CELL DEATH MECHANISM INDUCED BY 15-DEOXY PROSTAGLANDIN J_2 AND 17β-ESTRADIOL IN ER-POSITIVE AND ER-NEGATIVE BREAST CANCER CELL LINES

By

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MEKANISME KEMATIAN SEL CETUSAN 15-DEOXY PROSTAGLANDIN J_2 DAN 17β-ESTRADIOL DALAM SEL KANSER PAYUDARA ER-POSITIF DAN ER-NEGATIF

Oleh

RABAIL NASIR AZIZ

Tesis yang diserahkan untuk memenuhi keperluan bagi
Ijazah Doktor Falsafah

JANUARI 2011

DEDICATION

Especially for….

My beloved parents, Professor Dr. Nasir Aziz Kamboh and Naila Noureen.

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LIST OF ABBREVIATIONS

15-deoxy prostaglandin J_2	15d-PGJ_2
17β-estradiol	E2
Ammonium persulfate	APS
Adenosine 5'-triphosphate	ATP
American Type Culture Collection	ATCC
Apoptosis protease activating factor	Apaf
Apoptosis inducing factor	AIF
Arachidonic acid	AA
Base pair	bp
B-cell lymphoma	Bcl-2
Bcl-2 antagonist killer	BAK
Bcl-2-associated death promoter homologue	BAD
Bcl-2 -associated X protein	BAX
Beta-mercaptoethanol	β-ME
BH3-domain only death agonist	Bid
Bovine serum albumin	BSA
Calcium	Ca^{2+}
Caspase Associated Recruitment Death Domain	CARD
Complementary deoxyribonucleic acid	cDNA
Cyclin dependent kinase	CDK
Cyclin Dependent Kinase Inhibitor	CKI
Cysteinyl-aspartic acid proteases	Caspases
Cytokeratin-18	CK-18
Death Receptors	DR
Death Inducing Signaling Complex	DISC
Death Effector Domain	DED
Deoxyribonucleic acid	DNA
DNA binding domain	DBD
Diethylpyrocarbonate	DEPC
Dimethyl sulfoxide	DMSO
Dulbeco's Modified Eagle's Medium	DMEM
Effective concentration that causes 50 % drug response	EC_{50}
Endonuclease G	EndoG
Estrogen Receptor	ER
Estrogen Receptor alpha	ERα
Estrogen Receptor beta	ERβ
Ethidium bromide	EtBr
Ethylenediamine-tetra acetic acid	EDTA
Extracellular signal regulated kinase	ERK
Fetal bovine serum	FBS
FAS-associated death domain	FADD
Fas Ligand	FasL
FADD-like interleukin-1beta-converting enzyme	FLICE
Gap 1 phase	G1

Gap 2 phase	G2
Gram	g
Human epidermal growth factor receptor 2	HER2
Hour	h
Horseradish peroxidase	HRP
Inhibitor of Apoptosis Protein	IAP
Jun N-terminal Kinase	JNK
Kilo Dalton	kDa
Lactate dehydrogenase	LDH
Ligand binding domain	LBD
Litre	L
Messenger Ribonucleic acid	mRNA
Milligram	mg
Mitochondrial membrane potential	MMP
Mitogen activated protein kinase	MAPK
Mitosis	M
Molecular weight	MW
N,N,N'N'-Tetra-methylethylenediamine	TEMED
Necrosis factor-kappa B	NF-κB
Nomenclature committee on cell death	NCCD
Non-steroidal anti-inflammatory drugs	NSAIDs
Nuclear Receptor	NR
Peroxisome proliferator-activated receptor	PPAR
Peroxisome proliferator-activated receptor response element	PPRE
Peroxisome proliferator-activated receptor alpha	PPARα
Peroxisome proliferator-activated receptor beta	PPARβ
Peroxisome proliferator-activated receptor gamma	PPARγ
Phenylmethylsulfonyl fluoride	PMSF
Phosphate-buffered saline	PBS
Phosphoinositol-3-kinase	PI3K
Programmed cell death	PCD
Poly adenosine di-phosphate-ribose polymerase	PARP
Polyacrylamide gel electrophoresis	PAGE
Propidium iodide	PI
Prostaglandins	PG
Protein 53	p53
Quiescence phase	G_0
Reactive oxygen species	ROS
Receptor-interacting protein	RIP
Retinoid X receptor	RXR
Ribonucleic acid	RNA
Roselle's Park Memorial Institute Medium	RPMI
Serine/threonine kinase	AKT
Selective estrogen receptor modulator	SERM
Sodium dodecyl sulfate	SDS
Standard deviation	SD
Tumour Necrosis Factor	TNF

TNF Receptor-1	TNFR-1
TNF receptor-related apoptosis inducing ligand	TRAIL
TNFR-1 associated death domain protein	TRADD
Tris borate EDTA	TBE
Synthesis phase	S
Volume/volume	v/v
Weight/volume	w/v
X-galactosidase	X-gal

LIST OF SYMBOLS

Alpha	α
Approximately	~
Beta	β
Degree Celcius	°C
Delta	δ
Gamma	γ
Kappa	κ
Less than	<
Micro	μ
Psi	Ψ
Registered	®
Trademark	™

MEKANISME KEMATIAN SEL CETUSAN 15-DEOXY PROSTAGLANDIN J_2 DAN 17β-ESTRADIOL DALAM SEL KANSER PAYUDARA ER-POSITIF DAN ER-NEGATIF

ABSTRAK

Kanser payudara adalah kanser yang paling utama di kalangan wanita Malaysia. Peningkatan insiden penyakit ini di seluruh dunia menunjukkan kepentingan terhadap kajian untuk mengkaji dan mengenal pasti terapi yang lebih berkesan dan efektif untuk melawan kanser payudara. Penyelidikan terkini adalah kajian tindakan drug yang memberi kesan sampingan minimum dan juga memberi pemahaman yang lebih mendalam mengenai tindakan dan kerintangan sel tumor terhadap drug tersebut. Reseptor teraktif pemploriferasi peroksisom gamma (PPARγ) adalah reseptor nukleus bergantung pada ligand, yang dilaporkan nyatakan dalam pelbagai sel kanser termasuk payudara, prostat, kolorektal dan kanser pangkal rahim. Ligand untuk reseptor ini didapati telah merencat pertumbuhan sel kanser melalui apoptosis dan menghalang proliferasi akibat pengaktifan PPARγ. Walaupun begitu, peranan sebenar reseptor dan ligand ini, masih dalam kajian, terutama dalam sel kanser payudara. Kajian ini dilakukan untuk menyelidik kesan ligand endogenus PPARγ, iaitu 15 deoksi-prostaglandin J_2 (15d-PGJ_2) ke atas sel-sel kanser payudara manusia yang positif reseptor estrogen (ER) (MCF-7) dan negatif ER (MDA-MB-231) pada dengan kehadiran atau ketiadaan ligand ERα, iaitu 17 β-estradiol (E2). Kombinasi rawatan sel dengan 15d-PGJ_2 dan E2 bertujuan untuk mengkaji hubungan pengisyaratan antara PPARγ dan ERα. Kajian sitotoksik menunjukkan bahawa 15d-PGJ_2 menghalang proliferasi sel MCF-7 dan MDA-MB-231 pada nilai EC_{50} antara 15 dan 10 μM. Kajian berikutnya menunjukkan bahawa 15d-PGJ_2 menghalang

proliferasi kedua-dua sel secara apoptosis melalui pengaktifan mitokondria. E2 menggalakkan 15d-PGJ_2 untuk meningkatkan apoptosis dalam sel MCF-7 tetapi tidak pada sel MDA-MB-231. 15d-PGJ_2 menyekat kitaran sel pada fasa G2/M dalam sel MCF-7 dan fasa G_0/G1 dalam sel MDA-MB-231, tetapi E2 tidak memberi kesan kepada kitaran sel-sel tersebut. . Perbezaan pengekspresan mRNA dan protein ERα, PPARγ1 dan PPARγ2 dalam sel MCF-7 dan MDA-MB-231 kesan interaksi dua hala kedua-dua reseptor menyebabkan. Walaubagaimanapun, dengan menghalang pengaktifkan PPARγ, kami mendapati apoptosis yang diaruh oleh 15d-PGJ_2 dalam kedua-dua sel tidak bergantung kepada pengaktifan reseptor PPARγ. Kajian seterusnya untuk mengenal pasti mekanisme apoptosis aruhan oleh 15d-PHJ_2 menunjukkan bahawa BAX memainkan peranan penting dalam mekanisme apoptosis yang diaruh oleh 15d-PGJ_2 dengan kehadiran atau tanpa E2, tanpa melibatkan kaspase. Kajian terhadap molekul-molekul lain (Fas-Fasl dan p53) didapati memainkan peranan aktif dalam MDA-MB-231, tapi tidak dalam sel MCF-7. Kajian selanjutnya perlu dijalankan untuk mengkaji mekanisme kematian sel-sel payudara secara apoptosis cetusan 15d-PGJ_2 dengan kehadiran E2, yang tidak melibatkan PPARγ dan kaspase.

CELL DEATH MECHANISM INDUCED BY 15-DEOXY PROSTAGLANDIN J_2 AND 17β-ESTRADIOL IN ER-POSITIVE AND ER-NEGATIVE BREAST CANCER CELL LINES

ABSTRACT

Breast cancer is the most common malignancy in Malaysian women. An increase in the prevalence of this disease worldwide indicates the necessity to explore and identify more potent and effective therapies against breast cancer. A number of studies are investigating drugs that cause no or minimal adverse effects and also focus on better understanding of the drug response and resistance by the tumour cells. Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-dependent nuclear receptor which is reported to be expressed in various cancer cells including breast, prostate, colorectal and cervical cancer. Ligands for this receptor have been found to inhibit various cancer cell growth by inducing apoptosis and attenuating cellular proliferation following PPARγ activation. The exact role of this receptor and its ligands, however, remains to be elucidated, especially in breast cancer cells. The present study was carried out to explore the effect of an endogenous ligand of PPARγ, 15 deoxy-Prostaglandin J_2 (15d-PGJ_2) on the estrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cells in the presence and absence of an ERα ligand, 17β-estradiol (E2). The combined treatment of cells with 15d-PGJ_2 and E2 was aimed to explore the recently reported existence of signalling cross-talk between PPARγ and ERα. Cytotoxicity analysis showed that 15d-PGJ_2 inhibited MCF-7 and MDA-MB-231 cells proliferation at EC_{50} values of 15 and 10 μM, respectively. Furthermore, experiments revealed that 15d-PGJ_2 inhibited cell proliferation by inducing

apoptotic cell death in both cells with active involvement of mitochondria. E2 potentiated 15d-PGJ_2-induced apoptosis in MCF-7, but not in MDA-MB-231 cells. 15d-PGJ_2 arrested cell cycle at G2/M phase in MCF-7 and at G_0/G1 phase in MDA-MB-231, while E2 did not influence this cell cycle arrest in both cell lines. Differential mRNA and protein expressions of ERα and PPAR, γ1 and γ2 in MCF-7 and MDA-MB-231 cells treated with 15d-PGJ_2 in the presence and absence of E2 suggested the existence of a bidirectional signal cross-talk between these receptors. However, by blocking the activation of PPARγ, we found that 15d-PGJ_2 induced apoptosis in both cell lines independent of this receptor. Further experiments performed to identify the mechanism of 15d-PGJ_2-induced apoptosis in the presence and absence of E2 revealed caspase-independent apoptosis with a significant role of BAX in both cell lines. Other pro-apoptotic molecules investigated (Fas-FasL and p53) were found to play an active role in MDA-MB-231, but not in MCF-7 cells. Further experiments are needed to explore PPARγ- and caspase-independent apoptosis induced by15d-PGJ_2 in breast cancer cells and the influence of E2 on this cell death mechanism.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer or malignant neoplasm is a genetic disease characterized by the uncontrolled growth and spread of abnormal cells. It is a disease of somatic cells that results from the accumulation of mutations within a genetically unstable heterogeneous cell population, leading to the emergence of a malignant subclone that has accumulated all of the functions necessary in solid tumours, such as invasion, metastasis and defeating the hosts' defence. The three malignant properties of cancer cells; uncontrolled growth, invasion and metastasis, differentiate them from benign tumours, which are self limited and do not invade or metastasize (Chambers *et al.*, 2002).

Each subclone population of cells evolves independently from others, competing for space and resources, such as oxygen and nutrients (Farber, 1984). Hanahan and Weinberg (2011) recently reported an upgraded list of 'hallmarks of cancer' which are the minimum set of genotypes or phenotypes that a cancer cell must acquire to become malignant. These are; (a)sustaining proliferative signaling, (b) evading growth suppressors, (c) resisting cell death, (d) enabling replicative immortality, (e) inducing angiogenesis, (f) activating invasion and metastasis, (g) reprogramming of energy metabolism and (h) evading immune destruction. Cells that accumulate some, but not all of these hallmarks or other changes necessary for malignancy are referred to as partially transformed.

Almost all cancers are caused by abnormalities in the genetic material of the transformed cells. This can happen due to; *external factors*- tobacco smoke, radiation, chemicals, or infectious agents, and *internal factors*- mutations, hormones,

immune conditions and mutations that occur from metabolism (Meng and Riordan, 2005).

The genetic alterations observed in cancer cells are the manifestation of major chromosomal rearrangements (mutations) such as translocations, insertions, point mutations, deletions and/or gene amplification (Dixon and Kopras, 2004). Genes mostly affected by these mutations are oncogenes, tumour suppressor genes and the stability genes with the following characteristics (Strahm and Capra, 2005);

Oncogenes – Gain function and act in a dominant way.

One mutant allele is required to change cellular behavior.

Hyperactive growth and division.

Protection against PCD.

Loss of respect for normal cell boundaries.

Ability to become established in diverse tissue environment.

Example – Burkitt's lymphoma gene (c-myc)

Tumour suppressor genes – Inactivated and act in a recessive way.

Both alleles of the gene must be inactivated to change cellular behavior.

Loss of normal functions in those cells.

Inaccurate DNA replication.

Loss of control over cell cycle.

Loss of orientation and adhesion within tissues.

Loss of interaction with protective cells of the immune system.

Example – Retinoblastoma gene (Rb1)

Stability genes - Keep genetic alterations in general to a minimum.

Prevent mutations in tumour suppressor genes and oncogenes.

Alterations in both genes are required to result in a biological effect.

Example – Ataxia teleangiectasia gene (ATM)

The proteins altered by these genetic changes include growth factors (GF) and growth factor receptors (GFR), signal transducers, kinase inhibitors and transcriptional factors (Meng and Riordan, 2005).

1.2 Breast cancer

Breast cancer is the second most common cancer after lung cancer and the top most common cancer in women worldwide. It comprises 10.4 % of all malignancies and 18 % of all malignancies in women around the world, with the incidence ranging from an average of 95 per 100,000 in more developed countries to 20 per 100,000 in less developed countries (Imaginis Corporation, 2006). The incidence rate of breast cancer varies based on the variation in the risk factors. These factors can be broadly divided into *established factors* and *possible risk factors*. The established factors include a wide range of factors such as genetic susceptibility and family history, endogenous steroid hormone levels, age at menarche, age and type of menopause,

parity, age at first childbirth, height, weight, body size, and level of physical activity, lactation, alcohol consumption and use of exogenous hormone (hormone replacement therapy, combination oral contraceptives), while the possible risk factors mainly consist of insulin-like growth factors and dietary components including fat, fiber and soy (Henderson *et al.*, 2003).

Breast cancer was the commonest overall cancer (18 %) as well as the commonest cancer in women amongst all races from the age of 20 years in Malaysia for 2003 and 2005. It is most common in the Chinese women population (59.7 per 100,000), followed by the Indians (55.8 per 100,000) and then, Malays (33.9 per 100,000). Breast cancer formed 31.1 % of newly diagnosed cancer cases in women in 2003 (30.4 % in 2002) (National Cancer Registry, Malaysia, 2006).

1.2.1 Genes involved in breast cancer

Genetic mutations can be inherited (germline mutation) or acquired in a single cell during a person's lifetime (somatic mutation), which is passed on to all other cells (Rieger, 2004). Inherited breast cancers are less common and occur when gene mutations are passed within a family, from one generation to the next, while somatic mutations can be caused by environmental factors, such as cigarette smoke, or other environmental carcinogens leading to sporadic cancer (www.cancer.net).

Genetic mutations in certain types of genes are more likely to cause cancer. Several genes are linked to an increased risk of breast cancer. Mutations in these genes are associated with various hereditary syndromes. Some of the most common hereditary

cancer syndromes associated with breast cancer risk are described below (Greene, 1997):

Hereditary breast and ovarian cancer (HBOC) syndrome

The two tumour suppressor genes associated with HBOC are BRCA1 and BRCA2 (BReast CAncer 1 and 2). About 80 % of hereditary breast cancer is caused by mutations in these genes. Women who inherit BRCA1 and BRCA2 mutations have a 50 % - 85 % chance of developing breast cancer and a 15 % - 40 % chance of developing ovarian cancer.

Ataxia telangiectasia (A-T)

A-T is a rare recessive disorder inherited as an autosomal recessive condition. It is characterized by a progressive neurological problem that leads to difficulty in walking. The gene associated with A-T is called ATM (A-T mutated protein kinase). People with one altered copy of this gene, may have an increased risk of melanoma, breast, ovarian and stomach cancers. There is about a 40 % risk of cancer for people with A-T, the most common cancers being leukemia and lymphoma. With an increase in the lifespan of individuals with A-T, risk of other types of cancer, including melanoma, sarcoma, and breast, ovarian and stomach cancers, are increasingly reported.

Cowden syndrome (CS)

CS is a rare genetic condition caused by a mutation on the PTEN (phosphatase and tensin homologue deleted on chromosome 10) gene. Women with CS have a risk of developing breast cancer (25 % - 50 %) and also a risk of developing noncancerous

breast changes (65 %). People with CS also have a high risk of both non-cancerous and cancerous tumours of the thyroid and endometrium (lining of the uterus).

Li-Fraumeni syndrome (LFS)

LFS is a rare condition resulting from a mutation in a tumour suppressing gene, p53 (protein 53). Another gene, CHEK2 (checkpoint homologue), may cause LFS for some families. People with LFS have up to a 50 % chance of developing cancer by the age of 40 and a 90 % chance of developing cancer by the age of 60. Some of the most common cancers associated with LFS are osteosarcoma, breast cancer, soft tissue sarcoma, leukemia, brain cancer, and adrenal cortical tumours.

Peutz-Jeghers syndrome (PJS)

The gene associated with PJS is a tumour suppressor gene called STK11 (serine/threonine kinase 11). Women with PJS have a 50 % risk of developing breast cancer and about a 20 % risk of developing ovarian cancer. People with PJS often have multiple hamartomatous polyps, which are normal-appearing growths in the digestive tract (non-cancerous tumour). These polyps cause an increased risk of colorectal cancer.

1.2.2 Classification of breast cancer

Classification of breast cancer is performed in order to select which treatment approach should be taken to tackle this disease. Careful analysis of classification must be done so that these classifications can be tagged as true prognostic factors (estimate disease outcomes) or true predictive factors (estimate the likelihood of

response or lack of response to a specific treatment) (Gonzalez-Angulo *et al.*, 2007). Breast cancer classification divides the cancer into various categories based on multiple different schemes, such as histopathological type, grade and stage of tumour and expression of different proteins and genes. A general overview of these classifications is as follows (Filho *et al.*, 2011):

1. *Histopathology.* Most of the breast cancers are classified as mammary ductal carcinoma and are derived from the epithelial lining of the lobules or ducts. Carcinoma *in situ* refers to cancer within the epithelial tissue without invasion to surrounding tissues whereas invasive carcinoma invades the surrounding tissues. A more aggressive form of breast cancer is achieved when the cancer invades the perineural and/or lymphovascular spaces.
2. *Stage.* The breast cancer is staged based on TNM classification that measures the size of the cancer where it originally started and also the locations to which it metastasized. TNM refers to the size of tumour (T), if the tumour has spread to the lymph nodes (N) and if the tumour has metastazied (M). The main stages are:
   (a) Stage 0 – *in situ* disease. It is a pre-cancerous marker e.g. ductal carcinoma *in situ*, lobular carcinoma *in situ* etc.
   (b) Stage 1-3 – cancer restricted to the breast or regional lymph nodes but differes in size i.e., the higher the stage, the bigger the cancer.
   (c) Stage 4 – metastatic breast cancer with poor prognosis.

3. *Grade.* Grading refers to the appearance of breast cancer cells compared to the appearance of normal breast tissues. A well differentiated cancer is termed as low grade tumour, a moderately differentiated one as intermediate grade and a poorly differentiated cancer as high grade tumour. Poorly differentiated cancers have a worse prognosis.
4. *Receptor status.* Breast cancers are also classified based on various receptors either on the cell surface, cytoplasm and/or nucleus. The most important receptors in the breast carcinogenesis are estrogen receptors (ER), progesterone receptor (PR) and HER2/neu. Breast cancers that have these receptors are ER positive (+), PR+ or HER2+, and cancers that lack these receptors are classified as ER negative (-), PR- and HER2-. Cancer cells that lack all three receptors are called basal-like or triple negative which have a worse prognosis.

1.2.3 Treatment of breast cancer

With the advancements in research and development, a number of invasive and non-invasive treatments for breast cancer have been in practice. Early diagnosis makes these treatments more effective, increasing the survival period of patients, or even controlling or eliminating the disease as such. Some of the most widely practiced treatments are listed below (Dolinsky, 2002):

Surgery- Breast-conserving surgery (*lumpectomy*- removal of the tumour only and a small amount of surrounding tissue), *mastectomy* (removal of all of the breast tissue), and *lymph node removal* or *axillary lymph node dissection*, which can take

place during lumpectomy and mastectomy if the biopsy shows that breast cancer has spread outside the milk duct. *Cryotherapy*, also called cryosurgery, can also be performed that uses extreme cold to freeze and kill cancer cells. This treatment is an experimental treatment for breast cancer these days. *Prophylactic mastectomy* involves the removal of the breast to lower the risk of breast cancer in high-risk people. *Prophylactic ovary removal* is a preventive surgery that lowers the amount of estrogen in the body, making it harder for estrogen to stimulate the development of breast cancer.

Chemotherapy- This treatment involves usage of medicine to weaken and destroy cancer cells in the body, including cells at the original cancer site and cancer cells that may have metastasized to other parts of the body. Chemotherapy is a systemic therapy, affecting the whole body through the bloodstream. In many cases, a combination of two or more medicines (chemotherapy regimens) is used as chemotherapy treatment for breast cancer. Chemotherapy is used to treat the early-stage invasive breast cancer to get rid of any cancer cells that may be left behind after surgery and to reduce the risk of cancer recurrence, and in advanced breast cancer, chemotherapy regimens make the cancer shrink or disappear in about 30 % - 60 % of people treated (Figure 1.1). Chemotherapeutic drugs have been classified into two major groups; **Anthracyclines** are chemically similar to an antibiotic. Anthracyclines damage the genetic material of cancer cells, which makes the cells, die. This group includes drugs such as Adriamycin, Ellence, and Daunorubicin. **Taxanes** interfere with the proliferation of cancer cells. Taxol, Taxotere, and Abraxane are taxanes.

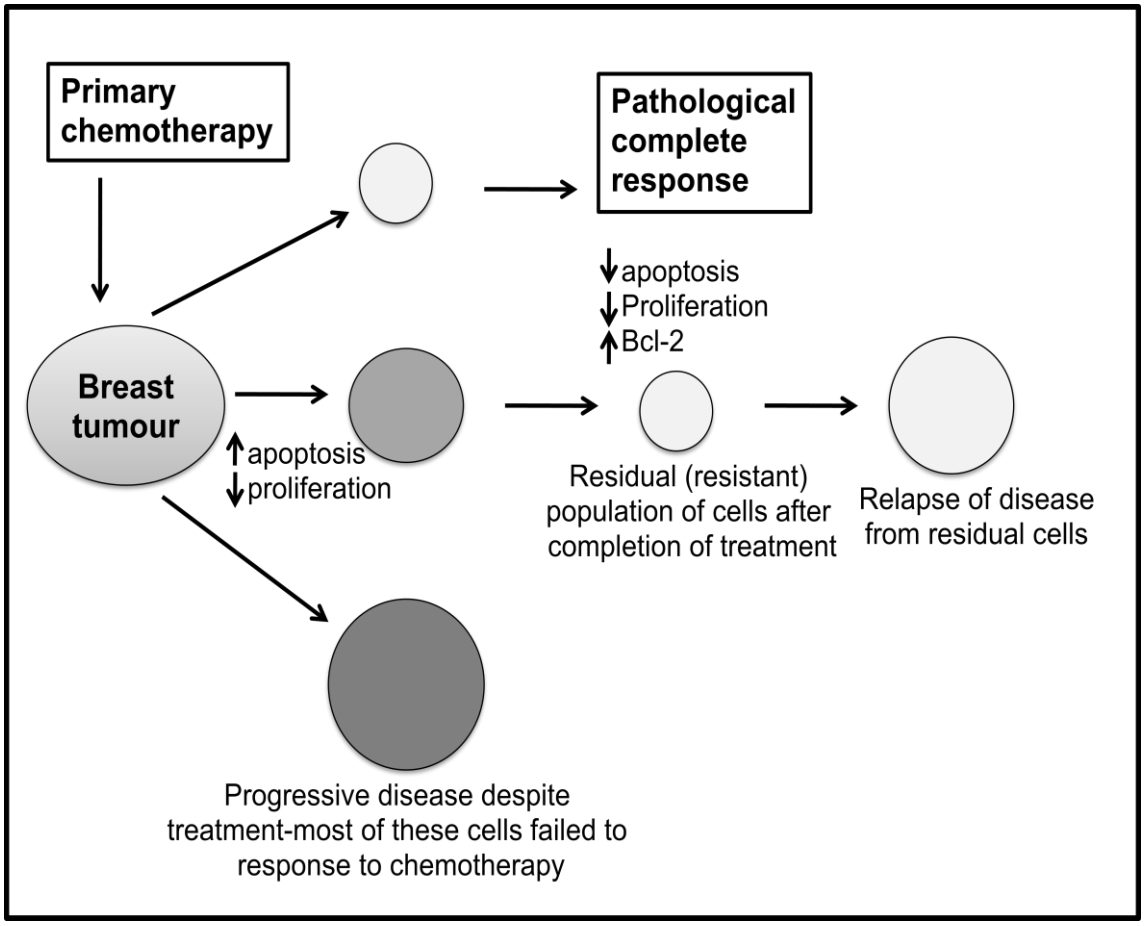

Figure 1.1 Schematic representation of tumours responding or resistant to chemotherapy and changes in growth control (Adapted from Parton *et al.*, 2001).

Hormonal therapy- Hormonal therapy medicines treat hormone receptor-positive breast cancers and are not effective against hormone receptor-negative breast cancers. These medicines work in two ways; lowering the amount of the hormone (estrogen) in the body, and by blocking the action of estrogen on breast cancer cells. Estrogen, mostly made by the ovaries, potentiates the growth of hormone receptor-positive breast cancers. Therefore, reducing the amount of this hormone or blocking its action can reduce the risk of early stage estrogen receptor (ER)-positive breast cancers recurring after surgery. Hormonal therapy medicines can also be used to help shrink or slow the growth of advanced stage or metastatic ER-positive breast cancers. There are several hormonal therapy medicines, including aromatase inhibitors (AI)- Arimidex, Aromasin and Femara, selective estrogen receptor modulators (SERMs)- Tamoxifen, Evista and Fareston, and estrogen receptor down regulators (ERDs)- Faslodex (Figure 1.2).

Radiation therapy- It is a highly targeted and effective way to destroy cancer cells in the breast that may be present after surgery. Radiation therapy is relatively easy to tolerate with side effects limited to the treated area and can reduce the risk of breast cancer recurrence by about 70 %. There are two main types of radiation; **external radiation** is the most common type of radiation, typically given after lumpectomy and sometimes, mastectomy, while, **internal radiation** is a less common method of giving radiation. It is being studied for use after lumpectomy.

Targeted therapy- Targeted cancer therapies are treatments that target specific features of cancer cells, such as a protein that allows the cancer cells to grow in a rapid or abnormal way. Generally, targeted therapies are less harmful to normal,

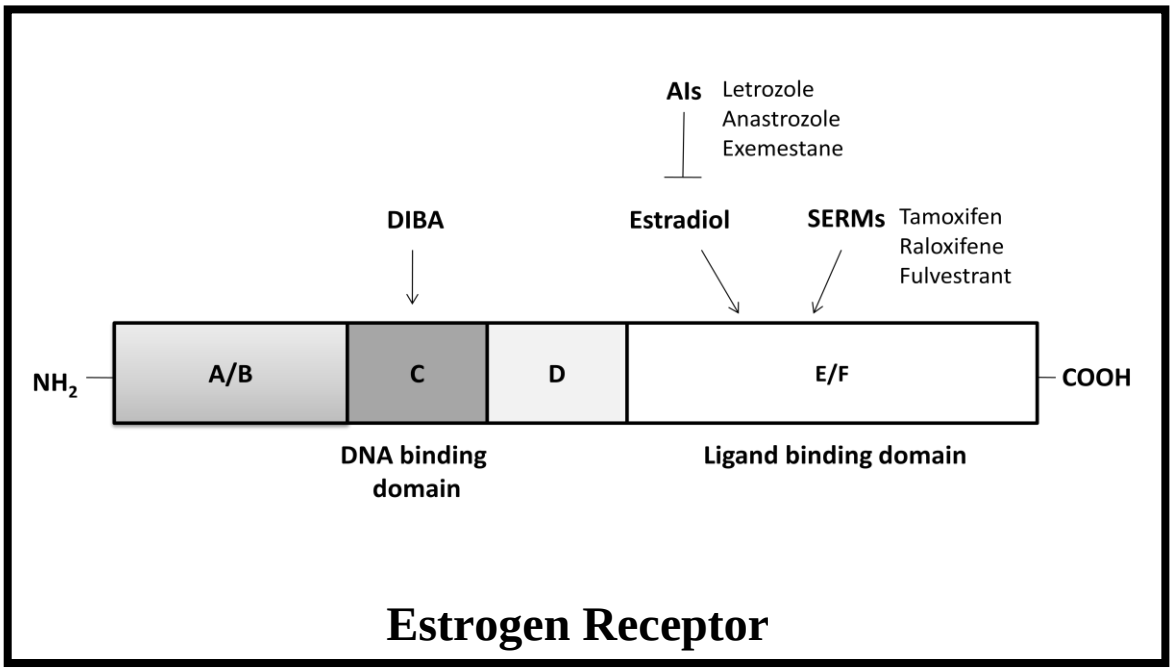

Figure 1.2 Treatments for ER-positive breast cancer and chemoprevention that target specific domains of the estrogen receptor (Adapted from Nichols, 2007).

The AIs inhibit the enzyme responsible for production of estradiol from androgenic precursors. SERMs compete with estradiol for binding to the ligand binding domain and alter the activator or repressor proteins that subsequently bind. Disulfide benzamide (DIBA) acts in a novel way, by interrupting the second zinc finger of the DNA binding domain, preventing receptor interaction at estrogen receptor response elements (Nichols, 2007).

healthy cells than chemotherapy. Some targeted therapies are antibodies that work like natural antibodies (immune targeted therapies). Currently three types of targeted therapies are under practice to treat breast cancer. These are; **Herceptin** (trastuzumab) works against HER2 (human epidermal GFR-2) -positive breast cancers by blocking the ability of the cancer cells to receive chemical signals that stimulate cell growth. **Tykerb** (lapatinib) works against HER2-positive breast cancers by interrupting with the HER2 pathway that can cause uncontrolled cell growth. **Avastin** (bevacizumab) works by blocking the growth of new blood vessels (angiogenesis) that cancer cells depend on to grow and function.

1.3 Programmed Cell Death

Balance between cell division and cell death is one of the most important mechanisms for the development and maintenance of multicellular organism. Disorders in either of these processes have pathological consequences that can lead to various diseases, such as cancer. This equilibrium between cell death and cell proliferation is tightly controlled by a process called programmed cell death or PCD (Broker *et al.*, 2005). PCD is a well defined and characterized set of events counteracting tumour growth and plays a critical role in a wide variety of physiological processes during fetal development and also in adults. In the past decades, PCD was held synonymous with apoptosis, which now has been classified as a type of PCD.

The Nomenclature Committee on Cell Death (NCCD) proposed a list of molecular or morphological criteria necessary for a cell to be considered dead (Kroemer *et al.*, 2009). These are;

1. The cell has lost the integrity of its plasma membrane.
2. The cell, including its nucleus, has undergone complete fragmentation into discrete bodies (referred to as apoptotic bodies).
3. The cell's fragments have been engulfed by an adjacent cell *in vivo.*

Thus, *bona fide* dead cells would be different from dying cells that have not yet concluded their demise (Table 1.1).

1.3.1 Classification of PCD

Participation of active cellular processes that can be intercepted by interfering with intracellular signalling is the foremost criterion for PCD (active cell death) (Leist and Jaattela, 2001). Due to its complex mechanism and intervening of different molecules, classification of PCD has always been a topic of debate among researchers. Only recently it has been found that PCD is not limited to the previously characterized apoptosis, but it can also occur in a well programmed manner in the complete absence of caspases (cysteinyl-aspartic acid proteases) or other apoptotic molecules without inducing accidental cell death (Broker *et al.*, 2005).

A number of studies have been carried proposing different types of PCD. Despite numerous models proposed to categorize PCD, exclusive definition of different

Table 1.1 Cell death methodology (Kroemer *et al.*, 2009).

Definition	Explanation	Methods of detection
Molecular or morphological criteria to define dead cells		
Loss of plasma membrane integrity	Plasma membrane has broken down, resulting in the loss of cell's identity	IF microscopy and/or FACS to assess the exclusion of vital dyes, *in vitro*
Cell fragmentation	The cell (including its nucleus) has undergone complete fragmentation into discrete bodies (usually referred to as apoptotic bodies)	IF microscopy, FACS quantification of hypodiploid events (sub-G1 peak)
Engulfment by adjacent cells	The corpse or its fragments have been phagocytosed by neighboring cells	IF microscopy, FACS colocalization studies
Proposed points-of-no-return to define dying cells		
Massive activation of caspases	Caspases execute the classic apoptotic programme, yet in several instances, caspase-independent death occurs. Moreover, caspases are involved in non-lethal processes including differentiation and activation of cells	Immunoblotting, FACS quantification by means of fluorogenic substrates or specific antibodies
$\Delta\Psi_m$ dissipation	Protracted $\Delta\Psi_m$ loss usually precedes MMPand cell death; however, transient dissipation is not always a lethal event	FACS quantification with $\Delta\Psi_m$ –sensitive probes, Calcein-cobalt techniques
MMP	Complete MMP results in the liberation of lethal catabolic enzymes or activators of such enzymes. Nonetheless, partial permeabilization may not necessarily lead to cell death	IF colocalization studies, Immunoblotting after subcellular fractionation
PS exposure	PS exposure on the outer leaflet of the plasma membrane often is an early event of apoptosis, but may be reversible. PS exposure occurs also in T-cell activation, without cell death	FACS quantification of Annexin-V binding

Table 1.1 Continued.

Definition	Explanation	Modes of detection
Operative definition of cell death, in particular in cancer research		
Loss of clonogenic survival	This method does not distinguish cell death from long-lasting or irreversible cell cycle arrest	Clonogenic assays

Abbreviations: $\Delta\Psi_m$- mitochondrial transmembrane permeabalization; FACS- fluorescence-activated cell sorter; IF- immunofluorescence; MMP- mitochondrial membrane permeabilization; PS- phosphotidylserine

types of PCD is difficult to due to the overlapping of signalling pathways between different death mechanisms. However, specific definitions of PCD have been put forth based on certain distinct features observed within the cells during PCD. The NCCD proposed a list of different types of PCD describing distinct modalities of cell death in their 2009 review of make Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009 (Kroemer *et al.,* 2009). They classified PCD into Typical (Table 1.2; Figure 1.3) and Atypical cell death modalities, which are briefly described below.

Typical cell death modalities

1. Apoptosis

Apoptosis or type I cell death is an inherent, controlled cell death programme characterized by cell death with specific morphological features, such as cell shrinkage, condensation of the chromatin and disintegration of the cells into small fragments that can be engulfed by nearby cells without inciting inflammation (Kerr *et al.*, 1972*;* Strasses et al., 2000; Ferri and Kroemer, 2001; Kaufmann and Hengartner, 2001). Apoptosis will be described in detail later in the current chapter.

2. Autophagy

Autophagy is a cellular catabolic degradation response to starvation or stress, whereby cellular proteins, cytoplasm and organelles are engulfed, digested and recycled to sustain cellular metabolism. It is a genetically programmed and evolutionary conserved process that degrades long lived cellular proteins and organelles (Clarke, 1990). Autophagic cell death is characterized by

Table 1.2 Distinct modalities of a typical PCD (Kroemer *et al.*, 2009).

Mode of PCD	Morphological features
Apoptosis	Rounding up of the cell Retraction of presudopods Reduction of the cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by neighboring phagocytes *in vivo*
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of double membrane, autophagic vacuoles Little or no uptake by phagocytic cells *in vivo*
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation or loss of corneocytes by protease activation
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condesation

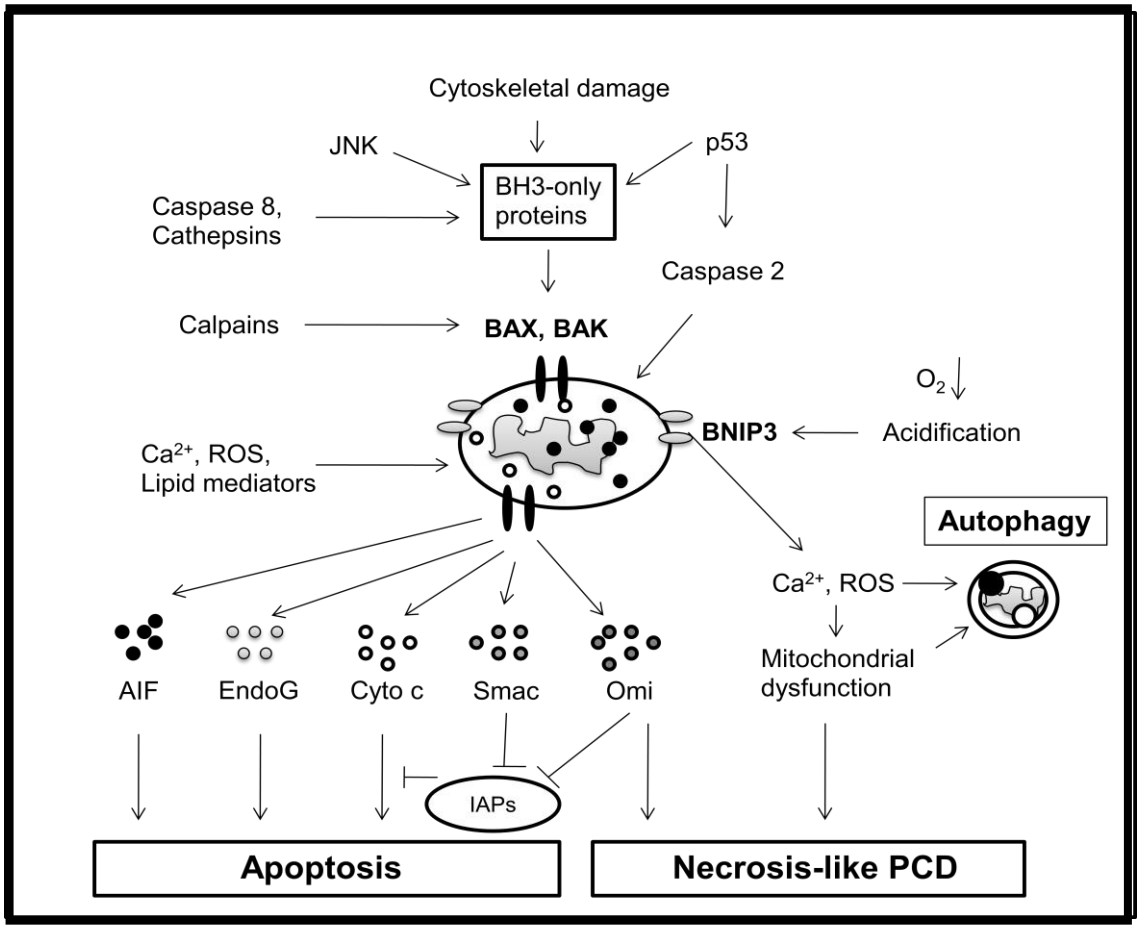

Figure 1.3 Distinct modalities of a typical PCD (Adapted from Jaattela *et al.*, 2004).

Caspase 8 and cathepsin can cleave and activate a BH-3 only protein, BID, while disruption of the cytoskeleton leads to the release of BH-3 only proteins BIM and BMF which can activate the pore forming proteins, BAX and/or BAK. Activation of JNK pathway increases the expression of BMF and HRK, whereas, DNA damage induces a p53-mediated transcription of genes encoding BAX as well as proteins involved in ROS generation. Endoplasmic reticulum stress results in the release of Ca^{2+}, which may cause mitochondrial damage directly, or activate BAX via calpain-mediated cleavage. BNIP3 is activated by acidosis, which is translocated to the mitochondrial membrane. Mitochondrial damage leads to the release of numerous mitochondrial proteins that trigger the execution of PCD, such as cyto c, Smac/Diablo, EndoG and Omi/HtrA2 which trigger caspase activation and classical apoptosis. AIF triggers caspase-independent apoptosis, while Ca^{2+} and ROS can lead to severe mitochondrial dysfunction and necrosis-like PCD and in certain cases, autophagy (Jaattela *et al.*, 2004).