ELUCIDATING THE EFFECTS OF RAPAMYCIN AND PF4 ON THE MNU INDUCED FEMALE RATS AND HUMAN BREAST CANCER CELLS (MCF-7)

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LAMPIRAN A : ABSTRAK

ELUCIDATING THE EFFECTS OF RAPAMYCIN AND PF4 ON THE MNU INDUCED FEMALE RATS AND HUMAN BREAST CANCER CELLS (MCF-7)

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Introduction: There is a strong evidence that tumour growth is not just a consequence of uncontrolled proliferation but also of reduced apoptosis. Bax and Bcl-2 are Bcl-2 family like apoptosis regulator. They function either as suppressor (Bcl-2) or a promoter (Bax) of apoptosis. As a member of the inhibitors of apoptosis (IAP), survivin is also a promoter of cellular proliferation and thus a key player in cancer progression. Beside, caspases also occupy a central role in maintaining cellular homeostasis.

Objectives: Therefore, this research aims to examine the apoptotic effects of Rapamycin and Platelet Factor 4 (PF4) on 1-methyl-1-nitrosourea (MNU)-induced mammary carcinoma through Bcl-2- survivin and caspase modulated pathways in female Sprague Dawley Rat (SDR) and *in vitro* MCF-7 breast cancer cell lines.

Methods: One hundred 21 days old female SDR were given an intraperitoneal injection (IP) of MNU to induce breast cancer formation. When tumour size reached 14.5±0.5mm, intratumoural injections of the following interventions were given; Group 1 (pre-intervention control, n=20 and post-intervention control, n=20), Group 2 (Rapamycin-treated, n=20), Group 3 (PF4-treated, n=20) and Group 4 (Rapamycin+PF4-treated, n=20). Tumour growth was then morphologically assessed using haematoxylin

and eosin (H&E) and immunohistochemistry (IHC) utilizing pro-apoptosis (Bax) and anti-apoptosis markers (Bcl-2), survivin and caspases-3,-6,-7,-8, and -9. Besides that, the MCF-7 cell line was used for *in vitro* assessment. Initially, half maximal inhibitory concentration (IC_{50}) of each drug was determined. The MCF-7 cell lines were then exposed to Rapamycin and PF4 and Rapammycin+PF4 at IC_{50} concentrations, after which they were subjected to flow cytometry and Western blot analyses.

Results: Bax was significantly expressed at higher levels in the rapamycin-treated and Rapamycin+PF4-treated groups than controls ($p<0.001$). Besides, survivin was significantly downregulated in the PF4-treated and Rapamycin+PF4-treated group when compared to controls ($p<0.001$). On the other hand, Bcl-2 expression was found not to be significantly altred in all treatment groups. Caspase-3, was significantly expressed at higher levels in both PF4-treated and Rapamycin+PF4-treated groups than controls ($p<0.001$), as well as rapamycin-only group when compared to Rapamycin+PF4-treated and PF4-treated groups (both $p<0.001$). Apart from that, caspase-6 was also significantly expressed in Rapamycin-treated, PF4-treated and Rapamycin+PF4-treated than the control groups ($p<0.001$). Besides, either PF4 or Rapamycin+PF4 combination was associated with increased caspase-7 expression, compared to the controls ($p<0.001$). However, Rapamycin-treated group showed a significantly higher caspase-8 expression when compared to PF4-treated ($p<0.05$). For caspase-9, higher caspase-9 expression was observed in Rapamycin-treated group when compared to control, Rapamycin+PF4-treated and PF4-treated cohorts ($p<0.001$). The IC_{50} Rapamycin, PF4 and Rapamycin+PF4 were 0.4 µg/ml, 6 µg/ml and 0.4 µg/ml+1.0 µg/ml respectively. Rapamycin and PF4, on the other hand, were non-toxic to the normal HMEC cells.

Furthermore, Rapamycin, PF4 and Rapamycin+PF4 induced a cell cycle arrest in MCF-7 cell lines at G0/G1, S and G0/G1 phases, respectively. Rapamycin, PF4 and Rapamycin+PF4 induced the upregulation of pro-apoptotic Bax and the downregulation of anti-apoptotic Bcl-2 and survivin. The expression levels of caspase-3 and caspase-8 were consistent in the treated group when compared with the control group. The expression of caspases -6, -7 and -9 protein were increased when treated with Rapamycin, Rapamycin + PF4 and PF4 when compared with the control group.

Conclusion, this study provides new insights on the mechanistic properties of Rapamycin and PF4 as anti-cancer agents in breast cancer animal model and *in vitro* MCF-7 cell lines. The results lend support to the notion that apoptotic induction by Rapamycin and PF4 and was mediated by both the intrinsic and extrinsic pathways through the activation of caspase-9, caspase-3 and caspase-8 activation.

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by

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DEDICATION

THIS THESIS IS ESPECIALLY DEDICATED TO MY FAMILY:

MY BELOVED PARENTS, KU (TENGKU DIN) AND MA (WAN HALIMAH),

MY BELOVED WIFE, AZIRAWATI BT ISMAIL, MY PRECIOUS DAUGHTERS, TENGKU NUR DANISYA AL-AWATIFF, TENGKU NURDARINA AL-AFIFAH, TENGKU NURDALIEYA AL-ALIYAH AND TENGKU NURDAMIA AL-ATHIRAH.

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“WITHOUT YOUR ENDLESS SUPPORT, SYMPATHETIC AND THOUGHTFUL ATTENTION, CONSTANT INSPIRATION AND FAITH IN ME OVER THE YEARS, I WOULD CERTAINLY NOT BE ABLE TO BE HERE TO PRESENT MY THESIS TO YOU”.

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4E-BP1	4E-Binding Protein 1
A20	Cys2/Cys2 Zinc Finger Protein
ABC	Avidin-Biotin Complex
AKT	Protein Kinase B
AMPK	AMP-Activated Protein Kinase
Apaf-1	Apoptosis Activating Factor
Apo3L	Apo3 Ligand
Apo2L	Apo2 Ligand
APS	Ammonium Persulfate
ARACS	Animal Research & Services Centre Of Universiti Sains Malaysia
ATCC	American Type Cell Collection
ATP	Adenosine 5'-Triphosphate
Bad	BCL2-Associated Agonist Of Cell Death
Bak	BCL2-Antagonist/Killer 1
Bax	Bcl-2-Associated X Protein
Bcl-xL	B-Cell Leukemia XL
Bcl-2	B Cell Lymphoma Gene-2

Bid	BH3 Interacting Domain Death Agonist
BH	Bcl-2 Homology
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
C. elegans	Caenorhabditis Elegans
c-Myc	Regulator Gene That Codes For A Transcription Factor
Caspases	Cysteine Proteases
DAB	Diaminobenzidine
DLBCL	Diffuse B-Cell Lymphomas
DISC	Death-Inducing Signaling Complex
dH_2O	Distilled Water
DCIS	Ductal Carcinoma In Situ
DMBA	7,12-Dimethylbenz[A] Anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DR3	Death Receptor 3
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor

EPR-1	Effector Cell Protease Receptor-1
ER-α	Estrogen Receptor Alpha
FACS	Fluorescent-Activated Cell Sorting
FADD	Fas-Associated Death Domain
FasL	Fatty Acid Synthetase Ligand
FasR	Fatty Acid Synthetase Receptor
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
FKBP12	FK506 Binding Protein 12
FITC	Fluorescein Isothiocyanate
FKBP12	12-Kda FK506-Binding Protein
FKBP38	FK506-Binding Protein 38
g	Gram
GAP	Gtpase Actativing Protein
GDP	Guanosine Diphosphate
GTP	Guanosine 5’ Triphosphate 2
HER-2	Human Epidermal Growth Factor Receptor
HIER	Heat-Induced Epitope Retrieval
HIF-1	Hypoxia Inducible Factor 1 Cancer

HMEC	Human Mammary Epithelial Cell
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis Protein
ICE	Interleukin-1–Converting Enzyme
IDC-NOS	Invasive Ductal Carcinomas Not Otherwise Specified
IDC-NST	Invasive Ductal Carcinomas No Special Type
IDP	Intraductal Proliferations
IGF-R	Insulin-Like Growth Receptors
IHC	Immunohistochemistry
ISS	Imunohistochemistry Scoring System
kD	Kilodalton
L	Liter
MCF-7	Michigan Cancer Foundation-7
MNU	1-Methyl-1-Nitrosourea
mTOR	Mammalian Target of Rapamycin
mTORC1	Rapamycin-Sensitive Complex
mTORC2	Rapamycin-Insensitive Complex
Myc	Myelocytomatosis Viral Oncogene Homolog
p70S6K	P70 Ribosomal S6 Kinase

p53	Tumour Protein P53
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
PDGF	Platelet Derived Growth Factor
PDK1	3-Phosphoinositide-Dependent Protein
PEG	*Polyethylene Glycol*
PF4	Platelet Factor 4
PI	Propidium Iodide
PIDD	Protein With A Death Domain
PI3K	The Phosphoinositide 3-Kinase
PS	Phosphatidylserine
Raptor	Rapamycin-Associated Protein Of Mtor
Rictor	Rapamycin-Insensitive Companion Of Mtor
RIPA	Radioimmunoprecipitation Assay
Ser/Thr	Serine/Threonine
SDR	Sprague Dawley Rat
SDS	Sodium Dodecyl Sulfate
SMAC	Second Mitochondria-Derived Activator of Caspases
STAT3	Signal Transducer And Activator of Transcription 3

TBS	Tris Buffer Saline
TBE	Trypan Blue Exclusion
TEB	Terminal End Buds
TEMED	Tetramethylethylenediamine
TGF	Tumour Growth Factor
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor Alpha
TNFR1	Type 1 TNF Receptor
TRADD	TNF Receptor-Associated Death Domain
VEGF	Vascular Endothelial Growth
VEGFR	Vascular Endothelial Growth Factor Receptor
WAP	Whey Acidic Protein

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KEBERKESANAN RAPAMYCIN DAN PF4 PADA MODEL HAIWAN INDUKSI MNU KANSER PAYUDARA DAN TITISAN SEL KANSER (MCF-7)

ABSTRAK

Terdapat bukti yang kukuh bahawa pertumbuhan tumor bukan sahaja akibat daripada proliferasi yang tidak terkawal tetapi juga apoptosis dikurangkan. Bax dan Bcl-2 adalah protein keluarga Bcl-2 yang mengawal proses apoptosis. Ia berfungsi sama ada sebagai anti-apoptosis (Bcl-2) atau pro-apoptosis (Bax). Perencat apoptosis (IAP), survivin juga sebagai anti-apoptotik, dimana ia mempromosi proliferasi sel dan memainkan peranan utama dalam perkembangan kanser. Disamping itu, caspase juga memainkan peranan penting untuk mengekalkan homeostasis sel. Oleh itu, kajian ini bertujuan untuk mengkaji kesan apapotik Rapamycin dan Platelet Faktor 4 (PF4) ke atas model kajian kanser payudara tikus betina melalui induksi 1-metil-1-nitrosourea (MNU) yang karsinogenik dan dalam titisan sel kanser MCF-7. Tindak balas ini seterusnya melihat kepada kesan laluan ekpresi keluarga Bcl-2, survivin dan keluarga caspase. Sebanyak seratus ekor tikus betina (SDR), berumur 21 hari diberikan MNU secara suntikan intraperitroneum (IP) untuk merangsang pembentukan kanser payudara. Apabila tumor mencapai saiz 14.5±0.5 mm, model tikus tersebut dibahagikan kepada beberapa kumpulan mengikut jenis rawatan yang diberikan seperti berikut; Kumpulan 1 (pra-intervensi, n=20 dan selepas intervensi, n=20), Kumpulan 2 (rapamycin, n=20), Kumpulan 3 (PF4, n=20) dan Kumpulan 4 (rapamycin+PF4, n=20). Perubahan morfologi tumor kemudian dinilai dengan menggunakan pewarna hematoksilin eosin (H&E) serta imunohistokimia (IHC) dengan menggunakan penanda pro-apoptosis (Bax), anti-apoptosis (Bcl-2), survivin

dan caspase-3, -6, -7, -8 dan -9. Di samping itu, MCF-7 telah digunakan untuk analisis secara *in vitro*. Kemudian MCF-7 diteruskan untuk analisis menggunakan sitometri aliran dan pemendapan western. Didapati bahawa taburan ekpresi Bax meningkat dengan tinggi dalam kumpulan yang dirawat dengan Rapamycin secara sangat signifikan ($p<0.001$) dan Rapamycin+PF4 secara signifikan ($p=0.007$) apabila dibandingkan dengan kontrol. Selain itu, ekpresi survivin menunjukkan penurunan yang sangat signifikan dalam kumpulan yang dirawat dengan PF4 dan Rapamycin+PF4 apabila dibandingkan dengan kontrol ($p<0.001$). Sebaliknya, ekspresi Bcl-2 didapati tidak berbeza dengan ketara dalam semua kumpulan rawatan. Caspase-3 menunjukkan ekspresi yang sangat signifikan dalam kumpulan yang dirawat dengan PF4 dan Rapamycin+PF4 secara sangat signifikan ($p<0.001$) apabila dibandingkan dengan kontrol dan juga kumpulan yang dirawat dengan rapamycin sahaja apabila dibandingkan dengan Rapamycin+PF4 dan kumpulan PF4 (masing-masing $p<0.001$). Selain itu, ekspresi caspase-6 menunjukkan taburan yang sangat signifikan dalam kumpulan rawatan Rapamycin, PF4 dan Rapamycin+PF4 apabila dibandingkan dengan kumpulan control ($p<0.001$). Selain itu, ekspresi caspase-7 menunjukkan ekspresi yang sangat signifikan dalam kumpulan yang dirawat sama ada dengan PF4 atau Rapamycin+PF4 apabila dibandingkan dengan kontrol ($p<0.001$). Walau bagaimanapun, kumpulan Rapamycin sahaja menunjukkan ekspresi caspase-8 yang lebih tinggi apabila dibandingkan dengan PF4 ($p<0.05$). Manakala ekspresi caspase-9 menunjukkan ekspresi yang signifikan ($p<0.05$) dalam kumpulan Rapamycin sahaja berbanding dengan kontrol dan Rapamycin+PF4 dan PF4 dirawat kohort ($p <0.001$). Rapamycin, PF4 dan Rapamycin+PF4 menunjukkan kesan perencatan pada IC_{50} 0.4 μg/ml, 6 μg/ml dan 0.4+1.0 μg/ml masing-masing. Rapamycin dan PF4, pada masa yang sama juga telah didapati tidak toksik kepada sel

HMEC. Tambahan pula, rapamycin, PF4 dan Rapamycin+PF4 menyebabkan gangguan kitaran sel di G0/G1, S dan G0/G1, masing-masing. Rapamycin, PF4 dan Rapamycin+PF4 mendorong peningkatan paras protein pro-apoptotik Bax dan penurunan paras protein anti-apoptotik Bcl-2 dan survivin. Paras protein caspases-3 dan caspase-8 konsisten dalam kumpulan yang dirawat apabila dibandingkan dengan kumpulan kontrol. Manakala paras protein -6, -7 dan -9 didapati telah meningkat apabila dirawat dengan Rapamycin, PF4 dan Rapamycin+PF4 apabila dibandingkan dengan kumpulan kontrol. Kesimpulan, kajian ini telah menemui hasilan baru tentang sifat mekanistik Rapamycin, PF4 dan Rapamycin+PF4 sebagai agen anti-kanser dalam kanser payudara model haiwan *in vivo* dan *in vitro*, MCF-7-bahagian sel. Pengenal pastian laluan isyarat kemungkinan melibatkan kematian sel secara apoptosis telah mengakibatkan gangguan kitaran sel telah dibuktikan sehingga ke peringkat molekular. Keputusan ini telah disokong melalui induksi apoptotik oleh Rapamycin, PF4 dan Rapamycin+PF4 yang mengaktifkan kedua-dua laluan intrinsik dan ekstrinsik melalui pengaktifan caspase-9, caspase-3 dan caspase-8. Selain itu, rawatan Rapamycin, PF4 dan Rapamycin+PF4 juga boleh merencatkan pertumbuhan kanser payudara dan mendorong apoptosis melalui caspase yang bergantung kepada laluan mitokondria dan mungkin menyebabkan kerosakan DNA.

ELUCIDATING THE EFFECTS OF RAPAMYCIN AND PF4 ON THE MNU INDUCED FEMALE RATS AND HUMAN BREAST CANCER CELLS (MCF-7)

ABSTRACT

There is a strong evidence that tumour growth is not just a consequence of uncontrolled proliferation but also of reduced apoptosis. Bax and Bcl-2 are Bcl-2 family like apoptosis regulator. They function either as suppressor (Bcl-2) or a promoter (Bax) of apoptosis. As a member of the inhibitors of apoptosis (IAP), survivin is also a promoter of cellular proliferation and thus a key player in cancer progression. Beside, caspases also occupy a central role in maintaining cellular homeostasis. Therefore, this research aims to examine the apoptotic effects of Rapamycin and Platelet Factor 4 (PF4) on 1-methyl-1-nitrosourea (MNU)-induced mammary carcinoma through Bcl-2- survivin and caspase modulated pathways in female Sprague Dawley Rat (SDR) and *in vitro* MCF-7 breast cancer cell lines. One hundred 21 days old female SDR were given an intraperitoneal injection (IP) of MNU to induce breast cancer formation. When tumour size reached 14.5±0.5mm, intratumoural injections of the following interventions were given; Group 1 (pre-intervention control, n=20 and post-intervention control, n=20), Group 2 (Rapamycin-treated, n=20), Group 3 (PF4-treated, n=20) and Group 4 (Rapamycin+PF4-treated, n=20). Tumour growth was then morphologically assessed using haematoxylin and eosin (H&E) and immunohistochemistry (IHC) utilizing pro-apoptosis (Bax) and anti-apoptosis markers (Bcl-2), survivin and caspases-3,-6,-7,-8, and -9. Besides that, the MCF-7 cell line was used for *in vitro* assessment. Initially, half maximal inhibitory concentration (IC_{50}) of each drug was determined. The MCF-7 cell lines were then exposed to Rapamycin and PF4 and Rapammycin+PF4 at IC_{50} concentrations, after

which they were subjected to flow cytometry and Western blot analyses. Bax was significantly expressed at higher levels in the rapamycin-treated and Rapamycin+PF4-treated groups than controls ($p<0.001$). Besides, survivin was significantly downregulated in the PF4-treated and Rapamycin+PF4-treated group when compared to controls ($p<0.001$). On the other hand, Bcl-2 expression was found not to be significantly altred in all treatment groups. Caspase-3, was significantly expressed at higher levels in both PF4-treated and Rapamycin+PF4-treated groups than controls ($p<0.001$), as well as rapamycin-only group when compared to Rapamycin+PF4-treated and PF4-treated groups (both $p<0.001$). Apart from that, caspase-6 was also significantly expressed in Rapamycin-treated, PF4-treated and Rapamycin+PF4-treated than the control groups ($p<0.001$). Besides, either PF4 or Rapamycin+PF4 combination was associated with increased caspase-7 expression, compared to the controls ($p<0.001$). However, Rapamycin-treated group showed a significantly higher caspase-8 expression when compared to PF4-treated ($p<0.05$). For caspase-9, higher caspase-9 expression was observed in Rapamycin-treated group when compared to control, Rapamycin+PF4-treated and PF4-treated cohorts ($p<0.001$). The IC_{50} Rapamycin, PF4 and Rapamycin+PF4 were 0.4 µg/ml, 6 µg/ml and 0.4 µg/ml+1.0 µg/ml respectively. Rapamycin and PF4, on the other hand, were non-toxic to the normal HMEC cells. Furthermore, Rapamycin, PF4 and Rapamycin+PF4 induced a cell cycle arrest in MCF-7 cell lines at G0/G1, S and G0/G1 phases, respectively. Rapamycin, PF4 and Rapamycin+PF4 induced the upregulation of pro-apoptotic Bax and the downregulation of anti-apoptotic Bcl-2 and survivin. The expression levels of caspase-3 and caspase-8 were consistent in the treated group when compared with the control group. The expression of caspases -6, -7 and -9 protein were increased when treated with Rapamycin, Rapamycin + PF4 and PF4 when compared with the control

group. In conclusion, this study provides new insights on the mechanistic properties of Rapamycin and PF4 as anti-cancer agents in breast cancer animal model and *in vitro* MCF-7 cell lines. The results lend support to the notion that apoptotic induction by Rapamycin and PF4 and was mediated by both the intrinsic and extrinsic pathways through the activation of caspase-9, caspase-3 and caspase-8 activation.

CHAPTER 1

INTRODUCTION

1.1 Overview On Breast Cancer

Breast cancer is the commonest malignancy in women and the second leading cause of cancer deaths. The latest 2012 report on the incidence and mortality from breast cancer showed that 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths are expected to arise among U.S. women (Ma & Jemal, 2013). On the other hand, the incidence and mortality of breast cancer rates over the past 20 years have sharply increased in economically less developed regions. Based on the GLOBOCAN report in 2008, it was estimated that half of the new worldwide breast cancer cases (1.38 million) and 60 % of the breast cancer deaths (458,000) occurred in developing countries (GLOBOCAN 2008 (IARC) 2008). Besides that, new data published by the National Cancer Institute in 2013 demonstrated that the number of new cases and death from breast cancer among women in the United States were 232,340 and 2,240, respectively. Based on this data the number of breast cancer cases is on the ascendancy.

As in Malaysia, breast cancer is the most common cancer in women because probably because men have less of the female hormones estrogen and progesterone, which can promote breast cancer cell growth. About 3,242 cases of breast cancer were diagnosed in women in 2007, contributing 18.1% of all reported cancer cases and 32.1% of total cancer cases in women (National Cancer Registry Report 2007). It has predilection for all major ethnic groups which include Malays, Chinese and Indians (Yip et al., 2006).

1.2 About The Breast

The breast is generally made up of fatty tissue. This tissue consists a set of network of lobes, which are composed of tiny, tube-like structures called lobules that houses milk glands (Azordegan et al., 2013; Rees & Bath, 2000). Tiny ducts fix the glands, lobules and lobes, carrying the milk from the lobes to the nipple, which is positioned at the middle of the areola (the darker spot that surrounds the nipple) (Figure 1.1). Blood and lymphatic vessels also run throughout the breast; blood nourishes the cells and the lymphatic channels drain physical waste products (Heys, 2011; Tse et al., 2013). The lymphatic vessels connect to lymph nodes, the tiny, bean-shaped organs that play paramount roles in immunological defense against infections.

The mammary glands of mammalians are unique since it evolved into an organ that is specialized in synthesizing, secreting, and distributing milk to the newborns which is critical for their optimal nutrients, protection, and development (Nguyen et al., 2011). In humans, the female mammary gland lifespan is characterized by harsh changes in architecture, composition, and functionality, mediated by obvious changes in gene appearance, that distinguishes its physiological stages of development, all of which are intended at allowing it to perform its function as a milk-producing organ upon the birth of the infant (Hassiotou & Geddes, 2013). The key developmental milestones of mammary glands consist of fetal growth, pubertal expansion, infant (pre-pubertal) growth, lactation and pregnancy associated remodeling, and post-lactation and post-menopausal involution (Hassiotou & Geddes, 2013). A vital knowledge on the anatomy, development, physiology, and regulation of the breast is important in the understanding of both the normal biology

and function of this organ and its benign or malignant pathologies which may ensure a successful implementation of relevant treatments on each malady.

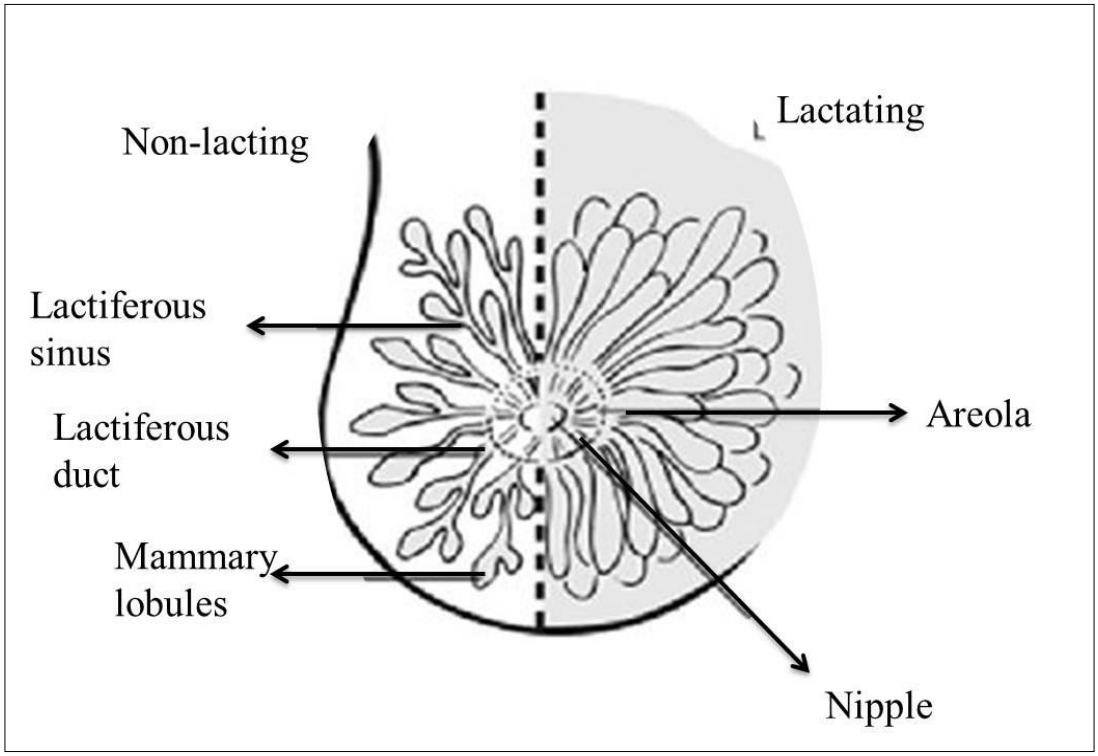

Figure 1.1 Anatomy of the breast and chest wall (Heys, 2011).

1.3 Breast Cancer Pathogenesis

Cancer exists as a result of mutations, or abnormal changes, in the genes responsible for regulating cellular growth and its physiological maintenance (Børresen-Dale et al., 2010; Voduc et al., 2010). The genes are in each cell's nucleus, which acts as the "control room" of each cell. Generally, the cells in human bodies restore themselves through an orderly process of cell growth: healthy new cells take over as old ones senesced and withered (Weigelt et al., 2010). However, as time goes by, mutations can activate certain genes and deactivate others in a cell. That genetically-altered cell gains the ability to keep proliferating in such a disorderly fashion, producing identical daughter cells which eventually culminate in the formation of a tumour (Weinberg, 2013).

There are two types of tumour; benign (not dangerous to health) and malignant (has the potential to be dangerous) (Bhattarai et al., 2011). The benign tumours are considered non-cancerous: their cells are genetically and morphologically similar to the normal ones; they grow much gradually and do not invade adjacent tissues or disseminates to the other parts of the body (Kennecke et al., 2010; Liu et al., 2011). Malignant tumours are, on the contrary, cancerous. Left unchecked, malignant cells can ultimately spread beyond the tumuor's primary site to other organs in the body (Chang et al., 2011; Kennecke et al., 2010).

Breast cancer therefore refers to any malignant tumour that originates from normal mammary cells (Bombonati & Sgroi, 2011; Chang et al., 2011). Generally, breast cancer either begins in the cells of the lobules, which are the milk-producing

glands, or the ducts, the passages that drain milk from the lobules to the nipple (Bombonati & Sgroi, 2011; Rakha et al., 2006). Less commonly, breast cancer may also begin in the stromal tissues, which include the fatty and fibrous connective tissues of the breast (Fentiman & D'Arrigo, 2004; Voduc et al., 2010).

Breast cancer can be classified into biologically and clinically different entities according to its histological type (Santagata et al., 2014) and histological grades, both carry different prognostic values (Horlings et al., 2013; Santagata et al., 2014). Tumour grade is measured by the degree of histological and cytological differentiation (i.e. tubule formation and nuclear pleomorphism) and the proliferative potential (i.e. mitotic index) of the tumour, both reflect its aggressiveness (Dinkel et al., 2000). It has also been included as one of the prognostic determinants in various validated prognostic staging systems such as the Nottingham Prognostic Index and Adjuvant! Online, from which the accurate tailoring of breast cancer treatment can be made possible (Horlings et al., 2013; Narbe et al., 2014). Interestingly, breast cancer histological grade has been shown to correlate well with its genetic and transcriptomic features and these observation has led to the devising of microarray-based genomic signatures which are useful for prognostic and research purposes (Garcia-Closas et al., 2013; Horlings et al., 2013).

Contrarily, histological type of breast cancer refers to the tumour growth patterns (Horlings et al., 2013). Pathologist has long been fascinated with the histological diversity of breast cancers and they have identified specific cytological and morphological patterns that are consistently and well associated with the spectrum clinical presentations. The most common type of breast carcinoma is

invasive ductal carcinomas not otherwise specified (IDC-NOS) or of no special type (IDC-NST) (Narbe et al., 2014; Weigelt et al., 2010) which is diagnosed by exclusion and comprises of adenocarcinomas that are devoid of any adequate histological characteristics that may allow them to be classified as one of the specific subtypes of breast cancers (Lacroix-Triki et al., 2010).

Specific types of breast cancer account for up to 25% of all breast cancer cases and in the latest edition of the World Health Organization classification for breast cancer recognizes the existence of at least 17 distinct histological special types (Lacroix-Triki et al., 2010). It is worth noted that information on tumour grade and type supplement each other (Bombonati & Sgroi, 2011). Even though grade identifies prognostic subgroups among special types of breast cancer, some histological types of breast cancer that are made of poorly differentiated cells (high histological grade) may have a relatively good prognosis (e.g. medullary carcinomas) (Dinkel et al., 2000; Huober et al., 2012).

1.4 Morphogenesis of Normal Mammary Gland

Mammary glands are epidermal appendages that are hypothetically developed from the evolutionarily-ancient apocrine glands which were connected to the skin (Hens & Wysolmerski, 2005). The major function of the mammary gland is to supply nutrition for the young in the form of milk fat and protein (Kent et al., 2013; Tiede & Kang, 2011). Apart from that, there are other benefits that are provided by lactation, for instance the provision of immune factors that are secreted into the milk and the nurturing of mother-infant during breastfeeding might confer developmental benefits to the newborns (Noel-Weiss et al., 2010). The mammary gland is a unique secretory organ that consists of a number of dissimilar cell types: epithelial cells that form the ductal network of the gland; adipocytes, which comprise the fat pad and in which the ductal network is implanted; vascular endothelial cells, which make up the blood vessels; stromal cells, including fibroblasts; and a variety of immune cells (Howlin et al., 2006). The mammary gland consists of two main epithelial types: luminal and basal (Sopel, 2010). The basal epithelium consists of myoepithelial cells, whereas luminal epithelium forms the ducts and the secretory alveoli (Sopel, 2010; Van Keymeulen et al., 2011). These two types of epithelium form a bi-layered structure of simple epithelium that is surrounded by the fatty stroma (Sakakura et al., 2013; Sopel, 2010).

There are three main steps of mammary gland development, both in rodents and humans: embryonic, pubertal and adult (Van Keymeulen et al., 2011). Growth factors and hormones play fundamental roles in these different steps of normal mammary development and its antipode, breast cancer (Brisken & O'Malley, 2010). The mammary gland is an ideal tissue to study human developmental processes. In

the embryo, the cellular signaling pathways that stimulate/induce the formation of mammary placodes from the skin are yet to be fully elucidated (Sakakura et al., 2013). Besides that, similar mechanistic pathways have also been discovered in the developing stages of other anatomical appendages, such as teeth and feathers (Forsmanl & Schwertfegerz, 2013), suggesting the crucial role they play. Once born, mammary development persists until puberty, during when extensive elongation of the ducts, accompanied by secondary branching, takes place. This provides a readily reachable system in which the study of branching morphogenesis is made feasible (Sakakura et al., 2013).

During pregnancy, mammary glands undergo further development which have these striking features; formation of tertiary branches, which end in alveolar buds and the quick proliferation of the luminal epithelium followed by subsequent differentiation and commitment to the secretory alveolar lineage (Forsmanl & Schwertfegerz, 2013) (Figure 1.2). A lactogenic key occurs during late pregnancy that is accompanied by the appearance of the milk proteins, whey acidic protein (WAP) and α-lactalbumin, and by the formation of lipid droplets (Barbieri, 2013; Kommagani et al., 2013). Following lactation, elimination of the now surplus alveolar cells is accomplished by cell death (apoptosis). Post-lactational deterioration, or involution, is the most remarkable example of physiologically regulated apoptosis in an adult tissue (Kommagani et al., 2013).

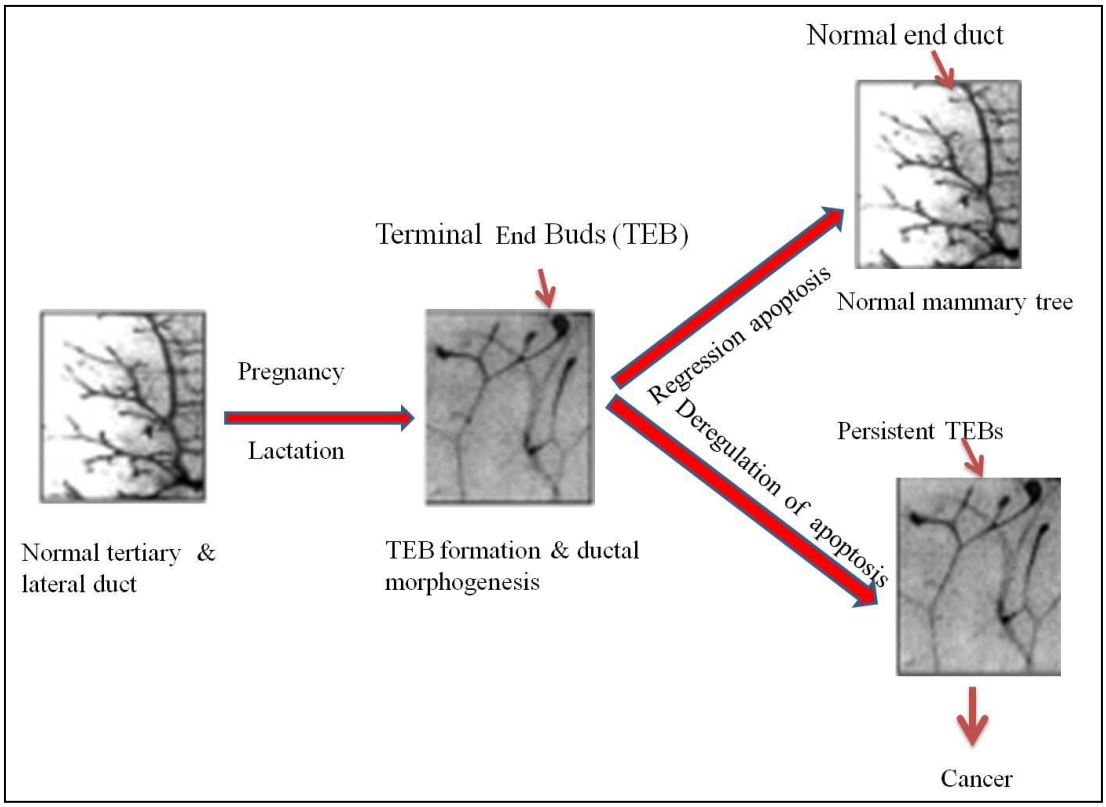

Figure 1.2 Schematic representation of various stages of mammary gland development (Forsmanl & Schwertfegerz, 2013).

1.5 1-Methyl-1-Nitosourea (MNU) Induced Breast Cancer In Rats

The use of animal models in the study of carcinogenesis is cardinal in investigating the mechanisms of carcinogenic interactions because the implicating agents can be clearly defined. The murine breast cancer model is a commonly used model for human breast cancer because of the similarities in hormonal dependence histopathological characteristics and the ease of tumour induction by the easily available breast cancer-causing carcinogens such as 7,12-dimethylbenz[a] anthracene (DMBA) and 1-methyl-1-nitrosourea (MNU) (Imaoka et al., 2009; Medina, 2007; Nandi, 2006). Therefore, the two carcinogenic substances are considered good choices for the induction of cancers in most studies investigating mammary carcinogenesis and breast cancer therapeutics.

The features of MNU-induced and DMBA-induced models have been previously reviewed (Imaoka et al., 2014; Macejova & Brtko, 2001; Shirai et al., 1997). They are alike in terms of reliability of tumour induction, organ site specificity and production of tumours with varying hormone responsiveness (Tsubura et al., 2011). Both provide the means for studying the attributes of tumour initiation, promotion and progression even though carcinomas induced by either carcinogen can rarely metastasize.

In spite of such similarities, there are some notable differences that set these two murine breast cancer models apart. The MNU model is used for investigating mammary tumourigenesis induced by a direct acting carcinogen, whereas the DMBA model provides a platform for studying mammary tumorigenesis induced by a pre-carcinogen requiring enzymatic-metabolic activation (Shirai et al., 1997). The MNU induced mammary carcinomas have a more aggressive histological pattern compared to those induced by DMBA and MNU-induced tumours have a higher incidence of adenocarcinomas (Medina, 2007). Furthermore, MNU-induced tumors appear to be more estrogen-dependent compared to DMBA-induced tumours which is more dependent prolactin for tumor progression (Medina, 2007). Since this study aims to examine the effects of both rapamycin and PF4 on the most ubiquitously prevalent human breast cancer, the MNU-induced murine breast cancer model is chosen for this study.

The administration of MNU at a dose of 50 mg/kg body weight in rats aged 21 days has been shown to be a rapid method for inducing mammary tumour with higher production of premalignant lesions including intraductal proliferations (IDP)

and ductal carcinoma in situ (DCIS) than other methods (Choudhuri et al., 2012; Nandi, 2006; Shirai et al., 1997). This latter feature has proved the model to be useful in studying breast cancer carcinogenesis at its earliest stage.

The times to the first appearance of cancerous lesions are varied among the many types of pre-malignant and malignant lesions (Tsubura et al., 2011). For instance, IDP occurs 14 days post MNU induction, whilst the DCIS and adenocarcinomas appear 21 days post MNU-induction (Choudhuri et al., 2012). This model has been used widely as a preclinical *in vivo* model for evaluating the potential efficiency of an agent in breast cancer treatment. Again, based on these favourable characteristics, MNU-induced murine breast cancer model is deemed the most suitable model for this study.

1.6 Programmed Cell-Death

Generally, programmed cell-death **(**PCD) is the death of the cell in any types, it was determined by an intracellular program (Fuchs & Steller, 2011). PCD is confessed out in a structured process, which frequently intended superior during an organism's life-cycle (Ouyang et al., 2012). For example, the toes differentiation and fingers in an emerging human embryo occurs because cells between the fingers apoptose; the effect is that the digits are separate. PCD explores fundamental functions during both plant and metazoa (multicellular animals) tissue development (Walsh, 2014). Apoptosis and autophagy are both forms of programmed cell death, but necrosis is a non-physiological process that occurs as a result of infection or injury (Walsh, 2014). The other types of programme cell death were included cornification, mitotic catastrophe, anoikis, excitotoxicity, paraptosis, pyroptosis, pyronecrosis and entosis (Kroemer et al., 2005).

In 2005, the The Nomenclature Committee on Cell Death (NCCD) has formulated recommendations in the Cell Death and Differentiation (Kroemer et al., 2005). The NCCD main function was provided the forum in describing the distinct modalities of cell death are critically evaluated and recommendations on their definition and use are formulated, hoping that a non-rigid, yet uniform, nomenclature will facilitate the communication among scientists and ultimately accelerate the pace of discovery. The NCCD also provided an updated guideline on the cell death study.

1.6.1 Necrosis

'Necrotic cell death' or 'necrosis' is morphologically distinguished by an acquire in cell volume (oncosis), plasma membrane rupture, swelling of organelles and subsequent loss of intracellular contents. For years, necrosis have been considered simply as an accidental uncontrolled form of cell death, but facts are accumulating that the execution of necrotic cell death may be delicately modulated by a set of signal transduction pathways and catabolic mechanisms (Golstein & Kroemer, 2007). For instance, death domain receptors (e.g., TNFR1, Fas/CD95 and TRAIL-R) and Toll-like receptors (e.g., TLR3 and TLR4) have been shown to induce necrosis, in particular in the presence of caspase inhibitors. TNFR1-, Fas/CD95-, TRAILR- and TLR3-mediated cell death, seemingly depends on the kinase RIP1 (Mocarski et al., 2011) as this has been demonstrated by its knockout/knockdown and chemical inhibition with necrostatin-1 (Degterev et al., 2013). Although there is no comprehensive agreement on the use of this expression, some researchers have suggested the term 'necroptosis' to specify regulated (as opposed to accidental) necrosis. At a biochemical level, necroptosis may be defined as a type of cell death that can be avoided by inhibiting RIP1 (either through genetic or pharmacological methods) (Ofengeim & Yuan, 2013) which may represent a convenient means to discriminate between programmed and fortuitous forms of necrosis. Thus far, however, there is no consensus on the biochemical changes that may be used to unequivocally identify necrosis. In the absence of a common biochemical denominator, necrotic cell death is still largely identified in negative terms by the absence of apoptotic or autophagic markers, in particular when the cells undergo early plasma membrane permeabilization (as compared with its delayed

occurrence, which is associated with late-stage apoptosis). For these reasons, caution should be used in classifying particular cell death routines as necrotic.

1.6.2 Apoptosis

Apoptosis or programmed cell death (PCD) is an active, energy-dependent process of cell death which occurs during development, in response to certain physiologic stimuli and secondary to cell injury and stress (Elmore, 2007; Gewies, 2003; Giansanti et al., 2011). The purpose of this type of cell death is for regulating the removal of damaged and unwanted cells in discrete tissues, a critical function in embryonic development and normal tissue homeostasis. It differs from necrotic cell death because the damaged cells are eliminated by PCD in a fashion without eliciting intense inflammatory response (Ashkenazi & Salvesen, 2014).

The term apoptosis had been coined in order to explain the morphological processes principal to controlled cellular self-destruction which was first discovered by Kerr, Wyllie and Currie (Czabotar et al., 2014). Apoptosis is of Greek origin, meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of a living matter is an important and often a necessary part of the life cycle of any organism.

Apoptosis is an active and clearly-defined process which has monumentally contributed to multicellular organisms's development and the regulation and preservation of the cell populations in tissues under physiological and pathological conditions. It should also be stressed that apoptosis is possibly the most common

form of programmed cell death, but other non apoptotic types of cell death might also be of biological significance (Portt et al., 2011).

Apoptosis is an orderly and tightly controlled series of events which culminates in coherent gene expression with specific proteomic signatures. It has a major function in normal growth and differentiation of organ systems and appears to be involved in a variety of human disorders (Delbridge et al., 2012).

There are two gene families which are critical in the control of apoptosis; interleukin-1–converting enzyme (ICE) family of cysteine proteases (caspases) and proto-oncogene Bcl-2-related genes. Both of these families are homologous to cell death genes in *Caenorhabditis elegans* (*C. elegans*) (Potts & Cameron, 2011). Proteolytic activity plays a special role in many apoptotic systems, the ICE family being of special importance, since it seems to be central in Fas-mediated and tumor necrosis factor (TNF)-induced apoptosis (Azahri & Kavurma, 2013; Waters et al., 2013).

New evidence suggests that Bcl-2 protein has two different functions: *1*) as an ion channel protein and *2*) as an adaptor/docking protein through its binding to several other proteins which are important in modulating apoptosis (Bortner & Cidlowski, 2014). However, the precise way in which these proteins modulate apoptosis remains unclear and conflicting theories have been proposed. Further, the gene product of Bcl-2 does not prevent apoptosis under all conditions (e.g., does not protect target cells from apoptosis induced by cytotoxic T cells) (Bagci et al., 2006).

Finally, genes involved in cellular differentiation and proliferation are also important in modulating the apoptotic process (e.g., the c-Myc, the p53, and the apoptosis suppressor gene A20). Both c-Myc and p53 are incriminated in the induction of apoptosis under certain conditions, whereas A20 is a cytokine-induced primary response gene involved in the inhibition of the apoptotic process (Portt et al., 2011).

1.7 Morphology And Biochemical Features

Apoptotic cells can be recognized by distinct morphological changes such as cell shrinkage, cellular deformation with the loss of contact with its neighbouring cells (Figure 1.3). Apart from those above, apoptotic cell chromatin condenses and marginates to the nuclear membrane with plasma membrane blebbing or budding, before culminating in cellular fragmentation and apoptotic bodies formation, a closely-compact membrane containing structure which is composed of cytosol, condensed chromatin, and organelles (Mariño et al., 2014). The apoptotic bodies are subsequently engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response (Suzanne & Steller, 2013).

Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most particularly the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Suzanne & Steller, 2013). Apoptosis is in contrast to necrosis in which

the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are uncontrollably released into the cell's microenvironment, resulting in severe damage to the surrounding cells and a strong inflammatory response by the necrotic tissues (Table 1.1) (Nanji & Hiller-Sturmhöfel, 1997).

Table 1.1 Differential features of apoptosis and necrosis.

Differential features of apoptosis and necrosis	
Apoptosis	Necrosis
Affects single cell	Affects groups of neighboring cells
No inflammatory response	Significant inflammatory response
Cell shrinkage	Cell swelling
Membrane blebbing with maintained cellular integrity	Loss of membrane integrity
Increased mitochondria membrane permeability release of proapoptotic protein and formation of apoptotic bodies	Organelle swelling and lysosomal leakage
Chromatin condensation and non-random DNA fragmentation	Random degradation of DNA
Apoptotic bodies ingested by neighboring cells	Lysed cell ingested by macrophage.

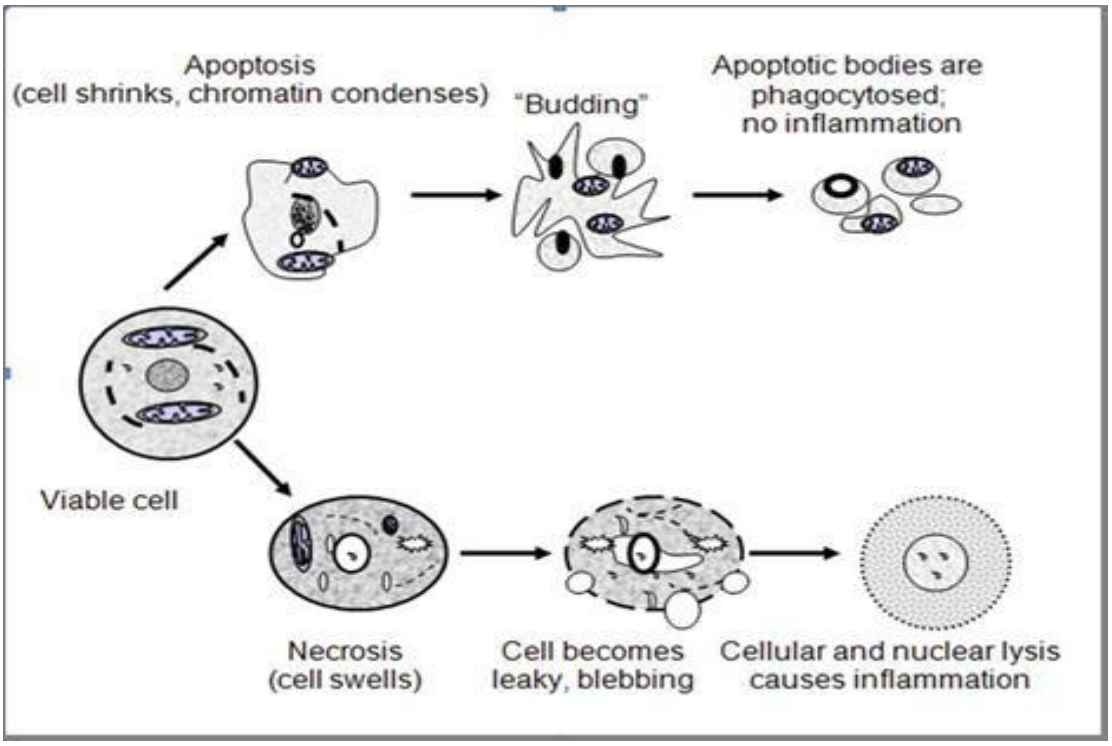

Figure 1.3 Morphological comparisons between apoptotic and necrotic cell deaths. Key features of apoptosis that includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies, comprising organelles, cytosol and nuclear fragments that are subsequently phagocytosed without triggering inflammatory processes. On the contrary, the necrotic cell swells and becomes leaky with the eventual disruption of membrane which results in the release of cellular contents into the surrounding tissue, triggering inflammation (Nanji & Hiller-Sturmhöfel, 1997).

Apoptotic cells usually display a distinctive constellation of biochemical modifications that underlie the structural pathology. Some of these features are shared by necrotic cells as well, but other alterations are more necrosis-specific. Caspases cleavage of the nuclear scaffold and cytoskeletal proteins (together with protein cross-linking) underlies the distinctive nuclear and cytoplasmic structural alterations solely seen in apoptotic cells. Caspase activity also triggers endonucleases (Ashkenazi & Salvesen, 2014).

Extensive protein cross-linking by transglutaminase activation converts cytoplasmic proteins into covalently linked shrunken shells that may break into apoptotic bodies (Szondy et al., 2011). Apoptotic cells demonstrates a characteristic feature of DNA break into large 50-kilobase to 300-kilobase pieces. Subsequently, there is internucleosomal cleavage of DNA into oligonucleosomes in multiples of 180 to 200 base pairs by Ca^{2+}- and Mg^{2+}- dependent endonucleases (Bortner et al., 1995). The fragments are visualizable by agarose gel electrophoresis as DNA ladders. Endonuclease activity also forms the basis for detecting cell death by cytochemical techniques that recognize the double-stranded DNA breaks. However, internucleosomal DNA cleavage is not specific for apoptosis (Nagata, 2000). Moreover, the "smear" pattern of DNA fragmentation thought to be indicative of necrosis may only be a late autolytic phenomenon, and typical DNA ladders can also be seen in necrotic cells as well (Widlak & Garrard, 2009).

Apoptotic cells have phosphatidylserine in the outer layers of their plasma membranes, the phospholipids having been "flipped" out from the inner layers. In some types of apoptosis, thrombospondin, an adhesive glycoprotein, is also expressed on the surfaces of apoptotic bodies (Hengartner, 2001). These alterations let the early detection of the dead cells by macrophages and adjacent cells for phagocytosis, without the discharge of proinflammatory cellular components. In this way, the apoptotic response disposes undesirable cells with minimal compromise to the surrounding tissue.

1.7.1 Mechanism Of Apoptosis Pathway

The apoptosis mechanisms are extremely multifarious and complicated, linking each key player into an energy-dependent cascade of molecular events (Figure 1.4). Currently, research shows that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Wong, 2011). Nevertheless, it is now verified that the two pathways are mutually linked and that a principal regulator in one pathway can also play a major part in the other pathway (Bagci et al., 2006; Prehn et al., 2013). Besides these two, there is also another pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cells.

The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways eventually converge on the same terminal apoptotic event in the form an execution pathway. This pathway is initiated by the cleavage of caspase-3 which leads to DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvalet et al., 2005).

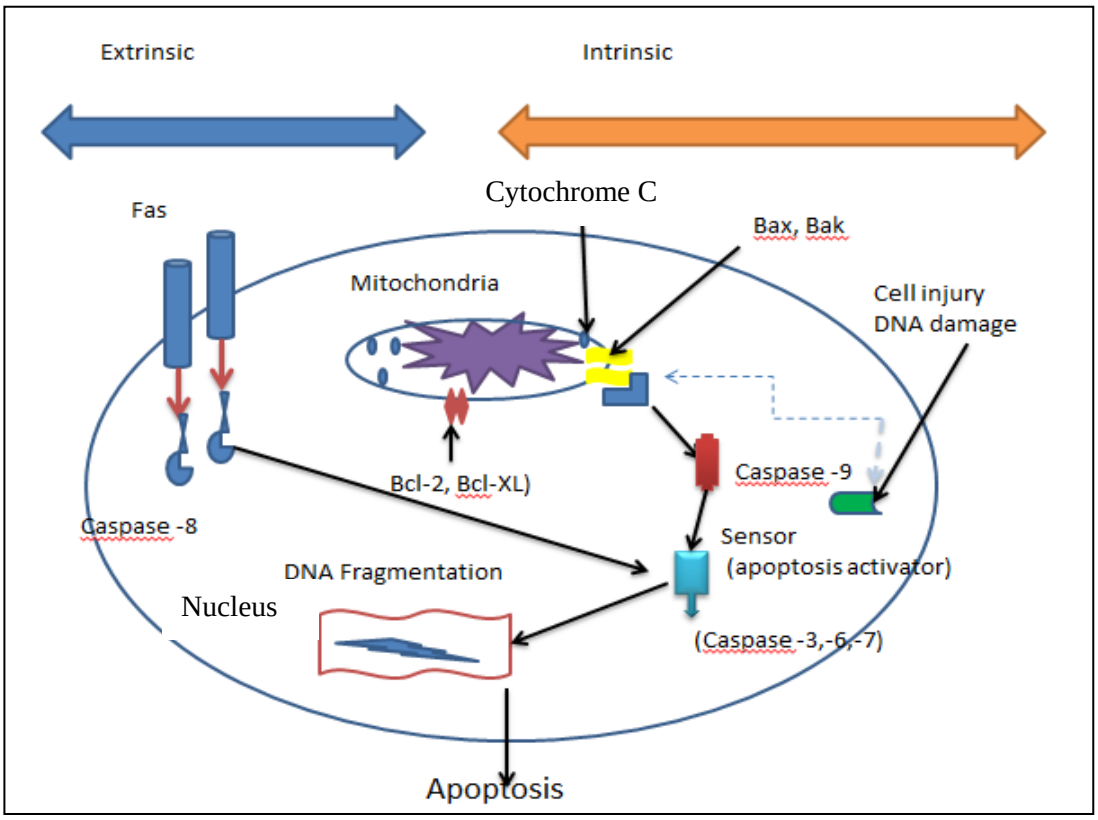

Figure 1.4 Schematic representation of apoptotic events (Dabbah & Rajei, 2013).

The major pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Every pathway requires particular triggering signals to initiate an energy-dependent cascade of molecular events. Every pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase-3. On the opposite, granzyme A pathway is executed in a caspase-independent style. The execution pathway results in typical cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Ashkenazi & Salvesen, 2014).

1.7.1.1 Extrinsic Pathway (Death receptor)

The extrinsic pathways of apoptosis involve transmembrane ligand-death receptor interactions. These death receptors are genetically belonged to the tumour necrosis factor (TNF) receptor gene superfamily members (Hengartner, 2001; Wang et al., 2012). TNF receptor family members share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids known as death domain (Aggarwal et al., 2012). This death domain plays an important role in transmitting the death signal from the cell surface to the intracellular signaling pathways. Among the best-characterized ligands and their corresponding death receptors include fatty acid synthetase ligand (FasL) / fatty acid synthetase receptor (FasR), Tumour necrosis factor alpha (TNF-α) / type 1 TNF receptor (TNFR1), Apo3 ligand (Apo3L) / Death receptor 3 (DR3), Apo2 ligand (Apo2L) /DR4 and Apo2L/DR5 (Dickens et al., 2012).

The sequences of events that describe the extrinsic pathway of apoptosis are best illustrated with the FasL/FasR and TNF-α/TNFR1 models (Rubio-Moscardo et al., 2005). In these models, there is a clustering of receptors when homologous trimeric ligands bind to them. Cytoplasmic adapter proteins are then recruited after successful binding, which possess corresponding death domains that bind with the death receptors. Accordingly, the binding of Fas ligand to Fas receptor to the adapter protein Fas-associated death domain (FADD) and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TNF receptor-associated death domain (TRADD) with

recruitment of FADD and RIP (Schneider & Tschopp, 2000). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this stage, a death-inducing signaling complex (DISC) is produced, an essential component in the auto-catalysis of procaspase-8 (Kischkel et al., 1995).

Once caspase-8 is activated, the execution phase of apoptosis is thus activated. Death receptor-mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective (Chang et al., 2002; Scaffidi et al.,1999). Another potential target for apoptosis inhibition involves a protein called Toso, which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (Hitoshi et al., 1998; Song & Jacob, 2005).

1.7.1.2 Intrinsic Pathway (Mitochondrial-dependent pathway)

The intrinsic pathway is regulated at the mitochondrial level and responsive to both extracellular stimuli and internal insults such as DNA damages (Elumalai et al., 2012). This pathway is set off via alterations in mitochondrial membrane potential and increased permeability of the membrane, resulting in the leakage of cytochrome *c*, a protein important to mitochondrial respiration. In the cytosol, cytochrome *c* binds to apoptosis activating factor (Apaf-1) to form a complex known as apoptosome, which in turn activates caspase-9 (Rogalska et al., 2013). At this juncture, if the balance between pro-apoptotic and inhibitory control is tipped in favor of cell death, the caspase cascade is initiated resulting in the withering of the cells (Tait & Green, 2010).

1.8 Bcl-2 Family

The Bcl-2 family proteins play a critical role in apoptosis regulation (Shamas-Din et al., 2013). Changes in the levels or bioactivities of these proteins are necessary for diverse physiological processes where programmed cell death are required for the maintenance and maturation of normal anatomical and physiological development such as during fetal growth, haematopoietic and immune cell differentiation, oogenesis, mammary gland involution and normal cell turnover in the epidermis, gut and other tissues (Carson & Ribeiro, 1993; Chipuk et al., 2010; Kerr et al., 1972). Moreover, pathological alterations in the expression of Bcl-2 proteins family have been identified as one of the main culprits in carcinogenesis, autoimmunity, immunodeficiency, heart failure, stroke and other pathologies (Favaloro et al., 2012; Kelly & Strasser, 2011; Nagata, 2010; Papathanasiou et al., 2012). Consequently, methods for assessing the relative levels and bioactivities of Bcl-2 proteins family are extremely important to scientists in a broad range of disciplines.

The Bcl-2 oncoprotein is an inhibitor of apoptotic cell death (Ola et al., 2011). Many experiments which involve cells that are genetically transfected with Bcl-2 mutations have shown that elevated levels of this protein can protect a wide variety of cells from various cell death stimuli ranging from growth factor withdrawal and cytotoxic lymphokines to virus infection and DNA-damaging, anticancer drugs and radiation (Fuchs & Steller, 2011). The 26-kD Bcl-2 resides on the cytoplasmic surface of the mitochondrial outer membrane,