THE EFFECTS OF TAMOXIFEN ON TNFR2 IN NMU-INDUCED BREAST CANCER RAT MODEL

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by

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LIST OF SYMBOLS

-	Negative
%	Percent
°C	Degree Celsius
+	Positive
±	Plus minus/Positive, negative or both
C	Standard value
d	Magnitude of difference
L	Liter
mins	Minutes
mL	Milliliter
N	Sample size
s	Standard deviation
µL	Microliter
µm	Micrometer

LIST OF ABBREVIATIONS

AECUSM	AECUSM
AIs	Aromatase Inhibitors
AKT	Ak strain transforming
AP1	Activator protein 1
ARASC	Animal Research and Services Centre
Bax	Bcl-2-Associated X Protein
Bcl-2	B Cell Lymphoma Gene-2
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
CACNG4	Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 4
CDK	cyclin-dependent kinase
CHRNA6	Cholinergic receptor nicotinic alpha 6 subunit
cIAP1	Cellular inhibitor of apoptosis protein-1
cIAP2	Cellular inhibitor of apoptosis protein-2
CO2	Carbon Dioxide
CO2	Carbon dioxide
DCIS	Ductal carcinoma in situ
DCIS	Ductal carcinoma in situ
DMDA	N,N-Dimethyldopamine
DNA	Deoxyribonucleic acid
DPX	Dibutylphthalate Polystyrene Xylene
E2	Estradiol
ER	Estrogen Receptor
ERK	Extracellular-signal-regulated kinase
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor alpha
Etk	Member of the Btk tyrosine kinase family
G1	First growth phase
H&E	Haematoxylin and Eosin
H&E	Hematoxylin and Eosin
HER2	Human Epidermal Factor 2

HR	Hormone receptor
HR	Hormonal receptor
HRP	Horseradish peroxidase
i.e.	That is
IACUC	Institutional Animal Care and Use Committee
IDC	Infiltrating ductal carcinoma
IDC	Infiltrating ductal carcinoma
IHC	Immunohistochemistry
IKK	IkB kinase
ILC	Infiltrating lobular carcinoma
IP	Intraperitoneally
IP	Intraperitoneally
IS	Intensity Score
Ki-67	Protein Ki-67
LCIS	Lobular carcinoma in situ
LCIS	Lobular carcinoma in situ
LSAB	Labeled Streptavidin-Biotin Complex
MAPK	Mitogen-activated protein kinases
MDSCs	Myeloid-derived suppressor cells
MEK	Mitogen-activated protein kinase/ERK kinase
mTNF	Membrane-bound TNF
mTNFR2	Membrane-bound TNFR2
mTOR	mammalian target of rapamycin
NF-kB	Nuclear factor kappa B
NMU	N-Nitroso-N-methylurea
P27kip1	Cyclin-dependent kinase inhibitor 1B
P53	Protein p53
pH	Potential of hydrogen
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI3K	Phosphoinositide 3-kinases
PKMYT1	Protein kinase, membrane associated tyrosine/threonine 1
PR	Progesterone Receptor
PS	Proportion Score
RAS	Rat sarcoma

RIPK1	Receptor-interacting serine/threonine-protein kinase 1
S	Synthesis phase
SD	Sprague Dawley
SERDs	Selective Estrogon Receptor Degraders
SERMs	Selective Estrogon Receptor Modulators
STAT5	Signal transducer and activator of transcription 5
sTNF	Soluble TNF
sTNFR1	Soluble TNFR1
sTNFR2	Soluble TNFR2
TAM	Tamoxifen
TNF	Tumor Necrosis Factor
TNFR1	Tumor Necrosis Factor 1
TNFR2	Tumor Necrosis Factor2
TNFα	Tumor Necrosis Factor α
TRAF2	TNF receptor-associated factor 2
Tregs	Regulatory T-cells
TS	Total Score
USM	Universiti Sains Malaysia
VIP	Vacuum infiltration processor

LIST OF APPENDICES

KESAN TAMOXIFEN TERHADAP TNFR2 DALAM MODEL TIKUS KANSER PAYUDARA TERARUH NMU

ABSTRAK

Reseptor faktor nekrosis tumor 2 (TNFR2) telah terlibat dalam keradangan berkaitan kanser dan tindak balas imun, namun fungsinya dalam kanser payudara masih tidak jelas. Kajian ini bertujuan untuk meneroka kesan Tamoxifen (TAM) pada ekspresi TNFR2 dalam model tikus kanser payudara yang disebabkan oleh N-methyl-N-nitrosourea (NMU), serta biomarker lain yang berkaitan (ER, PR, HER2, Bax, Bcl. -2). Tikus dibahagikan kepada tiga kumpulan: kawalan sihat, kumpulan positif yang disebabkan oleh NMU, dan kumpulan yang disebabkan oleh NMU yang dirawat dengan TAM. Imunohistokimia (IHC) dilakukan untuk menilai ekspresi biomarker. Keputusan menunjukkan bahawa TNFR2 tidak dapat dikesan dalam kedua-dua kumpulan Positif dan TAM yang dirawat, menunjukkan bahawa TNFR2 mungkin tidak memainkan peranan penting dalam model kanser payudara yang disebabkan oleh NMU ini. Tahap ER dan PR didapati menurun selepas rawatan TAM, menunjukkan pengaruh TAM terhadap isyarat reseptor hormon dalam model ini. Ekspresi HER2 hampir tidak dapat dikesan merentas semua kumpulan, manakala Bax, penanda pro-apoptosis, menunjukkan ekspresi berkurangan dalam kumpulan Positif manakala kumpulan TAM menunjukkan ekspresi sederhana. Sebaliknya, Bcl-2, protein anti-apoptosis, memaparkan corak ekspresi yang berbeza-beza merentas kumpulan. Penemuan ini menunjukkan bahawa TAM memberi kesan kepada isyarat reseptor hormon dan peraturan apoptosis tetapi nampaknya tidak mempengaruhi ekspresi TNFR2 dalam model tikus kanser payudara yang disebabkan oleh NMU ini. Ini adalah

kajian pertama yang menyiasat peranan TNFR2 dengan biomarker lain yang berkaitan dalam model tikus kanser payudara yang disebabkan oleh NMU.

THE EFFECTS OF TAMOXIFEN ON TNFR2 IN NMU-INDUCED BREAST CANCER RAT MODEL

ABSTRACT

The tumor necrosis factor receptor 2 (TNFR2) has been implicated in cancer-related inflammation and immune responses, yet its role in breast cancer remains unclear. This study aimed to explore the effect of Tamoxifen (TAM) on TNFR2 expression in an N-methyl-N-nitrosourea (NMU)-induced breast cancer rat model, as well as others associated biomarkers (ER, PR, HER2, Bax, Bcl-2). Rats were divided into three groups: healthy controls, NMU-induced positive group, and NMU-induced group treated with TAM. Immunohistochemistry (IHC) was performed to assess biomarkers expression. Results showed that TNFR2 was undetectable in both the Positive and TAM-treated groups, suggesting that TNFR2 may not play a significant role in this NMU-induced breast cancer model. ER and PR levels were found to decrease after TAM treatment, indicating TAM's influence on hormonal receptor signalling in this model. HER2 expression was almost undetectable across all groups, while Bax, a pro-apoptotic marker, showed reduced expression in the positive group meanwhile TAM group shows moderate expression. In contrast, Bcl-2, an anti-apoptotic protein, displayed varied expression patterns across the groups. These findings suggest that TAM impacts hormonal receptor signalling and apoptosis regulation but does not appear to influence TNFR2 expression in this NMU-induced breast cancer rat model. This is the first study that investigating the role of TNFR2 with others associated markers in NMU-induced breast cancer rat model.

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Tamoxifen (TAM) is the oldest and most commonly used selective estrogen receptor modulators (SERMs) to treat patients diagnosed with hormone receptor positive (HR +) breast cancer Jebahi et al. (2021). Research has demonstrated that high levels of endogenous estrogen are positively correlated with the incidences of breast cancer (Brown & Hankinson, 2015; Hankinson & Eliassen, 2007). Therefore, hormonal therapy particularly estrogen blocker is one of the important treatments in breast cancer. Mechanistically, TAM has dual actions. Firstly, it inhibits the hormonal impact of estrogen in breast tissue by interfering with estradiol (E2) in the receptor site. Secondly, it binds to DNA and inhibits carcinogenesis (Viedma-Rodríguez et al., 2014).

Tumor necrosis factor (TNF) has been implicated in almost all steps of tumorigenesis and can function as an angiogenic and antiangiogenic factor. TNF binds to two receptors: tumor necrosis factor 1 (TNFR1/p55-pro-apoptotic) and tumor necrosis factor 2 (TNFR2/p75-pro-survival) subsequently triggering distinct signalling pathways (i.e. pro-apoptotic and pro-survival) upon interaction with the ligand TNF (Sasi et al., 2014). To date, no studies have investigated on the mechanisms of TAM in regulating TNF-TNFR2 interaction. In this proposed study, we investigate the effects of TAM focusing on the expression of TNFR2 histologically. Moreover, other receptors also were studied such as estrogen receptor (ER), progesterone receptor (PR), human epidermal factor 2 (HER2), Bcl-2-Associated X Protein (Bax) and B cell lymphoma gene-2 (Bcl-2).

We utilized N-Nitroso-N-methylurea (NMU) induced breast cancer in rats as they are highly susceptible towards mammary carcinogen, easy to use, and highly reproducible compared to mouse model (Medina, 2007). Overall, this study investigated TAM regarding their respond in TNFR2 receptor for suppression or promotion of breast cancer in rat model.

1.2 Statement of Problem

In worldwide, 2.3 million women has been diagnosed with breast cancer and constitutes approximately 666,103 (15.4%) of death (Bray et al., 2024). Meanwhile, breast cancer cases in Malaysia have surpass all other types of cancer and this making them remains a significant health concern. Massive studies on breast cancer research have been done to lower these statistics, but the investment on this research in Malaysia is still relatively low compared to develop world, particularly on the novel cancer therapeutics.

TNF is generally considered a master pro-inflammatory cytokine. However, recent studies showed that TNF may also have anti- inflammatory effects, depending on the receptors (i.e. TNFR1 and TNFR2) localised at the cellular surface, which have unrelated intracellular region. A study in a model of inflammation-associated cancer indicated that TNFR2 is preferentially up-regulated over TNFR1, and therapy with the anti-TNF monoclonal antibody decreased both the amount and size of tumors. This shows that TNF can operate as a potent anti-inflammatory cytokine with important roles in innate and adaptive immunity, and it is thought to be an interaction between inflammation and cancer since long-term use of nonsteroidal anti-inflammatory drugs reduces the risk of cancer death. Therefore, TNF-TNFR2 axis was implicated in the suppression of immune response and affects tumor progression and growth.

This proposed study will be the first to examine the effects of TAM in an NMU-induced breast cancer rat model, focusing on TNF and its receptors histologically, while also providing a deeper understanding of how TAM helps breast cancer patients by reducing tumor growth and improving survival.

1.3 Hypotheses

We hypothesized that TAM treatment would provide protective effects by altering the expression TNFR2 on cancer cells.

1.4 Research Questions

The hypothesis on the targeted TNFR2 of TAM treatment is motivated for answering the following research question:

a) Is there any involvement of TNFR2 in TAM treatment?

b) To what extent TAM protect breast cancer induction in rats?

c) Does all NMU-induced breast cancer rat model are ER positive?

d) What are the pathological characteristics and degree of aggressiveness of the tumors?

e) Does TNFR2 receptor also have the same effects as others studied markers (ER, PR, HER2, Bax, and Bcl2)?

1.5 Objectives

1.5.1 General Objectives

To determine the effect of TAM on TNFR2 in NMU-induced breast cancer rat model.

1.5.2 Specific Objectives

1. To visualize the tumor morphology and to evaluate the pathological characteristics and degree of aggressiveness of such lesions (Haematoxylin and Eosin staining)

2. To assess the involvement of TNFR2 and association with other tumor markers in NMU-induced breast cancer rat model with and without TAM treatment (Immunohistochemistry)

1.6 Flowchart of the Study

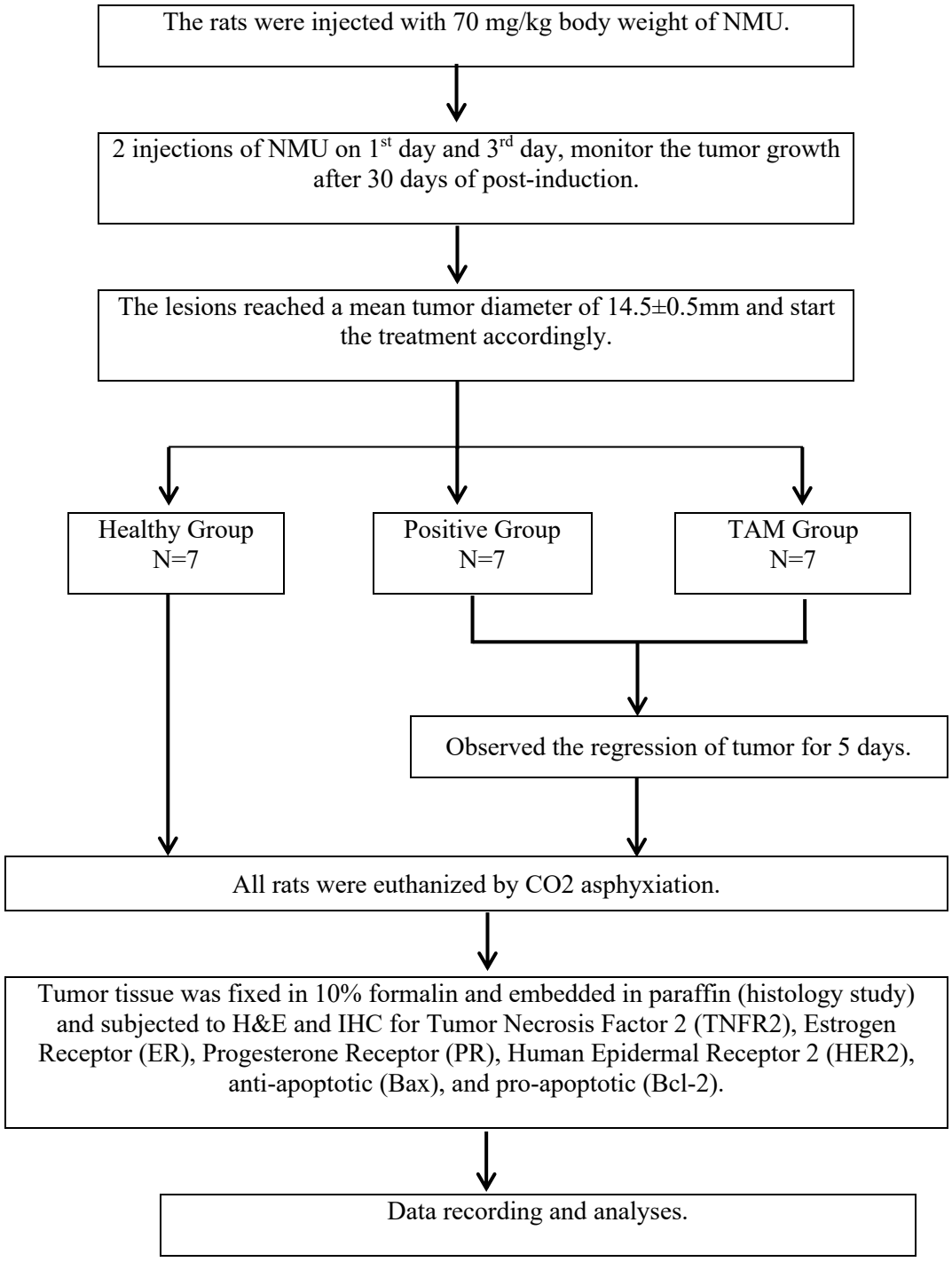

Figure 1.1 Flow chart of the animal study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview on Breast Cancer

Breast cancer in female is the highest cancer incidence in worldwide and has overtaken lung cancer as the most commonly diagnosed cancer globally (Bray et al., 2024). According to Globocan (2022), female breast cancer, with a predicted 2.3 million new cases had caused over 666,103 (15.4%) of death and this has resulted that female breast cancer becoming the second leading cause of cancer death.

Figure 2.1 shown the number of new cancer cases among females within all ages in Malaysia 2022. Breast cancer has contributed the highest cancer cases (8371) from 26 753 of total cases. From 8371 of these cases, breast cancer had caused 3526 of death (Figure 2.2). This has proved that breast cancer become one of the deadliest diseases in the world.

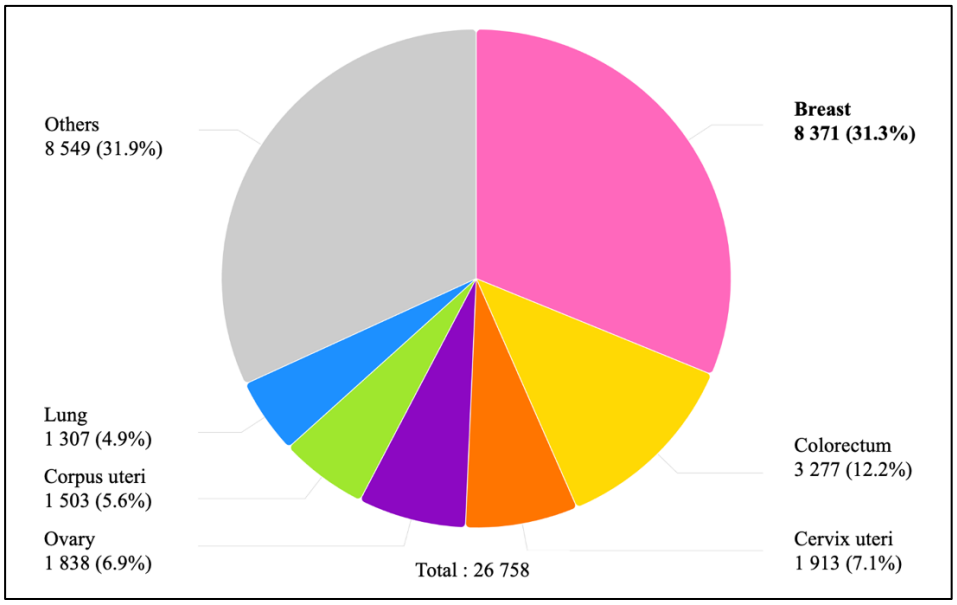

Figure 2.1 Number of new cancer cases in Malaysia 2022, females, all ages. Adapted from World Health Organization (2022b), Cancer Today (https://gco.iarc.who.int) with permission.

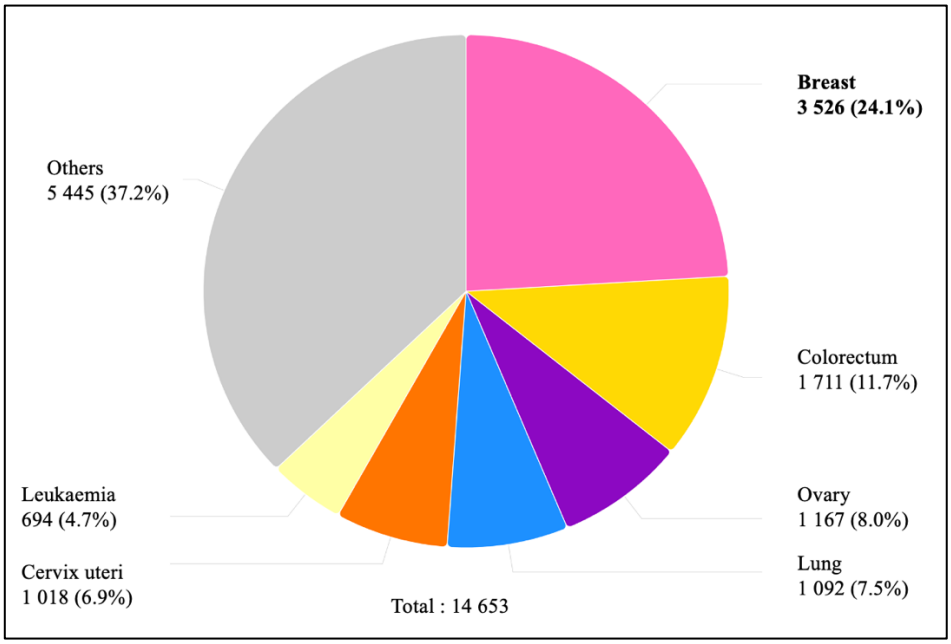

Figure 2.2 Number mortality of cancer cases in Malaysia 2022, females, all ages. Adapted from World Health Organization (2022a), Cancer Today (https://gco.iarc.who.int) with permission.

Breast cancer is defined as a type of cancer that starts when the cells in the breast grow out of control (World Health Organization, 2024). In other word, breast cancer also defined as a compilation of distinct malignancies that manifests in the mammary glands (Feng et al., 2018). Breast cancer can spread outside the breast via blood vessels and lymph vessels to different regions of the body, which is called metastasis (Center for Disease Control and Prevention, 2024). Metastatic breast cancer frequently spreads to distant organs such the lung, liver, bone, and brain, which is primarily responsible for its incurability (Sun et al., 2017). Thus, it is very important for the patient to have early diagnosis to have good prognosis and a high survival rate.

To understand the types of the breast cancer and where the breast cancer does originate from, it is very important to understand on the internal structure of the breast. Figure 2.3 shows the internal structure of the breast (Kammath, 2022). The breast is

the tissue that covers the chest (pectoral) muscles. Women's breasts are composed of up of both glandular tissue (which produces milk) and fatty tissue. The milk-producing area of the breast is divided into 15 to 20 sections called lobes. Each lobe comprises smaller structures called lobules, that are where milk originates. Milk flows through a system of microscopic tubes called ducts. The ducts connect and develop larger ducts that eventually exit through the skin in the nipple (Kammath, 2022).

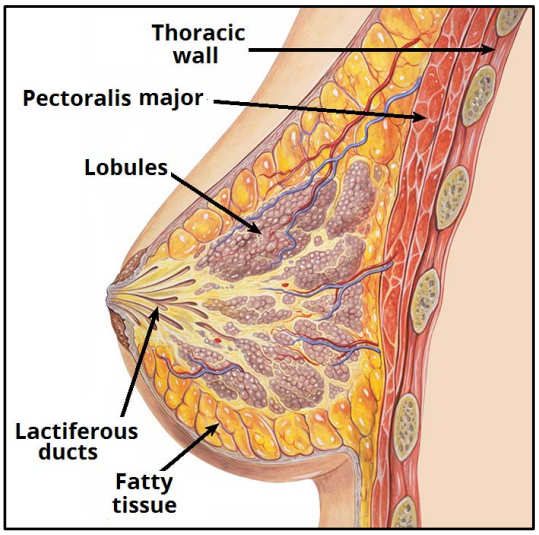

Figure 2.3 The internal structure of woman breast. Adapted from Kammath (2022), TeachMe Anatomy (www.teachmeanatomy.info) with permission.

2.1.1 Types of Breast Cancer

There are many types of breast cancer based on the pathology, invasiveness, and prevalence. But overall, breast cancer can be classified into two group, non-invasive breast cancer and invasive breast cancer. Non- invasive breast cancer is cancer that It has not spread beyond from the lobule or ducts in which it is located meanwhile invasive breast cancer is when the cancer cells grow into or invade normal tissues within or beyond the breast. To have a better understanding in the types of breast cancer, Table 2.1 summarised the list types of breast cancer respective to non-invasive and invasive with description (Akram et al., 2017).

Table 2.1 Types of breast cancer.

	Types of Breast Cancer	Description
Non-invasive breast cancer	Lobular carcinoma in situ (LCIS)	Develops into breast lobules but does not expand beyond the lobules into the breast tissue.
	Ductal carcinoma in situ (DCIS)	Most common non-invasive breast cancer that is limited to the breast duct.
Invasive breast cancer	Infiltrating lobular carcinoma (ILC)	Originates in lobules of the breast, but frequently extends to other areas of the body.
	Infiltrating ductal carcinoma (IDC)	Originates in the milk ducts of the breast and extends to the duct wall, invading the breast fatty tissues and probably other parts of the body.
	Medullary carcinoma	Designs a discrete margin normal tissue and medullary tissue.
	Mucinous carcinoma	Uncommon breast cancer created by the mucus-forming cancer cells.
	Tubular carcinoma	Usually small and consist of tube-shaped structures.
	Inflammatory breast cancer	Form of swollen breasts (red and warm) with wrinkles and/or wide grooves due to cancer cells blocking lymph veins or channels in the skin around the breast.
	Paget's disease of the breast	Usually displays apparent changes to the breast's nipple. Its symptoms include red, itchy rashes on the nipple, that can sometimes relocate to the normal skin. It resembles other skin diseases like eczema and psoriasis, but it may be distinguished. Usually only impacts one breast and originates with the nipple rather than the areola.

Table 2.1 continued

	Types of Breast Cancer	Description
	Phyllodes tumor	Develop in the connective tissues of the breast and can be either benign or malignant.
	Triple-negative breast cancer	Described by the deficiency of progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and estrogen receptor (ER) expression.

In addition, breast cancers have four molecular subtypes based on their genes expressed. Each of the subtypes has distinct behaviours and responses to therapy. The subtypes are being categorized based on the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 protein (Ki-67). Table 2.2 shows the molecular subtypes of breast cancer(Prat et al., 2017; Seho et al., 2012).

Table 2.2 The four molecular subtypes of breast cancer.

Molecular Subtypes	Molecular Characteristics
Luminal A	ER+, PR±, HER2-, Low Ki67
Luminal B	ER+, PR±, HER2±, High Ki67
HER2	ER-, PR-, HER2+
Triple Negative	ER-, PR-, HER2-

The prominence of breast cancer subtype is that it gives evidence to the pathologists on the prognosis and how cells may respond to treatment. Different breast cancer subtypes have different treatment accordingly. Luminal A and Luminal B may

undergone endocrine therapies and chemotherapy to block hormones from fuelling cancer growth (Di Leo et al., 2012). HER2 subtypes are frequently successfully treated with targeted therapies directed at the HER2 protein, whereas triple-negative breast cancer is usually managed by a combination of surgery, radiation therapy, and chemotherapy (Breastcancer.org, 2024).

2.1.2 Cause of Breast Cancer

There are numerous causes of breast cancer. The causes include age, reproductive variables, a family history of breast cancer, genetic predisposition, and environmental factors (Shah et al., 2014). People with unhealthy lifestyle such as consume excessive of alcohol, obesity and physical inactivity also tend to have breast cancer. In addition, research has shown that the damage to the deoxyribonucleic acid (DNA) and hereditary alteration associated with the exposure of estrogen are the main reasons of breast cancer to arise (Akram et al., 2017).

Some individuals inherit abnormalities in the DNA and genes, including the tumor protein p53 (*p53*), Breast Cancer gene 1 (*BRCA1*), and Breast Cancer gene 2 (*BRCA2*), among others (Shah et al., 2014). This related with RAS/MEK/ERK and PI3K/AKT pathways in which their function was to defend normal cells from cell suicide. When abnormalities occur in genes that are involved in the encoding of these protective processes, the cells become incapable of attempting apoptosis when they are no more required, which leads to the development of cancer (Akram et al., 2017). Figure 2.4 shows the PI3K/AKT/mTOR signalling pathways. This signalling pathway plays an essential role in modulating signal transmission and biological activities including cell proliferation, death, metabolism, and angiogenesis (Fei et al., 2020).

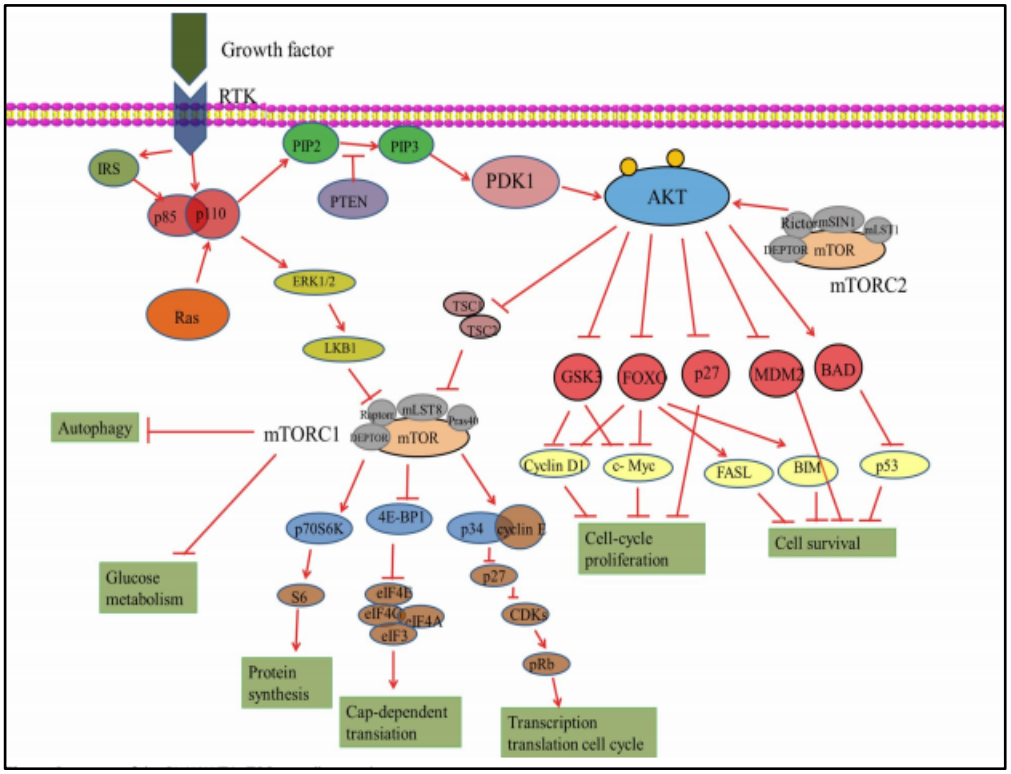

Figure 2.4 Overview PI3K/AKT/mTOR signalling pathways. Adapted from Fei et al. (2020) with permission.

The PI3K/AKT/mTOR signalling pathway participates in cell cycle activities and promotes growth and development of tumors. Molecules involve in the pathway such as activated AKT directly phosphorylates and thus activates mTOR. mTOR thus promotes the binding of cyclin D1 to cyclin-dependent kinase (CDK) to initiate cell division. High levels of cyclin D1 expression can induce cell cycle transition from the first growth (G1) to synthesis (S) phase, shorten the cell cycle and accelerate cancer development. CDK inhibitor protein family which is Cyclin-dependent kinase inhibitor 1B (P27kip1) work by inhibiting the activity of CDK to block cell proliferation. However, AKT suppresses the cell cycle blockage by phosphorylating P27kip1, which increases cell proliferation and differentiation (Riquelme et al., 2016). Furthermore, mTOR controls the synthesis of biological macromolecules such

proteins, nucleotides, and lipids, providing the components needed for cancer cell growth (Cheng et al., 2017).

Discussing on the pathogenesis of breast cancer, it also may occur through the induction of chemical such as N-Nitroso-N-methylurea (NMU), N,N-Dimethyldopamine (DMDA), and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Bazm et al., 2018). In this study, the chemical used to develop breast cancer is NMU. The topic of cancer induced NMU rat model was being reviewed further below.

2.2 NMU-Induced Animal Model

N-Nitroso-N-methylurea (NMU) or also known as 1-Methyl-1-nitrosourea is a chemical compound that was used in the past for the laboratory synthesis of diazomethane (PubChem, 2024). However, Since the early 1960s, NMU has been examined for mutagenicity and genetics, as well as for application as a cancer chemotherapeutic drug (Zetterberg, 1961). Study has been reported that NMU has been an effective therapy for mice that are intraperitoneally or intracerebrally implanted with L1210 leukaemia cells (Tsubura et al., 2014). However, NMU is currently used as a research chemical to develop animal models for human diseases as the effectiveness is more prominence.

2.2.1 Animal Research Model

NMU has been tested within many species of animal in developing tumors. It was been tested in mice, rats, rabbits, pigs, dogs, and monkeys (Guy, 2005). Besides, NMU has properties of alkylating, mutagenic, teratogenic, carcinogenic, and cytotoxic properties (Tsubura et al., 2014). The carcinogenic effect has been proven when it causes tumors at many sites, include the breast, nervous tissue, stomach, oesophagus,

pancreas, respiratory tract, intestine, lymphoreticular tissues, skin, and kidney (Bazm et al., 2018; Guy, 2005). NMU has been recorded to been widely used in developing breast cancer in animal model as the model is believed has an outstanding in screening and evaluating the potency of cancer supressing and promoting agents (Jaffar et al., 2020).

When NMU was induced, The chemical directly induces DNA alkylation, disrupting synthesis and repair (Bazm et al., 2018). Alkylation is the process of substituting one or more alkyl groups for hydrogen atoms in an organic molecule (Xu et al., 2017). Hence, NMU caused alkylation lesions in DNA and RNA. Besides, research showed that cells undergo apoptosis after DNA alteration through alkylation (methylation) (Bazm et al., 2018). However, the molecular mechanisms of transcriptional lesion identification, pausing, and bypass have remained unclear till now (Xu et al., 2017). Figure 2.5 illustrated on the effects of NMU on cells that causes DNA methylation and effects on cellular proteins, and ultimately the cell goes to tumor.

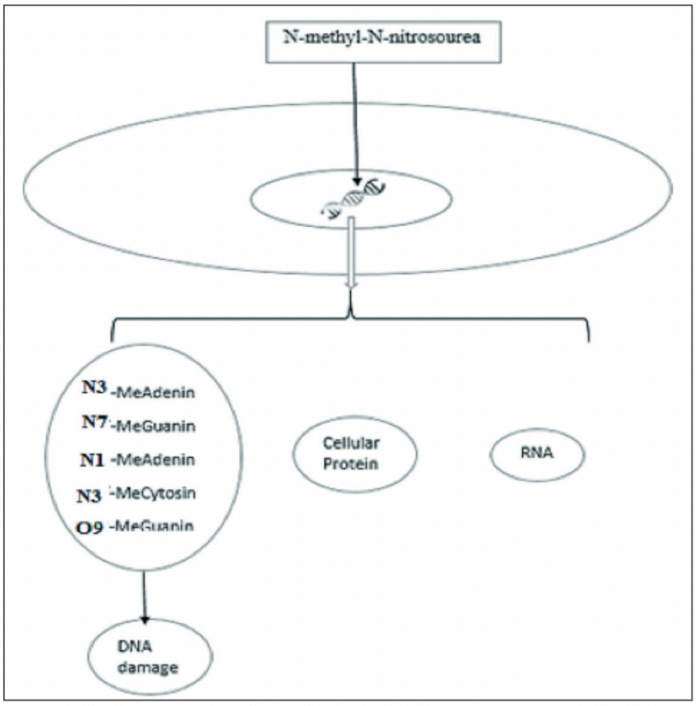

Figure 2.5 The Effects of NMU on cells. Adapted from Xu et al. (2017) with permission.

2.2.2 Rat as NMU Research Model

In this research, *Sprague Dawley* (SD) rat was used to develop NMU-induced breast cancer model. SD rat is one of the most widely used strains in the development of breast cancer models. Many factors that influenced on choosing rat as breast cancer model. Rat is more significances as they require less time of breeding, easy in handling, and less rearing care compare to other big mammals (Mukherjee et al., 2022).

Primarily, it was reported that rat is genetically and physiologically same as human. It's interesting to note that rat and human carcinomas have comparable growth and histological characteristics when it comes to cancer research, specifically in breast cancer research (Szpirer, 2020). Therefore, closely resemble of histological tumors between rat and human making them very useful in discovering breast cancer treatments. Moreover, rat also hormonal dependent towards breast cancer disease. The

similarity between rat and human making them very valuable in providing insights into histological and hormonal influences on breast cancer growth and response to treatments (Shen et al., 2007).

2.2.3 Dosage of NMU in Rat Model

Most previous studies used different dosages to induce breast cancer in rats. This variation is due to multiple factors that can influence tumor formation beyond NMU dosage. For example, factors such as the age of the rats at the time of NMU administration, the rat strain, hormonal influence, experimental conditions, and the stability of NMU based on the method of preparation can all impact tumor development.(Perše et al., 2009). Thus, this study cannot fully rely on previous studies to determine a suitable dosage for breast tumor formation. Therefore, a pilot study needs to be conducted.

A dose of 60 mg/kg has been commonly used as the minimum effective dosage for inducing mammary tumors(Russo, 2015). A dose of 60 mg/kg is considered the minimum effective dosage for inducing mammary tumors, ensuring sufficient tumor development while minimizing toxicity. Hence, higher doses of 70 mg/kg and 80 mg/kg were considered to evaluate dose-dependent effects, balancing tumor induction efficiency and animal survival at 70 mg/kg while assessing the impact of increased tumor aggressiveness at 80 mg/kg. Therefore, we selected NMU dosages of 60 mg/kg, 70 mg/kg, and 80 mg/kg for this pilot study.

2.3 Breast Cancer Treatments

Breast cancer can be treated using a variety of treatment. There are two different perspectives on the most effective approach to breast cancer treatment. First, some highlighted that treatment should be primarily guided by the stage of the disease

(Trayes & Cokenakes, 2021). There are 4 stages of breast cancer as shown as Table 2.3. Current treatment for breast cancer include surgery, radiation therapy, endocrine therapy, immunotherapy, and chemotherapy. Table 2.4 summarize on what treatments involved based on the stages of breast cancer. According to the table, the treatment approach was different since difference stages of cancer has difference objectives of therapy. For non-metastatic, the primary objectives are to remove the tumor from the breast and nearby lymph nodes and to prevent breast cancer recurrence. Meanwhile for metastatic, the objectives are extending life and relieving symptoms (Waks & Winer, 2019).

Table 2.3 The stages and type of breast cancer

Stage of Breast Cancer	**Breast Cancer Type**	**Aggressiveness**
0	Ductal carcinoma in situ (DCIS)	Non-invasive
I and II	Locally Advanced, Nonmetastatic Breast Cancer	Early invasive
III		
IV	Metastatic Breast Cancer	Invasive

Table 2.4 Breast cancer treatment base on stages

Breast Cancer Stage	**Breast Cancer Treatment**				
	Surgery	Radiation Therapy	Endocrine Therapy	Immunotherapy	Chemotherapy
0	Yes	Yes	Yes	No	No
I and II	Yes	Yes	Yes	Yes	Yes
III	Yes	Yes			
IV	Yes	Yes	Yes		

Although breast cancer stages can be considered as an approach on deciding the treatment, it is also clinically important to distinguish the breast cancer type molecularly. This is because molecular subtyping, such as distinguishing between Luminal A, Luminal B, HER2-positive, and triple-negative breast cancers, may provide more particular strategies. Hence, immunohistochemistry (IHC) expression of ER, PR, HER2, and Ki-67 are used to define surrogates of the four molecular subtypes of breast cancer (Table 2.2). Table 2.5 shows on how treatment being allocated based on stages and molecular subtypes of breast cancer.

Table 2.5 Breast cancer treatment base on stages and molecular subtypes. (A) Early-stage Invasive Breast Cancer, (B) Metastatic breast cancer.

A. Early-stage Invasive Breast Cancer					
Molecular Subtypes	Breast Cancer Treatment				
	Surgery	Radiation Therapy	Endocrine Therapy	Immunotherapy	Chemotherapy
Luminal A	Yes	Yes	Yes	No	Yes
Luminal B	Yes	Yes	Yes	No	Yes
HER2	Yes	Yes	No	Yes	Yes
Triple Negative	Yes	Yes	No	No	Yes

B. Metastatic Breast Cancer					
Molecular Subtypes	Breast Cancer Treatment				
	Surgery	Radiation Therapy	Endocrine Therapy	Immunotherapy	Chemotherapy
Luminal A	No	No	Yes	No	Yes
Luminal B	No	No	Yes	Yes	Yes
HER2	No	No	No	Yes	Yes

Table 2.5 continued

A. Metastatic Breast Cancer					
Molecular Subtypes	Breast Cancer Treatment				
	Surgery	Radiation Therapy	Endocrine Therapy	Immunotherapy	Chemotherapy
Triple Negative	No	No	No	No	Yes

In short, the current strategies of treatment are principally based on the stage of tumor to guide on tumor progression and also molecular subtypes in order to offer the most personalized treatment for breast cancer patients (Burguin et al., 2021). Not only stages and subtypes, but many other factors that also should be considered. Other than disease risk factors, the treatment also consider on patient risk factors (Drãgãnescu & Carmocan, 2017).

For example, phases in woman reproductive cycle also need to be considered whether they were premenopausal or postmenopausal as both have different level of woman hormonal level. Hence, different patient may receive different prescriptions of breast cancer treatment. As precision medicine continues to evolve, integrating both stage-based and subtype-based considerations may offer the most comprehensive and personalized approach to breast cancer management and treatment.

2.3.1 Tamoxifen as Hormonal Therapy

Hormonal therapy is one of the best ways used to treat breast cancer. It is very efficient in treating patient with hormonal receptor positive (HR +). HR + is referring to estrogen receptor positive (ER +), to progesterone receptor positive (PR +), or for both (Drãgãnescu & Carmocan, 2017). Most of breast cancer subtypes are ER + and

PR+. It was reported that 75% of breast cancer patients were diagnosed with ER + and PR+ (Patel & Bihani, 2018). Hence hormonal therapy would always become predominant breast cancer treatment strategy. In this study, hormonal therapy was chosen since previous studies reported that NMU-induced breast cancer rat model are mostly ER+ and PR+ in molecular subtypes (Antonieta Alvarado et al., 2017).

There are three well-known hormonal therapy in breast cancer treatment. First are selective estrogen receptor modulators (SERMs). SERMs work as estrogen agonists, antagonists, or both. Second are selective estrogen receptor degraders (SERDs). SERDs act as ER activity inhibitors (Drãgãnescu & Carmocan, 2017). Lastly are Aromatase Inhibitors (AIs). AIs prevent the synthesis of estrogen by inhibiting aromatase, which converts androgens to estrogen (Wang & Tang, 2022). All of them are hormonal receptors, but their mechanism works differently.

In this research, SERMs was chosen since it was more convenient compared to SERDs and AIs. SERMs can be found in tablets, and liquid forms. However, SERDs only available in injected form. Although there were studies showed the high efficacy of oral SERDs, they are still developing and undergoes clinical trial phase III (Neupane et al., 2024). In addition, studies also reported that AIs are most efficient at postmenopausal stage (Behan et al., 2015). Considering all factors, SERMs is the most suitable treatment approach in this study. TAM is the most prescribed SERMs over the time in breast cancer treatment. Hence, this study used TAM to treat NMU-induced breast cancer rat.

2.3.2 Mechanism of Action

As mentioned earlier, TAM in which being categorized as SERMs work as estrogen agonists, antagonists, or both (Drãgãnescu & Carmocan, 2017). To have a

clear idea on how TAM works as SERMs, the definition of agonists and antagonists must be understated. Agonists are drugs that attach to receptors and trigger a biological reaction by activating them. Meanwhile antagonists work in contrast with agonists. Antagonists are drugs that attach to receptors and dampen biological response by blocking them (Zamolodchikova et al., 2021). Figure 2.6 shows the mechanism of action SERMs when interact with ER (Patel & Bihani, 2018).

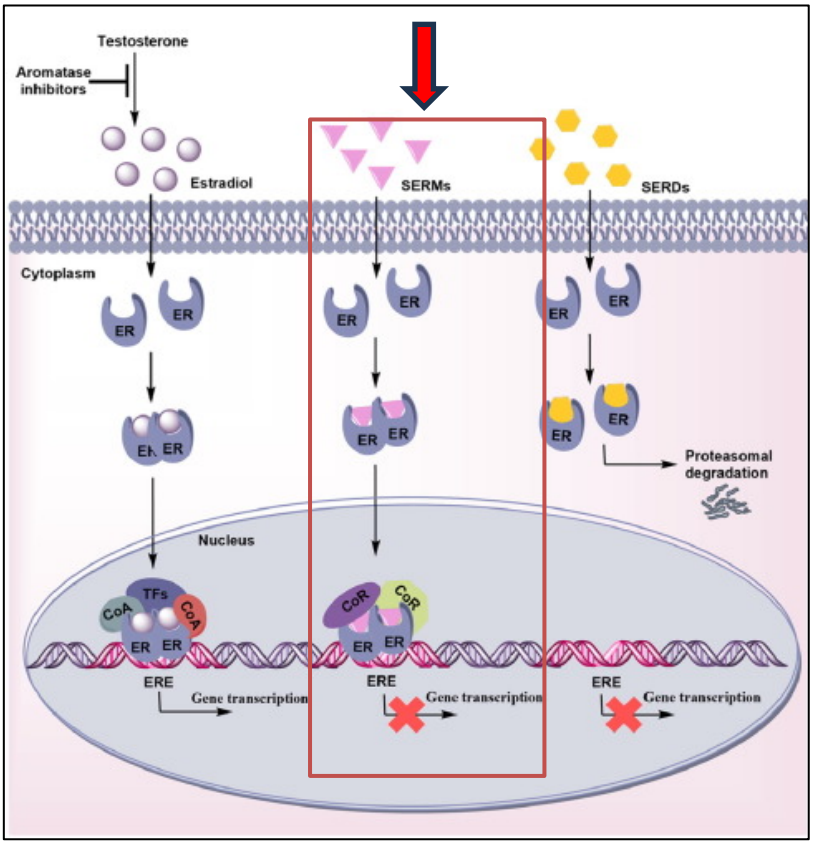

Figure 2.6 The mechanism of action of SERMs on the ER pathway in comparison with SERDs and AIs. Adapted from Patel and Bihani (2018) with permission.

Figure 2.6 give the illustration that when TAM bind to ER, it prevents ER signalling and cause an inactive complex. Hence, this mechanism is really needed since the development of tumor and breast cancer is depending on ER that become like a food supply to them. However, a whole disruption and blockage on ER pathway in the body are not good to the body. This is because ER also important for woman's

reproductive system, bone, cardiovascular and cognitive function (Deroo & Korach, 2006). This is why TAM is the perfect hormonal approach in breast cancer treatment. TAM is a drug that does not block all ER but it act accordingly whether as estrogen agonists, antagonists, or both (Drãgãnescu & Carmocan, 2017).

The functional variability of TAM is a result of the molecular and functional complexity of ER. ER exist in two forms, alpha and beta. These forms regulate transcriptional activation to provide physiological effects that are specific to receptor subtypes. Figure 2.7 shows how TAM works accordingly at different target tissues (Komm & Mirkin, 2014). TAM act as agonist in bone. As result, it can reduce the risk of osteoporosis (J. Lee et al., 2020). Besides, TAM act as partial agonist in endometrium that could lead to both beneficial and adverse effects. As result, TAM sustain the endometrial lining's thickness and health, but patient that consume TAM for long terms have high possibility of having endometrium cancer (Choi et al., 2021). The most remarkable effect of TAM is when it acts fully antagonist on breast tissues. Therefore, TAM is highly effective in treating breast cancer by binding to ER.

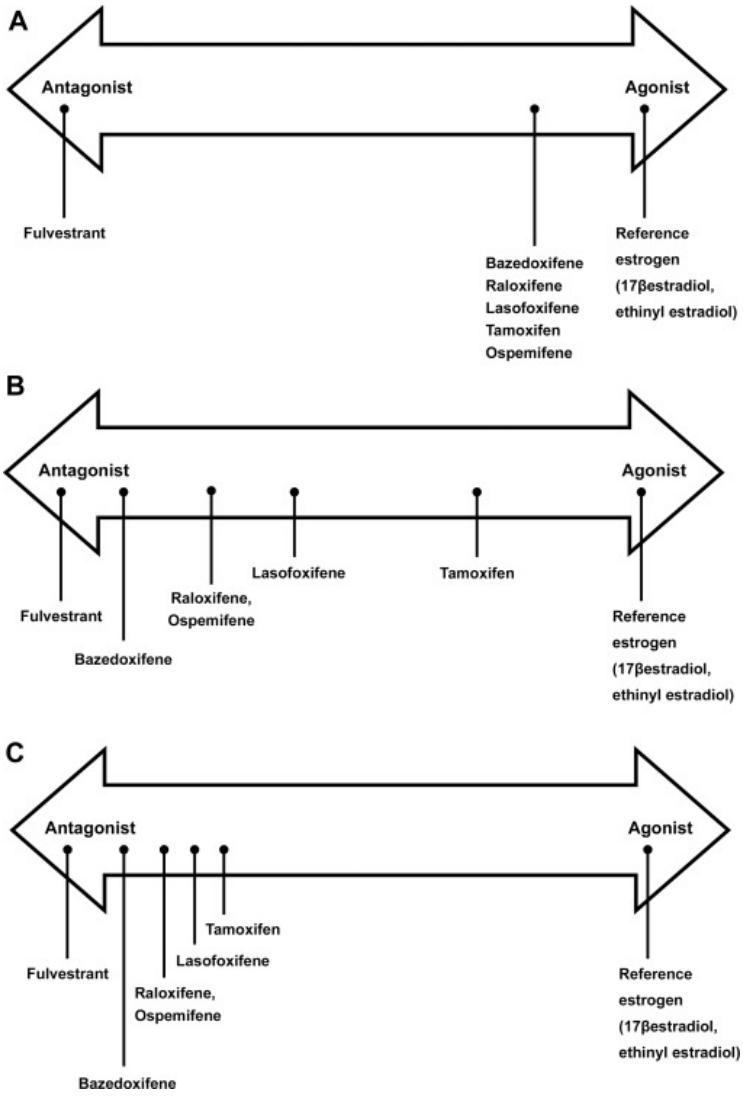

Figure 2.7 Activity of SERMs in different target tissues. (A) Bone. (B) Endometrium. (C) Breast. Adapted from Komm and Mirkin (2014) with permission.

2.3.3 Tamoxifen Limitations

Even though TAM is the most common SERMs used in treating breast cancer, there are still many limitations and gap in knowledges. As TAM works partial agonist towards endometrium, it causes adverse effect which is high chance of having endometrium cancer. Hence, this does not make TAM itself to be marked as a gold standard in breast cancer treatment. Further studies should focus on modifying and synthesizing TAM in combination with other agents to minimize or eliminate side

effects. That is why, combine therapy is the best treatment in breast cancer for now (Burguin et al., 2021).

In addition, focusing on conventional treatments is still not a promising approach. Although there is abundance of ongoing chemical trials and emerging therapies, the treatments of breast cancer are very complex and constantly evolving. This is because there are always new biomarkers uncovered by researchers over the time. For example, latest finding reported that there are four overexpressed genes in breast cancer patient in which three of them show a potential therapeutic target. They are Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 4 (*CACNG4*), Cholinergic receptor nicotinic alpha 6 subunit (*CHRNA6*), and protein kinase, membrane associated tyrosine/threonine 1 (*PKMYT1*) (Golestan et al., 2024).

The revealing of new biomarkers makes breast cancer research challenging but at the same time exhilarating for discovery. As well as new biomarkers, there are still many discovered markers that were not studied broadly such as Tumor Necrosis Factor Receptor 2 (TNFR2). TNFR2 is considered as significance biomarker in cancer biology. Hence, it is a must to discover this potential biomarker on how it works conjunction with the effect of TAM. TNFR2 biomarker was reviewed further below.

2.4 Breast Cancer Receptors and TNFR2

Cellular receptors are proteins that receive signals, either within or on the cell's surface. (Miller & Lappin, 2021). In any cancers including breast cancer, receptors are called as HR + or HR - based on whether they have these receptors (proteins) or not.