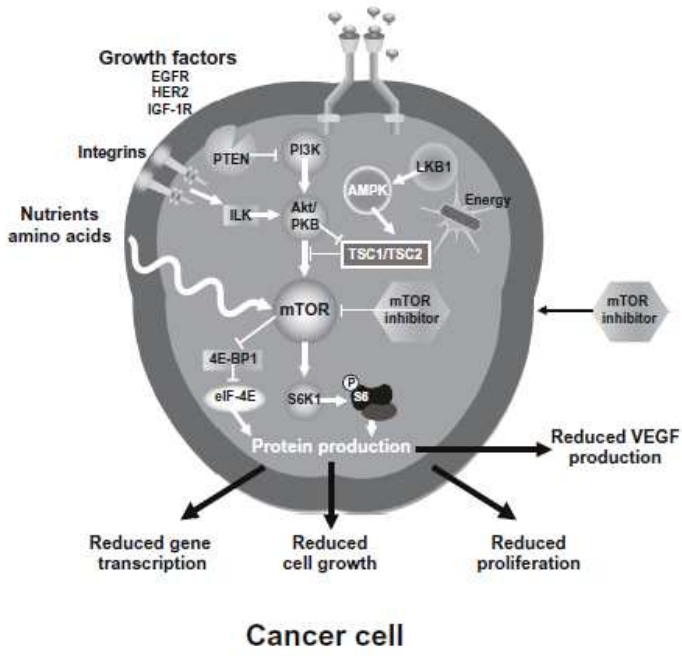

Figure 2.5 The PI3K/Akt/mTOR pathway. Abbrevations: 4E-BPs, 4 eukaryotic binding proteins; EIF4K, eukaryotic initiation factor 4K; mTORC, mammalian target of rapamycin complex; PTEN, phosphatase and tensin homolog; S6K1, ribosomal protein S6 kinase 1; S6, ribosomal protein S6; TSC1, tuberous sclerosis complex1; TSC2, tuberous sclerosis complex 2; PI3K, phosphoinositide 3-kinase; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; IGF-1R, insulin growth factor 1; Akt/PKB, protein kinase B; AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; ILK, integrin-linked kinase (Lane and Lebwohl, 2006).

2.5 Regulation of Estrogen Receptor in Breast Cancer

Cell proliferation, differentiation, apoptosis, and cell motility are promoted and regulated by several factors such as growth factors and hormones (Sherbet, 2005). The hormones related cancer such breast cancer shared different mechanism of carcinogenesis by driving cell proliferation, increase the number of cell division and opportunity for random genetic errors. Meanwhile, breast cancer risk is associated with prolonged exposure to female hormones such as estrogen which is either of ovarian or extra-ovarian origin. It was proven *in vivo* that the tumorigenic response is maximal when the carcinogen is administered to young and virgin intact animals in

which the mammary gland is undifferentiated and highly proliferating (Thompson *et al.*, 1995; Thompson *et al.*, 1998a). Increased proliferation could result in turn in accumulation of genetic damage and stimulation of the synthesis of growth factors that act on the mammary epithelial cells via an autocrine or paracrine loop (Russo and Russo, 1998).

Estrogen had clearly been shown to play a critical role in regulation of growth and differentiation of the normal mammary gland and mammary malignancy. Its Estrogenic activity in cells is mediated by high affinity binding protein receptor namely Estrogen Receptor (ER). Estrogen has direct mitogenic effects and promote paracrine mediators of estrogen action in mammary gland upon positivity of ER in mammary epithelial cell. Moreover, it was proven that ER is co-expressed with the proliferation antigen Ki67 in a population of normal primate mammary epithelial cells (Dimitrakakis *et al.*, 2006). ER expression could be a determinant of estrogen action in other tissues such as uterus and ovary. Studies of the ER expression and function in cancer are very important in improving the new strategies of prevention, diagnosis and therapy in estrogen-dependant cancers (Hayashi *et al.*, 2003). Therefore, ER expression is considered as prognosis factor in cancer therapy especially breast cancer.

There are two types of ER which distinctly regulated breast cancer progression. ERα and ERβ (as shown in Figure 2. 6) are the products of two separate genes that are differentially expressed in tissues. ERα is 66kD receptor protein located in cytoplasm and nucleus of cells which is responsible for estrogen-induced mitogenic signaling in epithelial cells in breast, uterine and ovarian tissues. Meanwhile, ERβ, 36kD protein located in cytoplasm and functionally controls cell proliferation and differentiation. Both ER isoforms are expressed at similarly low

levels in the normal breast, whereas ERα is predominantly expressed at high level rather than ERβ in breast cancer cells. Thus, the expression of ERβ is downregulated in lesions such as atypical ductal hyperplasia and DCIS compared with that in normal breast epithelium, as it is inversely correlated with proliferation. This is consistent with the suggestion that the ERβ negatively modulates the effects of the ERα (Roger P. et al., 2001).

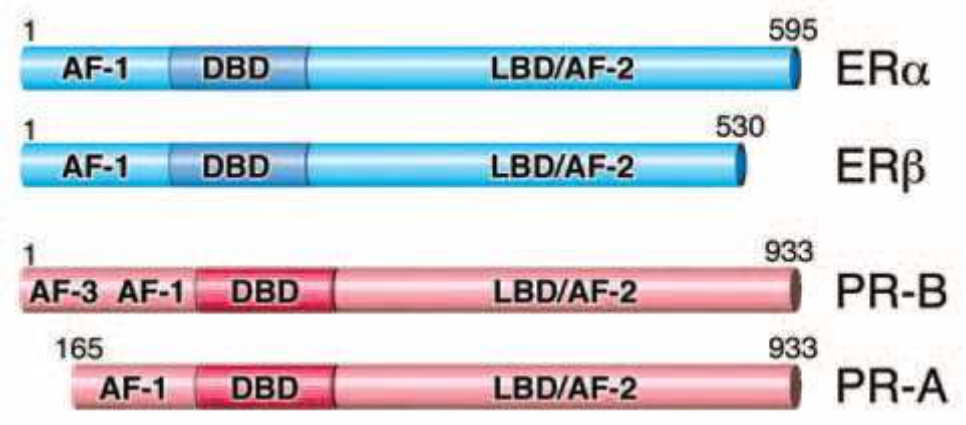

Figure 2.6 Structure of estrogen receptor (ER) and progesterone receptor (PR). ER consists of two isoforms ERα and ERβ that are transcribed from two genes. PR also consists of two isoforms PRA and PRB that are transcribed from a single gene using an alternative promoter and translation start site. Abbreviations: DBD, DNA-binding domain; LBD, ligand-binding domain. (Cui *et al.*, 2005).

In breast cancer treatment therapy, ER status has been used clinically almost 40 decades to identify those patients most likely to benefit endocrine therapies through its signaling pathway (Muscat *et al.*, 2013; Renoir *et al.*, 2013; Palmieri *et al.*, 2014). It has been a central to management in breast cancer as a prognosis factor acts as a guide to treatment (Osborne *et al.*, 1980). ERs are activated by two general mechanisms ligand dependent activation (the classic pathway or genomic pathway), in which estrogen binds to the ER and the resulting estrogen-ER unit then interacts directly with DNA to regulate gene transcription; and ligand independent activation

(non classical pathway or nongenomic pathway), in which an ER is activated, in part, after phosphorylation by growth factor receptors or other molecules with serine or tyrosine kinase domains. This pathway plays a central role in breast cancer development including cell survival, proliferation, and angiogenesis (Hasson *et al.*, 2013). Briefly, the summary of genomic and non-genomic ER signaling pathway is shown in Figure 2.7.

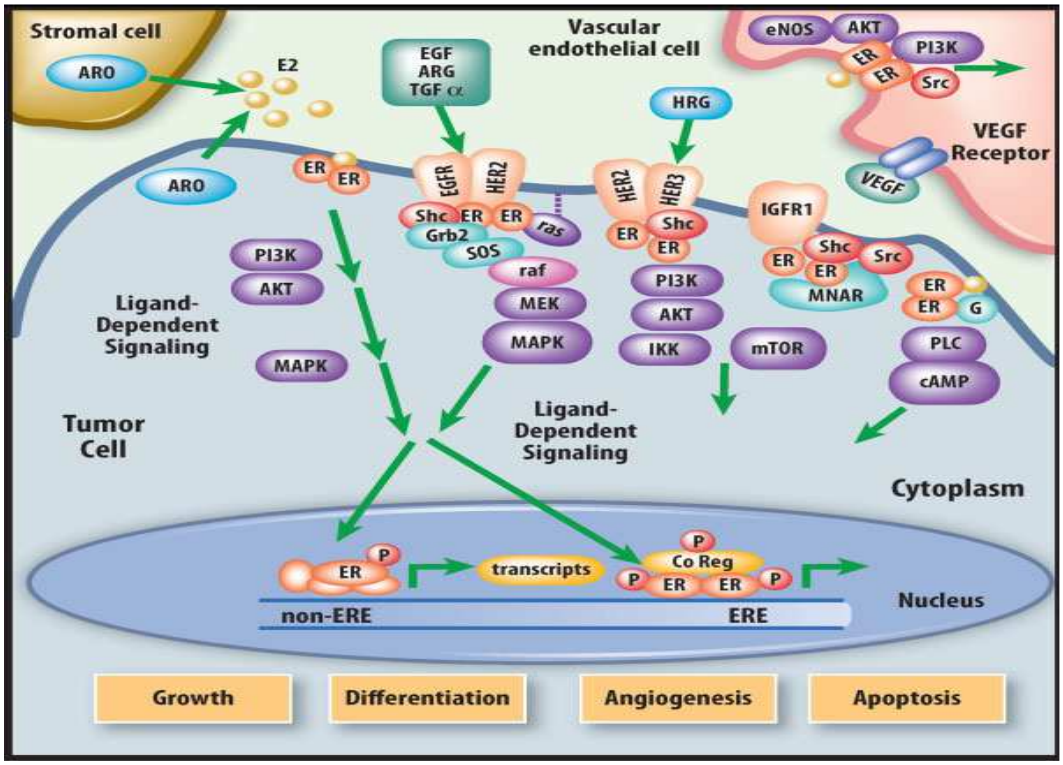

Figure 2.7 Genomic and nongenomic ER pathway in estrogen receptor (ER)-positive tumor cells. (Pietras and Marquez-Garban, 2007)

2.6 Regulation of Progesterone receptor in Breast Cancer

Progesterone is a critical regulator of normal female reproductive function, with diverse tissue-specific effects in the human. The effects of progesterone are mediated by its nuclear receptor (PR); a member of a large family of ligand-activated nuclear transcription regulators. The structure PR was described previously in Figure 2.6.

PR is expressed as two isoforms, PRA and PRB. Both are identical in sequence, except that PRA lacks 164 amino acids at the N-terminus, making it the shorter of the two proteins. In human physiology, the majority of PR positive cells express PRA and PRB at equivalent levels (Mote *et al.*, 2002; Mote *et al.*, 2007).

In vivo study demonstrated PR was important in development of alveoli. PR mediated the binding of progesterone then activated the ductal cells. Upon the crosstalk with the stroma, a signal was passed directly to the alveolar precursor cells. Mammary epithelial cells which lack of PR had a failure in side branching (Brisken *et al.*, 1998). The presence of PR in tumour is also an important predictor of tumour aggressiveness and responsiveness to endocrine therapy. Progesterone receptor has provided a mean in hormonal therapy in breast cancer therapeutics. Both ER/PR +ve and ER+ve/ PR- breast cancer sub typing are responsive to hormonal therapy; however the present of PR+ve enhanced the effectiveness of treatment (Bloom *et al.*, 1980; Osborne *et al.*, 1980; Bardou *et al.*, 2003) Thus the status of ER and PR appears as a prognostic index in the patient selection for the therapy.

2.7 Regulation of Peroxisome Proliferator Activation Receptorγ in Breast Cancer Cells.

The peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors which belong to the nuclear hormone receptor superfamily proteins. PPARs play essential roles in the regulation of carbohydrate, lipid, protein metabolism, cellular differentiation, development, and tumorigenesis.

There are three types of PPARs namely PPAR alpha (PPARα), PPAR beta/ delta (PPARδ/ PPARβ) and PPAR gamma (PPARγ) (Roberts-Thomson, 2000). Among the three PPARs subunit, PPARγ plays a critical role in regulating

tumourigenesis. In general, PPARγ can be found in adipose tissue, colonic epithelia, macrophages, endothelium, small intestine , liver, breast and others. The high expression of PPARγ and its RXRα ligand showed activation of this heterodimers potentially induce cell death in transitional bladder cancer (Guan *et al.*, 1999). Furthermore, there was an evidence that a cross-talk between ER and PPARγ by reciprocal interaction between PPARγ and ERβ significantly inhibits the proliferation and migration of thyroid cancer cells (Chu *et al.*, 2014).

In breast cancer, activation of PPARγ by respective ligand promotes terminal differentiation of the cancer cells (Mueller *et al.*, 1998); caused dramatic morphological and molecular changes to a less malignant state and well differentiated cells. For instance, a pilot study by (Yee et al., 2007) disclosed the activation of short-term rosiglitazone therapy in early-stage breast cancer patients leads to local and systemic effects on PPARγ signaling in inhibiting tumour growth and progression in human breast cancer. Meanwhile, estrogen biosynthesis in human breast adipose tissues also successfully inhibited by PPARγ ligands activity, suggesting as possible implication in breast cancer therapy (Rubin et al., 2000). Moreover, upregulation of BRCA1 and decreasing leptin secretion by activation of PPARγ in mammary stromal adipocytes regressed mammary tumourigenesis in vivo (Skelhorne-Gross *et al.*, 2012). PPARγ ligands also induced apoptosis and antiproliferation in human breast cancer cell lines (Crowe and Chandraratna, 2004; Lea *et al.*, 2004).

PPARγ activation mechanism involved the Retinoid-X-Receptor (RXR) and PPARγ Element (PPRE). The heterodimer of PPARγ and RXRα interacts with co-regulators. Binding of specific PPARγ ligand leads to coactivator recruitment and corepressor release. Upon the activation, PPARγ/ RXRα heterodimer binds to the

PPRE which is present in the promoters of target genes and activate gene transcription. Figure 2.8 shows the mechanism of PPARγ signaling.

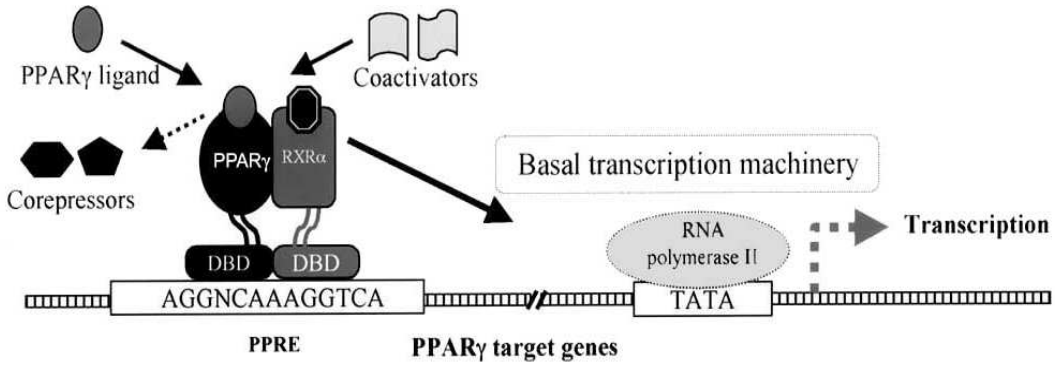

Figure 2.8 Schematic diagram of PPARγ signaling. Abbreviations: PPARγ: Peroxisome Proliferated Activation gamma; RXRα: Retinoid-X-Receptor alpha; DBD: DNA Binding Domain; RNA: Ribonucleic Acid. (Rumi *et al.*, 2004).

2.8 Histological grades in breast cancer

The purpose of breast cancer classification is to select the best treatment. Breast cancer can be classified based on tumour grade, tumour stage and histological type, receptor status and DNA testing of certain genes.

Grading focuses on the appearance of the breast cancer cells compared to the appearance of normal breast tissue. Cancerous cells are not well differentiated compared the normal cells which take on specific shapes and forms that reflect their function as part of that organ. In cancer, the breast cells become disorganized, cell division becomes uncontrolled and cell nuclei become less uniform. Poorly differentiated cancers have a worse prognosis. The Nottingham also called Elston-Ellis modification of the Scarff-Bloom-Richardson grading system is a grading system currently adopted by World Health Organisation (WHO) (Genestie *et al.*, 1998). This system grades breast carcinomas based on three parameters such as

tubule formation (how much of the tumour tissue has normal breast milk duct structures), nuclear pleomorphism (an evaluation of the size and shape of the nucleus in the tumor cells) and mitotic count (how many dividing cells are present, which is a measure of how fast the tumor cells are growing and dividing) respectively. These features were given 1 to 3 points each which is then added together to give an overall final score of 3 to 9. Tumour grade 3-5 represent well-differentiated cells have best prognosis compared grade 6-7 and 8-9 which represent moderate differentiated and poorly differentiated respectively. Therefore, lower-grade tumors, with a more favorable prognosis, can be treated less aggressively, and have a better survival rate (Bloom and Richardson, 1957; Elston and Ellis, 1991).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This in vivo study involving induction of mammary tumourigenesis in rat by a carcinogen, followed by histological features classification and determination of the effectiveness of anticancer agents through the steroid receptor analysis.

Throughout this study, 1-Methyl-1-Nitrosourea (MNU) was intervened into the female Sparague Dawley rats at the dose of 70mg/kg body weight to promote mammary tumour formation (Jaafar *et al.*, 2009). The tumors were divided into control and treatment groups. Treatment interventions of Rapamycin, Platelet-Factor-4 (PF4) and combination of Rapamycin and PF4 were done when tumour diameter size reached 14.5 ± 0.5 mm. Tumours regression was observed after 5 days of treatment interventions. Both anticancer agents were chosen based on their ability to inhibit cancer progression through anticancer properties towards cancer cells.

All tumour tissues were subjected to histopathological analysis and steroid receptor analysis. The expressions of Estrogen Receptor, Progesterone Receptor and Peroxisome Proliferator Activation Receptor-γ (PPAR-γ) were determined by semiquantitative protein expression of Immunohistochemistry and quantitative gene expression of Real-Time Polymerase Chain Reaction (RT-PCR) assays. Statistical inferences were then performed to justify the results. The study design was summarized as shown in Figure 3.1.