ELUCIDATION OF APOPTOTIC PATHWAY IN HUMAN BREAST CANCER, MCF-7 CELL, INDUCED BY VR-3848 ISOLATED FROM *EUPHOBIACEAE*

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ABSTRACT

VR-3848 is a plant-derived active compound purified from *Euphobiaceae*. Previous studies reported that this compound could inhibit various cancer cell proliferation such as lung (LU-1), breast (BCA-1), colon (COL-1), mouth epidermoid carcinoma (KB), and mouse lymphoid neoplasm (P388). VR-3848 also inhibits the growth of human breast cancer cell, MCF-7. This cancerous cell lacks functional caspase-3, which is believed to act as principal caspase. Lack of this enzyme significantly contributes to chemotherapeutic resistance. Growth of MCF-7 cells was inhibited by VR-3848 in a dose-dependent manner. The GI_{50} value was 8.20 nM. VR-3848 treated MCF-7 displayed elevated levels of apoptosis as demonstrated by DAPI staining and DNA fragmentation pattern. The DAPI nuclear-stained count showed 6.12%, 16.86%, 31.02%, and 49.06% of apoptosis at 6, 12, 18, and 24 hours, respectively, after drug treatment. In addition, DNA laddering patterns were shown at 18 hours and more clearly visualized at 24, 36, and 48 hours. To elucidate the apoptosis cell death pathway induced by VR-3848, we used cDNA analysis as a tool. The result from 205 apoptosis-related gene expression patterns of VR-3848 treated MCF-7 cell revealed that apoptosis was mediated via three different pathways, which were the receptor-mediated, the mitochondria-dependent and the ER-stress pathways. The mitochondrial pathway was confirmed by detection of the high level of AIF released from the mitochondria. The up-regulation of caspase-10 and caspase-8 at the initial time point was also observed. Moreover, it was shown that treatment of VR-3848-treated cells with caspase-10 and caspase-8 specific inhibitor can protect cell death by apoptosis so that these data support the idea of receptor-ligand mediated pathway. Moreover, the array results suggested ER-stress associated apoptosis gene. This ER-stress associated apoptosis pathway was confirmed by the upregulation of GADD153 gene and caspase-12 in the drug treatment group by PCR and western blot analysis. These mechanistic studies provide evidence that VR-3848 may be one of an anticancer lead compound targeting human breast cancer cells.

KEY WORDS: VR-3848 / MCF-7 CELL / APOPTOSIS / cDNA ARRAY / AIF

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การศึกษาวิถีการชักนำให้เกิดการตายแบบ Apoptosis ของสารสกัดจากพืชในวงศ์ยูโฟบีเอซีกับเซลล์มะเร็งเต้านมของมนุษย์ (ELUCIDATION OF APOPTOSIS PATHWAY IN HUMAN BREAST CANCER, MCF-7 CELL, INDUCED BY VR-3848 ISOLATED FROM *EUPHOBIACEAE*)

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บทคัดย่อ

จากการศึกษาที่ผ่านมาพบว่าสารวี อาร์ 3848 ซึ่งสกัดได้จากพืชในวงศ์ *Euphobiaceae* ให้ผลยับยั้งการเจริญของเซลล์มะเร็งในหลอดทดลองหลายชนิด เช่น เซลล์มะเร็งปอด (LU-1), เต้านม (BCA-1), ลำไส้ใหญ่ (COL-1), ปาก (KB) รวมทั้งมะเร็งในระบบน้ำเหลืองของหนู (P388) ในการวิจัยครั้งนี้มุ่งศึกษาผลยับยั้งการเจริญของเซลล์มะเร็งเต้านมมนุษย์ (MCF-7) กับสารวี อาร์ 3848 เนื่องจากเซลล์มะเร็งชนิดนี้เป็นเซลล์ชนิดที่พบในผู้ป่วยที่มีภาวะดื้อยารักษาโรคมะเร็ง ผู้ศึกษาก่อนหน้านี้ตั้งข้อสันนิษฐานว่าอาจเป็นผลจากเซลล์ MCF-7 มียีน caspase-3 ที่ไม่สมบูรณ์ โดยยีนชนิดนี้จำเป็นต่อการชักนำให้เกิดการตายแบบ apoptosis จากผลการทดลองพบว่า สารวี อาร์ 3848 สามารถยับยั้งการเจริญของเซลล์มะเร็งชนิดนี้ได้โดยชักนำให้เซลล์เกิดการตายแบบ apoptosis พิสูจน์ได้จากการนับจำนวนลักษณะนิวเคลียสที่ผิดปกติ ได้แก่ nuclear condensation และ nuclear fragmentation เปอร์เซ็นต์ apoptosis เทียบกับตัวอย่างที่ไม่ใส่ยาที่ระยะเวลา 6, 12, 18, 24 และ 36 ชั่วโมง ให้ผลดังนี้ 6.12 %, 16.86 %, 31.02 % และ 49.06 % ตามลำดับ นอกจากนี้ยังพบ DNA fragmentation ในตัวอย่างที่ใส่ยาในชั่วโมงที่ 18, 24, 36 และ 48 ชั่วโมงเช่นกัน จากการศึกษาต่อในระดับกลไกการชักนำให้เกิดการตายแบบ apoptosis โดยใช้เทคนิค cDNA analysis พบว่าสาร วี.อาร์ 3848 สามารถชักนำให้เซลล์ MCF-7 ตายแบบ apoptosis ได้ 3 แบบ คือ การชักนำโปรตีน AIF จากไมโตคอนเดรียออกมาทำลาย DNA จนเกิดการตายแบบ apoptosis ยืนยันได้จากการตรวจพบโปรตีน AIF มากขึ้นหลังจากเลี้ยงเซลล์ในอาหารที่มี สาร วี.อาร์ 3848 นานขึ้น นอกจากนี้พบว่ามีการแสดงออกของยีนต่อไปนี้ในระหว่างทดสอบยา ได้แก่ TNFR1, caspase-8 และ caspase-10 โดยพบว่าเมื่อเลี้ยงเซลล์ในอาหารที่มี caspase-8 และ caspase-10 inhibitor ขณะทดสอบยา พบว่าเปอร์เซ็นต์การเกิด apoptosis ลดลงเมื่อเทียบกับตัวควบคุมที่ใส่เฉพาะสารวี.อาร์ 3848 จากข้อมูลที่ได้อธิบายได้ว่าการเกิด apoptosis อาจได้รับสัญญาณผ่านทางตัวรับสัญญาณที่ผิวเซลล์และส่งผ่านมายัง initiator caspase ซึ่งสามารถกระตุ้นให้เอ็นไซม์ในกลุ่ม effector caspase ตัวอื่นๆ ทำงานได้ จากผล cDNA analysis ยังพบการแสดงออกของยีนที่ตอบสนองต่อความผิดปกติของ Endoplasmic reticulum (ER) รวมทั้งปริมาณโปรตีน caspase-12 ที่เพิ่มขึ้น เนื่องจากผลของสาร วี.อาร์ 3848 แสดงให้เห็นว่า สารวี.อาร์ 3848 สามารถกระตุ้นให้เซลล์ตายแบบ apoptosis ได้โดยใช้เส้นทางผ่านทาง Endoplasmic reticulum (ER) จากผลที่ได้รับทั้งหมดสามารถสรุปได้ว่าสารวี.อาร์ 3848 มีศักยภาพในการยับยั้งการเจริญของเซลล์มะเร็งเต้านมชนิดนี้ได้โดยกระตุ้นกระบวนการตายแบบ apoptosis อย่างน้อย 3 กระบวนการ ตามชนิดของสารตัวกลาง (mediators) ที่พบในการศึกษานี้

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LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
Apaf-1	apoptosis protease-activating factor 1
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 -associated x protein
BCA-1	breast cancer cell
Bcl-2	B cell leukaemia-2
Bcl-X_L	B cell leukaemia-x long
Bcl-X_S	B cell leukaemia-x short
BH	Bcl-2 homology
BIR	baculoviral inhibitory repeat
BOD	Bcl-2 -related ovarian death gene
Bok	Bcl-2 -related ovarian killer
bp	base pair
BRCA 1	breast cancer gene 1
cDNA	complementary deoxyribonucleic acid
CAD	caspase-activated DNase
CARD	caspase activation and recruitment domain
cAMP	cyclic adenosine 3', 5'-monophosphate
COL-1	colon cancer cell
DAPI	4', 6-diamidino-2-phenylindole
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	DNA ladder nuclease
E_2	estradiol
ER	endoplasmic reticulum

LIST OF ABBREVIATIONS (Cont'd)

ERK	extracellular signal regulated kinase
EST	expressed sequence tag
FADD	Fas-associated death domain
FasL	Fas ligand
FasR	Fas receptor
FBS	fetal bovine serum
FLICE	Fas ligand-interacting cell effector
FLIP	FLICE-inhibitory protein
GADD153	growth arrest DNA damage 153
IAP	inhibitor of apoptosis
ICAD	inhibitor of CAD
KB	mouth epidermoid carcinoma cell
kb	kilobase pair
kDa	kilodalton
Lu-1	non-small cell lung cancer cell
MAPK	mitogen-activated protein kinase
MCF-7	breast cancer cell
Mcl-1	myeloid cell leukaemia-1
MDR	multidrug resistance
μg	microgram
MKK	mitogen-activated protein kinase kinase
mRNA	messenger ribonucleic acid
NF-κ B	nuclear factor κ B
OD	optical density
P-388	mouse lymphoid neoplasm cell
PBS	phosphate-buffered saline
PI-3K	phophoinositide 3-kinase
PKC	protein kinase C
RIP	receptor-interacting protein

LIST OF ABBREVIATIONS (Cont'd)

RNA	ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S/N ratio	signal/noise ratio
SRB	sulforhodamine B
SSC	saline sodium citrate
TCA	trichloroacetic acid
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TNFRSF	tumor necrosis factor receptor superfamily
TRADD	TNFRSF1A-associated via death domain
TRAIL	TNF-related apoptosis inducing ligand
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
XIAP	X-linked inhibitor of apoptosis
Z-VAD-fmk	benzyloxycarbonyl-VAD-fluoromethylketone
$\Delta\Psi_m$	mitochondrial transmembrane potential

CHAPTER 1
INTRODUCTION

Cancer in Thailand is becoming a significant health problem. It is the leading cause of death in Thai population next to the cardiovascular disease and accident. Among those types of cancer, breast cancer is one of the most common cancers in Thai women and women in other developing countries. This is supported by the fact that the incidence of breast cancer in Thais is second to the cervical cancer (1). Routinely, once diagnosed, there are three major procedures for cancer treatment, including surgery, radiation, and chemotherapy but none of these procedures results in complete elimination of cancerous tissue. The resistance of cancer cells to multiple chemotherapeutic agents poses a major problem in the successful treatment of breast cancer. Most patients, who attain chemotherapy-induced remission, relapse and eventually die as a result of the emergence of subpopulations of tumour cells that are resistant to drugs. Single drug resistant cancerous cells frequently develop into a cross-resistance to a large variety of diverse drugs after exposure to only one drug, a phenomenon known as multi-drug resistance (MDR). Once encounter such problem, searching for potent chemotherapeutic agent is an alternative.

Thailand is one of those countries with tropical forest, which is well accepted that these natural resources possess invaluable natural medicinal products. Under the leadership of Prof. Vichai Reutrakul, his group at the Department of Chemistry, Faculty of Science, Mahidol University, has succesed in purify novel potential medicinal compounds from plants in Thai forest. Among these compounds, VR-3848, extracted from *Euphobiaceae*, is purified and structurally elucidated as a novel compound, 7-mer cyclic peptide. This compound strongly inhibits cell proliferation against various human cancer cell lines such as lung (LU-1) (2), breast (BCA-1), colon (COL-1), mouth epidermoid carcinoma (KB), and mouse lymphoid neoplasm (P388) (3). VR-3848 was shown herein to induce apoptosis at nanomolar concentrations in

the Leukemic Jurkat cell line. (4). The mechanism of inhibition in various cancer cell lines has not been investigated intensively.

Since VR-3848 exerts very potent anti-proliferation against various types of cancerous cells plus its novelty, we, therefore, would like to investigate activity of this compound in detail using MCF-7 cell as a model. The caspase-3-deficient human breast cancer, MCF-7 cells is one of an excellent *in vitro* model of human breast cancer. This cell line was established from the pleural effusion of a patient who was diagnosed with adenocarcinoma of the breast (5). There are several characteristics of differentiated mammary epithelium including the ability to respond to estrogen via cytoplasmic estrogen receptors (6), the widely used for studies of tumour biology and hormone mechanism of action. This cancerous cell is an interesting model to investigate the anti-cancer agent since MCF-7 lacks functional cardinal caspase suspecting of an endogenous characteristic of drug resistant (7).

cDNA array analysis is a recent technology that has been used to identify gene expression pattern in variety of organisms, in an interaction between host and pathogen. This technology is the driving force in recent antimicrobial discovery and development. With this technique several thousands of genes expression level can be investigated in parallel. Therefore, a network of gene expression can be composed. Basically, this technique helps us on target identification and hypothesis generation. Thus, in the present study, we proposed to elucidate molecular mechanism of MCF-7 cell death induced by VR-3848 using cDNA array analysis.

Objectives

According to the significances of VR-3848, we designed to investigate anti-cancer activity of this compound at molecular level. Two main objectives were proposed.

1. To investigate whether VR-3848 is able to inhibit growth of human breast carcinoma cells (MCF-7) via apoptosis
    a. To study the MCF-7 growth rate under normal growth condition.
    b. To investigate the effect of estrogen on the proliferation rate of MCF-7 cancer cell line.
    c. To study the cytotoxicity of VR-3848 against MCF-7 cancer cell line.
    d. To prove whether VR-3848 induces MCF-7 cell death by apoptosis.
2. To elucidate a cascade of apoptotic genes involved in response of MCF-7 to VR-3848.
    a. To elucidate the numbers of death-related gene in VR-3848-induced apoptosis.
    b. To validate the data obtained in 2a and use the result to construct the death pathway induced by VR-3848.

CHAPTER 2
LITERATURE REVIEWS

BIOLOGY OF CANCER CELLS

Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). The unregulated growth is caused by damage to DNA, resulting in mutation to vital genes that control cell division, among other functions. One or more of these mutations, which can be inherited or acquired, can lead to uncontrolled cell division and tumour formation. Tumour refers to any abnormal mass of tissue, but may be either malignant (cancerous) or benign (non-cancerous). Only malignant tumours are capable of invading other tissues or metastasizing. Metastasis requires that cells pick up the ability to chew through the basal lamina (by activating extracellular proteinases called metalloproteases), enter the bloodstream, exit the bloodstream, and survive in a new environment, often in the liver or the lungs (Figure 1). They must be able to tolerate a very different environment here in order to survive (8).

Typically, a series of several mutations is required before a normal cell transforms into a cancer cell (Figure 2). The process involves both proto-oncogenes and tumour suppressor genes. Proto-oncogenes are involved in signal transduction by coding for a chemical "messenger", produced when a cell undergoes protein synthesis. These messengers send signals based on the amount of them present to the cell or other cells, telling them to undergo mitosis, in order to divide and reproduce. When mutated, they become oncogenes and over express the signals to divide, and thus cells have a higher chance to divide excessively (9).

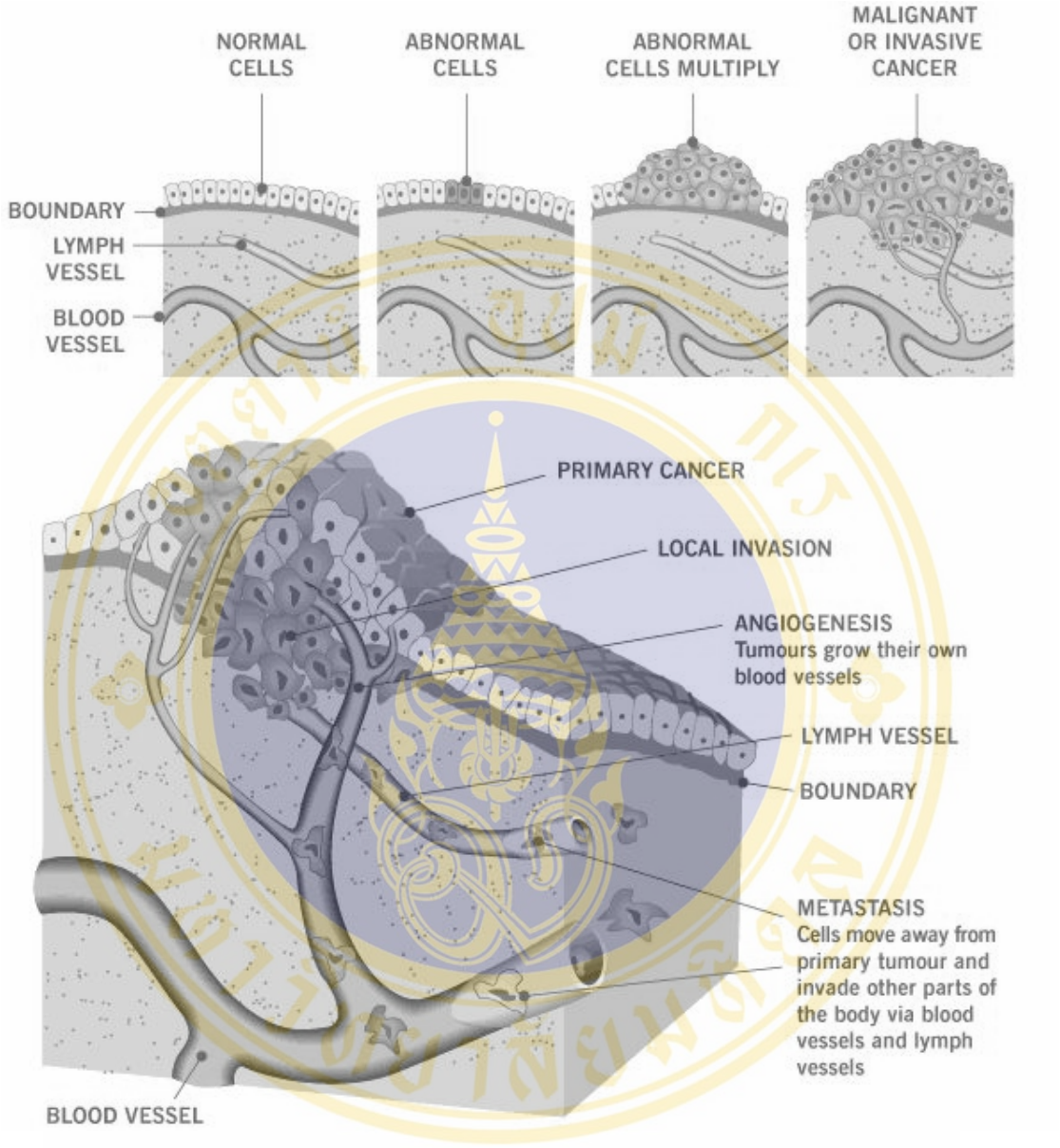

Figure 1. The metastasis development. (The cancer council Victoria 2005, What is cancer? Retrieved from http://www.cancervic.org.au/cancer1/students/what_is_cancer.htm).

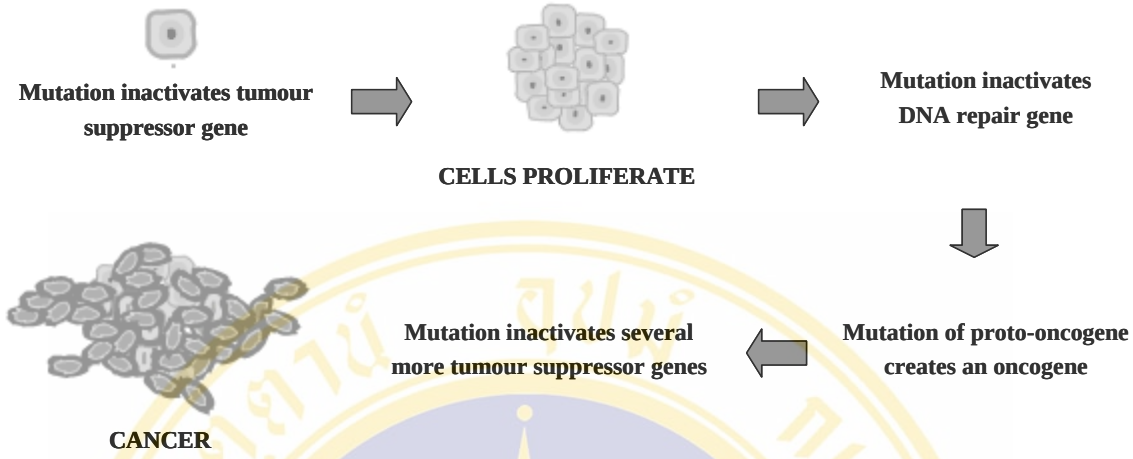

Figure 2. A series of mutation in cancer development, which each mutation alters the behavior of the cell (Wikipedia, the free encyclopedia. Carcinogenesis. Retrieved from http://en.wikipedia.org/wiki/Carcinogenesis).

TYPES OF GENE INVOLVED IN CANCER

Mutations in several different types of genes are involved in cancer. Some of these genes control growth or stop excessive growth of cancer cells while others control a cell's blood supply or its position in the body. In order for cells to start dividing uncontrollably, genes which regulate cell growth must be damaged. Proto-oncogenes are genes which promote cell growth and mitosis, a process of cell division, and tumour suppressor genes discourage cell growth, or temporarily halt cell division from occurring in order to carry out DNA repair. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell (10).

1. PROTO-ONCOGENES

A proto-oncogene is a gene, which is involved in signal transduction and execution of mitogenic signals, usually through its protein product. Upon activations, it becomes a tumour-inducing agent, an oncogene.

The proto-oncogene can become an oncogene by a relatively small modification of its original function. There are two main activation types, which are mutation within that proto-oncogene and increased in proto-oncogene activation.

Firstly, a mutation within a proto-oncogene can cause a change in the protein structure such as an increase in protein (enzyme) activity which responsible for cell growth activity or loss of regulation of cell growth proteins. Secondly, increase in proto-oncogene activation caused by increases of protein stability by evade from proteasome destroying which prolonging its activity in the cell or caused by gene duplication, resulting in an increased amount of protein in the cell (11). There are known classes of protein kinases and related proteins that can become an oncogene, for example, receptor tyrosine epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), cytoplasmic tyrosine kinases likes the Src-family, Syk-ZAP-70 family, the regulatory GTPases, for example, the Ras protein. Cytoplasmic Serine/Threonine kinases and their regulatory subunits are also related to oncogene, for example, the Raf kinase, and cyclin-dependent kinases (through overexpression). The adaptor proteins in signal transduction, for example *Shc* and *Grb2* can become an oncogene (11).

2. ONCOGENES

Oncogenes stimulate appropriate cell growth under normal conditions, as required for the continued turnover and replenishment of the skin, gastrointestinal tract and blood, etc. A mutation in an oncogene can causes cells continue to grow (or refuse to die) even when they are receiving no growth signals. Examples are Ras, activated in pancreatic and colon cancers, and Bcl-2, activated in lymphoid tumours. Many type of proteins have been grouped into oncoprotein such as growth factors, protein kinases and related proteins (11).

3. TUMOUR-SUPPRESSOR GENES

Tumour suppressor genes code for chemical messengers that command cells to slow or stop mitosis in order to allow DNA repair. This is done by special enzymes which detect any mutation or damage to DNA, such that the mistake is not carried on to the next generation. Tumour suppressor genes are usually triggered by signals that DNA damage has occurred. In addition to inhibiting mitosis, they can code for such enzymes themselves, or sending signals to activate such enzymes. However, a mutation can damage the tumour suppressor gene itself, or the signal pathway which

activates it, "switching it off". The invariable consequence of this is that DNA repair is hindered or inhibited. DNA damage accumulates without repair which in turn inevitably leading to cancer. Repression of genes is essential for the continuing of the cell cycle. If these genes are not expressed, the cell cycle will not continue, effectively inhibiting cell division (11). Mutations of *p53* and retinoblastoma *(Rb)* gene are common in a wide spectrum of tumours (12).

4. ANGIOGENIC GENES

These genes are coded for protein factor that control a cell's blood supply (13) such as vascular endothelial growth factors (*VEGF*, *VEGF-B*, *VEGF-C*), basic fibroblast growth factor (*bFGF*), angiopoetins (*Ang-1*, *Ang-2*), transforming growth factor (*TGF-a*) and platelet-derived growth factor alpha (*PDGF-*α). If a cell becomes cancerous and begins dividing rapidly, the daughter cells pile up in a mass. But as the mass expands, it needs to increase its supply of oxygen and nutrients; without such an increase, it will reach a steady-state size - about that of a pea - where the number of new cells is matched by dying cells. Such small carcinomas can stay in the body for months or years without gaining size. By the way, there is always the risk that one of the cancer cells will acquire a further mutation in a gene that regulates angiogenesis, the production of factors that promote the growth of new blood vessels. When this occurs, hundreds of new capillaries converge on the tumour, which gains the supplies it needs to grow in size.

5. METASTASIS GENES

These genes are coded for the protein factors that control the spread of cancer**.** Most cells are restricted to a particular place within a tissue, with their neighbours helping to define where that position should be. If a cell becomes detached from the tissue it usually stops proliferating and dies. But a cancer cell can acquire mutations in certain genes that allow it to spread to other parts of the body, for example, mutations in E-cadherin and the associated protein α-catenin have been associated with acquisition of the invasive phenotype (14).

Although in theory three mutations alone could lead to cancer, it is thought that most common at least five mutations, and probably more cause adult

cancers. But generally, mutations either in oncogene and tumour suppressor genes are required for cancer to occur. For example, a mutation limited to one oncogene would be suppressed by normal mitosis control and tumour suppressor genes. A mutation to only one tumour suppressor gene would not cause cancer either, due to the presence of many "backup" genes that duplicate its functions. It is only when enough proto-oncogenes have mutated into oncogenes, and enough tumour suppressor genes deactivated or damaged, that the signals for cell growth overwhelm the signals to regulate it, that cell growth quickly out of control (15).

TYPES OF CANCER

Cancer cells within a tumour are the descendants of a single cell, even after it has metastasized. Hence, a cancer can be classified into 5 types by the type of cell in which it originates and by the location of the cell (8).

1. Carcinomas

Carcinoma is any cancer that arises from epithelial cells. It invades surrounding tissues and organs, and may spread to lymph nodes and distal sites (metastasis). Carcinoma, like all neoplasia, is classified by its histopathological appearance. Adenocarcinoma and squamous cell carcinoma, two common descriptive terms for tumours, reflect the fact that these cells may have glandular or squamous cell appearances respectively. Severely anaplastic tumours might be so undifferentiated that they do not have a distinct histological appearance.

Sometimes a tumour is referred to by the presumptive organ of the primary (e.g. carcinoma of the prostate) or the putative cell of origin (hepatocellular carcinoma, renal cell carcinoma).

2. Lymphoma

Lymphoma is a general term for malignancies of lymphocytes or, more rarely, of histiocytes. Collectively, these cell types form the reticuloendothelial system and circulate in the vessels of the lymphatic system. Traditionally, Lymphoma is classified as Hodgkin's disease, discovered by Thomas Hodgkin in 1832, and non-

Hodgkin's lymphoma (all other types of lymphoma). Modern classifications of lymphoma have moved away from this artificial division.

3. Sarcoma

Sarcoma is a cancer of the bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Bone tumours (osteosarcomas) are also called sarcomas, but are in a separate category because they have different clinical and microscopic characteristics and are treated differently. Osteogenic sarcoma or osteosarcoma is one of the most common childhood bone cancers.

4. Melanoma

Melanoma is a malignant tumour of melanocytes. Melanocytes predominantly occur in the skin but can be found elsewhere, especially the eye. The vast majority of melanomas originate in the skin.

5. Teratoma

Teratoma is a type of tumour that derives from pluripotent germ cells. The word comes from a Greek term meaning roughly "monster tumour". Teratomas (more correctly teratomata) usually start from cells in the testes in men, the ovaries in women and in the sacrum in children. Teratomata involve cells from all three embryonic cell layers: ectoderm, mesoderm and endoderm. They can be benign or malignant.

BREAST CANCER

Breast cancer is the illness that many women fear most, though they're more likely to die of cardiovascular disease than they are of all forms of cancer combined. Still, breast cancer is second only to lung cancer as a cause of cancer deaths in American women. Although rare, breast cancer can also occur in men in the United States, more than 200,000 women and around 1,500 men develop the disease in 2005 (16).

CAUSE OF BREAST CANCER

Each of women breasts contains 15 to 20 lobes of glandular tissue, arranged like the petals of a daisy. The lobes are further divided into smaller lobules that produce milk during pregnancy and breast-feeding. Small ducts conduct the milk to a reservoir that lies just beneath the nipple. Supporting this network is a deeper layer of connective tissue called stroma.

The spaces between the lobes and ducts are filled with fat, which makes up about 80 - 85 % of women's breast during their reproductive years. Breasts also contain vessels that transport lymph, a colorless fluid that carries waste products and cells of the immune system - to lymph nodes located primarily under arm (axillary nodes) but also above the collarbone and in the chest. These nodes are collections of immune system cells that filter harmful bacteria and play a key role in fighting infection (17).

In breast cancer, some of the cells in breast begin growing abnormally. These cells divide more rapidly than healthy cells do and may spread throughout the breast, to the lymph or to other parts of the body (metastasize) (18). The most common type of breast cancer begins in the milk-producing ducts, but cancer may also occur in the lobules or in other breast tissue.

In most cases, it does not clear what triggers abnormal cell growth in breast tissue, but doctors do know that between 5-10 % of breast cancers are inherited. Defects in one of two genes, breast cancer gene 1 (BRCA1) or breast cancer gene 2 (BRCA2), put women at greater risk of developing both breast and ovarian cancer (19). Inherited mutations in the ataxia-telangiectasia mutation (ATM) (20) gene, the cell-cycle checkpoint kinase 2 (CHEK-2) genes (21) and the p53 tumour suppressor gene (22) also make it more likely that can be developed to breast cancer. Mutations of the CHEK-2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumours.

Yet most genetic mutations related to breast cancer are not inherited but instead develop during our lifetime. These acquired mutations may result from radiation exposure such as women treated with chest radiation therapy in childhood; for instance, have a significantly higher incidence of breast cancer than do women not exposed to radiation. Mutations may also develop as a result of exposure to cancer-

causing chemicals, such as the polycyclic aromatic hydrocarbons found in tobacco and charred red meats.

In the long run, establishing a link between genetic mutations and cancer is just the first step. Now researchers are trying to learn if a relationship exists between genetic makeup and environmental factors that may increase the risk of breast cancer. Although these studies are still preliminary, breast cancer eventually may prove to have a number of causes.

BREAST CANCER IN THAILAND

Breast cancer is one of the common malignancies in Thailand. The incidence is 16.3/100,000 population, second only to cervical cancer in women. The incidence of breast cancer in Thailand is low, with an age-specific rate similar to those in developing countries (Figure 3). From the statistical report of Siriraj Cancer Center in the year 2003, they found 552 cases of breast cancer or 19.99% of cancer in Thai female (Table 1). There was some report from the Faculty of Medicine Siriraj Hospital indicated during the year 1983 to 2001, there were 3,559 cases include 11 males about half of the cases were in the age between 40-54 years (Table 2). There have been no studies of breast cancer epidemiology in Thailand. The low risk may be a consequence of previously high frequency of fertility and low caloric intake. However, the incidence rate of breast cancer seems to have been increasing gradually over the past 5–10 years, which may be related to the change of lifestyle and diet (23).

Early detection and prompt treatment before lymphatic spread are key factors to a successful treatment result. Women who are over 40 years old, previously diagnosed as having or having a family history of breast carcinoma have an increased chance of developing breast cancer. Screening tests are recommended for these risk bystanders including breast self-examination and mammography.

Once diagnosed as positive, multimodalities treatment with surgery, radiation and chemotherapy are standard therapy. Adjuvant treatments with chemotherapy and/or hormonal therapy are recommended in certain cases. The objectives of adjuvant therapy are to increase overall and relapse–free survival and improve quality of life. There are various types of anti-cancer drug as shown in table 3.

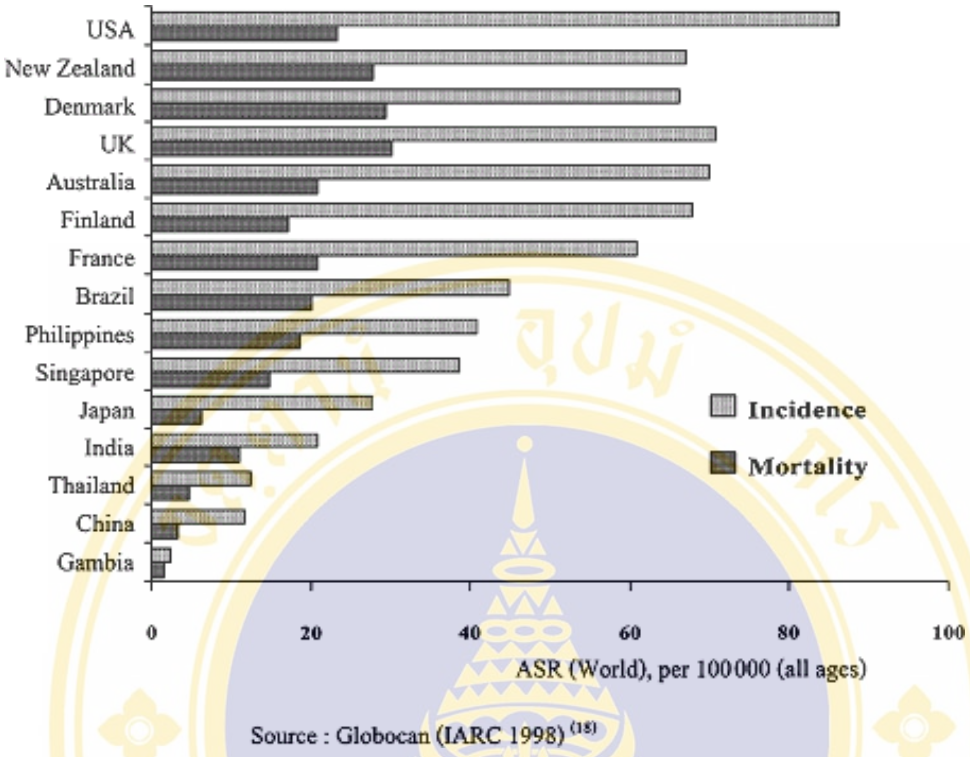

Figure 3. The world distribution of breast cancer patient.

Table 1. The ten leading sites of cancer (Modified from the statistic report from Siriraj Cancer Center in the year 2003).

Site	Total			Female			Male		
	Range	Number	Percent	Range	Number	Percent	Range	Number	Percent
All sites		4,663			2,727	58.48		1,936	41.52
Cervix	1	618	13.25	1	618	22.66		0	0.00
Breast	2	552	11.84	2	545	19.99		7	0.36
Colon Rectum	3	360	7.72	3	164	6.01	2	196	10.12
Lung	4	301	6.46	8	91	3.34	1	210	10.85
Lymphoma	4	301	6.46	4	151	5.54	4	150	7.75
Leukemia	5	294	6.30	5	148	5.43	6	146	7.54
Liver	6	213	4.57		65	2.38	5	148	7.64
Prostate	7	173	3.71		0	0.00	3	173	8.94
Oral cavity	8	147	3.15		62	2.27	8	85	4.39
Skin	9	131	2.81	10	74	2.71		57	2.94

Table 2. Breast cancer patients from 1983-2001 (Division of Head Neck and Breast Surgery).

Age	Number		Percent	
< 20	5			
20-24	10			
25-29	67		1.88	
30-34	193		5.42	
35-39	417		11.71	
40-44	603		16.94	
45-49	674	1,831	18.93	51.44
50-54	554		15.56	
55-59	369		10.36	
60-64	268		7.53	
65-69	172		4.83	
70-74	129		3.62	
75-79	61		1.71	
80-84	29			
85-89	10			

CLASSES OF ANTI-CANCER DRUGS

The anticancer drug has been grouped into 7 main groups according to their chemicals and drug mechanisms as shown in table 3.

Table 3. Classes of anti-cancer drugs

Anti-cancer drug	Action of drugs	Mechanism of resistant
1. ALKALOIDS		
Microtubule inhibitors		
- VINCA ALKALOIDS	Block mitosis with metaphase arrest	- decrease drug accumulation - over expression of the P-glycoprotein - alteration of tubulin structure
- PACLITAXEL (Taxol) and DOCETAXEL	Disrupt the equilibrium between free tubulin and microtubules	- same as occurred in VINCA ALKALOIDS
Chromatin function inhibitor		
- Topoisomerase inhibitors	Interfere the activity of topoisomerase	- over expression of the P-glycoprotein drug efflux transporter
- CAMPTOTHECIN	Target the nuclear enzyme topoisomerase I	-

Table 3. Classes of anti-cancer drugs (cont'd)

Anti-cancer drug	Action of drugs	Mechanism of resistant
2. ALKYLATING AGENTS		
Covalent DNA-binding drugs		
- NITROGEN MUSTARDS	Cross-linking guanine bases in DNA strands	- decrease permeability of the drug into cells - increase repair of DNA
- NITROSOUREAS	Drugs are converted non-enzymatically into a cytotoxic carbonium ion and isothiocyanate molecule	- cross-resistance between different alkylating agents
3. ANTIBIOTICS		
Non-covalent DNA-binding drugs		
- DACTINOMYCIN	DNA intercalating	- over expression of the P-glycoprotein
- ANTHRACYCLINE	DNA intercalating	- over expression of the P-glycoprotein
- BLEOMYCIN	Inhibit DNA synthesis	- degradation of drug by the activity of aminase enzymes
4. ANTIMETABOLITES		
Antifolates		
- METHOTREXATE	Inhibit dihydrofolate reductase(DHFR)	- decreased drug uptake - amplification of the DHFR gene - mutation in DHFR gene
- PURINE ANTIMETABOLITES	Inhibit nucleotide and nucleic acid synthesis	- alteration of phosphoribosyl transferase enzyme
- Chlorodeoxyadenosine	Inhibit DNA strand elongation	-
Pyrimidine antagonists		
- FLUOROPYRIMIDINES	Inhibit thymidylate synthase	- decreased conversion to the nucleotide form of drug
5. ENZYME		
L-asparaginase inhibitor		
- Hydroxyurea	Ribonucleotide reductase inhibitor	- change in ribonucleotide reductase
6. HORMONES		
Glucocorticoids		
- Antiestrogen: TAMOXIFEN	Estrogen competitive inhibitor to estrogen receptor	- not effected to the cell absent estogen receptor
Non-stearoidal antiandrogens		
- Aromatase inhibitor: ANASTROZOLE	Inhibit activity of aromatase	-
7. PLATINUM COMPOUNDS		
Cisplatin	DNA cross-linking	- increasing ability to repair intra-strand

NEW APPROACHES TO CANCER THERAPY

General outcome of chemotherapy is incomplete elimination of cancerous cells which results in drug and multi-drugs resistant cancerous cells. Two approaches have been developed in order to overcome this problem. The first approach is searching for new anticancer drugs. The second is developing new strategies for cancer treatment. Various strategies of cancer treatment are listed in table 4.

Table 4. The new approaches to cancer therapy.

Approach	**Mechanism**	**Ref.**
Monoclonal antibody	Inhibit cancer cell proliferation by using the antibody recognize specific antigenic determinants on cancer cells.	(24)
Biological response modifiers:-		
- Interferons	Direct cytotoxicity to cancer cells and NK cell and macrophage stimulation.	(25)
- Interleukin-2	Stimulate B lymphocyte proliferation	(26)
Adoptive immunotherapy	Activate person's own lymphocytes and inject them back into the patient	(27)
Hematopoietic growth factors	Use the granulocyte-macrophage stimulating factors to replenish blood after high dose chemotherapy and followed by bone-marrow transplant	(28)
Induction of tumour cell differentiation	Use differentiation agent to initiate cells in immature form into functional cells	(29)
Gene therapy	Incorporation of DNA coding for a particular gene to reprogramming of the cells	(30)
Antisense therapy	Introducing a small piece of DNA into the cell for regulating gene expression in cancer cells	(31, 32)

Table 4. The new approaches to cancer therapy (cont'd).

Approach	Mechanism	Ref.
Tumour vaccines	Stimulate immune response to specific antigenic determinants on cancer cells.	(33)
Therapy directed against tumour metastasis	Inhibit proteolytic enzymes which are known to play an important role in the invasive and metastatic process	(34)
Inhibitors of angiogenesis	Inhibit the growth of new blood vessels supplying tumour growth	(35)

CELL DEATH

Normal cell growth and development requires a well-controlled balance between the turnover rate of cell proliferation and cell death. There are three main forms of cell death have been described. First, any cells can be killed by a wide range of pathological stimuli, such as certain toxins or viruses. These cells die by a process known as necrosis, which involves disruption of membrane integrity and subsequent cellular swelling that results in lysis. Second, the programmed cell death (PCD), this is unlike necrosis. Programmed cell death (PCD) is an essential phenomenon in normal development of multicellular organisms. It involves the loss of individual cells at specific times during development (36). The last form of cell death is autophagy. The autophagic cell death appears to be activated when massive removal of cells or cytoplasm is demanded, for instance by developmental programmes. Autophagy preceeding cell death may also reflect a cell's adaptive response to sublethal (non-necrotic) conditions such as nutrient/growth factor deprivation or cell damage by cytotoxic drugs, hypoxia etc (37).

APOPTOSIS

Apoptosis is an active dedicated programme that leads to regulated destruction of a cell. If counter-balances cell division and cell migration in the upkeep of homeostasis and ensures proper function in a variety of tissues, such as reproductive organs and the inner system. The typical characteristics of cell undergoing apoptosis

are shown in table 5 and the morphological changes are diagrammed in figure 4. A cell undergoing apoptosis shows a characteristic morphology that can be seen under a microscope. The cell becomes round (circular) because the protein structures that conform the cytoskeleton are digested by specialized peptidases (called caspases) that have been activated inside the cell. Chromatin, the DNA and its packaging proteins in the nucleus of cell undergoes initial degradation and condensation. Chromatin undergoes further condensation into compact patches against the nuclear envelope. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented. The nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA. Plasma membrane blebbing and the cell break apart into several vesicles called apoptotic bodies, which are then phagocytosed.

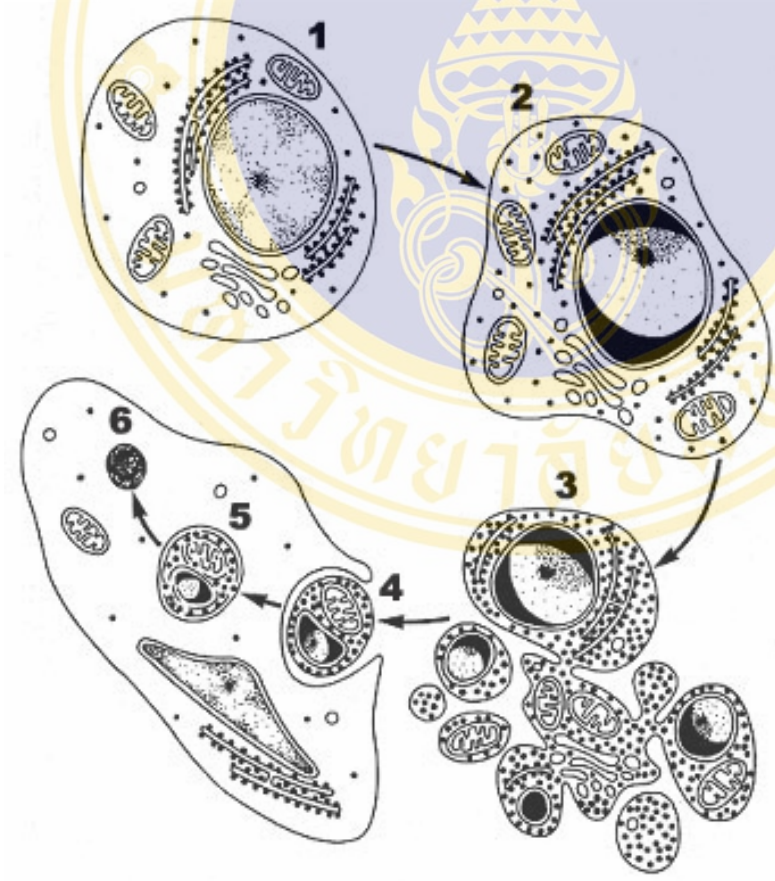

Figure 4. Sequence diagram of ultrastructural changes in apoptosis. (School of medicine, The University of Queensland, Australia 2003. Retrieved from http://www.som.uq.edu.au/Pathology/histotech/photos/Diagram.gif)

Table 5. Features that allow distinction of apoptosis from necrosis.

FEATURE	APOPTOSIS	NECROSIS
Stimuli	Physical/Pathological	Pathological
Occurrence	Single cells	Group of cells
Adhesion between cells and to basement membrane	Lost (early)	Lost (late)
Cytoplasmic swelling	Late stage	Very early
Damage to organelles	Late	Early
Release of lysosomal enzymes	Absent	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrhexis)	Disappearance of nuclei (karyolysis)
Nuclear chromatin	Compaction in uniformly dense masses	Clumping, not sharply defined
DNA breakdown	Internucleosomal (formation of DNA ladder)	Random (smearing)
Outcome	Formation of apoptotic bodies	Swelling and disintegration
Phagocytosis by other cells	Present	Absent

REGULATION OF APOPTOSIS

Cell survival is dependent on the variety of factors and the appropriate balance of both positive and negative signals. For example, some positive regulatory signals include growth factors in neurons and IL-2 for lymphocyte mitosis. Negative regulatory signals include increased intracellular oxidant levels, reactive oxygen species (ROS), DNA damage by oxidants or radiation (UV, X-rays, gamma irradiation), injury due to mechanical damage or exposure to chemotherapeutic drugs and toxins, the presence of mis-folded proteins, and 'death' activators (TNF, certain toxins, Fas ligand). Apoptosis-inducing signals or negative signals are carefully

processed and evaluated against anti-apoptotic factors or positive signals in the target cells. If the pro-apoptotic elements beat their counterparts, a dedicated death program is then activated and the cell will undergo apoptosis.

MAJOR FACTORS THAT REGULATE APOPTOSIS

1. CASPASES

Caspases are a family of cysteine proteases that cleave proteins after aspartic acid residues. They are the main effectors of apoptosis or programmed cell death (PCD). They are classified based on their pro-domain structure or primary function (Figure 5). Caspases-1, -2, -4, -5, -9, -11 and -12 contain a long pro-domain with a caspase activation and recruitment domain (CARD). Among these CARD-containing caspases, caspase-1, -4, -5 and -11 have functions in the inflammatory response, while caspases-2, -8, -9, -10, and -12 play roles in apoptosis. These apoptotic caspases can be further divided into ‘initiators’ (caspases-2, -8, -9, -10, -12) and ‘effectors’ (caspases-3, -6, -7, -14). Caspases-8 and -10 possess two death effector domains (DEDs) in their pro-domains. Caspases-3, -6, -7 and -14 has short pro-domains. Interestingly, this classification yields a close structure–function relationship among the caspases. All of initiator apoptotic caspases contain a large pro-domain whereas all effector caspases have a short pro-domain (38).

Caspase activation can be mediated by intrinsic factors such as Bcl-2 on the mitochondrial membrane or by external signals. Caspases activation via Bcl-2 is regulated by apoptosome formation and caspase-9 activation. External signals such as TNF and Fas receptor, triggers caspases cascade upon receptor-ligand interaction. Caspase-8 and caspase-10 are primary target of activation process (39).

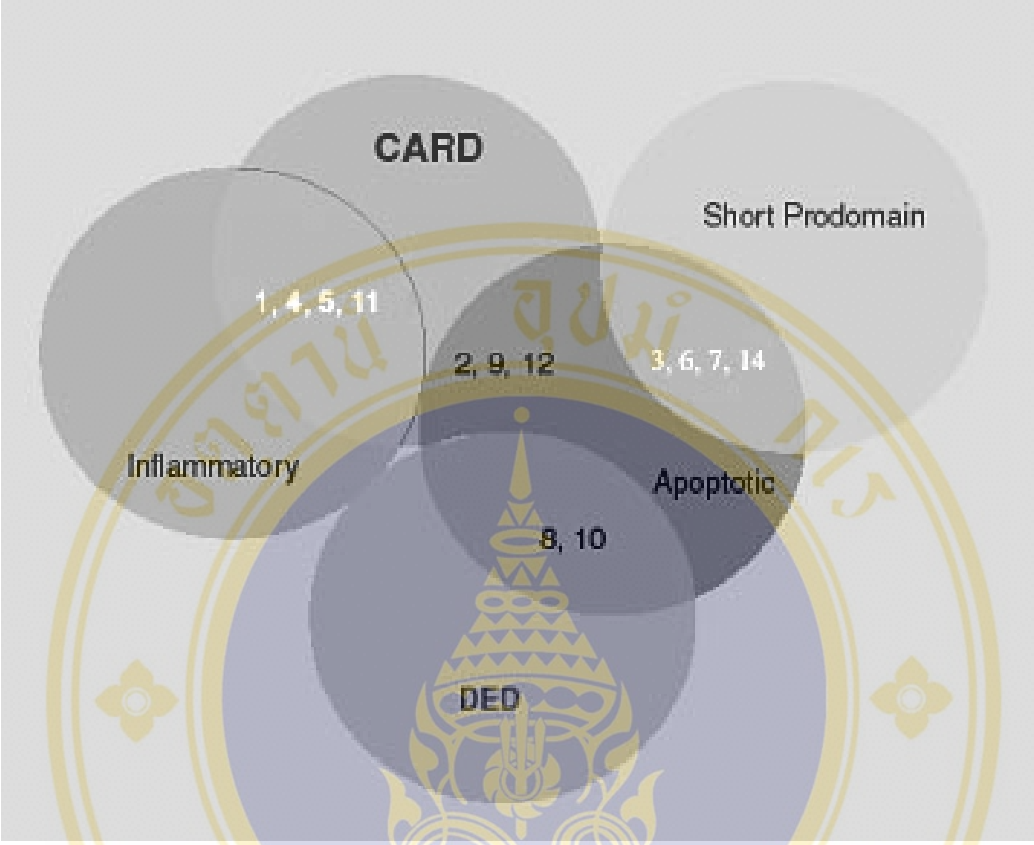

Figure 5. The classification of caspases (38).

2. BCL-2 FAMILY

Bcl-2 derives its name from *B-cell lymphoma 2*, as it is the second member of a range of proteins initially described as a reciprocal gene translocation in chromosomes 14 and 18 in follicar lymphomas. The Bcl-2 family consists of about 20 homologues of important pro- and anti-apoptotic regulators of programmed cell death (40). All Bcl-2 members were classified by one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) into 3 main groups as shown in figure 6-7.

1. Bcl-2 subfamily (anti-apoptotic): Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1
2. Bax subfamily (pro-apoptotic): Bax, Bak and Bok
3. BH3 subfamily (pro-apoptotic): Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL

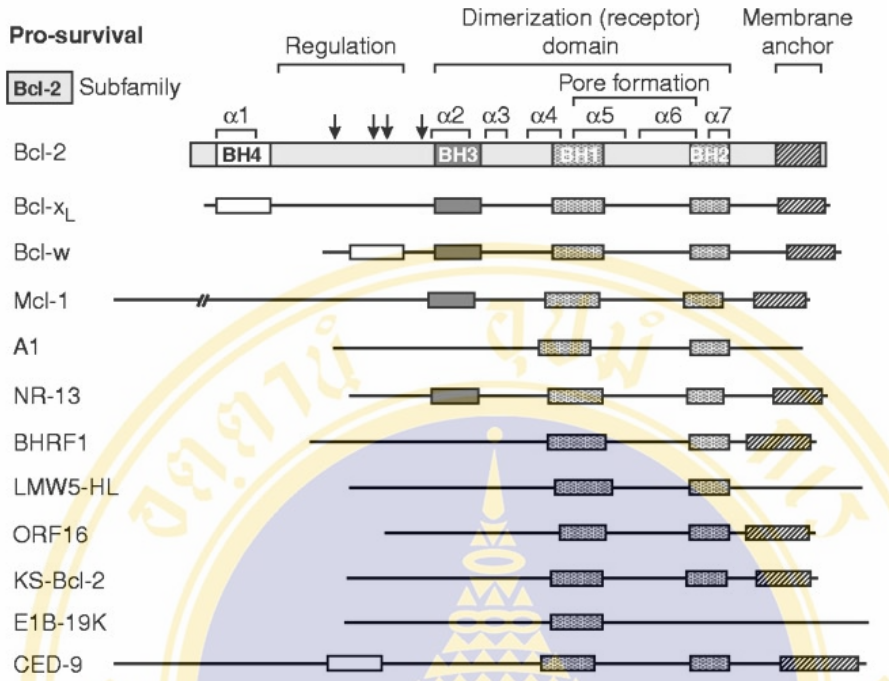

Figure 6. The homology domain of Pro-survival Bcl-2 family protein (41).

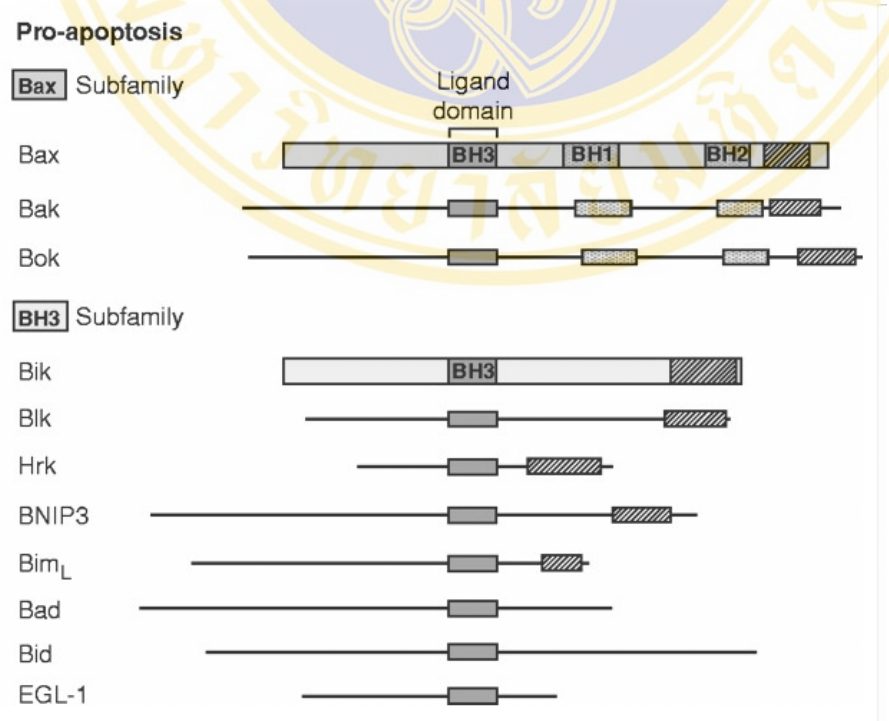

Figure 7. The homology domain of Pro-apoptosis Bcl-2 family protein (41).

Most pro-survival members, which can inhibit apoptosis in the face of a wide variety of cytotoxic insults, contain at least BH1 and BH2 (42), and those most similar to Bcl-2 have all four BH domains. The two pro-apoptotic subfamilies differ markedly in their relatedness to Bcl-2. Bax, Bak, and Bok which contain BH1, BH2, and BH3, resemble Bcl-2 fairly closely. In contrast, the seven other known mammalian "killers" possess only the central short (9 to 16 residue) BH3 domain (41).

Many members of the pro-apoptotic Bcl-2 family are present in cells at levels sufficient to induce apoptosis. However, these members do not induce apoptosis because their activity is maintained in a latent form. For example, Bax is present in the cytosol of live cells. After an appropriate signal, it causes release of mitochondrial cytochrome c into the cytosol which can activate other key regulatory proteins play role in apoptosis (43). BID is also present in the cytosol of live cells. After cleavage by caspase-8, it moves to the mitochondria where it causes release of cytochrome c possibly by altering the conformation of Bax (44). Similarly, BAK appears to undergo a conformational change that converts it from an inactive to an active state. Thus, understanding the molecular mechanisms responsible for regulating the Bcl-2 family activities creates the potential for pharmaceutical intervention to control apoptosis.

Since the Bcl-2 gene has been implicated in a number of cancers, including melanoma, breast, prostate and lung carcinomas. It is also thought to be involved in resistance to conventional cancer treatment (45). This supports a role for decreased apoptosis in the pathogenesis of cancer.

3. CYTOCHROME C

Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family such as BAX and BID, leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into cytosol (46). In the cytosol, or on the surface of the mitochondria, cytochrome c is bound by the protein called Apaf-1 (apoptotic protease activating factor), which also binds caspase-9 and dATP. Binding of cytochrome c triggers activation of caspase-9, which then accelerates apoptosis by activating other caspases.

4. SUPPRESSORS OF APOPTOSIS

The induction of apoptosis or progression through the process of apoptosis is inhibited by a group of proteins called Inhibitors of Apoptosis (IAPs). These proteins contain a BIR (baculovirus IAP repeat) domain near the amino-terminus. The BIR domain can bind some caspases (47). Many members of the IAP family of proteins block proteolytic activation of caspase-3 and -7. XIAP, cIAP-1 and cIAP-2 appear to block cytochrome c-induced activation of caspase-9, thereby preventing initiation of the caspase cascade (48). Since cIAP-1 and cIAP-2 were first identified as components in the cytosolic death domain-induced complex associated with the TNF family of receptors, they may inhibit apoptosis by additional mechanisms. FLIP/FLAME is highly homologous to caspase-8. It does not, however, contain the active site required for proteolytic activity. FLIP appears to compete with caspase-8 for binding to the cytosolic receptor complex (49), thereby preventing the activation of the caspase cascade in response to members of the TNF family of ligands. The exact *in vivo* influence of the IAP family of proteins on apoptosis is not clear.

APOPTOSIS PATHWAYS

There are four stages leading to apoptosis (figure 8). The first stage comprises of different potentially harmful stimuli that interact with a cell. In the second stage, an early signaling molecule is activated. This signal is processed by a regulatory mechanism, which evaluates the strength of the apoptosis inducing signal against anti-apoptotic signals in the third stage. If the death inducers prevail the cell commits to apoptosis and enters the fourth, the final stage where specific executor proteins are responsible for the organized destruction of the cell (50).

There are at least two broad pathways that lead to apoptosis, an "extrinsic" and an "intrinsic" pathway. The extrinsic and intrinsic pathways merge at caspase 3; subsequent processes result in many events, including phosphorylation of histone H2A.X at serine 149 and H2B at serine 14, cleavage of PARP, inter-nucleosomal degradation of DNA, and packaging of the cell into small units that are easily taken up by neighboring cells.

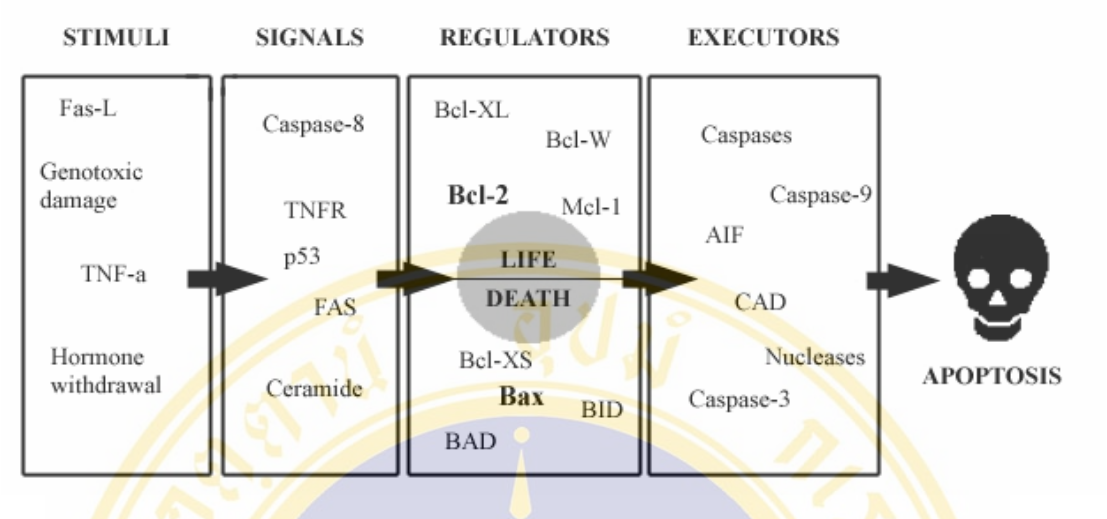

Figure 8. Four stages leading to apoptosis according to Morita and Tilly (50).

1. Extrinsic pathway

The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The cell surface death receptors play role in death signal transmitting with cooperation with death ligands which play a central role in instructive apoptosis (51). The best characterized death receptors are CD95 (also called Fas or Apo1) and TNFR1 (also called p55 or CD120a). Additional death receptors are death receptor 3 (DR3; also called Apo3), death receptor 4 (DR4), and death receptor 5 (DR5; also called Apo2, TRAIL-R2). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily (52). CD95 ligand (CD95L, also called FasL) binds to CD95; TNF and lymphotoxin α bind to TNFR1; Apo3 ligand (Apo3L, also called TWEAK) binds to DR3; and Apo2 ligand (Apo2L, also called TRAIL) binds to DR4 and DR5 (53).

As shown in figure 9, binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) results in clustering of receptors and initiates the extrinsic pathway. Activated Fas trimer recruits an adaptor protein called FADD, through interaction with their respective death domains (DDs). FADD functions as a bridge between Fas and downstream signal transduction, which is mediated by the N-terminal region of the protein, termed death effector domain (DED). The TNFR1 mediated pathway utilizes TRADD protein in recruitment of FADD. Concentration of pro-caspase 8 results in its

autocatalysis and activation; activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis. Activated caspase-8 is able to induce apoptosis through a mitochondrial pathway by cleaving BID, or directly by activating downstream effector caspases.

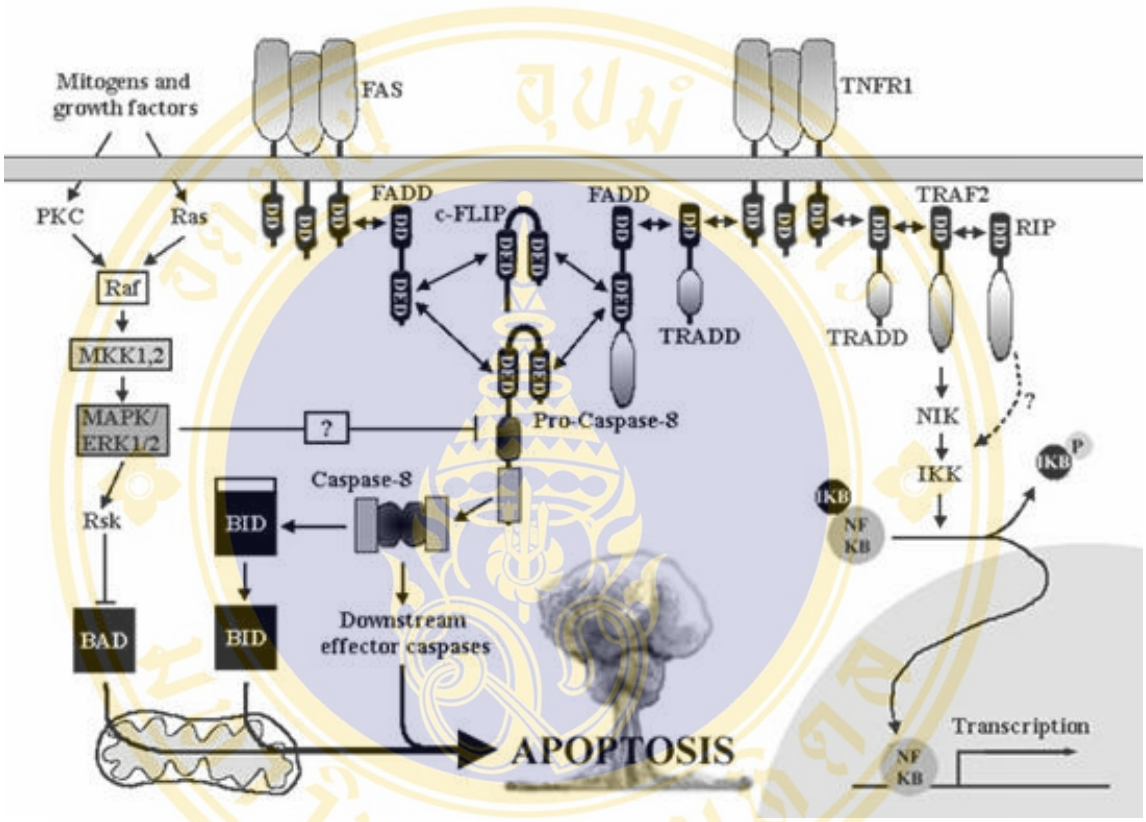

Figure 9. Regulation of death receptor signaling (54).

The apoptosis signaling by DR3 triggers responses that resemble those of TNFR1 (55). Like TNFR1, DR3 activates NF-κB through TRADD, TRAF2, and RIP and apoptosis through TRADD, FADD, and caspase-8 (figure 10). Apo3L activates NF-κB TRADD, TRAF2, RIP, and triggers apoptosis through TRADD and FADD, consistent with signaling through DR3 thus, with respect to the regulation of NF-κB and apoptosis.

A TNF family member, TRAIL or Apo2L shows the most similarity to CD95L (56). Apo2L triggers rapid apoptosis in many tumour cell lines unlike expression of CD95L, which is restricted mainly to activated T cells and NK cells

(57). The DR4 and DR5 receptors contain death domains in their intracellular domain, but as yet no adapter molecule (such as FADD or TRADD) has been identified that associates with the receptor to initiate apoptosis. Work in FADD-deficient mice has indicated that FADD is not essential for triggering apoptosis via these receptors (58). Since DR4 and DR5 mRNA has been shown to be expressed constitutively in several tissues, it has been suggested that there are mechanisms that protect cells from apoptosis. One possible mechanism of protection is based on a set of decoy receptors that compete for binding of TRAIL with the DR4 and DR5 receptors. The decoy receptors are called DcR1 and DcR2 (see figure 11). Both of these receptors are capable of competing with DR4 or DR5 receptors for binding to the TRAIL ligand.

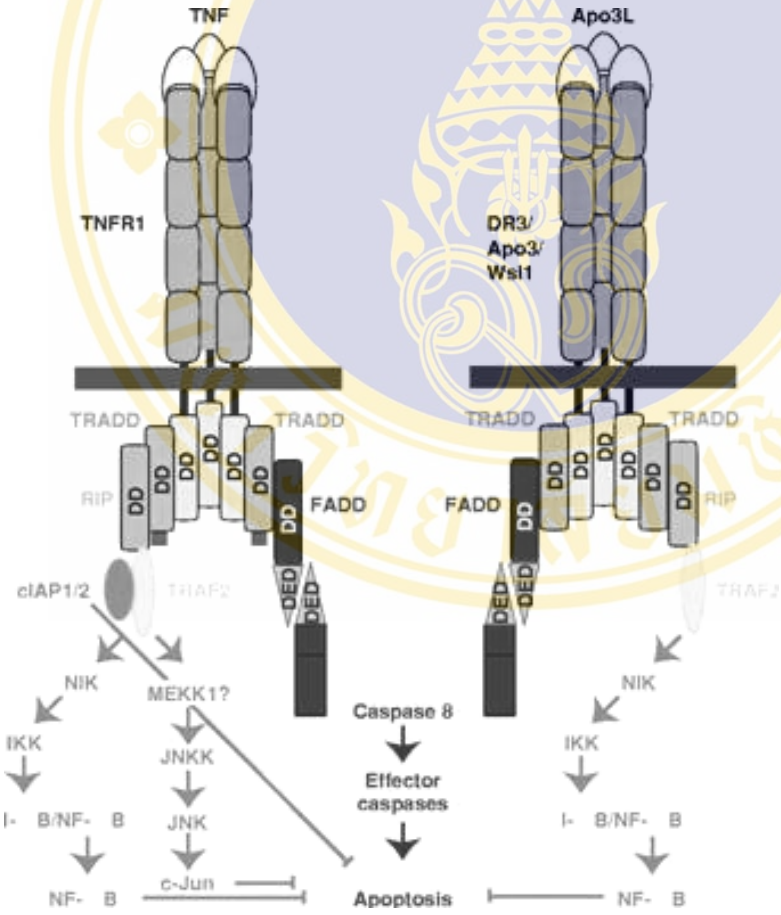

Figure 10. Proapoptotic and antiapoptotic signaling by TNFR1 and DR3 (51).

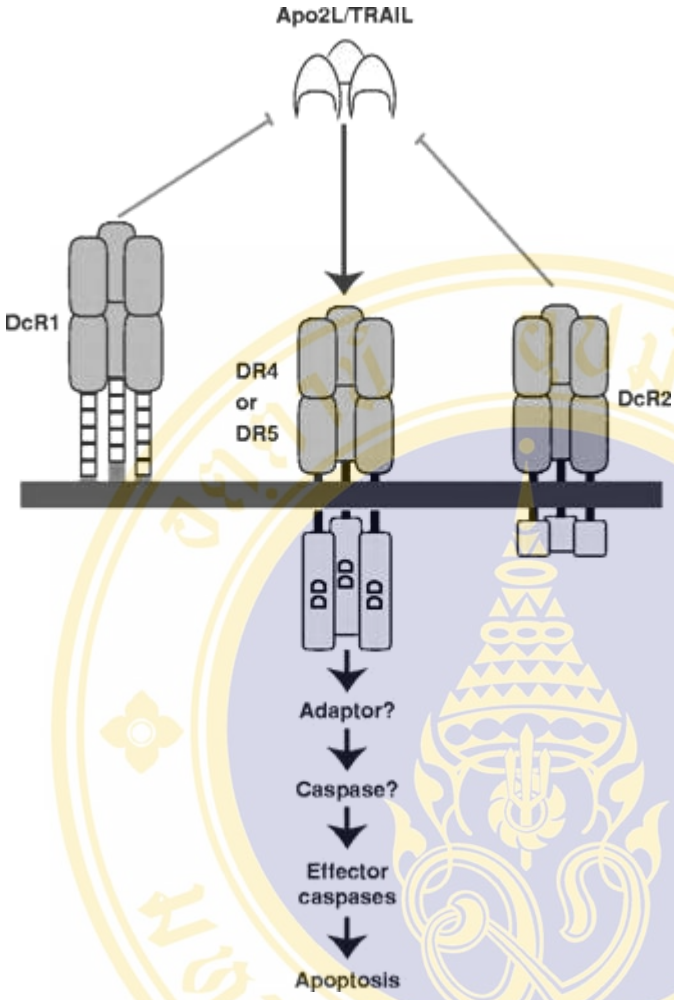

Figure 11. Apoptosis signaling by DR4 and DR5 and its modulation by decoy receptors (51).

2. Intrinsic apoptosis

The intrinsic apoptosis pathway begins when an injury occurs within the cell. The injury could result in necrosis and produce an inflammatory response, but the apoptotic machinery is in place to ensure that the damaged cell is packaged and removed cleanly, in order to prevent inflammation. Mitochondrial damage, for example, can initiate the intrinsic pathway, overcoming the effect of pro-survival protein Bcl-2. Cytochrome c, released from damaged mitochondria, binds Apaf1, which then activates an initiator caspase; in this case caspase 9, which then activates the effector caspase, caspase 3. Other proteins released from damaged mitochondria, Smac/DIABLO and Omi/HtrA2, counteract the effect of IAPs (inhibitor of apoptosis

proteins), which normally bind and prevent activation of caspase 3. The dance between Bcl family members, IAPs, Smac/DIABLO and Omi/HtrA2 is central to the intrinsic apoptosis pathway.

As illustrated in figure 12, apoptotic effectors are stored in the mitochondrial intermembrane space in healthy cells (left). When it receives appropriate apoptotic stimuli, the mitochondrion releases these factors into cytosol (right). Released caspases may act mainly in amplification of the caspase cascade. Apoptosis-inducing factor (AIF) is one of death protein, which can be released from the intermembrane space. It travels and concentrates into the nucleus, where it seems to be essential for nuclear disassembly (59).

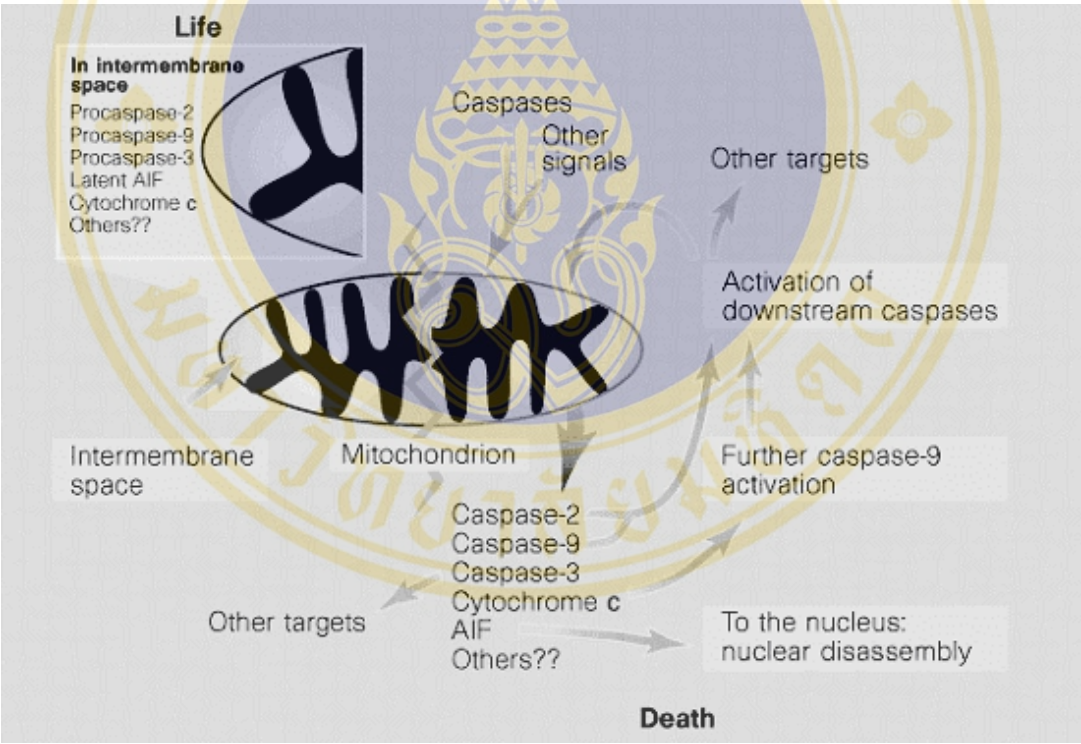

Figure 12. The balance between life and death-promoting factors in the mitochondria (59).

ER Stress-mediated Apoptosis Pathway

Endoplasmic reticulum (ER) is the site of synthesis and folding of secretory proteins. Perturbations of ER homeostasis affect protein folding and cause ER stress. ER can sense the stress and respond to it through translational attenuation, upregulation of the genes for ER chaperones and related proteins and degradation of unfolded proteins by a quality-control system. One of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) (60). Three apoptosis pathways, triggered by ER stress, have been reported (figure 13).

The first is transcriptional induction of the gene for CHOP (C/EBP homologous protein)/GADD153, a member of the C/EBP family of transcription factors (61). CHOP is barely detected under physiological conditions, but is strongly induced in response to ER stress (62). Over-expression and targeted disruption of the CHOP gene has demonstrated that CHOP promotes apoptosis in response to ER stress (63-67). Transcription of the CHOP gene is mediated by three distinct upstream components, Ire1(68), PERK (69) and ATF6 (70). Ire1 transmits signals by processing a transcription factor XBP-1 mRNA, which is frame switched by removing a small intron. PERK transmits signals by phosphorylating eIF-2α, which can induce the translational upregulation of ATF4 by uORF bypass scanning system. ATF6 is cleaved by ER stress-induced proteolysis and releases an N-terminal cytosolic domain (p50ATF6). XBP-1, ATF4 and p50ATF6 work as a transcription factor to increase CHOP mRNA. Compared with the great progress in understanding the upstream of CHOP induction, search for the downstream of CHOP has been stalled. A stress-induced form of carbonic anhydrase VI was identified as one of the downstream target genes of CHOP by using representational difference analysis (71). It was suggested to promote apoptosis by decreasing intracellular pH because the membrane pore-forming activity of the pro-apoptotic regulator Bax is higher at lower pH (72). McCullough et al reported that CHOP down-regulates Bcl-2 protein and increases the production of reactive oxygen species (73). Thus, several genes that respond to CHOP have been identified but none was shown directly be involved in the process of apoptosis, and further investigation is needed.

The second apoptotic pathway in ER stress is activation of the cJUN NH_2-terminal kinase (JNK) pathway. JNKs constitute a family of signal transduction proteins, regulating gene expression and participating in determination between apoptosis and survival in response to stresses. Activated Ire1 recruits the TRAF2 and ASK1, which results in formation of the Ire1-TRAF2-ASK1 complex leading to JNK activation (74, 75).

The third is activation of the ER-localized cysteine protease, caspase-12 (76). Caspase-12 is activated by ER stress, but apparently not by death receptor-mediated or mitochondria-targeted apoptotic signals. It was reported that caspase-12 is activated through activation of m-calpain (77), IRE1α/TRAF2 (78) or caspase-7 (79). Although targeted disruption of the caspase-12 gene in mice clearly demonstrated that caspase-12 mediates ER stress-induced apoptosis.

As previous described in many studies about the activation of human caspase-12 and the pathway linking ER stress apoptosis. Fischer *et al* reported that human caspase-12 acquires multiple stop codons, suggesting the lack of functional human homologue (80). Cells expressing caspase-12 were more vulnerable to ER stress than cells expressing the empty vector, concomitant with increased activation of caspase-3. These findings suggested that activation of ER-resident caspase-12 indirectly activates cytoplasmic caspase-3 and might be important in ER stress-induced neuronal apoptosis (81). In contrastly, some previous studies reported that caspase-12 plays a pivotal role in cisplatin-induced apoptosis in renal tubular epithelial cells (LLC-PK1) (82). Further studies on the human caspase-12 and the pathway linking ER stress to activation of caspase-12 are necessary.

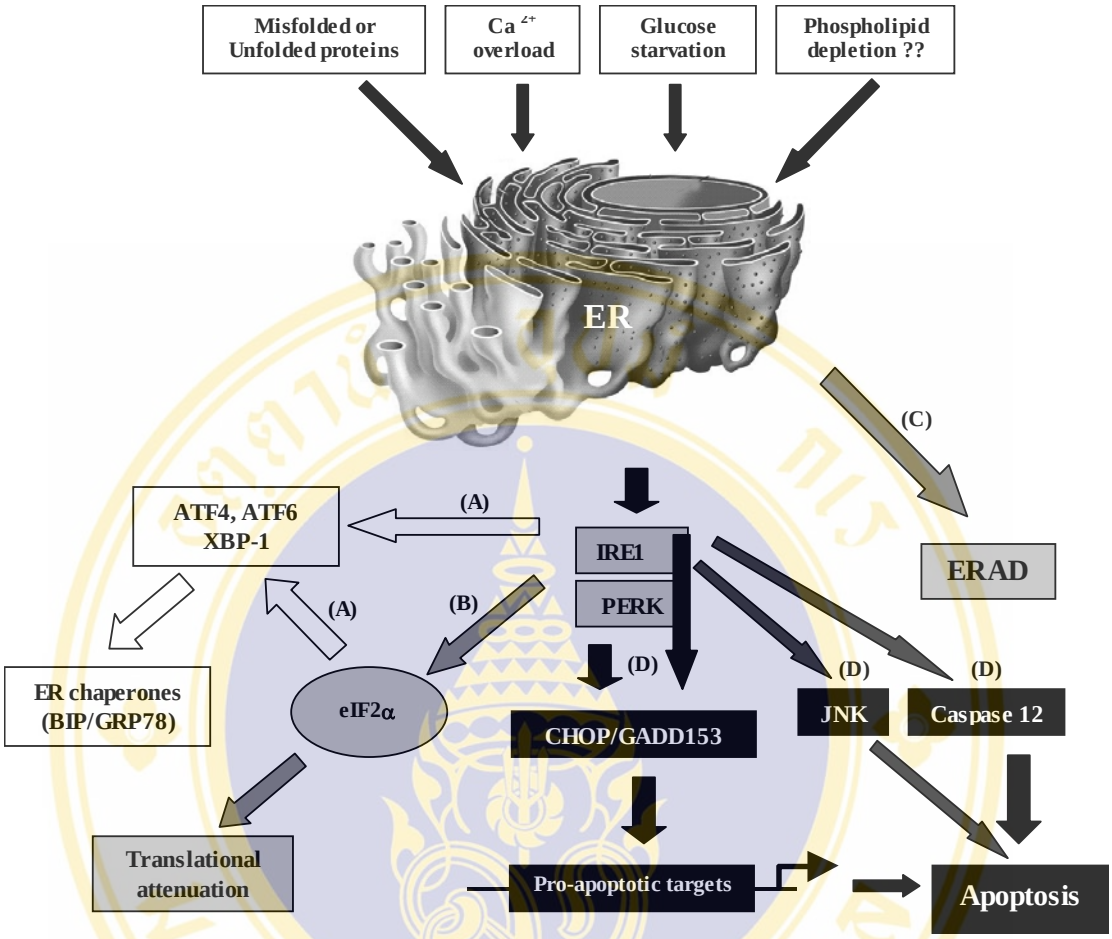

Figure 13. ER stress mediated apoptosis pathway (Modified from Sanden, 2004) (83).

CASPASE-3-DEFICIENT BREAST CANCER (MCF-7)

Caspase-3 is a member of the cysteine protease family, which plays a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins. Caspase-3 can be activated by diverse death-inducing signals, including the chemotherapeutic agents. Primary breast tumour and normal breast parenchyma obtained from patients undergoing breast surgery. Approximately 75% of the tumour as well as morphologically normal peritumoural tissue samples lacked the caspase-3 transcript and caspase-3 protein expression (84). In addition, the caspases-3 mRNA levels in commercially available total RNA samples from breast, ovarian, and cervical tumours were either undetectable (breast and cervical) or substantially decreased (ovarian). Despite the complete loss of caspase-3, the expression levels of other caspases, such as caspase-8 and caspase-9, were normal in the entire tumour samples studied. The

sensitivity of caspase-3-deficient breast cancer (MCF-7) cells to undergo apoptosis in response to doxorubicin and other apoptotic stimuli could be augmented by reconstituting caspase-3 expression. These results suggest that the loss of caspases-3 expression may represent an important cell survival mechanism in breast cancer patients (7).

VR-3848

As part of ongoing searching for chemotherapeutic compound from tropical plants in Thailand, Prof. Vichai Reutrakul and colleagues have discovered a novel peptide named VR-3848 which was purified from *Euphobiaceae* known in Thai name as **Takhe khumwang** (ตะเข้คุมวัง), and **Tao tua mia** (เต่าตัวเมีย). Local people use the root part for face whitening.

This novel compound exerts potent anti-cancer activity against many tumour cell lines. For example, VR-3848 kills Lu-1, a non-small cell lung cancer cell line, 10 folds greater than vinblastine does (2). This novel peptide is not only killing cancer cells in an *in vitro* system but also exerts its effect in mouse model using Hollow Fiber technology. Therefore, it is interesting to investigate molecular mechanism of anti-cancer exerted by this high potential chemotherapeutic agent.

DNA HYBRIDIZATION ARRAYS

Hybridization arrays have created a wave of interest and skepticism in the past five years. While many scientists view the use of arrays to monitor gene expression for thousands of genes as the dawn of functional genomics (85, 86). Although gene expression studies using multiplex hybridization arrays have been performed on a wide range of research topics including cell biology, aging, cancer, environmental toxicity and drug abuse (87-89), performing these experiments in a manner that yields accurate results presents a unique technical challenge.

In the past, analysis of gene expression (through measurement of steady state levels of mRNA) was conducted one gene at a time. Northern blotting, dot blots and quantitative RT-PCR were the common methods for investigating such changes in gene expression. Northern blot analysis works with sample RNA that has been

resolved by agarose electrophoresis and bound to a membrane. A gene-specific probe is then labeled and hybridized to the immobilized RNA, but large amounts of RNA are frequently required, only one gene is analyzed at a time and the approach requires the production of an individual and specific probe for each gene of interest (90). Dot blots are an attempt to increase the throughput of northern blots by eliminating the need for electrophoretic resolution, but they suffer from the same problems of single-gene analysis (91). The quantitative RT-PCR was developed in the hope that it would increase the throughput and reduce the sample size needed to measure gene expression. This method is more sensitive and therefore requires less RNA than blotting methods, but this procedure presents unique problems in the form of designing appropriate amplification standards and characterizing reaction kinetics for each gene of interest (92). The differential display (93), serial analysis of gene expression (SAGE) (94) and total gene expression analysis (TOGA) (95) offer great promise because they are multiplex technologies, but they have limited development and acceptance. The very advantage of these approaches is the source of their greatest limitation in that identified genes must then be sequenced, identified and analyzed in a serial fashion. These procedures can be time and labor intensive and prone to false positives. However, these techniques are still the best for gene dicovery – that is, finding unknown genes. Hybridization array technology, on the other hand, offers to bypass many of the limitations of these techniques by simultaneously creating labeled copies of multiple sample RNAs and then hybidizing them too many different gene-specific fixed DNA molecules. Most hybridization arrays are not designed to differentiate between alternatively spliced transcripts of the same gene and, in some cases, between highly homologous members of a gene family. Finally, a change in mRNA does not necessarily correlate with a change in protein (96), and the translated protein often requires further modification to realize its full activity. However, until proteomic technologies become universally accessible to the research community, hybridization arrays are the best opportunity for studying gene expression on a genomic scale.

ARRAY TYPES

Current array formats can be categorized into 4 groups: macroarrays, microarrays, high-density oligonucleotide arrays (Gene Chips) and microelectronic arrays. The difference depends on their matrix, probe number/density, array size and type of label. An understanding of the strengths and weaknesses of each platform is necessary to decide which is appropriate for an individual investigator's research aims.

1. Macroarrays

Macroarrays are generally defined as those arrays that rely on robotically spotted probes that have been immobilized on a membrane-based matrix. The term macroarray as opposed to microarray refers to the generally lower probe density on these arrays. Theses arrays, typically containing between 200 and 5,000 genes, are commercially available for a wide variety of organisms and genes. The idea of macroarrays grew from early hybridization experiments against cDNA libraries (97). These experiments were combined with improvements in molecular biology and the use of robotic workstations to spot individual cDNA clones onto membranes. Macroarray are unique among hybridization arrays in that they use radioactive target labeling, although chemiluminescent labeling has also been described. A typical gene array experiment involves in these steps (figure 14).

1. Isolating RNA from the samples to be compared.
2. Converting the RNA samples to labeled cDNA via reverse transcription.
3. Hybridizing the labeled cDNA to identical membrane or glass slide arrays.
4. Removing the unhybridized cDNA.
5. Detecting and quantitating the hybridized cDNA
6. Comparing the quantitative data from the various samples

2. Microarrays

Microarrays can be differentiated from macroarrays in three ways. First, microarrays use a glass or plastic slide as a matrix. Second, they use fluorescent dye-labeling detection. Third, they tend to have a larger number and higher density of

probes than macroarrays. This approach has advantages in that hybridization takes place in a flow cell or small hybridization chamber, which uses a much smaller hybridization solution volume as compared to macroarrays, thereby increasing the relative target concentration. A competitive fluorescent scheme allows both sample groups (control and treated) to be hybridized to the same array (98).

3. High-density oligonucleotide arrays (Gene Chips)

High-density oligonucleotide arrays (Gene Chips) differ from other formats in that the probe is generated *in situ* on the surface of the matrix. The leader in these arrays is Affymetrix and their combinatorial synthesis method. This method use a process called photolithography to construct probes on the array surface by making oligonucleotides one base at a time. These arrays contain between 40,000 and 60,000 probes (99).

4. Microelectronic arrays

Microelectronic chips are one of the newer formats for hybridization arrays. They are the result of a combination of advances in molecular biology and semiconductor microfabrication techniques. Instead of a membrane or a glass slide platform, these arrays consist of sets of electrodes covered by a thin layer of agarose coupled with an affinity moiety (permitting biotin-avidin immobilization of probes). The incorporation controllable electric field gives a new degree of control over probe deposition and target hybridization. This technology is still under development by Nanogen (San Diego, CA, USA) but has great potential because of its ability to move nucleic acids (and proteins) around on the surface of the chip (100).

Data Analysis

Where once the bottleneck in gene expression analysis was the benchwork, with array analysis, it is the computer work. Because a single array experiment can generate thousands of data points, the primary challenge of the technique is making sense of the data. Many commercial companies provide image analysis software, including BioDiscovery (ImaGene) and Imaging Research (ArrayVision). Furthermore, many array manufacturers offer software specifically for the analysis of

their arrays and offer the analysis as a service. For membrane array analysis, a file of the data is generated by phosphoimaging and that file is then analyzed using software. The software will correlate spots to genes and can compare spot intensities for differential expression studies. Glass array data is treated in much the same way, but the image's fluorescence is scanned and the software allows detection of each samples' fluorescence individually or simultaneously for analysis. Most software packages can analyze several arrays simultaneously.

Validation

Differences in expression of specific sequences are often validated by another method of analysis such as RT-PCR, Northern analysis or nuclease protection assays. These same methods can be used for relative or absolute quantitation of specific messages of interest identified by array analysis.

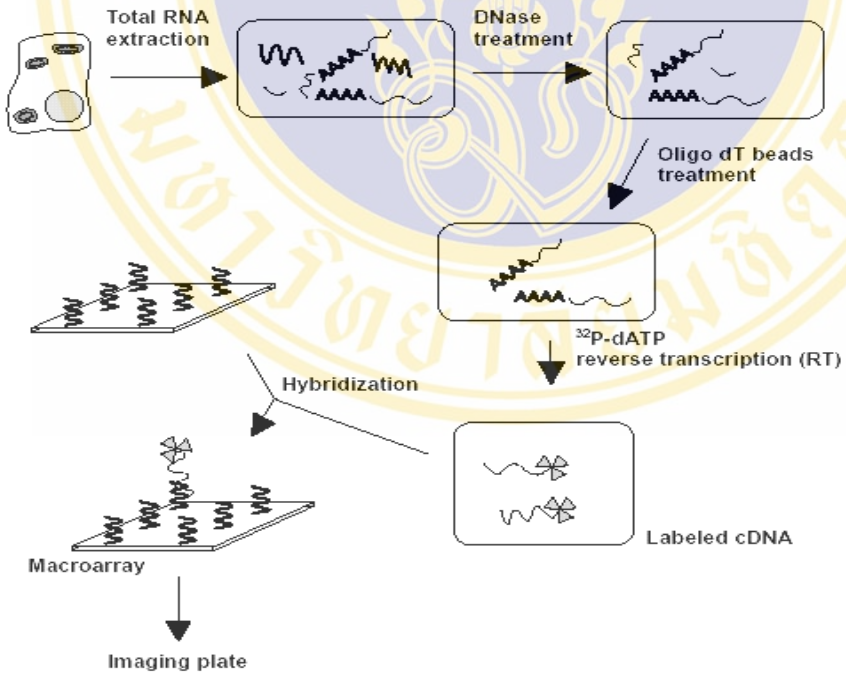

Figure 14. Conceptual view of macroarray method (BD Biosciences Clontech, Macroarray using membrane from CLONTECH Laboratories. Application notes No. 15, p.3)

CHAPTER 3
MATERIALS AND METHODS

MATERIALS

1. Chemicals and Kits

VR-3848, the novel peptide was kindly provided by Professor Vichai Reutrakul (Department of Chemistry, Faculty of Science, Mahidol University, Thailand). Briefly, this compound was isolated from *Euphobiaceae* in Thailand using bioassay-directed fractionation. Minimum essential medium (MEM), trypsin, sodium pyruvate, and faetal bovine serum were obtained from GIBCO BRL (Grand Island, NY, USA). Sulforhodamine B, dimethylsulfoxide (DMSO) and bovine insulin were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was purchased from Roche Diagnostics (IN, USA). Total RNA isolation Kit (Nucleospin®RNA II) was purchased from MACHEREY-NAGEL Inc. (PA, USA). Atlas™ Human Apoptosis Array and Atlas™ Pure Total RNA Labeling System were purchased from BD Biosciences Clontech (CA, USA). The [α-^{32}P] dATP (10 μCi/μl; 3,000 Ci/μmol) was purchased from Amersham Biosciences (Kwai Chung, New territories, HK). Caspase-8 inhibitor (Z-I-E (OMe)-T-D (OMe)-FMK), Caspase-10 inhibitor (Z-A-E (OMe)-V-D (OMe)-FMK) and AIF (Rabbit-Anti-human AIF antibody) were purchased from R&D Systems (Minneapolis, MN). The Rabbit anti-caspase-12 polyclonal antibody was purchased from CHEMICON International, Inc., Temecula, CA.

2. Cell line and cell maintenance

Breast cancer cell line, MCF-7 (ATCC number is HTB-22) was kindly given by Assist. Professor Kulawee Sujarit (Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand). Cells were grown in Eagle's

minimal essential medium (MEM, Gibco) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids. The culture medium was supplemented with 1 mM sodium pyruvate (Gibco), 0.01 mg/ml bovine insulin (Sigma) and 10% of heat inactivated (56^{o}C, 30 min) fetal bovine serum. The confluent cells were passaged every 3 days with 0.05% trypsin/EDTA solution.

METHODS

1. To determine the effect of estrogen (β-estradiol) on the proliferation rate of MCF-7 cancer cell

This experiment was performed in 6-well tissue culture plate. MCF-7 cells were cultured in various medium conditions at the start density of 1x10^{5} cells per well. To avoid the interfering of hormones, which always presence in normal serum and the medium containing phenol red, therefore, phenol red-free MEM (MEM*) supplemented with charcoal-treated fetal bovine serum was used. The viable cell number was performed by using trypan blue staining method (see appendix B). The number of viable cell was plotted against culture day. Five different medium conditions were used in this experiment which were MEM complete medium supplemented with 0.01 mg/ml bovine insulin, MEM* (phenol red free) complete medium supplemented with 0.01 mg/ml bovine insulin, MEM* complete medium supplemented with 100 nM of estrogen, MEM* complete medium supplemented with 100 nM of estrogen and 0.01 mg/ml bovine insulin and MEM* complete medium without bovine insulin and estrogen.

2. Cytotoxicity test

The cytotoxicity effect of VR-3848 on MCF-7 cells was performed by using SRB assay for determining the number of viable cells (101). The assay was evaluated after MCF-7 exposing to various concentrations of VR-3848 for 72 hours.

Briefly, MCF-7 cells at the exponential phase were plated into 96-well plates at the density of 19,000 cells/well and allowed to grow overnight. This seeding density was used to ensure an exponential growth during a 4-day assay, which will yield an absorbance reading not exceeding a value of 2.0. Monolayer of MCF-7 cells

in 96-well plate was then treated with either various concentration of VR-3848, vinblastine and 0.1% DMSO, a negative control. A purified VR-3848 was diluted in DMSO as a stock solution which was further diluted in medium to yield a working concentration. At 3 days after drug treatment, culture medium was removed and cells were fixed by means of protein precipitation with cold 20% trichloroacetic acid at 4^{o}C for 30 min (100 µl/well). After five washings with tap water, the cells were stained for at least 30 min with 0.4% SRB dissolved in 1% acetic acid (100 µl/well) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried and bound protein stain was solubilized with 200 µl/ well of 10 mM Tris-base (pH 10) for 5 minutes on a gyratory shaker. The optical density was read at 510 nm. The percentage of cell survival was calculated from the following formula:

$$\% \text{ Growth inhibition} = \frac{\text{Mean } OD_{510} \text{ treated cells on day 3} - \text{Mean } OD_{510} \text{ starting cells}}{\text{Mean } OD_{510} \text{ control on day 3} - \text{Mean } OD_{510} \text{ starting cells}} \text{ X } 100$$

To obtain a dose-response curve of VR-3848 on MCF-7, the percentage of cell survival was drawn against the log concentration of test compound. Subsequently, the GI_{50} (the concentration producing 50% inhibition of cell growth) of VR-3848 and vinblastine were obtained using a non-linear regression analysis. The data are means of three independent experiments.

3. Detection of Apoptosis

In the present study, cells undergoing apoptosis were detected using a hallmark of apoptosis such as chromatin condensation and DNA fragmentation. These changes were detected by DAPI staining and agarose gel electrophoresis as previously described (102).

3.1 Fluorescence microscopy of DAPI stained apoptotic nuclei

After drug treatment, both detached and attached cell were harvested, centrifuged and washed with PBS. The resuspended cells were then fixed with 0.5 ml of ice-cold methanol. The fixed cells were stained with 0.5 ml of DAPI (4′-6-diamidino-2-phenylindole) at the concentration of 1 µg/ml in PBS. After 15

minutes staining at 37°C, cells were centrifuged and washed with ice-cold methanol. The cell pellet were diluted with small amount of ice-cold methanol, mounted on a glass slide, covered with a drop of 50% glycerol and examined for apoptotic cells with condensed chromatin or fragmented chromatin using fluorescence microscopy. Number of apoptotic cells by comparing with the control (DMSO) at each time point were counted and expressed in percentage from a total of at least 500 cells count.

3.2 Analysis of DNA fragmentation

Nuclear morphology changes characteristic of apoptosis appear within the cell together with a distinctive biochemical event. The endonuclease-mediated cleavage of nuclear DNA cause the formation of DNA fragments of oligonucleosomal size (180-200 bp) which is a hallmark of apoptosis in many cell types.

The MCF-7 cells at the concentration of 6 x 10^6 cells were treated with 100 nM of VR-3848 and 0.1% DMSO at several time points. At each time point, both control and treated cells were collected, washed and used for DNA extraction by using Phenol: chloroform method. In apoptotic cells specific DNA cleavage becomes evident in electrophoresis analysis as a typical ladder pattern due to multiple DNA fragments (103). The DNA samples were run on 1.8 % agarose gel electrophoresis at 80 volts for 1.5 hours, stained with ethidium bromide, visualized and photographed under UV illumination to determine ladder patterns of fragmented DNA.

4. Macroarray: This analysis is composed of 5 major procedures as follows

4.1 Total RNA extraction

Total cellular RNA was isolated from both 6 x 10^6 treated and non-treated MCF-7 cells at each time point using a Nucleospin® column (Clontech). The treated cells were lysed with lysis buffer, which is immediately inactivates RNases, which are present in virtually all biological materials. This lysis buffer also served as good condition which favor adsorption of RNA to the silica membrane. To eliminate the contaminating DNA, which was also bound to the silica membrane, DNase I was directly applied onto the silica membrane during the preparation. In order to clean the

extracted RNA, RNA samples were washed with two different buffers for removing salts, metabolites and macromolecular cellular components. The purified RNA samples were finally eluted under low ionic strength conditions with RNase-free water.

The integrity of extracted RNA was examined by using agarose gel electrophoresis. The purity of RNA can be determined using $A_{260/280\ nm}$ ratio, generally exceeds 1.9. Good RNA product should appear as two bright bands of 28S and 18S ribosomal RNA at approximately 4.5 and 1.9 kb. The ratio of intensities of the 28S and 18S rRNA bands should be 1.5-2.5:1. The additional bands or a smear lower than the 18S rRNA band, including very small bands corresponding to 5S rRNA and tRNA. RNA is very sensitive to trace contaminations of RNases, often found on general labware, fingerprints and dust. To ensure RNA stability, RNA samples were frozen at -20°C for short term or –70°C for long-term storage.

4.2 Poly A^+ RNA enrichment

For each total RNA samples, 30-50 µg of the total RNA was subjected to poly A^+ RNA enrichment using Oligo-dT beads (Clontech). The system was used a biotinylated oligo (dT) primer to hybridize at high efficiency in solution to the 3' poly A^+ region which is present in most mature eukaryotic mRNA species. The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The magnetic bead, which has captured mRNA, was resuspended in 2 µl ribonuclease-free deionized water and was used in cDNA probe synthesis.

4.3 ^{32}P-labeled single-strand cDNA synthesis

The 10 µl reaction described in table 6 below converts 1 µg of poly A^+ RNA into ^{32}P-labeled first-strand cDNA. Combine the following reagents in a 0.5-ml microcentrifuge tube at room temperature.

For each reaction, the mRNA that attached to the magnetic beads was combined with 1 µl of CDS primer mix which is apoptosis gene-specific primer (supplied in apoptosis array kit) and then added deionized water to final volume of 3.0 µl. Mix well by pipetting and each reaction was incubated in a pre-heated PCR

thermal cycler. The reaction was incubated at 70°C for 2 min and was reduced to 50°C for 2 min. During this time, 1 µl per reaction of MMLV Reverse transcriptase was added to the master mix and keep at room temperature. The 8 µl per reaction of master mix was added after completion of 2 min at 50°C and incubated at this temperature for 25 min. The reaction was stopped by adding 1 µl per reaction of 10x termination mix.

Table 6. The component of master mix for labeling reaction

Reagents	**Volume per reaction**
5x Reaction buffer	2.0 µl
10x dNTP Mix (for dATP label)	1.0 µl
[α-^{32}P] dATP (3,000 Ci/mmol, 10 µCi/µl)	3.5 µl
DTT (100 mM)	0.5 µl
Total volume	**7.0 µl**

4.4 Probe purifying and assess label incorporation

The unincorporated ^{32}P-labeled nucleotides and small cDNA fragments were removed from probe synthesis reaction by using column chromatography. The ^{32}P-labeled probe was eluted from the chromatography column by using elution buffer. The probe was then checked the radioactivity by using scintillation counting. Probe synthesized, which efficiently detected by cDNA array should have a total of 1-10 x 10^6 cpm.

4.5 cDNA array hybridization.

The highly labeled single-strand cDNA, synthesized from the MCF-7 treated with DMSO or VR-3848 poly A^+ RNA, were directly hybridized with the Atlas™ Human Apoptosis Arrays (Clontech) according to manufacturer's recommendations. In the hybridization bottles, the arrays were pre-hybridized with the mixture of hybridization solution and heat-denatured sheared salmon testes DNA for 30 minutes with continuous agitation at 68°C. To prepare the probe for hybridization, Cot-1 DNA was added to entire pool of labeled probe, incubated in a boiling (95–100°C) water bath for 2 minutes. Probes were incubated on ice for 2 minutes before

pouring into the hybridization bottle containing with the pre-warmed hybridization solution and cDNA array. Probes were hybridized overnight with continuous agitation at 68°C.

On the next day, the arrays were washed 3 times with wash solution (2x SSC, 1% SDS) for 10 minutes at room temperature, 15 minutes of pre-warmed wash solution at 68°C and 20 minutes of pre-warmed wash solution at 68°C. The damped arrays were immediately wrapped with plastic bag. The plastic-wrapped arrays were mounted on Whatman paper (3 MM Chr). The hybridized membranes were autoradiographed by exposing to Kodak BioMax MS x-ray film at –70°C with a corresponding BioMax MS intensifying screen. The ratio of gene expression comparing between drug treatment and control at more than and less than 2 folds were considered as significantly changed and will be further analyzed. This data was acquired and further analyzed by using the Atlas Image version 2.7 (Clontech).

5. The alteration of expressed genes confirmation

5.1 Reverse transcription and polymerase chain reaction.

Total cellular RNA of MCF-7 treated cells and control at each time point was isolated by using Nucleospin column (Clontech). The precipitated RNA samples were dissolved in DEPC-treated water and were then subjected to the first strand cDNA synthesis before being further amplified by PCR using specific primers. GADD153 gene primers were sense 5'GAA ACG GAA ACA GAG TGG TCA TTC CCC 3' and antisense 5' GTG GGA TTG AGG GTC ACA TCA TTG GCA 3'. Cytotoxic TRAIL receptor 2 (TRICK2A) gene primers were sense 5' CAC CAG GTG TGA TTC AGG TG 3' and antisense 5' CCC CAC TGT GCT TTG TAC CT 3'. Human β-actin gene primers were sense 5' ATC TGG CAC CAC ACT TCT ACA 3' and antisense 5' GTT TCG TGG ATG CCA CAG GAC T 3'. The level of gene expression was estimated by using densitometry and expressed as a percentage relative to β-actin gene, an internal control.

5.2 Apoptosis inhibition by specific caspases (caspase-8 inhibitor (Z-IETD.FMK) and caspase-10 inhibitor (Z-AEVD.FMK)

Cell suspension (50,000 cells per well) were plated into 24-well plate and incubated for 29 hours before treatment. The treatment starting with pre-incubated the cells for 2 hours with inhibitors, caspase-8 or caspase-10 or caspase-8 plus caspase-10 inhibitors. After pretreatment with inhibitors, cultures were treated with 100 nM VR-3848 in the present of inhibitors. Caspase-8 and caspase-10 inhibitors at the concentration of 20 μM and 40 μM were used, respectively. The apoptosis inhibition was detected by DAPI nuclear staining at 6, 12 and 18 hours compared with the culture medium without addition of specific caspase inhibitors.

6. Quantitative analysis of apoptosis inducing factor (AIF) by western blotting method

6.1 Preparation of AIF cytosolic fraction

To detect the AIF released from the mitochondria by using western blotting technique. MCF-7 cells at 3 x 10^6 cells per sample were treated with 100 nM of VR-3848 or 0.1% DMSO (negative control) at 6, 12, 18, 24 and 36 hours, respectively. At each time point, cells were harvested, washed twice with ice-cold PBS, resuspended in ice-cold transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1mM glycoletherdiaminetetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 1 μg/ml each of aprotinin, leupeptin, and pepstatin) containing 300 μg/ml digitonin and allowed to permeabilize at 37°C for 5 min (104). After centrifugation at 14,000 g for 15 min at 4°C, the supernatants were stored at -80°C, and used as the cytosolic fractions for western blot analysis.

6.2 Western blot analysis

To detect the 57 kDa of AIF protein, the cell cytosolic fraction of both control and treatment at each time point were treated with lysis buffer, heated at 95-100 °C for 5 minutes and then spinned down at 10,000 rpm for 5 minutes. The samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide and 3% acrylamide, which was served as separating and stacking gel, respectively. After complete run, proteins were transferred onto nitrocellulose membranes (Amersham, Arlington, Heights, IL) by

using electroblotting method. The blot was blocked with blocking solution (Roche Ltd, Basel, Switzerland), probed for 1 hour with the primary antibody, rabbit-anti-human AIF antibody (R&D Systems, Inc., Weisbaden, Germany) which was stored in phosphate buffered saline (PBS) containing 0.01% NaN_3 and 1% bovine serum albumin and visualized using a secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, California, U.S.A.) conjugated with horseradish peroxidase. Chemiluminescence was performed with chemiluminescence blotting (POD) reagent (Roche Ltd, Basel, Switzerland). The protein bands were observed by comparing with the standard protein bands. The level of protein was estimated by using densitometry band analyzer and expressed as a ratio relative to β-actin gene, an internal control.

7. Qualitative analysis of caspase-12 by western blotting method

7.1 Preparation of caspase-12 cytosolic fraction

The same sample preparation procedure was used as described in AIF sample preparation.

7.2 Western blot analysis

To detect a band at ~53-49 kDa, the cell cytosolic fraction of both control and treatment at each time point were treated with lysis buffer, heated at 95-100°C for 5 minutes and then spinned down at 10,000 rpm for 5 minutes. The samples were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide and 3% acrylamide, which were served as separating and stacking gel, respectively. After complete run, proteins were transferred onto nitrocellulose membranes (Amersham, Arlington, Heights, IL) by using electroblotting method. The blot was blocked with blocking solution (Roche Ltd, Basel, Switzerland), probed for 1 hour with the primary antibody, rabbit-anti-human caspase-12 antibody (CHEMICON International, Inc., Temecula, CA) which was stored in PBS containing 0.02% sodium azide and visualized using a secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, California, U.S.A.) conjugated with horseradish peroxidase. Chemiluminescence was performed with chemiluminescence blotting (POD) reagent (Roche Ltd, Basel, Switzerland). The protein bands were observed by comparing with the standard protein bands. The level of protein was

estimated by using densitometry band analyzer and expressed as a ratio relative to β-actin gene, an internal control.

CHAPTER 4
RESULTS

Effect of estrogen on the proliferation rate of MCF-7 cancer cell line

There is no single cause for breast cancer development, but one major factor is estrogen. The previous studies have reported that estrogen could act as breast cancer cell growth factor. In this study, we investigated to find out whether estrogen promotes the growth of MCF-7 cell line *in vitro* as reported *in vivo*. This cancer cell line retains several characteristics of differentiated mammary epithelium including capability of forming foci (figure 15). Demonstrating shown in figure 16, the doubling time of MCF-7 in our hand was 29 hours which was similar to doubling time of this cell line reported by ATCC. MCF-7 cells were cultured under various medium conditions to studying the effect of hormone on MCF-7 cell growth. Five conditions of medium were tested (see materials and methods). To avoid the effect of any other hormone, which always found in serum, thus charcoal treated serum was used in our experiment. Moreover, the culture medium was phenol red free due to the fact that the chemical structure of phenol red is similar to the structure of estrogen (105).

As demonstrated in figure 17, the higher growth rate of MCF-7 could be seen in the routine used culture medium (MEM) containing only bovine insulin (closed rectangular) comparing with MEM (phenol red free) supplemented with only bovine insulin (opened circle). Moreover, the estrogen supplemented in growth medium (opened rectangular) could not promote higher growth rate of this cancer cell line when compared with normal growth medium (closed rectangular). These results suggest that estrogen did not affect on the proliferation rate of MCF-7 in *vitro*. Therefore, normal culture medium without estrogen supplementation was chosen for culturing MCF-7 cell for the rest of our studies.

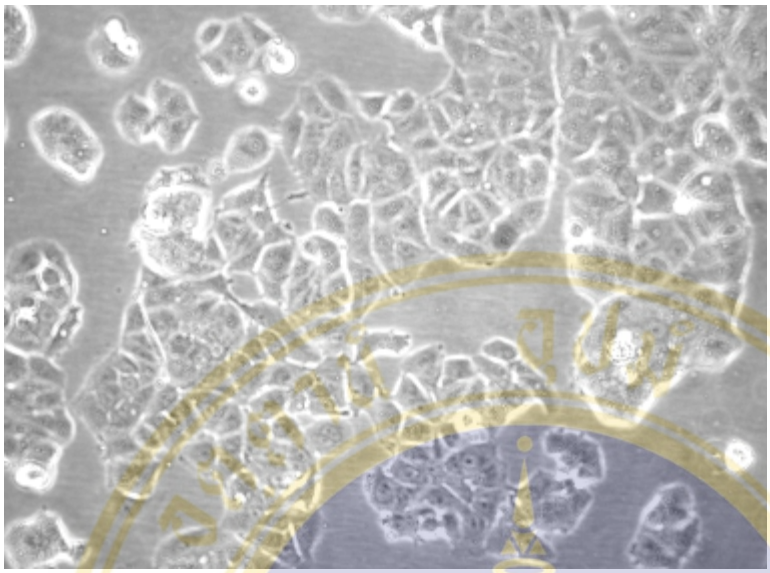

Figure 15. The morphology of normal MCF-7 cell line under phase contrast microscope at the magnification of 400 X.

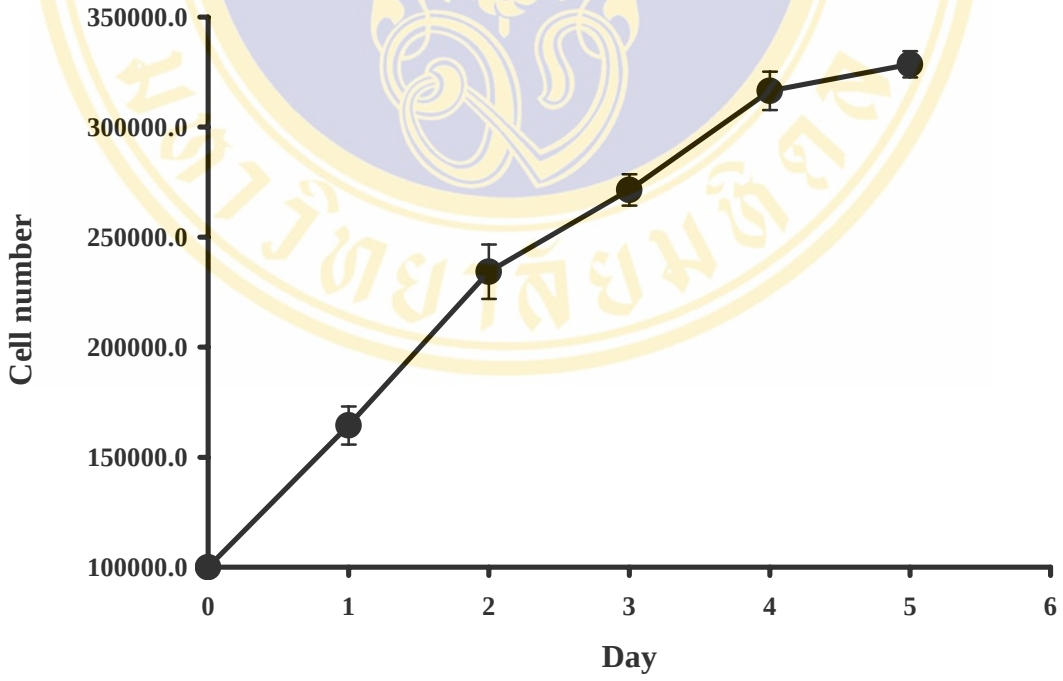

Figure 16. The growth kinetic of MCF-7 cell. The x-axis represents the cultured day while the y-axis represents the cell counting number. Results are mean ± SEM from three independent experiments.

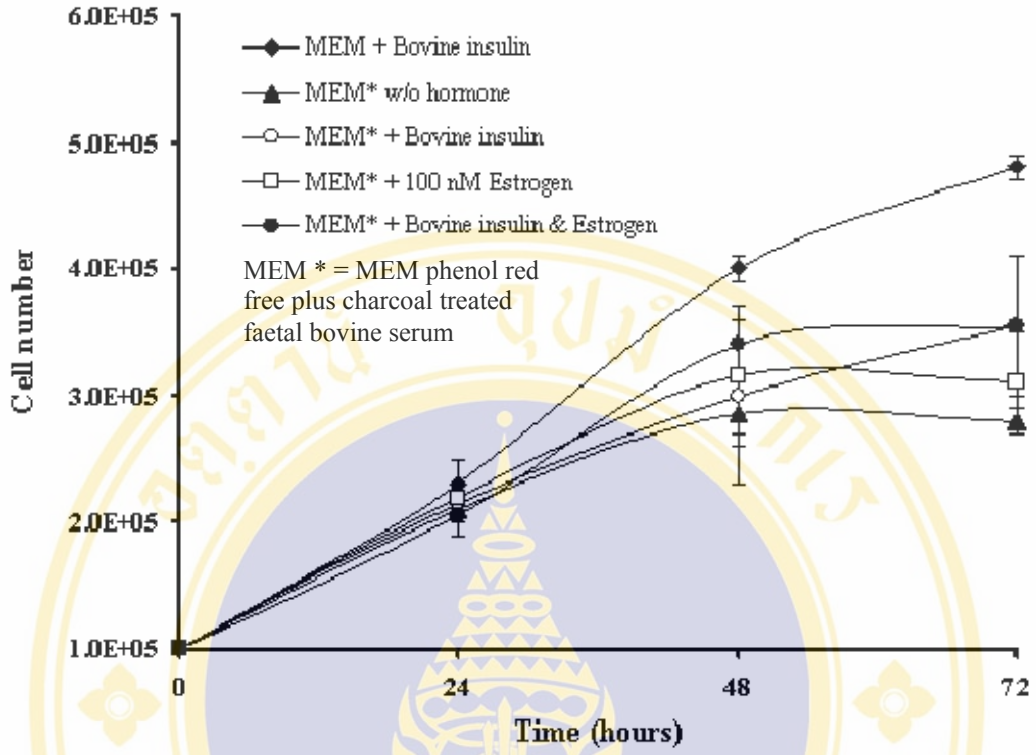

Figure 17. The growth kinetic of MCF-7 cell under various medium conditions. The x-axis represents the cultured day while the y-axis represents the cell counting number. Results are mean ± SEM from three independent experiments.

VR-3848 exerts MCF-7 cell growth inhibition

Whether VR-3848 kills MCF-7 was first question to be answered. To answer this question, MCF-7 cells were treated with various concentration of VR-3848 and cell growth inhibition was determined at 72 hours after treatment using SRB assay. As shown in figure 18, growth of MCF-7 cells was inhibited by VR-3848 in a dose-dependent manner. In order to compare the efficiency of VR-3848 to standard anti-cancer drug, vinblastine, on MCF-7 cell growth inhibition, the anti-cancer potency which was evaluated by determining the 50% growth inhibitory value (GI_{50}) of VR-3848 and vinblastine were studied. The GI_{50} value of VR-3848 and vinblastine were 8.20 nM and 1.53 nM, respectively. Therefore, VR-3848 has 5.3 folds less cytotoxicity than vinblastine against MCF-7 cells.

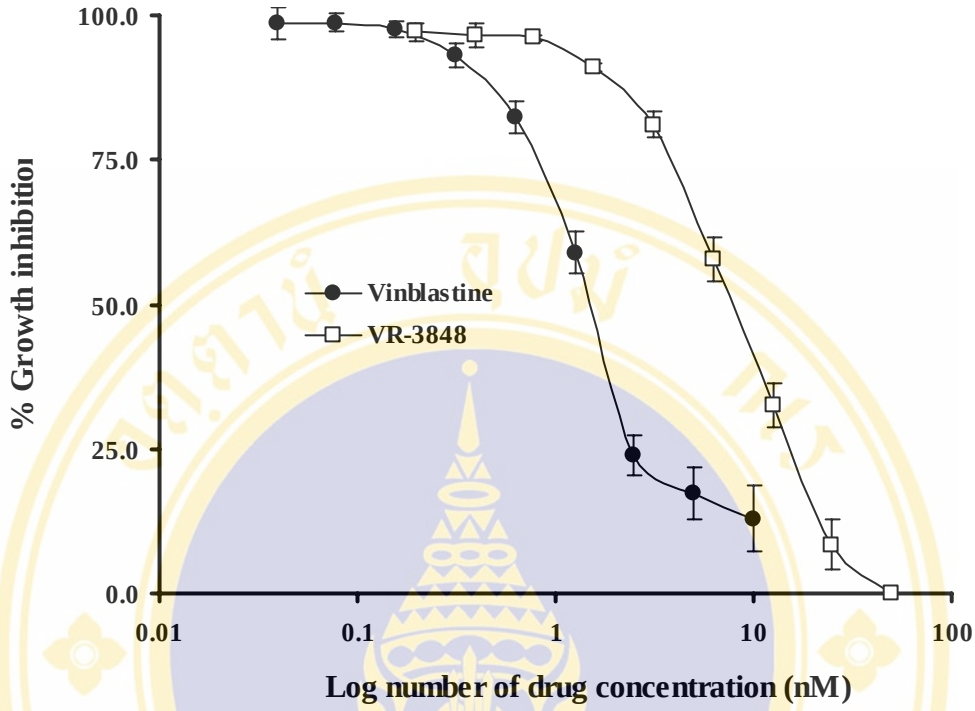

Figure 18. Dose response curve of VR-3848 and anti-cancer drug (vinblastine) against MCF-7 cell. The x-axis represents concentration (nM) of VR-3848 while the y-axis represents the percentage of growth inhibition. Results are mean ± SEM from three independent experiments.

Apoptosis induction by VR-3848 using DAPI nuclear staining

We next assessed the effect of VR-3848 on the induction of apoptosis in MCF-7 cells. The apoptotic hallmark, nuclear fragmentation or nuclear condensation was detected by using DAPI nuclear staining. In this experiment, MCF-7 cells were treated with 100 nM of VR-3848 (12 folds of GI_{50} value) and apoptotic nuclei were monitored at each time point (6, 12, 18, and 24 hrs). As shown in Figure 19, VR-3848 treatment induced nuclear condensation and fragmentation indicating that this compound inhibits growth of MCF-7 via apoptosis induction. The number of apoptotic nuclei were 6.12%, 16.86%, 31.02%, and 49.06% at 6, 12, 18, and 24 hrs after drug treatment, respectively, while the control (0.1% DMSO treated cells) was approximately 1.0-2.0 % apoptosis at each time point (Figure 20). This data

demonstrated that VR-3848 exerts MCF-7 cell growth inhibition in a dose dependent manner.

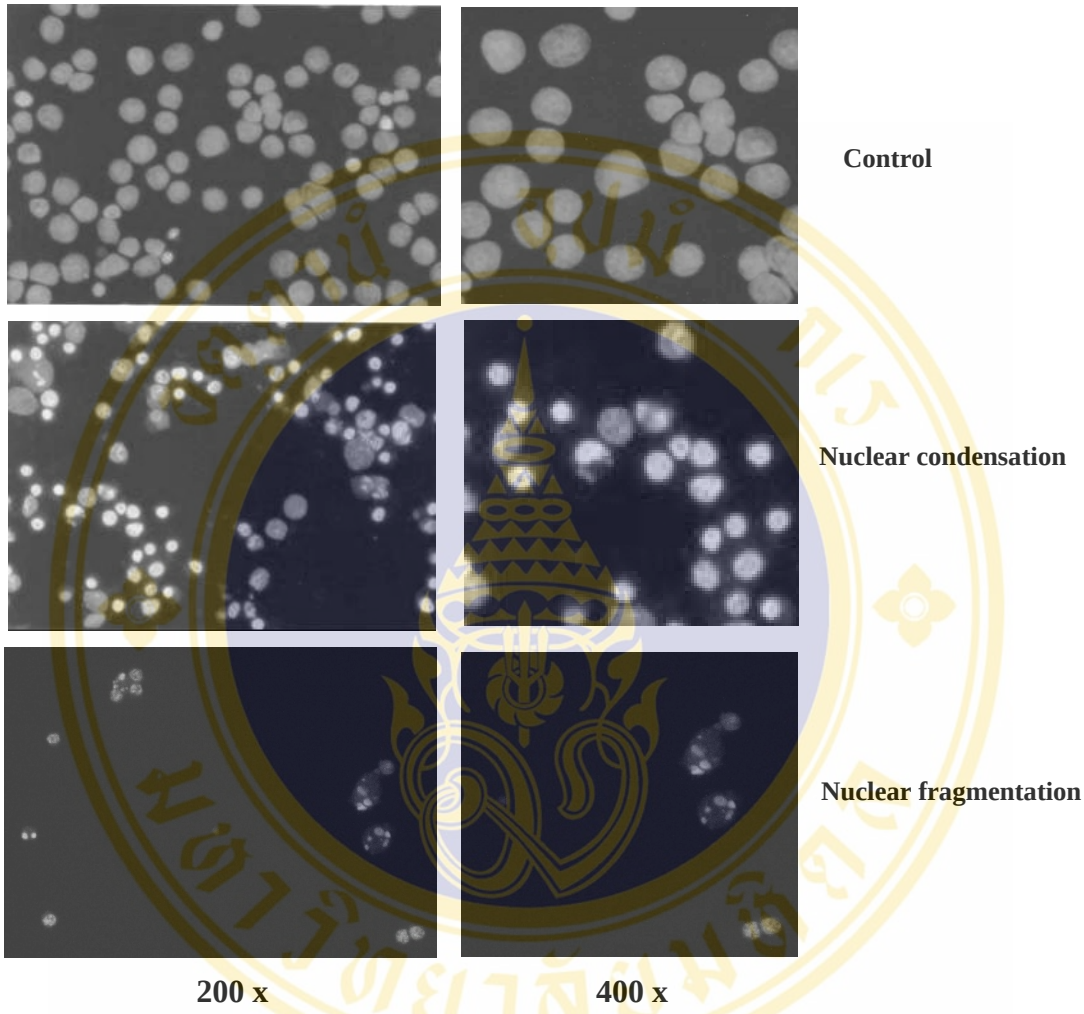

Figure 19. Detection of VR-3848-induced MCF-7 apoptosis using DAPI staining. The cells were treated with 100 nM of VR-3848 and apoptotic nuclei were monitored at 24 hours. The apoptotic nuclei were demonstrated by chromatin condensation and nuclear fragmentation, photographed by a fluorescence microscope at the magnification of 200 x (left panel) and 400 x (right panel).

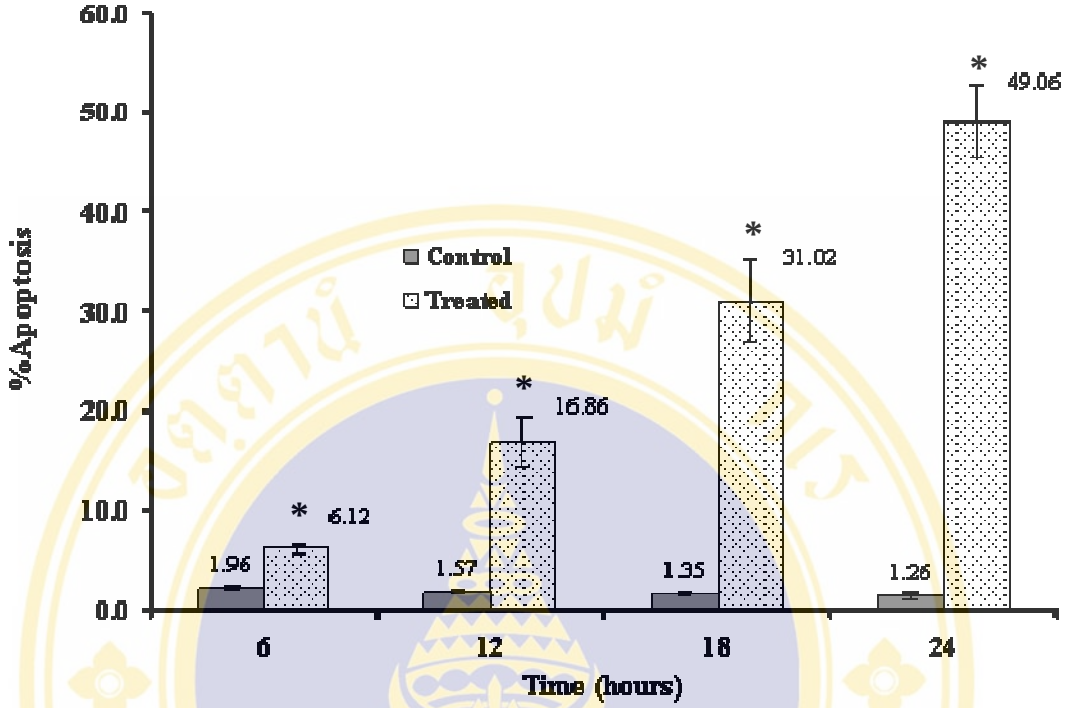

Figure 20. Percentage of chromatin condensation plus chromatin fragmentation nuclei treated with 100 nM of VR-3848 compared with DMSO treatment. Results are mean ± SEM from three independent experiments.

* *P<0.05*; significantly different from the control group at the corresponding time (Student's paired *t-test*)

Detection of chromosomal cleavage in VR-3848-treated MCF-7 cells

Apoptosis induced by VR-3848 was demonstrated using DAPI. To confirm this data, chromosomal cleavage or DNA laddering, another apoptosis hallmark was detected. As illustrated in figure 21, DNA laddering was first detected at 18 hours after treatment and more clearly visualized at 24, 36, and 48 hours in MCF-7 treated with VR-3848 compared with the control. These results indicated that the endogenous endonucleases may be activated and caused nuclear DNA double strand breaks at 180 to 200 base pairs which is a hallmark of apoptosis in many cell types.

Figure 21. Electrophoretic patterns of DNA ladder in VR-3848-treated MCF-7.

Human apoptosis cDNA array analysis

Since VR-3848 has high potential to be a new chemotherapeutic agent. Therefore, it is worth to elucidate the molecular mechanism of apoptosis induced by this compound. Two hundred and five apoptotic-related genes were screened in order to identify population of these genes that response to VR-3848 stimulation. A total of 48, 49, 49, 48 and 54 apoptotic-related genes were responded to VR-3848 at 6, 12, 18, 24 and 36 hours after treatment, respectively (table 7).

Table 7. Numbers of apoptosis-related genes response to VR-3848 stimulation at various times.

Time after drug application (hour)	**6**	**12**	**18**	**24**	**36**
Upregulation	32	19	9	23	42
Downregulation	16	30	40	25	12
Total number of gene expressed	**48**	**49**	**49**	**48**	**54**

From the acquired data analysis, gene which expression level was two folds higher or lower than the control cells were listed in table 8-10. Genes which expression level was altered were categorized based on their function and was fell into 3 major groups. That were receptor-ligand mediated apoptotic, stress response related apoptosis genes and cell cycle related apoptosis genes.

In the group of receptor-ligand mediated apoptotic and apoptotic mediator genes, we found the initiator caspases, caspase-8 and caspase-10, were upregulated at 6 hours until 18 hours of drug treatment. We found some of death receptors were upregulated, for example, tumour necrosis factor receptor superfamily (TNFRSF) 10B which related to TRAIL receptor (DR5) was upregulated at the beginning time of drug treatment. The TNFRSF1B related to TNFR2, the TNFRSF1A related to TNFR1 and TNFRSF25 which related to death domain of receptor 3 (DDR3) were also upregulated at the early phase of our study. Surprisingly, death ligands such as TNF-alpha, SARP1 and SARP3 were upregulated at the late phases. The stress response genes such as growth arrest and DNA-damage-inducible protein 153 (GADD 153) and GADD 45 were consistency upregulated almost all the time of drug treatment. As demonstrated in table 10, most of cell cycle genes including some proto-oncogenes were downregulated such as G2/mitotic-specific cyclin A, B1, G1, c-jun and c-raf. The CDC-like kinase 3 (CLK3) and CDK6 which has been grouped into survival kinase, that play a role during G1 to S-phase of cell cycle were downregulated. Some of extracellular signaling proteins were upregulated at the beginning of the treatment such as MAPKK 1, MAPKK 5, ERK1, ERK 2, ERK 4, ERK 5 and ERK 6. The pro-apoptotic bcl-2 family was either downregulated or unalterred (Bak and Bax), however, antiapoptotic bcl-2, Bcl-w, was suppressed.

Table 8. List of receptor-ligand mediated apoptotic and apoptotic mediator genes at different time points after VR-3848 stimulation.

Apoptosis related genes	Drug exposure time (hour)				
	6	12	18	24	36
Caspases					
- Caspases-4	-2.8	-	-2.4	-2.6	-
- Caspases-6	1.1	-	2.0	-2.4	-5.4
- Caspases-8	2.4	1.6	-	-1.8	-2.9
- Caspases-10	5.0	2.3	4.4	-1.5	-1.4
Bcl-2 family					
- Bcl-w (anti-apoptotic gene)	-2.2	-2.7	-	-1.4	-1.5
- BAK (pro-apoptotic gene)	-2.7	-	-6.4	-2.9	-3.4
- Bax (pro-apoptotic gene)	-	-2.1	-3.8	-	2.0
Death receptors					
- tumour necrosis factor receptor 1 (TNFR1) ; **TNFRSF1A**	1.6	-4.6	-3.5	-4.1	-
- tumour necrosis factor receptor 2 (TNFR2) ; **TNFRSF1B**	8.2	5.1	2.4	-7.7	-2.8
- death domain receptor 3 **(DDR3) ; Apo-3** ; **TNFRSF25**	2.5	-	-3.4	-7.3	-4.7
- death receptor 5 **(DR5)** ; cytotoxic TRAIL receptor 2; **TNFSF10B**	2.8	1.6	-3.2	-2.6	-
Death adaptors					
- DAXX	-	-2.2	-6.2	-3.2	-
Death ligands					
- **TNF-alpha**; TNFA; cachectin	-	-	-	-	1.6
- secreted apoptosis related protein 1 **(SARP1)**	-	-	-3.5	-2.5	2.5
- secreted apoptosis related protein 3 **(SARP3)**	-	-	-4.8	-2.3	10.2

Table 9. List of stress response genes at different time points after VR-3848 stimulation.

Apoptosis related genes	Drug exposure time (hour)				
	6	12	18	24	36
Stress response genes					
- growth arrest & DNA-damage-inducible protein 153 **(GADD153)**	4.6	2.1	1.9	1.3	-
- growth arrest & DNA-damage-inducible protein **(GADD45)**	3.4	3.5	6.0	2.4	-

Table 10. List of cell cycle-related apoptosis genes at different time points after VR-3848 stimulation.

Cell cycle associated genes	Drug exposure time (hour)				
	6	12	18	24	36
CDKs					
- CDK-2	3.2	8.0	-	-8.1	-16.2
- CDK-4	-	-6.9	-4.0	-3.1	-
- CDK-5	-	-	-2.5	-	-4.1
- CDK-7	3.2	4.8	-	-	-3.8
- CDK-9	2.7	-	-3.0	-2.1	8.9
- CDC-like kinase 3 (**CLK3**)	-	-8.6	-	-2.7	-
- serine/threonine-protein kinase **PLK1** (STPK13)	-2.3	-9.1	-5.4	-4.1	-
CYCLINs					
- **cyclin H**	3.2	1.1	1.3	-2.7	-1.8
- G2/mitotic-specific **cyclin A**	-	-7.9	-2.4	-1.1	17.1
- G2/mitotic-specific **cyclin B1**	-	-	-2.2	-2.1	-
- G2/mitotic-specific **cyclin G1**	-3.3	-19.3	-	-8.6	-
- G1/S-specific **cyclin C**	2.1	-	-	-	-
Proto-oncogenes					
- c-jun proto-oncogene; transcription factor AP-1	-	-	-	5.1	-61.8
- c-raf proto-oncogene	-	-5.9	-	-7.6	-4.5
MAPKs pathway-related genes					
- growth factor receptor-bound protein 2 (GRB2) isoform	-	-3.5	-2.4	-6.5	-2.1
- MAPK/ERK kinase kinase 3 (MEK kinase 3; MEKK3)	-	-	-	-1.8	-4.5
- MAP kinase kinase 1; MAPKK 1	2.6	-	-2.4	-	-2.1
- MAP kinase kinase 5; MAPKK 5	5.1	-	-	-	-4.2
- extracellular signal-regulated kinase 1 (ERK1)	-	-	-4.1	-6.4	-
- extracellular signal-regulated kinase 2 (ERK2)	-	2.1	-	-2.6	-
- extracellular signal-regulated kinase 4 (ERK4)	3.9	3.8	-2.7	-3.14	-
- extracellular signal-regulated kinase 5 (ERK5)	2.8	1.8	-	-2.3	-
- extracellular signal-regulated kinase 6 (ERK6)	1.8	-	-	-	-

- = There was no alteration of gene expression detected.

Mitochondrial apoptosis inducing factor (AIF) releasing may contribute to apoptosis.

Data from cDNA array analysis surprised us since there was no alteration of mitochondrial-dependent apoptosis gene expression. As previous studies, there are many factors that cause DNA strand break during apoptosis induction. One of the pathways is mitochondrial-dependent pathway, which is separated into two different pathways based on the differences in proteins released from mitochondria. These two pathways are simply known as caspase-dependent and caspase-independent pathway. As we all known that MCF-7 is caspase-3 deficient breast cancer cell, so the death protein, which released from mitochondria should not be related to caspase-3. The apoptosis inducing factor (AIF) protein was one of death protein candidate that serves in mitochondrial-related caspase-independent pathway. Therefore, detection of AIF in VR-3848-treated MCF-7 was performed using specific antibody to AIF. As demonstrated in figure 22A and B, AIF released could be significantly detected after 18 hours of drug treatment. The higher amount of AIF releasing correlated to the longer time of VR-3848 treatment.

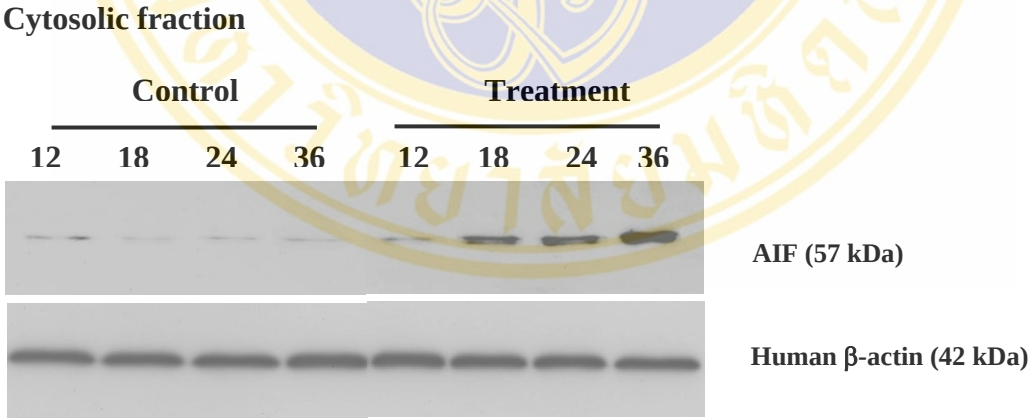

Figure 22A. Kinetic of AIF proteins released from intermembrane space of mitochondria detected by western blotting.

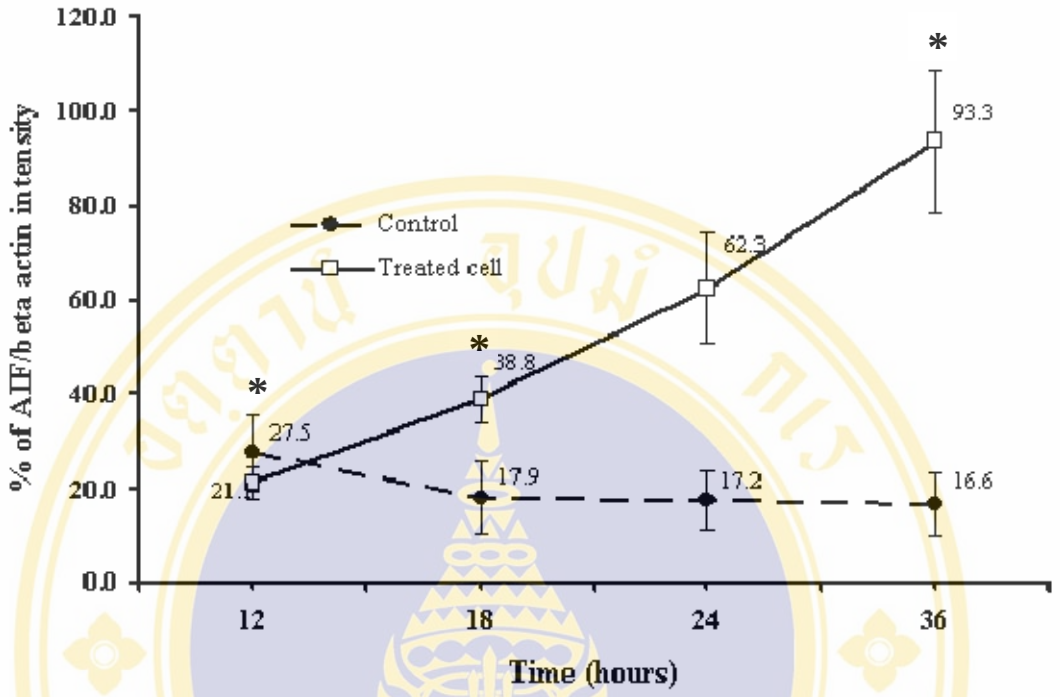

Figure 22B. The intensity of bands shown in figure 22A was semiquantitated by densitometer and expressed in a percentage to the expression level of an internal control gene, β-actin. Results are mean ± SEM from two independent experiments.
* *P<0.05*; significantly different from the control group at the corresponding time (Student's paired *t-test*)

Validation of gene expression alteration by RT-PCR and protein functional test

From the cDNA array analysis, these responsive genes can be grouped into three major apoptotic pathways, which were receptor-ligand mediated death or extrinsic pathway, endoplasmic reticulum stress response pathway, and caspase-independent mitochondrial pathway. To validate the alteration of apoptotic gene expression detected by cDNA array analysis, RT-PCR of upregulated genes and protein functional test were performed.

For receptor-ligand mediated pathway, the major mediators are receptor such as TNF receptor (TNFR) family, death ligand (TNF-α, FasL or TRAIL), initiator caspases such as caspase-8, caspase-10 etc. To confirm role of this pathway in VR-3848 induced cell death, upregulation of cytotoxic TRAIL receptor 2 gene was

evaluated by RT-PCR and function of caspase-8 and caspase-10 were inhibited with caspase specific inhibitors. As demonstrated in figure 23, the cytotoxic TRAIL receptor 2 gene was upregulated significantly.

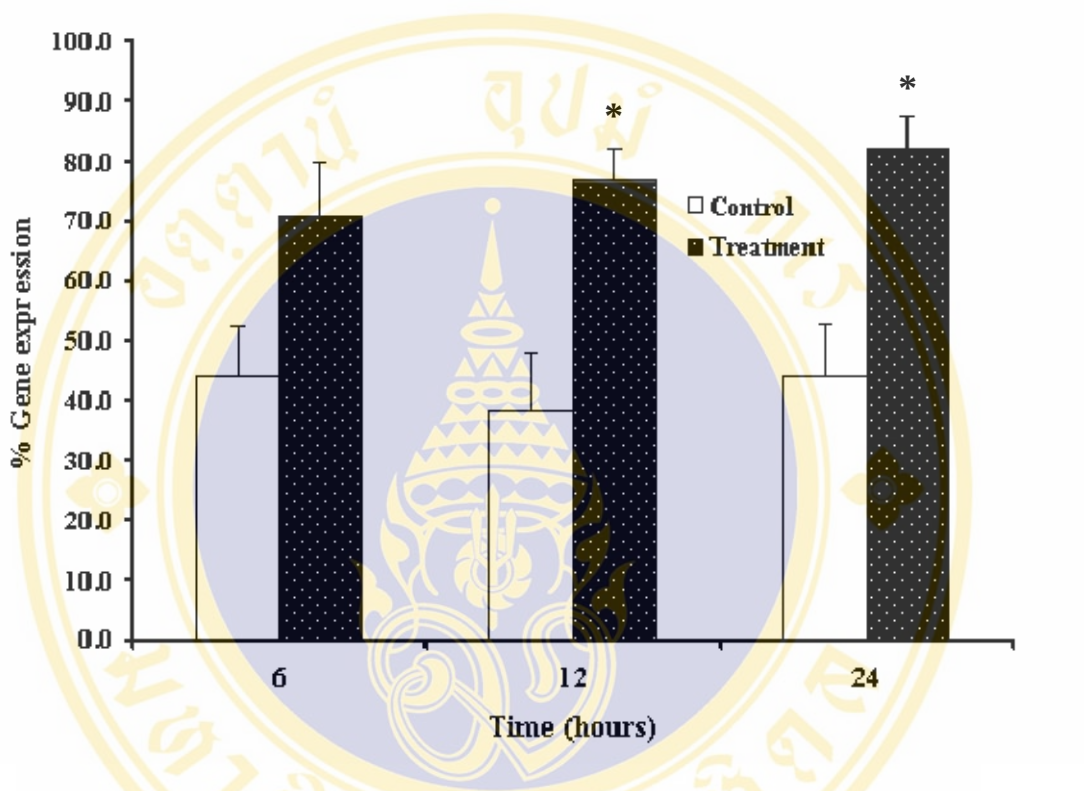

Figure 23. Upregulation of cytotoxic TRAIL receptor 2 in VR-3848 treated cells detected by RT-PCR. Results are mean ± SEM from two independent experiments.
* $P<0.05$; significantly different from the control group at the corresponding time (Student's paired *t-test*).

From the array results, caspase-8 and caspase-10 were up regulated significantly at the initial time point. To functionally investigate role of caspase-8, -10 in VR-3848 induced MCF-7 apoptosis, the effect of caspase-8, and -10 inhibitors on VR-3848 treated MCF-7 cells was investigated. As demonstrated in figure 24, the specific inhibitors were able to block apoptosis induced by VR-3848 with the time dependent manner. Indicating that VR-3848 induced death in MCF-7 cells via caspase-8, -10 mediated pathway or receptor-ligand pathway.

To validate the endoplasmic reticulum stress response pathway, the up regulated stress response gene was detected by RT-PCR. From the array result, *GADD153* was consistently upregulated as shown in table 9. As demonstrated in figure 25, the longer time of drug treatment, the higher expression level of *GADD153* gene.

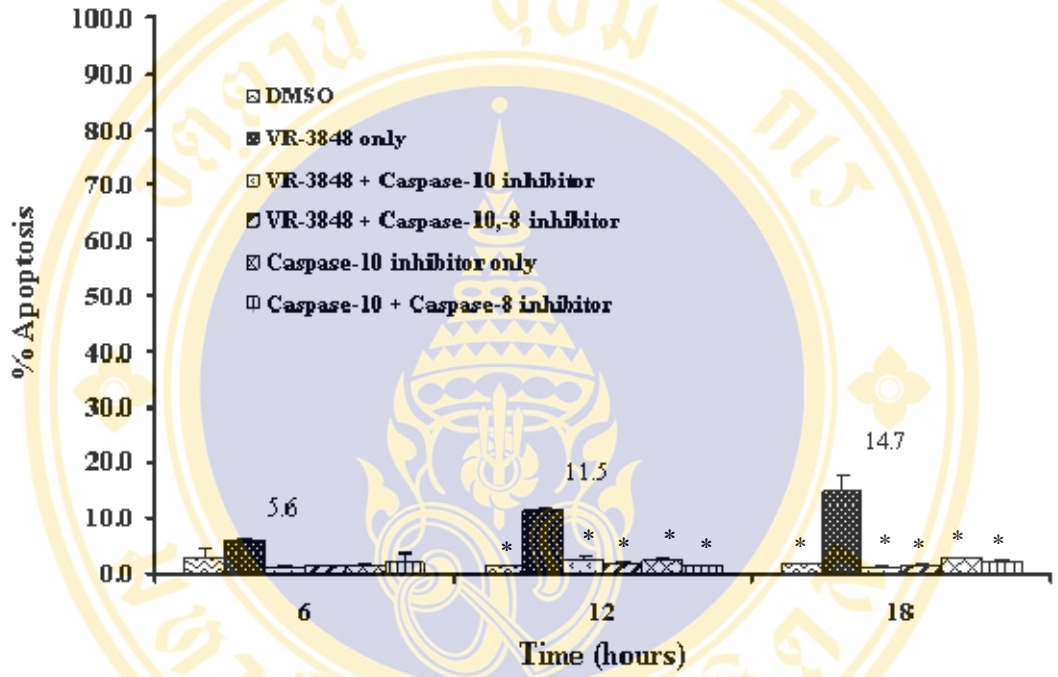

Figure 24. The effect of initiator caspase inhibitors on the percentage of apoptosis. Results are mean ± SEM from two independent experiments.
* *P<0.05*; significantly different from VR-3848 treatment group at the corresponding time (Student's paired *t-test*).

Moreover, we also monitored the alteration of caspase-12 protein synthesis, which is a stress-induced caspase localized to the ER and is activated upon ER stress during VR-3848 treatment. As shown in figure 26, the longer time of drug treatment, the higher level of caspase-12 protein was detected compared with the control experiment (no drug).

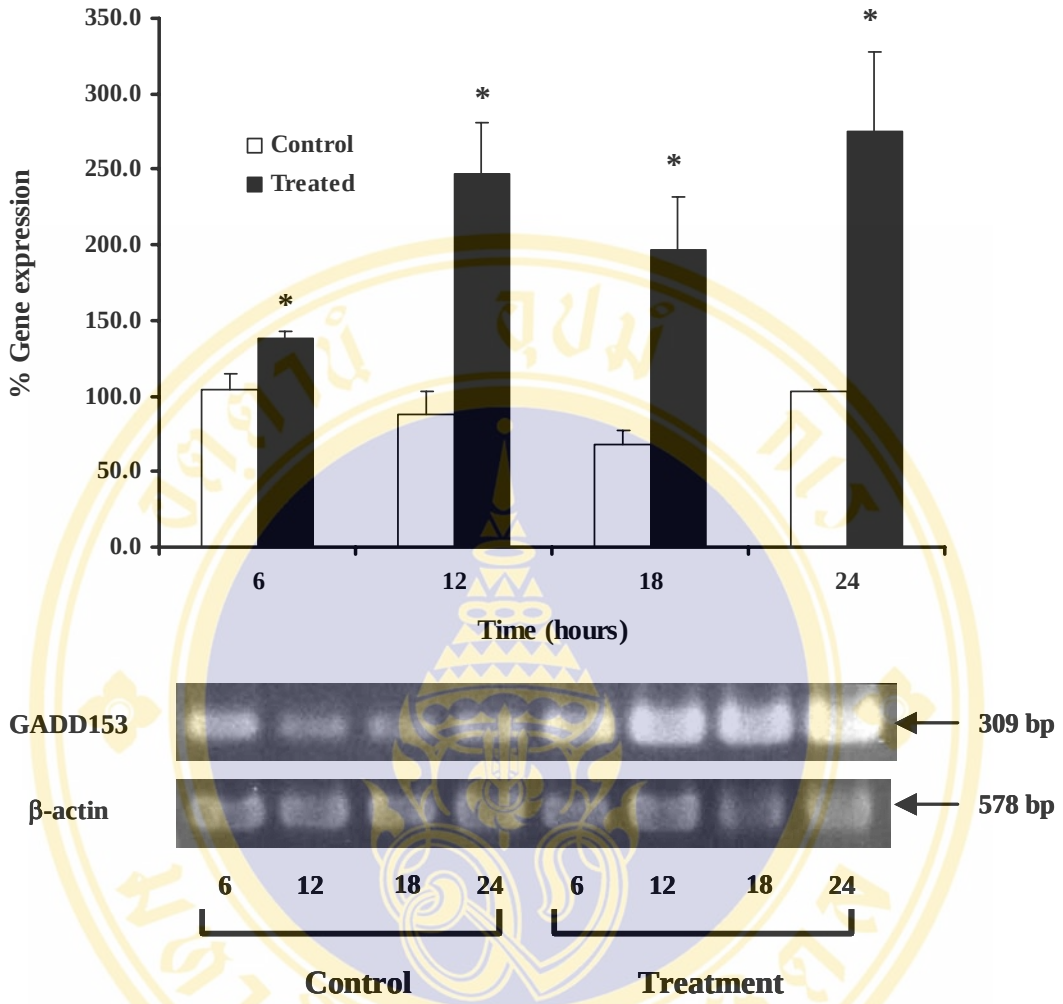

Figure 25. The upregulation of *GADD153* gene expression response to VR-3848 stimulation detected by RT-PCR. Results are mean ± SEM from three independent experiments. * *P<0.05*; significantly different from the control group at the corresponding time (Student's paired *t-test*).

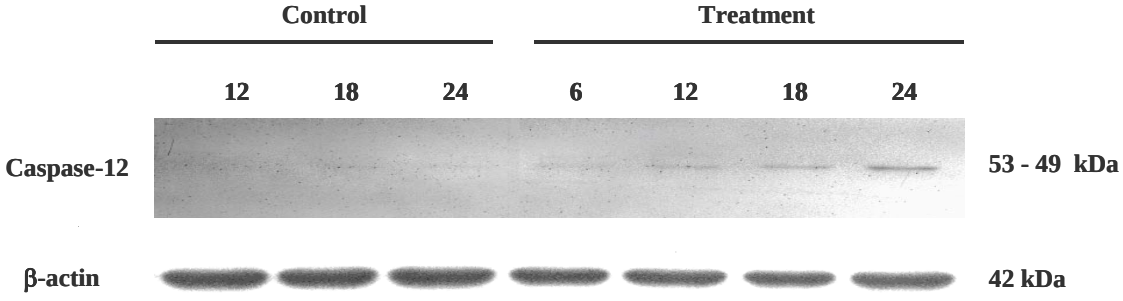

Figure 26. The level of caspase-12 production in VR-3848-treated MCF-7 versus untreated MCF-7 cells analyzed by Western blotting technique.

CHAPTER 5
DISCUSSION

Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms. Vincristine, etoposide and paclitaxel are examples of plant-derived compounds that are being employed in cancer treatment. Dactinomycin, Bleomycin and Doxorubicin are anti-cancer agents derived from microbial sources. Citarabine is an example of an anti-cancer agent originating from a marine source. Bryostatin-1, Aplidine, Dolastatin 10 and ET-743, which have recently entered phase I and II clinical trials are originating from marine sources (106).

Many natural product compounds have been reported to contain anti-cancer property. VR-3848 is one of them which exerts anti-proliferating effect to various cancer cells including Lu-1, leukemia cells etc (2, 4). VR-3848 kills Lu-1, non-small lung cancer cell line, 10 folds greater than vinblastine does but vice versa for MCF-7 killing. This information demonstrated that an anti-cancer activity of VR-3848 is dependent on type of cancer cell and also suggested that VR-3848 and vinblastine may induce a different mode of killing.

MCF-7 has been classified into estrogen-responsive cell type but in our studies estrogen seems not to be required for cell proliferation. This conflicting result has often been obtained in MCF-7 studies as reported from different laboratories. This may due to the fact that four different MCF-7 cell lines were used, MCF-7 (ATCC), MCF-7, MCF-7 (KO), and MCF-7 (S). These cell lines show similar morphology in monolayer culture but different phenotypes. For example, they contain variable amounts of estrogen receptor (ER) and progesterone receptor (PgR). The growth rate of MCF-7 (ATCC) was 50% slower than that of the other lines. Unlike the other three lines, MCF-7 (ATCC) cell proliferation was unaffected by estrogen or anti-estrogen treatment despite the presence of receptors. These data demonstrate that MCF-7 lines

from different laboratories may have unique biological properties (107). Our data demonstrated that bovine insulin is necessary for MCF-7 proliferation, indicating that both bovine insulin and estrogen can be used as a growth-promoting factor for MCF-7 cells. This information was supported by array data in which IGFBP (insulin growth factor binding protein) -2 and IGFBP-5 gene expressions were up-regulated before VR-3848 treated cells had committed to die (data not shown). The physiological role of IGFBPs is incompletely characterized. Under various conditions IGFBPs have been observed to either enhance or inhibit the biological activity of insulin-like growth factors (108). Among the incidence of chemoresistance in many cancer cell type, this situation often accompanies the progression of breast cancers from a hormone-dependent, non-metastatic or anti-estrogen-sensitive phenotype to a hormone-independent, invasive, metastatic or anti-estrogen-resistance phenotype (109). Whether termination of IGFBPs gene expression during VR-3848 treatment is one of the anti-cancer process required further investigation.

To describe the mechanism of VR-3848 cell killing, nuclear morphological changes of treated cells were observed through DAPI nuclear staining and the pattern of chromosomal fragmentation under gel electrophoresis. Well defined nuclear condensation and fragmentation were demonstrated by DAPI staining however chromosomal DNA fragmentation was poorly detected. Our data is supported by previous investigation that MCF-7 cell variants show difference pattern of nuclear DNA laddering. This result was demonstrated by detection of sub-G1 DNA of doxorubicin induced MCF-7-ATCC and MCF-7-MG. At the same fold increase in apoptosis, MCF-7 (ATCC) did not show DNA laddering while MCF-7 (MG) did. Therefore, the detection of DNA ladders is not an accurate indicator of apoptosis in MCF-7 cells as fragmentation of DNA appears to vary between strains (110).

Chemotherapy, radiation therapy, and immunotherapy all rely heavily on apoptosis to kill cancer cells (111, 112). Understanding the mechanism of apoptosis could be the way to specifically induce cancerous cell eradication. The molecular events of apoptosis can be divided into three steps; initiation by an apoptosis-inducing agent, activation of the caspases by a signal transduction cascade, and proteolytic cleavage of cellular components. Ligand-receptor induces apoptosis is extensively studied in various system such as Fas-FasL, TNF-TNFR interaction etc (113). This

death pathway involved various type of death signaling proteins such as death ligands (FasL, APO-2L/TRAIL, TNF-α or APO-3L), death receptors (Fas, DR4/DR5, DR3 or TNFR; Tumour necrosis factor receptor). Upon receipt of a death signal, initiator caspases are recruited to a multiprotein activating complex, the DISC (death-inducing signaling complex) for the extrinsic initiator caspases-8 and -10, and the apoptosome for the intrinsic initiator caspase-9. The activation of caspases-8 and -10 occurs independently of cleavage of either the caspase or FLIP(L). FLIP(L) activates caspase-8 by forming heterodimeric enzyme molecules with substrate specificity and catalytic activity indistinguishable from those of caspase-8 homodimers (114). Apoptosis occurring through this pathway need some key regulators to play role in the process of death. Activation of the initiator procaspases is a key regulatory step in apoptosis, and to date all proteins known to promote this activation directly are death adaptors including the mammalian proteins FADD (Fas-associated death domain protein), TRADD (TNFR-associated death domain protein) and Apaf-1. During apoptosis, these adaptors bind to the N-terminal prodomain region of procaspases, and facilitate the oligomerization of the C-terminal protease domain (115).

Our cDNA synthesis displayed the upregulation of several genes categorized as death receptors (TNFRSF1A, DR5 and DDR3) and death ligands (TNF-α or TRAIL). Not only these initiators were expressed but initiator caspases, caspase-8, -10, were also stimulated. Upregulation of these upstream mediators led us to hypothesize that VR-3848 drove MCF-7 cells into apoptotic processes via production of apoptotic factor which acts as an autocrine. Interaction of this factor to its receptor activated caspase-8 and –10 which subsequently turned on effector caspases follows by stimulation of destruction processes. To prove our hypothesis, function of caspase-8 and –10 were blocked by specific inhibitors. Blocking of these two caspases significantly suppressed apoptosis in VR-3848 treated MCF-7 cells. These informations indicated that one of the apoptotic death induced by VR-3848 is mediated via receptor-ligand interaction.

Mitochondria also play a central role in apoptosis through the release of pro-apoptotic proteins contained in the intermembrane space. Mitochondrial membrane permeabilization induces the release of death mediated proteins such as cytochrome c, Smac/DIABLO and AIF (apoptosis inducing factor) which are regulated by

proapoptotic and antiapoptotic proteins such as Bax/Bak and Bcl-2/xL in caspase-dependent and caspase-independent apoptosis pathways (116). Moreover, mitochondrial permeability changes may also release endonuclease G (EndoG)-triggering cell death. Both of AIF and EndoG represent caspase-independent pathways that can lead to apoptosis-like features of cells following transfer into nuclei (117, 118).

According to our cDNA array screening, none of genes involve in caspase-3-dependent mitochondrial pathway such as caspase-9, caspase-3 was stimulated. This data agree to the fact that MCF-7 cell lacks functional caspase-3. In the absent of functional caspase-3, MCF-7 cell can undergo apoptosis via an activation of other caspases such as caspase-7, and caspase-6 (119). This notion is supported by our data which caspase-6 was stimulated during VR-3848 treatment. An alternative pathway is caspase-independent mitochondrial pathway through an activation of AIF and Endonuclease G (117). We demonstrated that VR-3848 treatment triggered AIF production in cytoplasm. This data showed that VR-3848 induced death involved AIF secretion and caspase-3 independent activation.

The endoplasmic reticulum (ER) stress is the third apoptotic pathway. The ER serves several functions, including folding and assembly of newly synthesized transmembrane and secretory proteins, and their post-translational modification. Various conditions can disturb ER functions and are collectively termed “ER stress” (120) and can sense the stress and respond to ER through translation attenuation, upregulation of the genes for ER chaperones and related proteins, and degradation of unfolded proteins by a quality-control system. However, when the ER function is severely impaired, the organelle elicits apoptotic signals (60). Upon ER-stress response, some core components can participate in ER stress-induced apoptosis as well. For example, mammalian Ire1 can activate JNK and downstream proapoptotic kinases such as ASK1 (74, 121). CHOP (C/EBP homologous protein), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), it has also been shown to promote apoptosis. The function of CHOP can be blocked by BiP overexpression, indicating that CHOP-activated apoptotic pathways are downstream from the ER (62). CHOP can transcriptionally downregulate the antiapoptotic protein Bcl-2 and upregulate DR5, a member of the death receptor protein family (73, 122).

ER stress can also activate well-known general regulators of mammalin apoptosis, including the Bcl-2 and caspase family proteins. It has long been known that a pool of endogenous Bcl-2 resides in the ER membrane, and while Bcl-2 family members are thought to function principally at the mitochondrial outer membrane (123). The caspase family also plays a critical role in ER stress-induced apoptosis. Once activated, caspase-12 can initiate downstream apoptotic pathways. For example, ER stress can induce the activation of caspase-9 independent of Apaf-1, the usual mediator of caspase-9 activation (124). This probably occurs via the direct cleavage of caspase-9 by caspase-12 (125). As demonstrated in our results, GADD153 was dominantly expressed during VR-3848 treatment and the expression was prolonged up to 24 hours. From the western blot analysis, the level of caspase-12 in cytosolic fraction was increased during VR-3848 treatment. This is suggesting that VR-3848 induces ER-stress pathway.

However, the understanding of the molecular mechanisms that lead to inappropriate proliferation of cancer cells will lead to the identification of target that can be therapeutically manipulated to arrest or kill tumour cells. The RAS-MAPK is another signaling pathway which has long been viewed as an attractive pathway for anticancer therapies, based on its central role in regulating the growth and survival of cells from a broad spectrum of human tumours (126). The genes that are most often found when tumour and normal tissues are compared by gene expression are those involved in proliferation. Contained in this list are genes that are expressed in each cell cycle phase: G1/S, S, G2 and M phases. The cell-cycle-regulated genes identified by microarray analysis provide biomarkers of proliferation in both normal cells and tumours (127). The increased expression of the proliferation genes in tumours is often associated with poor prognoses in cancer patients. During cell cycle, kinases and phosphatases control the reversible process of phosphorylation and are dysregulated in many diseases, such as cancer. In many cancerous cells, survival kinases are selectively upregulated, resulting in cellular resistance to conventional cancer therapies. Notably, downregulation of survival signaling sensitizes the cell to concentrations of chemotherapeutic agents that alone do not induce significant cell death (128). Moreover, cyclin-dependent protein kinases (CDKs) have essential roles in cell proliferation, and considerable interest has been directed towards developing

specific CDK inhibitors for cancer therapy (129). From the previous studies (128), 73 kinases were identified as survival kinases such as CDK6 (Cyclin-dependent kinase 6), CDK8 (Cyclin-dependent kinase 8) or CLK3 (CDC-like kinase 3). Disruption against these survival kinase genes may contribute to apoptosis. As shown in our array result (table 10), some survival kinases such as CDK6, CDK8 and CLK3 were downregulated during VR-3848 treatment. More studies about the exact role of kinases or phosphatases during cell cycle control needed to be done before any clear conclusion could be drawn.

On the basis obtained from our results, this study demonstrated that VR-3848, a novel peptide extracted from plant, induced cell death by apoptosis in caspase-3 deficient human breast cancer cell, MCF-7. The results from cDNA analysis, we propose that there are at least three possible pathways were drawn by which VR-3848 leading this cancer cell to die by apoptosis (shown in figure 27).

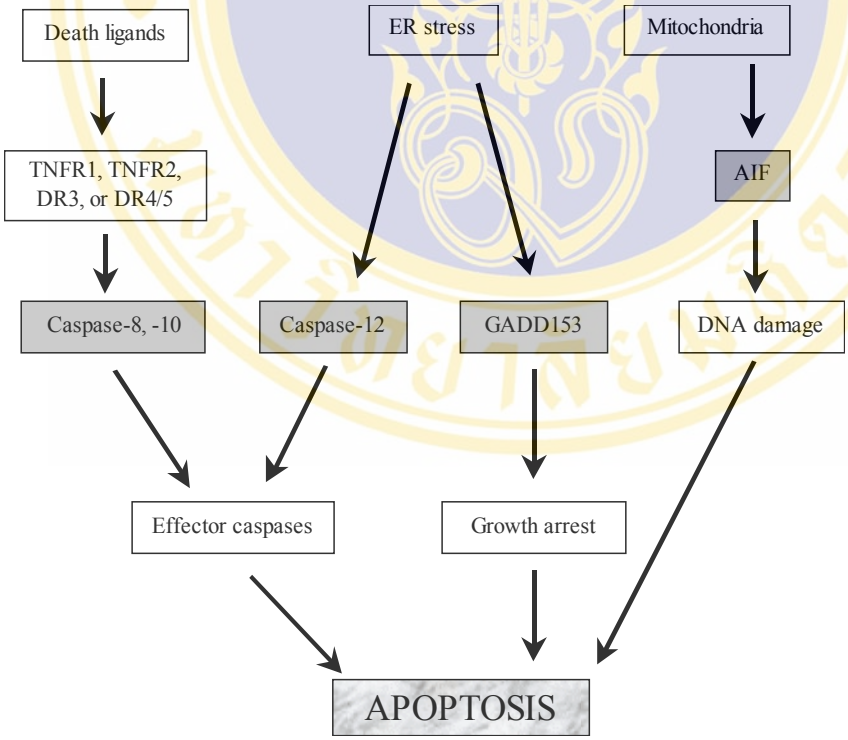

Figure 27. The three possible pathways, which lead MCF-7 cell apoptosis by induction of VR-3848.

CHAPTER 6
CONCLUSION

Based on the results collecting together, we conclude that even though MCF-7 was classified in the group of hormone responsive cancer cell but our MCF-7 (ATCC) cell line has not been used estrogen as the growth supplement.

VR-3848 can induce MCF-7 cell death by apoptosis, which was confirmed by the observation of apoptosis hallmark such as nuclear condensation and fragmentation. This compound can induce damaging of DNA which was confirmed by the pattern of DNA ladder. The destroying of DNA integrity then affects to the survival of MCF-7 cancer cell and cell eventually committed to die by apoptosis.

The data from protein and apoptosis-related cDNA array analysis has helped us to partially elucidate the mechanisms of apoptosis induction by VR-3848. The three proposed apoptosis pathways for this study are receptor-ligand mediated apoptosis pathway, mitochondrial caspase-3-independent apoptosis pathway and ER-stress mediated apoptosis pathway.

Our data demonstrated that at nanomolar concentration of VR-3848, a 7-mer cyclic peptide exerts a potent anti-proliferating activity against breast cancer cell line, MCF-7. This compound thus holds the promise of being a future alternative anti-cancer drug which deserves further exploration.

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APPENDIX

APPENDIX A
MEDIUM FOR CELL MAINTENANCE

1. Preparation 1 litre of MEM (Minimum essential medium) containing 10% faetal bovine serum

	Weight (gm)	Volume (ml)
1. $NaHCO_3$	1.5	-
2. Powder MEM medium contains	9.5	-
- 2 mM L-glutamine		
- 0.1 mM non-essential amino acids		
3. 100 mM sodium pyruvate	-	10
4. 1.0 mg/ml bovine insulin	-	10
5. Nanopure distilled water	-	880
6. Heat inactivated fetal bovine serum	-	100

Dry powder media are manufactured and packaged in a humidity-controlled environment. This ensures maximum dehydration and product stability. The end product is extremely hygroscopic. Dry powder media must be protected from atmospheric moisture and must be hydrated immediately after opening.

Procedure

1. Add 80 - 90% of the final volume of water to an appropriate size-mixing vessel. Use deionized or cell culture grade water that is between 15 and 30^{o} C.
2. While mixing, slowly add the dry powder medium to the water. Rinse the original package with a small volume of water to remove all traces of medium and add the rinsed material to the mixing vessel.
3. Allow the medium to mix for at least 30 minutes.

4. Add the appropriate amount of sodium bicarbonate to the medium and allow it to mix for at least 10 minutes.
5. While mixing, adjust the pH of the medium to 0.1 - 0.2 units below the desired final pH. Adjust with 1N HCl or 1N NaOH.
6. Stop mixing and add water to bring the volume up to the final desired level. Cover the vessel and mix for at least 30 minutes.
7. Filter the medium through a sterile filter with a cellulose acetate or other low protein-binding membrane with a porosity of 0.2 µm. Dispense the medium into sterile vessels and store protected from light at 2 to 8^{o} C.
8. Other supplements such as serum, antibiotics, L-glutamine may be added aseptically at this point.

2. Preparation of phosphate-buffered saline (PBS), Mg^{++} and Ca^{++} free

Components	Final concentration (mM)	Amount (gm/L)
NaCl	136.75	8.0
KCl	2.67	0.2
Na_2HPO_4	7.99	1.15
KH_2PO_4	1.47	0.2

Dissolved distilled water up to 1 litre. The solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes at 15 pound/inch^2. The pH should be adjusted at 7.4 and was kept at room temperature.

APPENDIX B
MICROSCOPIC COUNT OF VIABLE CELLS

Performing cell count

A sample of cells was mixed with trypan blue, a dye that is commonly used to distinguish viable from nonviable cells. Viable cells exclude the dye, while nonviable cells absorb the dye and appear blue when viewed with a microscope. Cells should be in suspension as single cells in medium or a buffered saline before counting. The cells are placed on a kind of gridded slide called a hemocytometer, and are counted manually under a microscope

Materials

- Hemocytometer (improved Neubauer type), cleaned and dried each time.
- Hemocytometer coverglass (reusable) cleaned and dried each time.
- 0.4% trypan blue (w/v) in PBS
- Counter
- Cells in suspension, either a suspension culture or a trypsinized adherent culture. Make sure that the adherent cells are trypsinized enough that they are single cells and that you swirl the flask and take a representative sample.
- Pipette and tips.
- Phase-contrast microscope, upright or inverted, with 10x objective.
- Sterile culture medium for dilutions.

Procedure

1. Place the coverslip evenly on the middle of the hemocytometer.
2. Aseptically withdraw a sample of the cell suspension and prepare 1:1 ratio in trypan blue solution 0.4%. The optimal concentration of cells for counting is 5-10 x 10^5 cells /ml (50 - 100 cells per large hemocytometer square) after dilution in the trypan blue solution.

3. After being stained with trypan blue, the cells should be counted within 3 minutes; after that time viable cells will begin to take up the dye.
4. Using a pasteur pipette, add 20 µl of stained cell suspension to each side of the coverslip by allowing a drop held at the end of the tip to be taken under the slide by capillary action. Do not overfill, lift or move the coverslip.
5. Place the hemocytometer on the stage of an inverted microscope. Adjust focus and power until a single counting square fills the field.
6. Count the viable cells in the four counting squares and record the numbers.

    6.1) the middle of the triple lines separating each primary square is the boundary. Cells that touch the upper or left boundaries are included; those that touch the bottom or right boundaries are excluded (Figure 28).

    6.2) if greater than 10% of particles are cell clusters, attempt to disperse the original cell suspension further and start again.

    6.3) if there are too many cells present to realistically count perform a dilution using a buffer or dye (if a viable count is being performed). Any diluents must be isotonic.

    6.4) for more accurate and reproducibility, counts must be carried out in the same manner each time.
7. Calculate the average number of viable cells per square (total cells in all four squares divided by 4).
8. The viable cell number is calculated using the formula:

$$\text{Viable Cell Number/mL} = \frac{\text{Number of Viable Cells Counted}}{\text{Number of Squares Counted}} \times 10^4 \times \text{Dilution Factor}$$

The following formula is used to calculate the percentage of viable cells:

$$\% \text{ Viability} = \frac{\text{Number of Viable Cells Counted}}{\text{Number of Viable Cells + Nonviable Cells}} \times 100$$

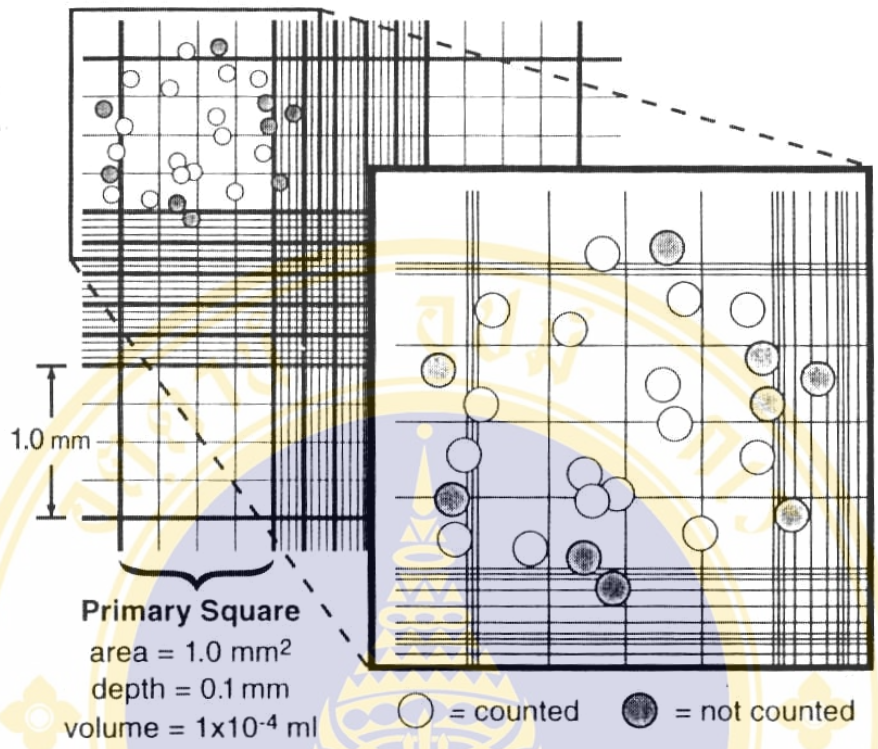

Figure 28. Diagram showing hemocytometer chamber counting area. The chamber contains 9 squares (1 mm^2 each). The counted cells are presented in white circles and the uncounted cells are shown in black circles. The total cell count per ml is obtained by multiplying the average cell counts from 4 squares by 10^4 (130).

Reference:

Barker, K., At the bench: A laboratory navigator, cell maintenance, p.227-230. 2004.

APPENDIX C
SULFORHODAMINE B ASSAY

PROCEDURE

1. Cell fixation

Briefly, after removal the growth medium, cells in each well of 96-well culture plate were fixed with 100 µl of 20% ice-cold trichloroacetic acid (TCA) for 30 minutes at 4^{o}C. Excess TCA was removed by washing five times with distilled water; the plate was then allowed to dry and stored at room temperature or 4^{o}C until use. Background optical densities were measured in wells incubated with growth medium without cells.

2. SRB assay

After that, the TCA-fixed cells were stained with 100 µl of 0.4% (w/v) SRB in acetic acid for 30 minutes at room temperature, washed with 1% acetic acid for five times to remove excess SRB and air dried again. Finally, the stained cells were solubilized in 200 µl of 10 mM Tris base (pH 10) for 5 minutes on a gyratory shaker. The amount of bound SRB released was analyzed by measuring the absorbance at 510 nm using a microtitre plate reader.

Reference:

Skehan P, Storeng R, Dominic S, Anne M, James M, and David V, et al. New colorimetric cytotoxicity assay for anti-cancer-drug screening. J Natl Cancer Inst 1990; 82: 1107-12.

APPENDIX D

Qualitative analysis of DNA fragmentation by agarose gel electrophoresis

Principle

During apoptosis a series of re-organization occur in the cell; chromatin condensation, loss of cell volume and membrane blebbing are some of the most evident morphological changes of apoptotic cells. Although the molecular mechanisms leading to such changes are not completely known, many of them seem to proceed in parallel with biochemical events. This is the case, for example, of chromatin condensation and nuclear envelope breakdown. In fact, in parallel with them occurs DNA fragmentation, a biochemical hallmark of apoptosis in the majority of cells. Responsible for DNA cleavage is believed to be an endogenous Ca^{++}- and Mg^{++}-dependent endonuclease able to break double strand DNA at internucleosomal sites. Therefore, apoptotic DNA cleavage results in characteristic fragments of oligonucleosomal size (180-200 bp). Such phenomenon, described for the first time by Wyllie (1980), can be visualized by an agarose gel electrophoresis analysis. The present protocol provides a method for qualitative determination of DNA fragmentation.

Procedure

1. Approximately 6x 10^6 cells in drug treated experiment were collected both detached cells and attached in culture vessel then washed with PBS and trypsinized.
2. The cell suspension was centrifuged at 3,000 rpm, 4^{o}C for 15 minutes to remove spent medium and twice with PBS.
3. The cell was lysed with lysis buffer containing 20 mg/ml proteinase K and was incubated at 50 ^{o}C overnight.

4. The solution should be clear after cell was completely lysed. Extract the DNA with equal volume of mixture of Phenol: Chloroform (1:1).
5. Thoroughly mixed for 5 minutes by repeated inversion and centrifuged at 12,000 rpm for 15 minutes.
6. The upper aqueous phase was then carefully transferred into fresh clean tube and repeated this phenol: chloroform extraction again.
7. After finish repeated extraction step, 0.2 volume of 3 M sodium acetate was added to the upper aqueous phase and nucleic acid was subsequently precipitated with 2 volumes of absolute ethanol. Mixed by inversion for 10 minutes.
8. The fragmented DNA was obtained by centrifuge at 12,000 rpm for 15 minutes and washed in 70% ethanol. Allow the DNA pellet to dry and dissolved in TE buffer containing 1 mM EDTA and 10 mM Tris-Cl, pH 8.0.
9. The DNA solution was then treated with 1 mg/ml of RNase A at 37^{o}C for 1 hour.
10. To visualize the DNA fragmentation, the DNA samples were run on 1.8% (w/v) horizontal agarose gel. Samples mixed with loading dye buffer and loaded 30 μl per lane.
11. The electrophoresis was run in 1x TBE buffer at 80 constant voltage.
12. After electrophoresis, the gel was stained in 1 μg/ml of ethidium bromide for 15 minutes and destained for 10 minutes.
13. The gel was visualized under UV transilluminator and photographed.

Reference:

Ubol, S., et al., Temporal changes in chromatin, intracellular calcium, and poly(ADP-ribose) polymerase during Sindbis virus-induced apoptosis of neuroblastoma cells. J Virol, 1996. 70 (4): p. 2215-20.

APPENDIX E

Western blot analysis (sample preparation)

Preparation of cytosolic AIF (for MCF-7)

Principle

Mitochondria, treated with atractyloside, release a soluble factor into the supernatant that can induce nuclear apoptosis in isolated nuclei. This factor is a 57-kDa mitochondrial intermembrane protein and was called AIF (apoptosis inducing factor). The primary transcript code for a 67 kDa pro-peptide contains a mitochondrial localization sequence (MLS) within its first 120 amino acids. This propeptide is processed during import into the mitochondria, resulting in the mature 57 kDa AIF and also contains putative nuclear localization sequence (NLS). Induction of apoptosis (e.g. with staurosporine) induces translocation of AIF into the cytosol and to the nucleus. Recombinant AIF, added to cytosol, induces also mitochondrial swelling (i.e. mitochondrial PT) and release of cytochrome c and caspase-9.

Preparation of cytosolic AIF (for MCF-7)

1. Collect culture medium (~5 ml) in 15 ml centrifuge tube- to collect non-adherent cells.
2. Wash the culture flask (25 cm^2) with 5 ml PBS then add 0.5 ml of 0.05 % trypsin/EDTA and incubate for 3 min.
3. After cells were all detached, collected and resuspended with the non-adherent cells in culture medium (same tube at the step 1).
4. Spin down at 1,000 rpm for 5 min then discard supernatant and tap the cell pellet.
5. Wash the pellet twice with 5 ml ice-cold PBS, spin down, discard supernatant and tap the cell pellet. (take some small number of cell for DAPI staining)
6. Resuspend cells pellet (~ 3 x 10^6 cells/sample) with 150 µl of transport buffer and allow permeabilizing at 37^{o}C for 5 min.
7. Resuspend and transfer the cell suspension into 1.5 ml microcentrifuge tube.

8. Spin down at 14,000 xg, 4^{o}C for 15 min.
9. Separate supernatant (cytosolic fraction) and cell pellet (mitochondrial fraction) keep in -20^{o}C until used.

2. Preparation of cytosolic caspase-12 (for MCF-7)

Principle

Three distinct signaling pathways lead to apoptosis. The death receptor and mitochondrion pathways are the two mains, which involve respectively caspase-8 and caspase-9. The endoplasmic reticulum stress is the third apoptotic pathway and involves caspase-12. Localized to the ER but not to cytoplasm or mitochondria, caspase-12 is activated by ER stress, including disruption of ER calcium homeostasis.

Preparation of cytosolic caspase-12 (for MCF-7)

Detection of caspase-12 protein contains in cytoplasm, we used the same procedure as done before in AIF preparation.

APPENDIX F

Assessing yield and purity of total RNA

Principle

RNA degradation was such a serious problem that, for years, people would avoid all RNA work. Some labs still religiously keep all RNA reagents separate from all other reagents in the lab. But a few rules, similar to those of aseptic technique, keep everything running smoothly.

A. Determining $A_{260\ nm}$ and $A_{280\ nm}$

1. Thoroughly mix RNA and measure the total RNA sample volume.
2. Transfer 5 µl of total RNA sample to a 1.5-ml tube.
3. Bring volume up to 495 µl with DEPC treated water and mix by pipetting.
4. Transfer contents (500 µl) to a 1 ml glass cuvette with a 1-cm path length.
5. Measure A_{260} and A_{280} using DEPC treated water as a reference blank.
6. Calculate RNA yields as follows:
   - RNA constant for 1-cm path length. One A_{260} unit of RNA = 40 µg/ml
   - Total A_{260} = (A_{260} of diluted sample) x (dilution factor)
   - Concentration (µg/ml) = (total A_{260} x 40 µg/ml)
   - Yield (µg) = (total sample volume) x (concentration)
7. Calculate the A_{260}/A_{280} ratio. Pure RNA exhibits a ratio of 1.9-2.1.

*****Equipment should be reserved for RNA work only*****

Table 11. Representative total RNA yields

Tissue/Cell source	Amount of starting material	Yield of total RNA	Yield after DNase (70% recovery)
Rat liver	100 mg	600 μg	420 μg
Rat skeletal muscle	100 mg	90 μg	60 μg
Mouse brain	100 mg	125 μg	90 μg
Mouse spleen	100 mg	245 μg	170 μg
Mouse testes	100 mg	240 μg	170 μg
Mouse thymus	100 mg	85 μg	60 μg
Human cerebellum	100 mg	85 μg	60 μg
Human prostate tumour	100 mg	100 μg	70 μg
MCF-7 cell line	1 x 10^7 cells	70 μg	50 μg
Mouse fibroblasts	1 x 10^7 cells	800 μg	560 μg
U251 cell line	1 x 10^7 cells	95 μg	65 μg

Table 12. Effects of pH on A_{260}/A_{280} ratio.

BLANK/DILUENT	A_{260}/A_{280} RATIO
DEPC-treated water (pH 5-6)	1.60
Nuclease-free water (pH 6-7)	1.85
TE (pH 8.0)	2.14

B. Gel Electrophoresis

1. Run gel at 4-5 V/cm (equivalent to 50-60 V on a mini-gel box).
2. Examine gel when dye has migrated 3-4 cm from the wells.

C. Expected Results

Total RNA from mammalian sources should appear as two bright bands (28S and 18S ribosomal RNA) at approximately 4.5 and 1.9 kb (shown in figure 29). The ratio of intensities of the 28S and 18S rRNA bands should be 1.5-2.5:1. Lower ratios

are indicative of degradation. You may also see additional bands or a smear lower than the 18S rRNA band, including very small bands corresponding to 5S rRNA and tRNA.

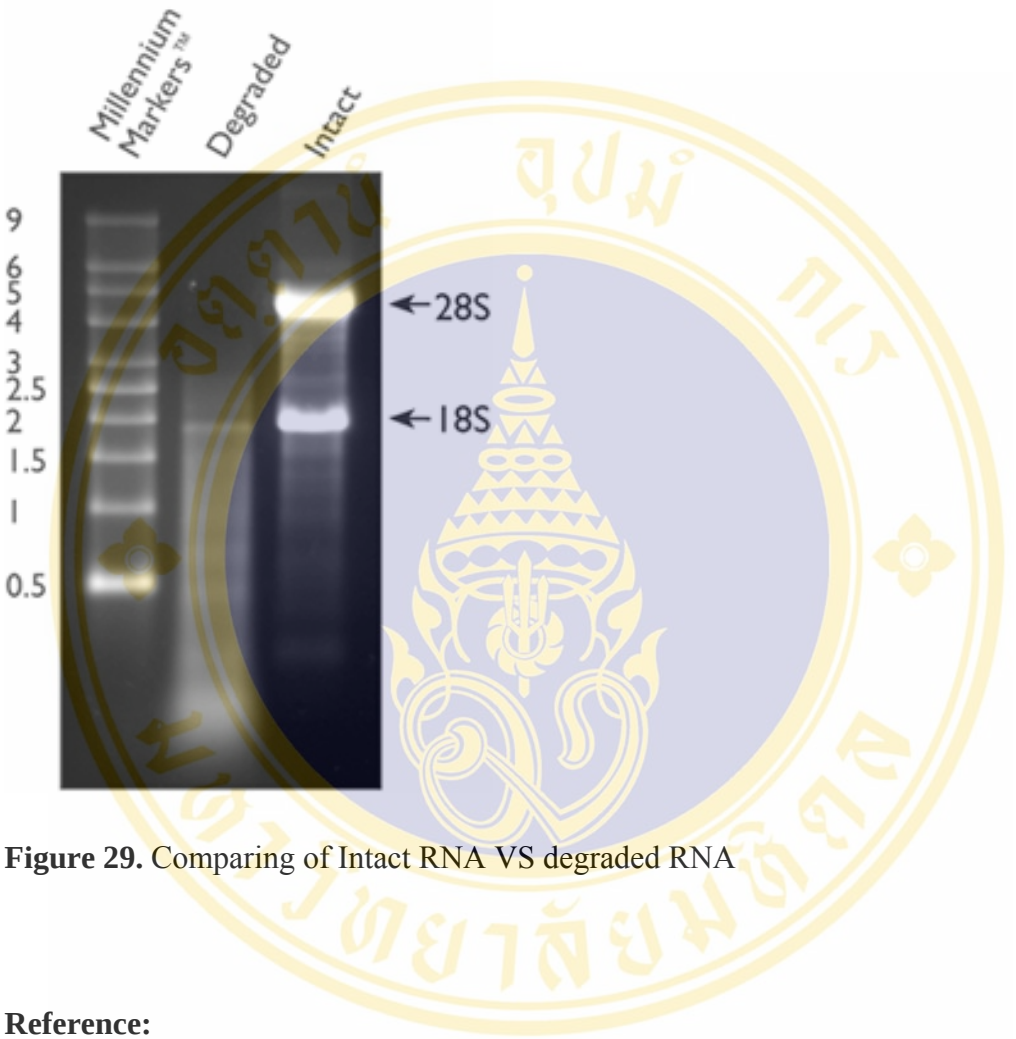

Figure 29. Comparing of Intact RNA VS degraded RNA

Reference:

Ambion, Inc 2005. TechNotes 8 (3). Is your RNA intact? Methods to check RNA integrity. Retrieved from http://www.ambion.com/techlib/tn/83/8313.html.

APPENDIX G

Poly A+ RNA Enrichment and Probe Synthesis

A. Streptavidin Magnetic Bead Preparation

1. Resuspend Magnetic Beads by inverting and gently tapping tube.
2. Aliquot 15 µl of beads per probe synthesis reaction into one 0.5-ml tube.
3. Separate beads on magnetic particle separator.
4. Pipette off and discard supernatant.
5. Wash beads with 150 µl of 1x Binding Buffer; pipette up and down.
6. Separate beads on magnetic particle separator.
7. Pipette off and discard supernatant.
8. Repeat Steps 5-7 three times.
9. Resuspend the beads in 15 µl 1x Binding Buffer per reaction.

B. Poly A+ RNA enrichment

Perform the following steps for each total RNA sample.

Important: Do not pause between any of these steps.

1. Preheat a PCR thermal cycler to 70°C.
2. Aliquot 10-50 µg total RNA into a 0.5 ml tube.
3. Add deionized H_2O to 45 µl.
4. Add 1 µl biotinylated oligo (dT), thoroughly mix by pipetting.
5. Incubate at 70°C for 2 min in the preheated thermal cycler.
6. Remove from heat and cool at room temperature for 10 min.
7. Add 45 µl 2x Binding Buffer, mix well by pipetting. Resuspend the washed beads by pipetting up and down, and add 15 µl to each RNA sample.

8. Mix on a vortexer or shaker at 1,500 rpm for 25-30 min at room temp.
9. Separate beads using the magnetic separator. Carefully pipette off and discard supernatant.
10. Gently resuspend beads in 50 µl 1x wash buffer.
11. Being careful not to lose particles, separate beads and then pipette off and discard supernatant.
12. Repeat Steps 11 and 12 one time.
13. Gently resuspend beads in 50 µl 1x Reaction Buffer.
14. Separate beads, then pipette off and discard supernatant.
15. Resuspend beads in 3 µl dH_2O (for Atlas Nylon 1.2 Arrays).
16. Proceed with the appropriate protocol for Atlas Nylon Membrane.

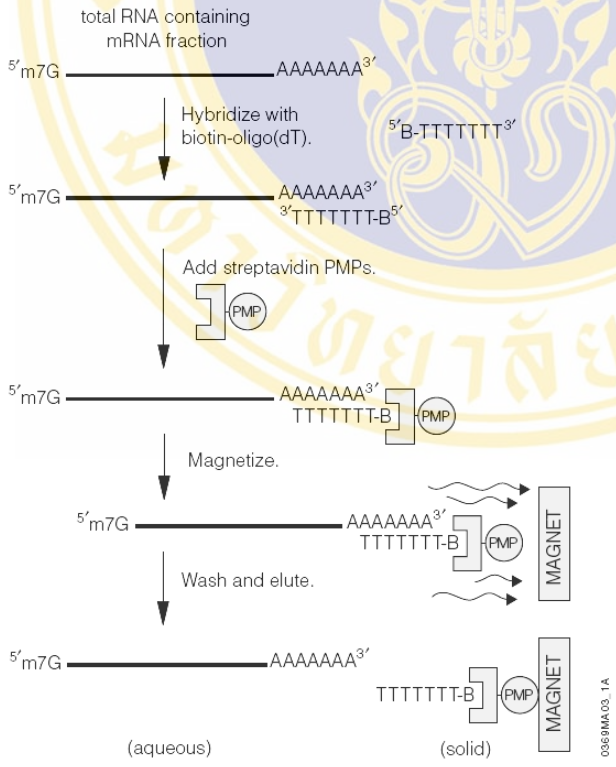

Figure 30. Schematic diagram of the PolyA$^+$ mRNA isolation procedure (Promega corporation, MA, USA. The PolyATtract® mRNA isolation systems. Technical Manual No. 021, p.2)

C. cDNA Probe Synthesis—Atlas Nylon Membrane Arrays

Proceed with the following steps, using reagents included with your Atlas

cDNA expression arrays

Prepare a Master Mix for all labeling reactions plus one extra reaction (to ensure that you have sufficient volume). Combine the following in a 0.5-ml microcentrifuge tube at room temperature:

REAGENTS	per rxn
5x Reaction Buffer	2.0 µl
10x dNTP Mix (for dATP label)	1.0 µl
[α-^{32}P] dATP (3,000Ci/mmol, 10 µCi/µl)	3.5 µl
DTT (100 mM)	0.5 µl
Total volume	7.0 µl

Preheat a PCR thermal cycler to 70°C.

1. For each experimental poly A+ RNA and the Control Poly A+ RNA, combine the following in a labeled 0.5 ml PCR tube:

   poly A$^+$ RNA sample 1 µg (1-2 µl)

   CDS Primer Mix 1 µl

   - If you have observed weak signals in previous hybridizations, use 2 µl CDS Primer Mix, but do not exceed 3 µl total volume.
   - To each tube, add deionized H_2O to a final volume of 3 µl (if necessary).

2. Mix well by pipetting and spin tubes briefly in a microcentrifuge.
3. Incubate tubes in preheated PCR thermal cycler at 70°C for 2 min.
4. Reduce the temperature of the thermal cycler to 50°C (or 48°C if you are using an unregulated heating block or water bath) and incubate tubes for 2 min. During this incubation, add 1 µl MMLV Reverse Transcriptase per

reaction to the Master mix. Mix by pipetting, and keep the Master mix at room temperature.

5. After completion of the 2 min incubation at 50 ^{o}C, add 8 µl of Master Mix to each reaction tube (Do not remove the RNA samples from the thermal cycler for longer than is necessary to add the Master mix).
6. Mix the contents of the tubes by pipetting and immediately return them to the thermal cycler.
7. Incubate tubes in the PCR thermal cycler at 50 ^{o}C (or 48 ^{o}C) for 25 min.
8. Stop the reaction by adding 1 µl of 10x Termination Mix.
9. Proceed with the column chromatography. If necessary, you can store your probe on ice or at 4^{o}C for a few hours.

D. Column Chromatography

To purify the labeled cDNA from unincorporated ^{32}P-labeled nucleotides and small (<0.1kb) cDNA fragments, follow this procedure for each reaction tube. Before use, be sure to add 95% ethanol directly to Buffer NT 3 as specified on the bottle label.

1. Separate beads and pipette the supernatant (~20 µl) into 180 µl Buffer NT2, mix well by pipetting.
2. Place a NucleoSpin Extraction Spin Column into a 2 ml Collection Tube, and pipette the sample into the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough into the appropriate container for radioactive waste.
3. Insert the NucleoSpin column into a fresh 2 ml Collection Tube. Add 400 µl Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough.
4. Repeat Step 3 twice.

5. Transfer the NucleoSpin column to a clean 1.5 ml microcentrifuge tube. Add 100 µl Buffer NE, and allow column to soak for 2 min.
6. Centrifuge at 14,000 rpm for 1 min to elute purified probe.
7. heck the radioactivity of the probe by scintillation counting:
    a. Add 4 µl of each purified probe to 5 ml of scintillation fluid in separate scintillation-counter vials.
    b. Count ^{32}P-labeled samples on the ^{32}P channel, and calculate the total number of counts in each sample. (Multiply counts by a dilution factor of 25)

Probes synthesized should have a total of 5-20 x 10^6 cpm for probes made from poly A^+ RNA or 2-10 x 10^6 cpm for probes made from total RNA. Probes made using the BD Atlas Pure Total RNA Labeling System typically have an activity of 1-10 x 10^6 cpm. Store probes at –20°C. Discard flowthrough fractions, columns, and elution tubes in the appropriate container for radioactive waste.

E. Hybridizing cDNA probes to the BD Atlas™ Array

1. Prepare a solution of BD ExpressHyb and sheared salmon testes DNA
    a. Pre-warm 5 ml of BD ExpressHyb at 68°C.
    b. Heat 0.5 mg of the sheared salmon testes DNA at 95-100°C for 5 min, and then chill quickly on ice.
    c. Mix heat-denatured sheared salmon testes DNA with pre-warmed BD ExpressHyb. Keep at 68°C until use.
2. Fill a hybridization bottle with deionized H_2O. Wet the array by placing it in a dish of deionized H_2O, and then place the membrane into the bottle. Pour off all the water from the bottle; the membrane should adhere to the inside walls of the container without creating air pockets. Add 5 ml of the solution prepared in Step 1. Ensure that the solution is evenly distributed over the membrane. Perform this step quickly to prevent the array

membrane from drying. Pre-hybridize for 30 min with continuous agitation at 68°C.

Notes:

- Do not remove array from the container during the pre-hybridization, hybridization, or washing steps.
- If performing the hybridization in roller bottles, rotate at 5-7 rpm during pre-hybridization and hybridization steps.

3. To prepare your probe for hybridization, add 5 µl Cot-1 DNA to your entire pool of labeled probe and incubate in a boiling (95-100°C) water bath for 2 min. Then incubate probe on ice for 2 min.
4. Being careful to avoid pouring the concentrated probe directly on the surface of the membrane, add the mixture prepared in Step 4 directly to your array and pre-hybridization solution. Make sure that the two solutions are mixed together.
5. Hybridize overnight with continuous agitation at 68°C. Be sure that all regions of the membrane are in contact with the hybridization solution at all times. If necessary, add an extra 2-3 ml of pre-warmed BD ExpressHyb.
6. The next day, wash each membrane with 50 ml Wash Solution 1 for 10 min and wash with pre-warm Wash Solution 1(2x SSC, 1% SDS) at 68°C for 15 and 20 min, respectively.
7. Using forceps remove the array from the container and shake off excess Wash Solution. Do not blot dry or allow the membrane to dry.

*** If necessary, place the array in H_2O while preparing an adequate sized piece of plastic wrap. If the membrane dries even partially, subsequent removal of the probe (stripping) from the BD Atlas ™ Array will be difficult.

8. Immediately wrap the damp membrane in plastic wrap. If possible, seal the edges of the wrapped array using a heat sealer to prevent the array from drying.
9. Mount the plastic-wrapped array on Whatman paper (3 MM Chr). Expose the array to x-ray film at –70°C with an intensifying screen. When setting up your exposure, make sure you are aware of the orientation of the array by noting the position of the orientation notch at the upper right-hand corner of the membrane. Also, be sure to try several exposures for varying lengths of time (e.g., 3-6 hr, overnight, and 3 days). Alternatively, use a phosphorimager. When exposing the array to a phosphorimaging screen at room temperature, be sure to seal the array membrane in plastic to prevent drying.

F. Stripping cDNA probes from the BD Atlas™ Array

To re-use the array after exposure to x-ray film or phosphorimaging, you may remove the cDNA probe by stripping.

Note: Perform all steps in a fume hood with appropriate radiation protection.

1. In a 2 L beaker, heat 500 ml of 0.5% SDS solution to boiling.
2. Remove the plastic wrap from the array and immediately place the membrane into the boiling solution. Avoid prolonged exposure of the membrane to air.
3. Continue to boil for 5-10 min.
4. Remove the solution from heat and allow cooling for 10 min.
5. Rinse the array in wash solution1 (2x SSC, 1% SDS).
6. Remove the array from the solution and immediately wrap the damp membrane in plastic wrap. Check the efficiency of stripping with a Geiger hand counter and by exposure to x-ray film. If radioactivity can still be detected, repeat the stripping procedure (steps1-5).

7. Place the array into a hybridization container and proceed with the next hybridization experiment. Alternatively, the array can be sealed and stored in plastic wrap at -20^{o}C until needed. Do not allow the membrane to dry, even partially.

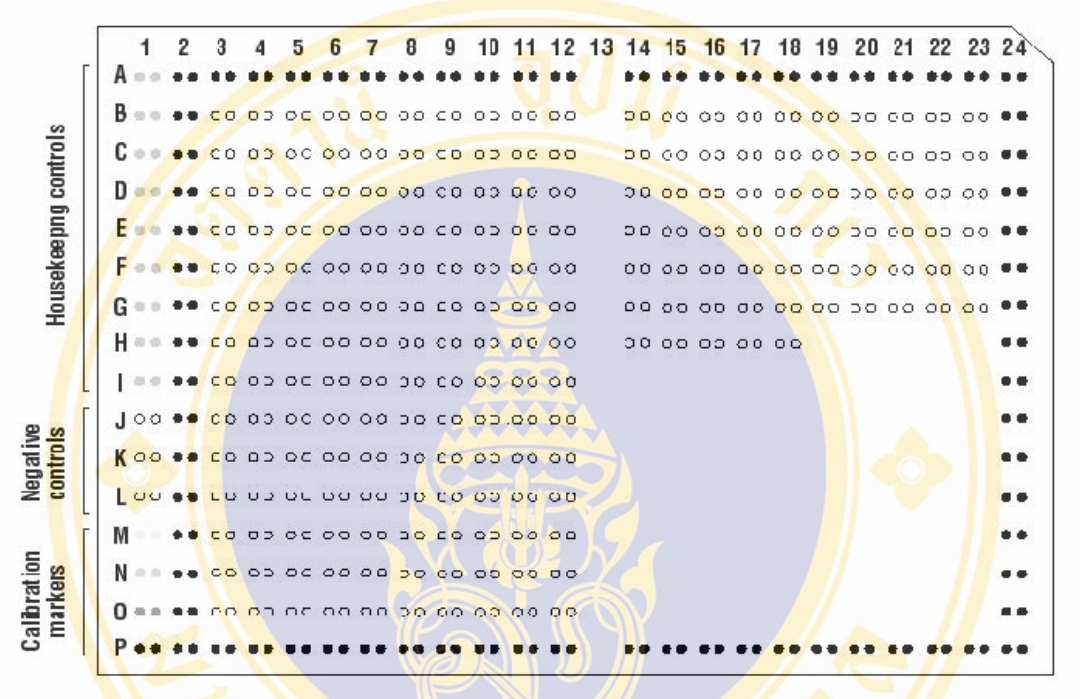

Figure 31. Distribution of genes on Atlas™ Human Apoptosis Arrays. The array including of 205 human apoptosis cDNAs, 9 housekeeping and negative control immobilized in duplicate dots on a nylon membrane. (BD Biosciences Clontech. Atlas human apoptosis array user manual. Protocol number PT3545-3, p.1).

APPENDIX H
PCR PRODUCTS DETECTION

Polymerase chain reaction the gene of interested was used to confirm the alteration of expressed genes which came from cDNA analysis. In this study the Cytotoxic TRAIL receptor 2 (TRICK2A), GADD153 (growth arrest DNA damage 153) and human β-actin were determined by using the RNA samples (the same stock which were used in cDNA macroarray) as the template. The level of gene expression was estimated by using densitometer band detection and expressed as a ratio relative to human β-actin for internal control.

1. cDNA synthesis (Reverse transcription)

Procedure (perform these step in ice box)

1. The master mix was prepared by mixed the component as listed in table 13.
2. Dissolved 2 μg of each RNA samples with DEPC-treated sterile water in 10 μl total volume.
3. Mix the RNA sample with master mix and briefly spinned down. The total volume is 20 μl.
4. The reactions were incubated at 42^{o}C for 1 hour.
5. Added nuclease free water into the synthesized cDNA to make final volume of 50 μl. This cDNA were used in step of PCR reaction.

Table 13. The component of master mix for cDNA synthesis.

Master mix for cDNA synthesis	Volume per reaction (µl)
5x AMV Reverse transcriptase buffer	4.0
0.1 M DTT	1.0
10 mM dNTP mix	2.0
200 µg/ml oligo dT_{15}	1.0
200 µg/ml random primer	1.0
40 Unit/µl RNase inhibitor	0.2
10 Unit/µl AMV RT enzyme	0.8
Total volume	**10.0**

2. Polymerase chain reaction (PCR)

The genes of interested and internal control gene (human β-actin in this study) were amplified by PCR.

1. The master mix was prepared by mixed the component as listed in table 14.
2. Mix 2.5 µl of cDNA with 22.5 µl of PCR master mix to make a total volume of 25.0 µl. Mixed well and briefly spinned down.
3. The reactions were performed in PCR thermal cycler. The temperature programmes of each gene were indicated below.

Table 14. The component of master mix for PCR reaction.

Master mix for cDNA synthesis	Volume per reaction (µl)
Distilled water	16.625
10 x buffer	2.5
10 mM dNTP mix	0.5
20 µM sense primer	0.625
20 µM anti-sense primer	0.625
25 mM $MgCl_2$	1.5
Taq polymerase (5Unit/µl)	0.125
Total volume	**22.5**

3. Primer sequences used in PCR reaction

3.1 Human β-actin

- Product size 578 bp
- Primer (sense) 5’ ATC TGG CAC CAC ACT TCT ACA 3’
- Primer (anti-sense) 5’ GTT TCG TGG ATG CCA CAG GAC T 3’
- PCR condition

94°C	5 min	Pre-heated step
94°C	30 sec	Denaturing step
55°C	30 sec	Annealing step
72°C	30 sec	Pre-heated step
72°C	7 min	Extension step
4°C	5 min	Cooling step
Cycle	30 cycles	

3.2) Cytotoxic TRAIL receptor 2 (TRICK2A)

- Product size 221 bp
- Primer (sense) 5’ CAC CAG GTG TGA TTC AGG TG 3’
- Primer (anti-sense) 5’ CCC CAC TGT GCT TTG TAC CT 3’
- PCR condition

94°C	5 min	Pre-heated step
94°C	30 sec	Denaturing step
56°C	30 sec	Annealing step
72°C	30 sec	Pre-heated step
72°C	7 min	Extension step
4°C	5 min	Cooling step
Cycle	30 cycles	

3.3) GADD153 (growth arrest DNA damage 153)

- Product size 309 bp
- Primer (sense) 5’ GAA ACG GAA ACA GAG TGG TCA TTC CCC 3’

- Primer (anti-sense) 5’ GTG GGA TTG AGG GTC ACA TCA TTG GCA 3’
- PCR condition

94°C	5 min	Pre-heated step
94°C	30 sec	Denaturing step
61°C	30 sec	Annealing step
72°C	30 sec	Pre-heated step
72°C	7 min	Extension step
4°C	5 min	Cooling step
Cycle	30 cycles	

4. Agarose gel electrophoresis

Run the PCR product on 1.8% agarose, 10 µl of PCR product per lane was loaded and electrophoresed in 1x TBE buffer at 80 constant voltage for 1 hour and 30 minutes. The gel was stained in 1 µg/ml of ethidium bromide solution for 10 minutes and destained in distilled water for 5 minutes. The bands of PCR product were visualized on a UV transilluminator and photographed.

APPENDIX I

APOPTOTIC cDNA GENES LIST

Principle

BD Atlas™ cDNA expression arrays include hundreds of cDNAs spotted on positively charged nylon membranes. Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several housekeeping cDNAs as positive controls for normalizing mRNA abundance. This complete list is also available on CLONTECH's web site (atlas.clontech.com).

Table 15. The apoptotic cDNAs gene list spotted on nylon membrane

Code	Gene name	Classification
1A	Ubiquitin	Protein turnover, stress response proteins, chromatin proteins, cytoplasmic proteins, nuclear proteins
1B	Phospholipase A2	Phospholipases and phosphoinositol kinases, complex lipid metabolism, cytoplasmic proteins
1C	Hypoxanthine-guanine phosphoribosyltransferase (HPRT)	Nucleotide metabolism, cytoplasmic proteins
1D	Liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Complex carbohydrate metabolism, energy metabolism, DNA damage signaling/repair proteins and DNA ligases, cytoplasmic proteins, nuclear proteins
1E	Brain-specific tubulin alpha 1 subunit (TUBA1)	Cytoskeleton/motility proteins, cytoplasmic proteins, cytoskeletal proteins
1F	HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	Major histocompatibility complex, plasma membrane proteins
1G	Cytoplasmic beta-actin (ACTB)	Cytoskeleton/motility proteins, cytoplasmic proteins, cytoskeletal proteins
1H	23-kDa highly basic protein; 60S ribosomal protein L13A (RPL13A)	Chaperones/ heat shock proteins, other immune system proteins, cytoplasmic proteins, plasma membrane proteins

Code	Gene name	Classification
1I	40S ribosomal protein S9	Chaperones/ heat shock proteins, cytoplasmic proteins
3B	Cell division control protein 2 homolog (CDC2); p34 protein kinase; cyclin-dependent kinase 1 (CDK1)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3C	Cyclin-dependent protein kinase 2 (CDK2); p33 protein kinase	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3D	Cell division protein kinase 3	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3E	Cell division protein kinase 4; cyclin-dependent kinase 4 (CDK4); PSK-J3	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3F	Cell division protein kinase 5 (CDK5); tau protein kinase II catalytic subunit (TPKII catalytic subunit); serine/threonine protein kinase PSSALRE	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3G	Cell division protein kinase 6 (CDK6); serine/threonine protein kinase PLSTIRE	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3H	Protein serine/threonine kinase STK1; cell division protein kinase 7 (CDK7); CDK-activating kinase (CAK); 39-kDa protein kinase	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
3I	Cyclin-dependent kinase 5 activator isoform p39I precursor (CDK5 activator; NCK5AI)	Other cell cycle proteins, cytoplasmic proteins
3J	Cyclin-dependent kinase 5 activator precursor (CDK5 activator); tau protein kinase II 23-kDa subunit; TPKII regulatory subunit	Other cell cycle proteins, cytoplasmic proteins
3K	Cell division cycle protein 25A (CDC25A); M-phase inducer phosphatase 1	Other cell cycle proteins, intracellular protein phosphatases (non-receptor phosphatases), nuclear proteins
3L	CDC25B; CDC25HU2; M-phase inducer phosphatase 2	Other cell cycle proteins, intracellular protein phosphatases (non-receptor phosphatases), nuclear proteins
3M	CDC25C; M-phase inducer phosphatase 3	Other cell cycle proteins, intracellular protein phosphatases (non-receptor phosphatases), nuclear proteins
3N	CDC-like kinase 1 (CLK1)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins

Code	Gene name	Classification
3O	CDC-like kinase 2 (CLK2)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
4B	CDC-like kinase 3 (CLK3)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
4C	Serine/threonine-protein kinase KKIALRE	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
4D	Serine/threonine-protein kinase PCTAIRE 1 (PCTK1)	Intracellular kinase network members (non-receptor protein kinases), death kinases, cytoplasmic proteins, nuclear proteins
4E	Serine/threonine-protein kinase PCTAIRE 2 (PCTK2)	Intracellular kinase network members (non-receptor protein kinases), apoptosis associated proteins, cytoplasmic proteins, nuclear proteins
4F	Serine/threonine-protein kinase PCTAIRE 3 (PCTK3)	Intracellular kinase network members (non-receptor protein kinases), apoptosis associated proteins, cytoplasmic proteins, nuclear proteins
4G	Cell division protein kinase 9 (CDK9); serine/threonine protein kinase PITALRE	Cell cycle-regulating kinases, intracellular kinase network members (non receptor protein kinases), nuclear proteins
4H	CDC2-related protein kinase CHED	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
4I	Cdc2-related protein kinase PISSLRE	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
4J	G2/mitotic-specific cyclin A (CCNA; CCN1)	Cyclins, nuclear proteins
4K	G2/mitotic-specific cyclin B1 (CCNB1)	Cyclins, nuclear proteins
4L	G1/S-specific cyclin C	Cyclins, nuclear proteins
4M	G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	Cyclins, nuclear proteins
4N	G1/S-specific cyclin D2 (CCND2) + KIAK0002	Cyclins, nuclear proteins
4O	G1/S-specific cyclin D3 (CCND3)	Cyclins, nuclear proteins
5B	G1/S-specific cyclin E (CCNE)	Cyclins, nuclear proteins
5C	G2/mitotic-specific cyclin G1 (CCNG1; CYCG1)	Cyclins, nuclear proteins

Code	Gene name	Classification
5D	Cyclin G2 (CCNG2)	Cyclins, nuclear proteins
5E	Cyclin H (CCNH); MO15-associated protein	Cyclins, nuclear proteins
5F	Cyclin-dependent kinase inhibitor 1 (CDKN1A); melanoma differentiation-associated protein 6 (MDA6); CDK-interacting protein 1 (CIP1); WAF1	CDK inhibitors, kinase activators and inhibitors, kinase activators and inhibitors, nuclear proteins
5G	Cyclin-dependent kinase inhibitor 1C (CDKN1C); p57-KIP2	CDK inhibitors, kinase activators and inhibitors, kinase activators and inhibitors, nuclear proteins
5H	Cyclin-dependent kinase 4 inhibitor (CDK4I; CDKN2); p16-INK4; multiple tumour suppressor 1 (MTS1)	CDK inhibitors, kinase activators and inhibitors, kinase activators and inhibitors, nuclear proteins
5I	Cyclin-dependent kinase 4 inhibitor B (CDKN2B); p14-INK4B; multiple tumour suppressor 2 (MTS2)	CDK inhibitors, kinase activators and inhibitors, kinase activators and inhibitors, nuclear proteins
5J	Cyclin-dependent kinase 4 inhibitor D (CDKN2D); p19-INK4D	CDK inhibitors, kinase activators and inhibitors, kinase activators and inhibitors, nuclear proteins
5K	P35 cyclin-like CAK1-associated protein	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
5L	Wee1Hu CDK tyrosine 15-kinase; wee-1-like protein kinase	CDK inhibitors cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), kinase activators and inhibitors, nuclear proteins
5M	Serine/threonine-protein kinase PLK1 (STPK13)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), nuclear proteins
5N	Phospholipase D1 (PLD 1); choline phosphatase 1; phosphatidylcholine-hydrolyzing phospholipase D1	Phospholipases and phosphoinositol kinases, general trafficking, cell cycle, cytoplasmic proteins, plasma membrane proteins
5O	NEDD5 protein homolog; DIFF6; KIAA0158	Other cell cycle proteins, GTP/GDP exchangers and G-protein GTPase activity modulators, cytoplasmic proteins
6B	CDC10 protein homolog	Other cell cycle proteins, cytoplasmic proteins
6C	CDC27HS protein	Other cell cycle proteins, nuclear proteins
6D	Ubiquitin-conjugating enzyme E2 32-kDa complementing protein; ubiquitin-protein ligase; ubiquitin carrier protein; CDC34	GTP/GDP exchangers and G-protein GTPase activity modulators, cytoplasmic proteins, nuclear proteins
6E	CDC16HS	Other cell cycle proteins, cytoskeletal proteins

Code	Gene name	Classification
6F	CDC37 homolog	Other cell cycle proteins, intracellular transducers/effectors/modulators, cytoplasmic proteins
6G	CDC6-related protein	Other cell cycle proteins, intracellular transducers/effectors/modulators, nuclear proteins
6H	Extracellular signal-regulated kinase 1 (ERK1; p44-ERK1); microtubule-associated protein 2 kinase; insulin-stimulated MAP2 kinase; MAP kinase 1 (MAPK1; p44-MAPK); PRKM3	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins, cytoskeletal proteins
6I	Extracellular signal-regulated kinase 2 (ERK2); mitogen-activated protein kinase 2 (MAP kinase 2; MAPK 2); p42-MAPK	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins
6J	Extracellular signal-regulated kinase 3 (ERK3); MAP kinase 3 (MAPK3; p97-MAPK); PRKM5	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins
6K	Extracellular signal-regulated kinase 4 (ERK4); MAP kinase 4 (MAPK4; p63-MAPK); PRKM4	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins
6L	Extracellular signal-regulated kinase 5 (ERK5); BMK1 kinase	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins
6M	Extracellular signal-regulated kinase 6 (ERK6); stress-activated protein kinase-3; mitogen-activated protein kinase p38 gamma; (MAP kinase p38 gamma)	Cell cycle-regulating kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins, nuclear proteins
6N	Mitogen-activated protein kinase p38 (MAP kinase p38); cytokine suppressive anti-inflammatory drug binding protein (CSAID binding protein; CSBP); MAX-interacting protein 2 (MXI2)	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins
6O	c-jun N-terminal kinase 1 (JNK1); JNK46	Intracellular kinase network members (non-receptor protein kinases), stress response proteins, cytoplasmic proteins
7B	c-jun N-terminal kinase 2 (JNK2); JNK55	Intracellular kinase network members (non-receptor protein kinases), stress response proteins, cytoplasmic proteins
7C	c-jun N-terminal kinase 3 alpha2 (JNK3A2); PRKM10 + MAP kinase p493F12	Intracellular kinase network members (non-receptor protein kinases), stress response proteins, cytoplasmic proteins
7D	Dual specificity mitogen-activated protein kinase kinase 5 (MAP kinase kinase 5; MAPKK 5)	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins

Code	Gene name	Classification
7E	Dual-specificity mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1; MAPKK 1; MKK1); extracellular signal-regulated kinase 1; ERK activator kinase 1	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins
7F	Dual-specificity mitogen-activated protein kinase kinase 6 (MAP kinase kinase 6; MAPKK 6; MKK6); MAPK/ERK kinase 6; SAPKK3	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins
7G	MAPK/ERK kinase kinase 3 (MEK kinase 3; MEKK3)	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins
7H	Proliferating cyclic nuclear antigen (PCNA); cyclin	DNA replication: polymerases, replication factors, and topoisomerases, nuclear proteins
7I	Peptidyl-prolyl cis-transisomerase nima-interacting 1 (PIN1)	Other cell cycle proteins, other post-translational modification proteins, nuclear proteins
7J	Retinoblastoma-associated protein (RB1); PP110; P105-RB	Kinase activators and inhibitors, cell cycle, intracellular transducers / effectors/modulators ,nuclear proteins
7K	Retinoblastoma-like protein 2 (RBL2; RB2); 130-kDa retinoblastoma-associated protein	Kinase activators and inhibitors, cell cycle, intracellular transducers / effectors/modulators ,nuclear proteins
7L	Chromatin assembly factor 1 p48 subunit (CAF1 p48 subunit); retinoblastoma-binding protein 4 (RBBP4); RBAP48; msi1 protein homolog	Chromatin proteins, histone acetyltransferases / deacetylases, nuclear proteins
7M	RBP2 retinoblastoma binding protein	Basic transcription factors, other cell cycle proteins, nuclear proteins
7N	Retinoblastoma-binding protein 1 (RBP1) isoform I + isoform II	Other cell cycle proteins, transcription, nuclear proteins
7O	RBQ-1 retinoblastoma binding protein	Other cell cycle proteins, transcription, nuclear proteins
8B	RBQ-3	Other cell cycle proteins, transcription, nuclear proteins
8C	PRB-binding protein E2F1; retinoblastoma-binding protein 3 (RBBP3); retinoblastoma-associated protein 1 (RBAP1); PBR3	Transcription activators and repressors, other cell cycle proteins, nuclear proteins
8D	E2F-3	Transcription activators and repressors, other cell cycle proteins, nuclear proteins
8E	Transcription factor E2F5	Transcription activators and repressors, cell cycle, nuclear proteins

Code	Gene name	Classification
8F	E2F dimerization partner 1; DRTF1-polypeptide 1 (DP1)	Other cell cycle proteins, intracellular transducers / effectors/modulators transcription activators and repressors, nuclear proteins
8G	Transcription factor DP2 (Humdp2); E2F dimerization partner 2	Other cell cycle proteins, intracellular transducers / effectors / modulators transcription activators and repressors, nuclear proteins
8H	Abl interactor 2 (Abi-2) + Abl binding protein 3 (AblBP3) [ArgBPIB]	Kinase activators and inhibitors, adaptors and receptor-associated proteins, cytoplasmic proteins, nuclear proteins
8I	Growth factor receptor-bound protein 2 (GRB2) isoform; GRB3-3; SH2/SH3 adaptor GRB2; ASH protein + epidermal growth factor receptor-bound protein 2 (EGFRBP-GRB2)	Adaptors and receptor-associated proteins, death receptor-associated proteins and adaptors, cytoplasmic proteins
8J	GRB-IR / GRB10	Kinase activators and inhibitors, adaptors and receptor-associated proteins, kinase activators and inhibitors, cytoplasmic proteins
8K	c-raf proto-oncogene	Kinase activators and inhibitors, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins
8L	b-raf proto-oncogene (RAFB1)	Kinase activators and inhibitors, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins
8M	c-jun proto-oncogene; transcription factor AP-1	Kinase activators and inhibitors transcription activators and repressors, intracellular transducers / effectors / modulators, nuclear proteins
8N	jun-B	Kinase activators and inhibitors transcription activators and repressors, intracellular transducers / effectors / modulators, nuclear proteins
8O	jun-D	Kinase activators and inhibitors transcription activators and repressors, intracellular transducers / effectors / modulators, nuclear proteins
9B	N-myc proto-oncogene	Kinase activators and inhibitors transcription activators and repressors, intracellular transducers / effectors / modulators, apoptosis associated proteins, nuclear proteins
9C	c-myc binding protein MM-1	Kinase activators and inhibitors, other transcription proteins, nuclear proteins
9D	c-myc purine-binding transcription factor puf; nucleoside diphosphate kinase B (NDP kinase B; NDKB) + nm23-H2S	Kinase activators and inhibitors, apoptosis associated proteins transcription activators and repressors, nuclear proteins, cytoplasmic proteins
9E	proto-oncogene tyrosine-protein kinase abl; p150; c-abl	Kinase activators and inhibitors, adaptors and receptor-associated proteins, cytoplasmic proteins, nuclear proteins

Code	Gene name	Classification
9F	p53 cellular tumour antigen	Kinase activators and inhibitors, intracellular transducers / effectors / modulators apoptosis associated proteins, nuclear proteins
9G	p53-associated mdm2 protein	Kinase activators and inhibitors, intracellular transducers / effectors / modulators, apoptosis associated proteins, cell cycle, nuclear proteins
9H	MDM2-like p53-binding protein (MDMX)	Kinase activators and inhibitors, intracellular transducers / effectors / modulators, apoptosis associated proteins, cell cycle, nuclear proteins
9I	p73 (monoallelically expressed p53-related protein)	Transcription activators and repressors, cell cycle, nuclear proteins
9J	p53-induced protein	Kinase activators and inhibitors, amino acid metabolism, cytoplasmic proteins
9K	PIG3	Functionally unclassified, kinase activators and inhibitors, unclassified localization
9L	PIG7	Functionally unclassified, kinase activators and inhibitors, unclassified localization
9M	PIG10	Functionally unclassified, kinase activators and inhibitors, unclassified localization
9N	PIG11	Functionally unclassified, kinase activators and inhibitors, unclassified localization
9O	PIG12	Functionally unclassified, kinase activators and inhibitors, unclassified localization
10B	Glutathione-S-transferase (GST) homolog	Other stress response proteins, apoptosis associated proteins, intracellular transducers / effectors / modulators, metabolism of cofactors, vitamins, and related substances, cytoplasmic proteins
10C	p53-dependent cell growth regulator CGR19	Other cell cycle proteins, unclassified localization
10D	p33ING1	Kinase activators and inhibitors, nuclear proteins
10E	Growth-arrest-specific protein 1 (GAS1)	Other cell cycle proteins, plasma membrane proteins
10F	Apoptosis regulator bcl-2	BCL2 family proteins, kinase activators and inhibitors, endoplasmic reticulum, nuclear membranes / nuclear matrix / nuclear pores, mitochondrial proteins
10G	Bcl2 & p53 binding protein Bbp/53BP2 (BBP/53BP2)	Other cell cycle proteins, apoptosis associated proteins, cytoplasmic proteins

Code	Gene name	Classification
10H	Apoptosis regulator bax	BCL2 family proteins, cytoplasmic proteins, mitochondrial proteins
10I	Apoptosis regulator bclw; KIAA0271; BCL2L2	BCL2 family proteins, cytoplasmic proteins
10J	Induced myeloid leukemia cell differentiation protein MCL-1	BCL2 family proteins, unclassified localization
10K	BCL-2-related protein A1 (BCL2A1); BFL1 protein; hemopoietic-specific early response protein; GRS protein	BCL2 family proteins, cytoplasmic proteins
10L	Bcl-2 interacting killer (BIK); NBK apoptotic inducer protein; BP4; BIP1	BCL2 family proteins, nuclear membranes / nuclear matrix / nuclear pores, endoplasmic reticulum
10M	Bcl2 homologous antagonist/killer (BAK)	BCL2 family proteins, kinase activators and inhibitors, endoplasmic reticulum, nuclear membranes / nuclear matrix / nuclear pores, mitochondrial proteins
10N	E. coli hypothetical sugar kinase in HIPB-UXAB intergenic region; YDEV (originally described as human brain-related apoptosis gene [BRAG-1])	Energy metabolism BCL2 family proteins, cytoplasmic proteins
10O	BAD protein; bcl-2 binding component 6 (BBC6); bcl-2L8	BCL2 family proteins, endoplasmic reticulum, nuclear membranes / nuclear matrix / nuclear pores, mitochondrial proteins
11B	BCL-2 binding athanogene-1 (BAG-1); glucocorticoid receptor-associated protein RAP46	BCL2 family proteins, endoplasmic reticulum, nuclear membranes / nuclear matrix / nuclear pores, mitochondrial proteins
11C	Activator of apoptosis harakiri (HRK); neuronal death protein 5 (DP5); BID3	BCL2 family proteins, cytoplasmic proteins
11D	Interleukin-1 beta convertase precursor (IL-1BC); IL-1 beta converting enzyme (ICE); p45; caspase-1 (CASP1)	Caspases, cysteine proteases, cytoplasmic proteins
11E	Caspase-2 precursor (CASP2); ICH-1L protease + ICH-1S protease	Caspases, cysteine proteases, cytoplasmic proteins
11F	Caspase-3 (CASP3); apopain precursor; cysteine protease CPP32; YAMA protein; SREBP cleavage activity 1; SCA-1	Caspases, cysteine proteases, cytoplasmic proteins
11G	Caspase-4 precursor (CASP4); ICH-2 protease; TX protease; ICE(REL)-II + caspase-5 precursor (CASP5); ICH-3 protease; TY protease; ICE(REL)-III	Caspases, cysteine proteases, cytoplasmic proteins

Code	Gene name	Classification
11H	Caspase-6 precursor (CASP6); cysteine protease MCH2 isoforms alpha + beta	Caspases, cysteine proteases, cytoplasmic proteins
11I	Caspase-7 precursor (CASP7); ICE-like apoptotic protease 3 (ICE-LAP3); apoptotic protease MCH-3; CMH-1	Caspases, cysteine proteases, cytoplasmic proteins
11J	Caspase-8 precursor (CASP8); ICE-like apoptotic protease 5 (ICE-LAP5); MORT1-associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease (FADD-like ICE; FLICE); apoptotic cysteine protease MCH-5	Caspases, cysteine proteases, cytoplasmic proteins
11K	Caspase-8 precursor (CASP8); ICE-LIKE apoptotic protease 5; MORT1-associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease; FADD-like ICE (FLICE); MCH5; CAP4	Caspases, cysteine proteases, cytoplasmic proteins
11L	Caspase-9 precursor (CASP9); ICE-like apoptotic protease 6 (ICE-LAP6); apoptotic protease MCH6; apoptotic protease activating factor 3 (APAF3)	Caspases, cysteine proteases, cytoplasmic proteins
11M	Caspase-10 precursor (CASP10); ICE-LIKE apoptotic protease 4 (ICE-LAP4); apoptotic protease MCH4; fas-associated death domain protein; interleukin-1-beta-converting enzyme 2 (FLICE2)	Caspases, cysteine proteases, cytoplasmic proteins
11N	Tumour necrosis factor receptor 1-associated death domain protein (TNFR1-associated death domain protein; TRADD)	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
11O	TRAF5	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12B	TRAF6	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12C	TRAF-interacting protein (I-TRAF) + TRAF family member-associated NF-kappa B activator TANK	Transcription activators and repressors, nuclear proteins
12D	TRAF-interacting protein (hTRIP)	Other apoptosis-associated proteins, intracellular transducers / effectors / modulators, cytoplasmic proteins
12E	NIK serine/threonine protein kinase	Death kinases, intracellular kinase network members (non-receptor protein kinases), cell cycle, cytoplasmic proteins

Code	Gene name	Classification
12F	Casper, a FADD- and caspase-related inducer of apoptosis (CASH-alpha + CASH-beta); FLAME-1; FLICE-like inhibitory protein	Other apoptosis-associated proteins, inhibitors of proteases, cytoplasmic proteins
12G	Caspase & rip adaptator with death domain (CRADD); rip-associated protein with death domain (RAIDD)	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12H	Receptor interacting protein; serine/threonine protein kinase RIP transferase; serine/threonine-protein kinase ATP-binding apoptosis; cell death protein RIP	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12I	DAXX	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, plasma membrane proteins, cytoplasmic proteins
12J	Tumour necrosis factor type 2 receptor associated protein (TRAP3)	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12K	CD40 receptor-associated factor 1 (CRAF1)	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
12L	Inhibitor of apoptosis protein1 (HIAP1; API1) + IAP homolog C; TNFR2-TRAF signaling complex protein 1; MIHC	Other apoptosis-associated proteins, cytoplasmic proteins
12M	Inhibitor of apoptosis protein 2 (HIAP2; IAP2) + IAP homolog B; TNFR2-TRAF signalling complex protein 2; MIHB	Other apoptosis-associated proteins, cytoplasmic proteins
12N	Inhibitor of apoptosis protein 3 (API3; IAP3); X-linked inhibitor of apotosis protein (X-linked IAP; XIAP); IAP-like protein (HILP)	Other apoptosis-associated proteins, cytoplasmic proteins
12O	Tumour necrosis factor precursor (TNF-alpha; TNFA); cachectin	Death receptor ligands, growth factors, cytokines, and chemokines, plasma membrane proteins cell signaling, extracellular communication proteins
14B	Lymphotoxin-alpha precursor (LT-alpha); tumour necrosis factor-beta (TNF-beta; TNFB)	Death receptor ligands, growth factors, cytokines, and chemokines cell signaling, extracellular communication proteins
14C	Lymphotoxin-beta (LT-beta; LTB); tumour necrosis factor C (TNFC)	Death receptor ligands, growth factors, cytokines, and chemokines, plasma membrane proteins
14D	TNF-alpha converting enzyme (TACEA); transmembrane metalloproteinase/disintegrin; adamalysin	Metalloproteinases, apoptosis associated proteins cell signaling, extracellular communication proteins

Code	Gene name	Classification
14E	Fas antigen ligand (FASL); apoptosis antigen ligand (APTL; APT1LG1); TNFSF6	Death receptor ligands, growth factors, cytokines, and chemokines, plasma membrane proteins cell signaling, extracellular communication proteins
14F	TNF-related apoptosis inducing ligand (TRAIL); APO-2 ligand (APO2L)	Death receptor ligands, growth factors, cytokines, and chemokines, plasma membrane proteins
14G	Secreted apoptosis related protein 1 (SARP1)	Death receptor ligands, other extracellular communication proteins cell signaling, extracellular communication proteins
14H	Secreted apoptosis related protein 3 (SARP3)	Death receptor ligands, other extracellular communication proteins cell signaling, extracellular communication proteins
15B	Tumour necrosis factor receptor 1 (TNFR1); tumour necrosis factor binding protein 1 (TBP1); CD120A antigen	Death receptors, intracellular transducers / effectors / modulators, growth factor & chemokine receptors, eath domain receptors, cell surface antigens, plasma membrane proteins
15C	Tumour necrosis factor receptor (TNFR) + tumour necrosis factor receptor 2 (TNFR2); tumour necrosis factor binding protein 2 (TBP2)	Death receptors, intracellular transducers / effectors / modulators, growth factor & chemokine receptors, death domain receptors, cell surface antigens, plasma membrane proteins
15D	Fas soluble protein; Apo1	Death receptors, growth factor & chemokine receptors, death domain receptors, cell surface antigens, plasma membrane proteins
15E	Cytotoxic ligand TRAIL receptor	Death receptors, other receptors, other receptors (by ligands), plasma membrane proteins
15F	Death receptor 5 (DR5); cytotoxic TRAIL receptor 2 (TRICK2A)	Death receptors, death domain receptors, other receptors (by ligands), plasma membrane proteins
15G	WSL protein + TRAMP + Apo-3 + death domain receptor 3 (DDR3)	Death receptors, growth factor & chemokine receptors, death domain receptors, plasma membrane proteins
15H	Insulin-like growth factor IA precursor (IGF1A); IGFBP1; somatomedin C + insulin-like growth factor I (IGF1)	Growth factors, cytokines, and chemokines cell signaling, extracellular communication proteins
16B	Insulin-like growth factor II (IGF2); somatomedin A	Growth factors, cytokines, and chemokines cell signaling, extracellular communication proteins
16C	Insulin-like growth factor I receptor (IGF1R)	Death receptors, growth factor & chemokine receptors, intracellular transducers / effectors / modulators, protein kinases, plasma membrane proteins

Code	Gene name	Classification
16D	Cation-independent mannose-6-phosphate receptor precursor (CI man-6-P receptor; CI-MPR); insulin-like growth factor II receptor (IGFR II)	Growth factor & chemokine receptors, other receptors, endocytosis, kinase activators and inhibitors, plasma membrane proteins, golgi complex, lysosomal proteins
16E	IGFBP complex acid labile chain	Functionally unclassified, unclassified localization
16F	Insulin-like growth factor binding protein 2 (IGFBP2)	Kinase activators and inhibitors, growth factor & chemokine receptors cell signaling, extracellular communication proteins
16G	Insulin-like growth factor-binding protein 3 precursor (IGF-binding protein 3; IGFBP3; IBP3)	Hormones cell signaling, extracellular communication proteins
16H	Insulin-like growth factor binding protein 4 precursor (IGF-binding protein 4; IGFBP4; IBP4)	Extracellular transport / carrier proteins, other extracellular communication proteins cell signaling, extracellular communication proteins
17B	Insulin-like growth factor binding protein 5 precursor (IGF-binding protein 5; IGFBP5; IBP5)	Extracellular transport / carrier proteins, other extracellular communication proteins cell signaling, extracellular communication proteins
17C	Insulin-like growth factor binding protein 6 precursor (IGF-binding protein 6; IGFBP6; IBP6)	Extracellular transport / carrier proteins, other extracellular communication proteins cell signaling, extracellular communication proteins
17D	Ionizing radiation resistance-conferring protein + death-associated protein 3 (DAP3)	Other apoptosis-associated proteins, unclassified localization
17E	Death-associated protein kinase 1 (DAP kinase 1; DAPK1)	Death kinases, intracellular kinase network members (non-receptor protein kinases), cytoplasmic proteins
17G	PDCD2	Other apoptosis-associated proteins, transcription, nuclear proteins
17H	rac-alpha serine/threonine kinase (rac-PK-alpha); protein kinase B (PKB); c-akt; akt1	Death kinases, intracellular kinase network members (non-receptor protein kinases), kinase activators and inhibitors, cytoplasmic proteins
18B	rac-beta serine/threonine kinase (rac-PK-beta); akt2	Intracellular kinase network members (non-receptor protein kinases) cell cycle-regulating kinases, cytoplasmic proteins
18C	Seven in absentia homolog	Other apoptosis-associated proteins, transcription, cell cycle, nuclear proteins, cytoplasmic proteins
18D	RATS1	Other apoptosis-associated proteins, cell cycle, kinase activators and inhibitors transcription activators and repressors, nuclear proteins
18E	DNA fragmentation factor 45 (DFF45)	DNA fragmentation proteins, intracellular transducers / effectors / modulators, cytoplasmic proteins
18F	Apoptosis-related protein TFAR15	Other apoptosis-associated proteins, unclassified localization

Code	Gene name	Classification
18G	Calcium / calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B (CAM-PDE1B); HCAM2	Adenylate / guanylate cyclases and diesterases, cytoplasmic proteins
18H	CD27BP (Siva)	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
19B	Cellular apoptosis susceptibility protein (CAS); chromosome segregation gene homolog	Other apoptosis-associated proteins, cell cycle, cytoplasmic proteins
19C	Apoptosis inhibitor survivin	Other apoptosis-associated proteins, cytoplasmic proteins
19D	Transforming protein rhoA H12 (RHO12; ARH12; ARHA)	Kinase activators and inhibitors, G-proteins, plasma membrane proteins
19E	Inducible nitric oxide synthase (INOS); type II NOS; hepatocyte NOS (HEP-NOS)	Other apoptosis-associated proteins, cytoplasmic proteins
19F	Nuclear factor kappa-B DNA binding subunit (NF-kappaB; NFKB)	Transcription activators and repressors, nuclear proteins
19G	I-rel (RELB)	Transcription activators and repressors, nuclear proteins
20B	Transcription factor NF-ATc	Cell cycle-regulating kinases, nuclear proteins
20C	Defender against cell death 1 (DAD1)	Other apoptosis-associated proteins, plasma membrane proteins
20D	Clusterin precursor (CLU); complement-associated protein SP-40,40; complement cytolysis inhibitor (CLI); apolipoprotein J (APO-J); TRPM-2; sulfated glycoprotein 2	Other apoptosis-associated proteins, immune system proteins, cell signaling, extracellular communication proteins
20E	DNA-binding protein inhibitor ID-1; Id-1H	Other cell cycle proteins, intracellular transducers / effectors / modulators transcription activators and repressors, nuclear proteins
20F	Glutathione reductase (GRase; GSR; GR)	Xenobiotic transporters, apoptosis associated proteins, cytoplasmic proteins
20G	Microsomal glutathione S-transferase 12 (GST12; MGST1)	Xenobiotic transporters, apoptosis associated proteins, cytoplasmic proteins
21B	Glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	Other stress response proteins other apoptosis-associated proteins, cytoplasmic proteins
21C	Glutathione S-transferase pi (GSTP1; GST3)	Xenobiotic transporters, apoptosis associated proteins, cytoplasmic proteins
21D	Glutathione S-transferase A1 (GTH1; GSTA1); HA subunit 1; GST-epsilon	Other stress response proteins other apoptosis-associated proteins, cytoplasmic proteins
21E	Glutathione peroxidase (GSHPX1; GPX1)	Xenobiotic transporters, apoptosis associated proteins, cytoplasmic proteins
21F	Glutathione S-transferase theta 1 (GSTT1)	Xenobiotic transporters, apoptosis associated proteins, cytoplasmic proteins

Code	Gene name	Classification
21G	Cytochrome P450 reductase	Other apoptosis-associated proteins, stress response proteins, endoplasmic reticulum
22B	Growth arrest & DNA-damage-inducible protein 153 (GADD153); DNA-damage-inducible transcript 3 (DDIT3); C/EBP homologous protein (CHOP)	Other apoptosis-associated proteins, intracellular transducers / effectors / modulators, kinase activators and inhibitors, stress response proteins, nuclear proteins
22C	Growth arrest & DNA-damage-inducible protein (GADD45); DNA-damage-inducible transcript 1 (DDIT1)	Apoptosis associated proteins, other DNA synthesis, recombination, and repair proteins, kinase activators and inhibitors, stress response proteins, nuclear proteins
22D	NIP1 (NIP1)	BCL2 family proteins, mitochondrial proteins
22E	NIP3 (NIP3)	BCL2 family proteins, mitochondrial proteins
22F	CD40 ligand (CD40-L); tumour necrosis factor (TNF)-related activation protein (TRAP); T-cell antigen GP39	Death receptor ligands, growth factors, cytokines, and chemokines, plasma membrane proteins cell signaling, extracellular communication proteins
22G	CD27 ligand (CD27LG); CD70 antigen	Death receptor ligands, growth factors, cytokines, and chemokines, cell surface antigens, plasma membrane proteins
23B	FAN protein	Death receptor-associated proteins and adaptors, adaptors and receptor-associated proteins, cytoplasmic proteins
23C	Retinoic acid receptor beta (RXR-beta; RXRB)	Death receptors transcription activators and repressors, intracellular transducers / effectors / modulators, hormone receptors, nuclear receptors, nuclear proteins
23D	Retinoic acid receptor epsilon (RAR-epsilon); retinoic acid receptor beta 2 (RAR-beta2; RARB); HAP	Death receptors transcription activators and repressors, intracellular transducers / effectors / modulators, hormone receptors, nuclear receptors, nuclear proteins
23E	Protein-tyrosine phosphatase zeta precursor (R-PTP-zeta)	Death receptors, intracellular transducers / effectors / modulators, other receptors (by ligands), protein phosphatases, plasma membrane proteins
23F	Excision repair protein ERCC6; Cockayne syndrome protein CSB	DNA damage signaling / repair proteins and DNA ligases, stress response proteins, nuclear proteins
23G	*Xeroderma pigmentosum* group C repair complementing protein p58/HHR23B	DNA damage signaling / repair proteins and DNA ligases, stress response proteins, nuclear proteins

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