A POTENTIAL ANTI-BREAST CANCER MECHANISM OF THE CYCLIC PEPTIDE VR-3848, EXTRACTED FROM *EUPHOBIACEAE*

PROMSIN MASRINOUL

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Thesis

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.....

Mr. Promsin Masrinoul
Candidate

.....

Assoc. Prof. Sukathida Ubol, Ph.D.
Major-Advisor

.....

Assoc. Prof. Samaisukh Sophasan, Ph.D.
Co-Advisor

.....

Assist. Prof. Jeerayut Chaijaruwanich, Ph.D.
Co-Advisor

.....

Prof. Dr.M.R.Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies

.....

Prof. Peerapan Tan-ariya, Ph.D.
Chair
Master of Science Programme
in Microbiology
Faculty of Science

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Entitled

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for the degree of Master of Science (Microbiology)
on
August 30, 2006

.....
Mr. Promsin Masrinoul
Candidate

.....
Assist. Prof. Chariya Hanvajanawong, Ph.D.
Chair

.....
Assoc. Prof. Sukathida Ubol, Ph.D.
Member

.....
Assist. Prof. Jeerayut Chaijaruwanich, Ph.D.
Member

.....
Assoc. Prof. Samaisukh Sophasan, Ph.D.
Member

.....
Prof. Dr.M.R.Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

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Promsin Masrinoul

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A POTENTIAL ANTI-BREAST CANCER MECHANISM OF THE CYCLIC PEPTIDE VR-3848, EXTRACTED FROM *EUPHOBIACEAE*

PROMSIN MASRINOUL 4736165 SCMI/M

M.Sc. (MICROBIOLOGY)

THESIS ADVISORS: SUKATHIDA UBOL, Ph.D., SAMAISUKH SOPHASAN, Ph.D., JEERAYUT CHAIJARUWANICH, Ph.D.

ABSTRACT

The 7-mer cyclic peptide VR-3848, a natural compound extracted from the Thai tropical plant *Euphobiaceae*, has the ability to kill several cancerous cell types at nanomolar concentrations. However, the target and mechanism corresponding to the action of this compound has only been partially elucidated. To investigate the target and mechanism of VR-3848, the MCF-7 breast cancer cell was chosen as a model. The gene expression pattern at various time points during the treatment of MCF-7 cells with VR-3848 were obtained by using human whole genome microarray analysis. The VR-3848 treated cells were analyzed by using Significance Analysis of Microarray (SAM) at estimating 5% false discovery rate and using two-fold analysis. 4,075 genes whose expression significantly altered during treatment were identified. These genes were further analyzed by k-means clustering analysis. Most of the down-regulated genes functioned metabolically and controlled cell proliferation whereas most of the up-regulated genes regulated apoptosis and cell proliferation negatively. To identify the early gene response, the significantly altered genes were analyzed at one hour of treatment. The results showed that 155 genes were significantly altered. Among these genes, 52 genes had known cellular functions. Transcription and translation genes were major groups that early responded to VR-3848 treatment. Only the SRCAP gene was significantly altered at 30 minutes after treatment. It has been identified as a coactivator for steroid mediated transcription. The study did not reveal whether or not VR-3848 can interact with steroid receptor. Unfortunately, approximately 66% of gene expression at one hour of treatment revealed unknown functions. It is suggested that VR-3848 may induce cell death by an unexplored pathway.

Elucidating the anti-cancer mechanism, gene expression from the microarray analysis revealed that VR-3848 stimulated apoptosis-related genes including receptors, mitochondria and ER stress mediated pathways and suppressed cell cycle related genes. One possible anti-cancer mechanism, an inhibin mediated pathway was found. The strong up-regulation of INHBA and signaling cascade genes such as GADD45β, GADD153, ATF4 and ARTS suggest that the inhibin stimulated pathway is one among various death pathways stimulated by VR-3848. The finding indicates that VR-3848 exerts a potent anti-breast cancer activity by stimulation of multiple apoptotic pathways and suppression of cell cycle progression.

KEYWORDS: VR-3848/ CYCLIC PEPTIDE/ MCF-7 CELL/ MICROARRAY/ APOPTOSIS/ CELL CYCLE/ INHIBIN

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การศึกษา gene responses ของเซลล์มะเร็งเต้านมต่อ cyclic peptide ซึ่งสกัดจากพืชในสกุล *Euphobiaceae* (A POTENTIAL ANTI-BREAST CANCER MECHANISM OF THE CYCLIC PEPTIDE VR-3848, EXTRACTED FROM *EUPHOBIACEAE*)

พร้อมสิน มาศรีนวล 4736165 SCMI/D

วท.ม. (จุลชีววิทยา)

คณะกรรมการควบคุมวิทยานิพนธ์: ศุขธิดา อุบล, Ph.D., สมัยศึก โสภาสรรค์, Ph.D., จีรยุทธ ไชยจารุวณิช, Ph.D.

บทคัดย่อ

จากการศึกษาที่ผ่านมาพบว่าสารวีอาร์ 3848 ที่สกัดจากพืชในสกุล *Euphobiaceae* มีฤทธิ์ในการยับยั้งเซลล์มะเร็งในหลอดทดลองหลายชนิดที่ระดับความเข้มข้นนาโนโมล่าร์ อย่างไรก็ตามกลไกการออกฤทธิ์ของสารนี้ได้มีการศึกษาเพียงบางส่วน ดังนั้นการวิจัยนี้มุ่งศึกษาถึงเป้าหมายของยา และกระบวนการยับยั้งการเจริญเติบโตในเซลล์มะเร็งเต้านม MCF-7 โดยศึกษาการตอบสนองของยีนในเซลล์ MCF-7 ที่ถูกกระตุ้นด้วยสารวีอาร์ 3848 ที่ระยะเวลาต่างๆ โดยใช้เทคนิค DNA microarray analysis ซึ่งวิเคราะห์การเปลี่ยนแปลงการแสดงออกของยีนด้วยวิธี significance analysis of microarray (SAM) ที่อัตราการพบความผิดพลาด (false discovery rate) 5% และ two fold difference analysis ผลการทดลองพบว่า 4,075 ยีนมีการเปลี่ยนแปลงอย่างมีนัยสำคัญ เมื่อทำการจัดกลุ่มตามการแสดงออกของยีนโดย k-mean clustering analysis พบว่าวีอาร์ 3848 ยับยั้งยีนในกลุ่มเมตาบอลิซึมและวงจรชีวิตของเซลล์ (cell cycle) และกระตุ้นยีนในกลุ่ม apoptosis และกลุ่มยังยั้งวงจรชีวิตของเซลล์ นอกจากนี้พบว่า 155 ยีนมีตอบสนองโดยเร็ว (early response genes) ต่อสารวีอาร์ 3848 โดยมีการเปลี่ยนแปลงที่เวลา 1 ชั่วโมงหลังจากกระตุ้น ซึ่งยีนที่เปลี่ยนแปลงส่วนใหญ่จัดอยู่ในกลุ่ม transcription และ translation ในที่นี้พบว่ายีน SRCAP ซึ่งทำหน้าที่เกี่ยวข้องกับ steroid receptor mediated transcription มีการเปลี่ยนแปลงการแสดงออกของยีนที่เวลา 30 นาทีหลังจากการกระตุ้น ดังนั้นการกระตุ้นของสารดังกล่าวผ่านทาง steroid mediated pathway จึงควรได้รับการศึกษาต่อไป

เมื่อทำการศึกษาการตอบสนองการแสดงออกของยีนที่เวลา 6, 12, 18 และ 24 ชั่วโมงหลังการกระตุ้น พบว่าสารวีอาร์ 3848 สามารถกระตุ้นให้เกิดการตายแบบ apoptosis โดยพบการเปลี่ยนแปลงของยีนทั้งในกลุ่มการกระตุ้นการตายผ่านตัวรับสัญญาณบนผิวเซลล์ กระตุ้นการตายผ่านทางไมโตคอนเดรีย กระตุ้นการตายผ่านทาง endoplasmic reticulum และการยับยั้งวงจรชีวิตของเซลล์ ในการทดลองนี้ได้แสดงให้เห็นถึงกลไกของสารวีอาร์ 3848 ในการกระตุ้นการตายของเซลล์ MCF-7 ผ่านทาง inhibin mediated pathway โดยพบการเพิ่มการแสดงออกของยีนในกลุ่ม inhibin signailing ได้แก่ INHBA, GADD45β, GADD153, ATF4 และ ARTS จากผลการทดลองนี้แสดงให้เห็นว่าสารวีอาร์ 3848 มีฤทธิ์ในการยับยั้งเซลล์มะเร็งเต้านมทั้งการขัดขวางวงจรชีวิตของเซลล์และกระตุ้นผ่านกลไกการตายแบบ apoptosis ในหลายรูปแบบ

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LIST OF ABBREVIATIONS

%	Percent
ºC	Degree Celsius
/	Per, in, or
±	Plus or minus
AMV	Avian Myeloblastosis Virus
ARTS	Apoptosis related protein in TGF-β signaling pathway
ATCC	American Type Culture Collection
APC	Anaphase promoting complex
Bad	BCL2-antagonist of cell death
Bak	BCL2-antagonist/killer
Bax	BCL2-associated X protein
Bcl-2	B-cell leukaemia 2
Bcl-X_L	B-cell leukaemia X-long
bHLH	Basic helix loop helix
Bid	BH3 interacting domain death agonist
Bik	BCL2-interacting killer (apoptosis-inducing)
Bim	Bcl-2 interacting mediator of cell death
BIR	Baculoviral inhibitory repeat
Blk	B lymphoid tyrosine kinase
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
Bok	BCL2-related ovarian killer
bZIP	Basic region leucine zipper
cDNA	Complementary deoxyribonucleic acid
CDC2	Cell division cycle 2, G1 to S and G2 to M
C/EBP	CAAT-enhancer binding protein
cRNA	Complementary ribonucleic acid

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Cy	Cyanine
Cyt C	Cytochrome C
DAPI	4'-6-diamidino-2-phenylindole
dbEST	Database of expressed sequence tags
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dNTP	Deoxyribonucleotide triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribo nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
Endo G	Endonuclease G
eIF2α	Eukaryotic translation initiation factor 2A
FBS	Fetal bovine serum
FDR	False discovery rate
g	Force of gravity
hr or hrs	hour or hours
Hrk	Harakiri, BCL2-interacting protein (contains only BH3 domain)
IAP	Inhibitor of apoptosis protein
IL-2	Interleukin 2
Ire1α	Inositol-requiring 1 alpha
Log2	Base 2 logarithm
LU1	Non-small lung cancer cell
MAPK	Mitogen activated protein kinase
MCL1	Myeloid cell leukaemia 1
NCBI	National center for biotechnology information

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LIST OF ABBREVIATIONS

(continued)

PBS	Phosphate buffer saline
RefSeq	Reference sequences
RT-PCR	Reverse transcription- polymerase chain reaction
SAM	Significance analysis of microarray
SE	Standard error
SERM	Selective estrogen receptor modulators
siRNA	Small interfering RNA
SNR	Signal to noise ratio
SOM	Self organizing map
SSC	Standard saline citrate
TGF-β	Transforming growth factor beta
TRAILR2	Tumor necrosis factor-related apoptosis inducing ligand receptor 2
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UV	Ultraviolet
XBP1	X-box binding protein 1
XIAP	X-linked inhibitor of apoptosis
μg	Microgram
μl	Microliter
ml	Mililiter
μM	Micromolar
mM	Milimolar
nM	Nanomolar

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CHAPTER 1
INTRODUCTION

Breast cancer is one of the three leading cancers that cause cancer death among women in the United State. Unfortunately, standard breast cancer therapies including chemotherapy, radiation therapy, surgery and immunotherapy are unable to completely eliminate cancerous cells. These failures result in emerging of the single or multiple drug resistant cancerous cells. Thus, the development of new therapeutic approaches and searching for new chemotherapeutic drugs which target an unexplored molecule, are urgent to prevent the increase in breast cancer mortality.

Professor Vichai Reutrakul and colleagues at Department of Chemistry, Faculty of Science, Mahidol University, have succeeded in purification of novel potential anti-cancer compounds from the tropical Thai plants. Among these compounds, VR-3848, a cycloheptapeptide, is extracted and purified from *Euphobiaceae*. This compound exerts a growth inhibitory activity against several types of cancerous cells. For instance, it inhibits growth of human leukemia Jurket cell and non small lung cancer cell line (LU-1) (1, 2). Moreover, the cytotoxic effect of VR-3848 is specific for tumor cell since human IL-2 activated T lymphocytes retard the killing activity of VR-3848 (2). However, the potential target and mechanism of this compound have not been thoroughly elucidated. This proposal proposed to elucidate the molecular anticancer mechanism(s) of VR-3848 using MCF-7 cell as a model and DNA microarray screening as a tool.

MCF-7 cell line is well-studied in vitro system for breast cancer cell transformation and proliferation. This cell line was derived from a pleural effusion of metastatic breast cancer patient since 1970 (3). It exhibits some specific characteristics such as estrogen sensitive and caspase-3 deficiency cells. The lack of caspase-3, a cysteine protease, which is known as a cardinal caspase in apoptosis pathway may be responsible for irradiation and several chemotherapeutic drug resistances of this cell line (4, 5). The former Master degree student, Jarin Kramyu, in our laboratory has

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partially elucidated apoptosis mechanism in VR-3848 induced MCF-7 cell death using cDNA macroarray analysis focusing on genes involved in apoptosis. From his observation, this compound induced MCF-7 cell death via three modes of apoptosis which were receptor mediated pathway as a consequence of activation of cytotoxic TRAILR2 and caspase-8 and-10, mitochondrial mediated but caspase-3 independent pathway and ER stress pathway via GADD153 and caspase-12 (6). However, the apoptotic pathways that mentioned above may be a late stage of the suicide process. To reveal the potential target and to complete the death mechanism, the responsive genes remain to be investigated.

One of advantages of microarray analysis is the ability to study the thousands of gene expression at the same time. On the basis of comparing the differential expression in two or more conditions, DNA microarray is the powerful technique that can be used in several applications especially clinical studies such as cancer classification, molecular process in host and pathogen interaction, and drug target discovery (7). Using the human whole genome microarray analysis, the potential targets and global effect of gene expressions that respond to VR-3848 treatment can be constructed. Therefore, the objectives of this proposal are

1. To identify the gene or the set of genes that respond in the early phase of VR-3848 treatment. Results from this study may give us the clue on primary target of this novel anticancer candidate.

2. To search for, as many as possible, genes that involve in downstream of apoptosis commitment process in order to compose a complete apoptotic pathway stimulated by VR-3848.

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CHAPTER 2
LITERATURE REVIEWS

Breast cancer

Breast cancer is one of the three leading diagnosed cancers among women in the United State and is the second leading cause of cancer death. Although, the incidence rate of breast cancer was slowed in the 1990s, it is estimated that there are approximately 213,000 new cases of invasive breast cancer and 41,000 deaths among women in 2006 (8). The estimated new cases and deaths of ten leading cancer types are shown in table 1.1-1.2.

In Thailand, breast cancer is one of the common diseases among adult women. The incidence rate in Thailand is 16.3/100,000 population. Although the occurrence is low, breast cancer is second leading cancer, 28.4%, next to cervical cancer among Thai women in the year 2003. The incidence of breast cancer has been increased gradually over the past 5-10 years that may relate to the change of lifestyle and diet (9).

Cause of breast cancer

The mammary gland consists of two tissue compartments: the epithelium including duct and milk glands (lobules) that are responsible for producing milk, and the connective tissue or stroma (10).

Homeostasis of breast epithelium requires the balance of cell proliferation and cell death. However, changes in genetic of cell death regulatory mechanisms result in an increase number of cells with genetically abnormal. The accumulation of these cells begins the process called tumorigenesis (11). The breast cancer cells can spread throughout the breast as well as other parts of body. Most common type of breast cancer arises in the milk-producing duct, and may also occur in the other breast tissue. The causes of breast cancer result from several factors. One of factors is hereditary factor in which mutation occurs in genes that generally regulate the normal cellular

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mechanism. For examples, the mutation of the breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2) increases the risk of breast cancer up to 80% and ovarian cancer up to 40%.(12) Also, the defects in genes such as ataxia-telangiectasia mutation (ATM) (13), ATR (ataxia-telangiectasia- and Rad3-related) (14), the cell-cycle checkpoint kinase 2 (CHEK-2) gene, (15) and the p53 tumor suppressor gene (16) have been identified to breast cancer cell development.

In contrast to hereditary factor, the acquired mutation by exposing to cancer causing agents (carcinogens) such as benzopyrene in cigarette smoke also leads to cancer development. Moreover, women who used to be treated with chest radiation therapy increase the risk of breast cancer (17).

Table 1.1 The estimated new cancer cases of ten leading cancer types for women in USA 2006

Cancer types	**Estimate New Case**	
Breast	212,920	31%
Lung and Bronchus	81,770	12%
Colon and Rectum	75,810	11%
Uterine Corpus	41,200	6%
Non-Hodgkin Lymphoma	28,190	4%
Melanoma of the Skin	27,930	4%
Thyroid	22,590	3%
Ovary	20,180	3%
Urinary Bladder	16,730	2%
Pancreas	16,580	2%
All sites	679,510	100%

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Table 1.2 The estimated deaths of ten leading cancer types for women in USA 2006.

Cancer types	**Estimated Deaths**	
Lung and Bronchus	72,130	26%
Breast	40,970	15%
Colon and Rectum	27,300	10%
Pancreas	16,210	6%
Ovary	15,310	6%
Leukemia	9,810	4%
Non-Hodgkin Lymphoma	8,840	3%
Uterine Corpus	7,350	3%
Multiple Myeloma	5,630	2%
Brain and Other Nervous Systems	5,560	2%
All sites	273,560	100%

Table 2. The leading cancer types in Thai women in 2003

Cancer site	**Number of death people**	
Cervix	618	32.2%
Breast	545	28.4%
Colon Rectum	164	8.6%
Lymphoma	151	7.9%
Leukemia	148	7.7%
Lung	91	4.7%
Skin	74	3.8%
Liver	65	3.4%
Oral Cavity	62	3.2%
All sites	1,918	100%

Table 1 and 2 demonstrate the significance of breast cancer in women in USA and Thailand. Breast cancer is second to cervical cancer among Thai women. However, breast cancer is the first incidence of American women.

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Breast cancer therapy

Early screening tests by self examination and mammography are recommended for women who have high risk in developing breast cancer. When screening tests are positive, the treatments with surgery, radiation therapy, hormone therapy, and chemotherapy are used as standard therapies. A number of chemotherapeutic drugs and strategies have been developed in order to eliminate cancerous cell (Table 3, 4). Unfortunately, none of mentioned strategies is able to completely eliminate cancerous cells. These failures result in emerging of the resistant form of cancerous cell against one or many drugs. Therefore, the development of new therapeutic approaches and searching for new chemotherapeutic drugs are urgent for preventing the increase in breast cancer mortality.

Table 3. Classes of breast cancer drug. (http://imaginis.com/breasthealth/drugs.asp)

Classes	Drug action	Examples
Anthracyclines	Deform DNA structure of cancer cells	Adriamycin (doxorubicin) Ellence (epirubicin)
Taxanes	Prevent cancer cells from dividing	Taxol (paclitaxel) Taxotere (docetaxel)
Alkylating Agents	Target DNA of cancer cells	Cytoxan (cyclophosphamide)
Vinca alkaloids	Prevent cancer cell from dividing	Velban (Vinblastine)
SERM (Selective Estrogen-Receptor Modulators)	Bind to estrogen receptors in breast cancer cells, starving cancer cells	Tamoxifen (Nolvadex) Evista (raloxifene) Fareston (toremifene)
Aromatase Inhibitors	Prevent production of estrogen in adrenal glands	Aromasin (exemestane) Arimidex (anastrozole)
Biologic Response Modifiers	Bind to certain proteins on breast cancer cells preventing their growth	Herceptin (trastuzumab)
Other Hormonal Therapies	Treat breast cancer that is dependent on estrogen for survival	Zoladex (goserelin acetate) Faslodex (fulvestrant)

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Table 4. The new approaches to breast cancer therapy.

Strategies	Mechanism	Ref.
Monoclonal antibody	Inhibit cancer cell proliferation by using the antibody which recognizes specific antigenic determinants on cancer cells.	(18, 19)
Biological response modifiers:-		
- Interferons	NK cell and macrophage stimulation and direct cytotoxicity to cancer cells	(20)
- Interleukin-2	Stimulate B lymphocyte proliferation	(21)
Adoptive immunotherapy	Activate person's own lymphocytes and inject them back into the patient	(22)
Genetically modified Dendritic cell therapy	Enhance immune activation against tumor antigen by gene engineering dendritic cell	(23)
Gene therapy	Incorporation of DNA coding for a particular gene to reprogramming of the cells	(24)
Antisense therapy	Introduce a small piece of DNA into the cell for regulating gene expression in cancer cells	(25, 26)
Inhibitors of angiogenesis	Inhibit the growth of new blood vessels supplying tumour growth	(27)

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Cell death

Cell death plays many essential roles in the maintenance of homeostasis during normal cell growth as well as pathogenesis. Several distinct mechanisms of cell death have been described based on the morphological, biochemical and molecular hallmarks. To date, cell death can be subdivided into several categories that are apoptosis (type I), autophagic cell death (type II), and necrosis (type III). However, the boundaries are not always distinct (28). The discrimination of cell death features is shown in figure 1.

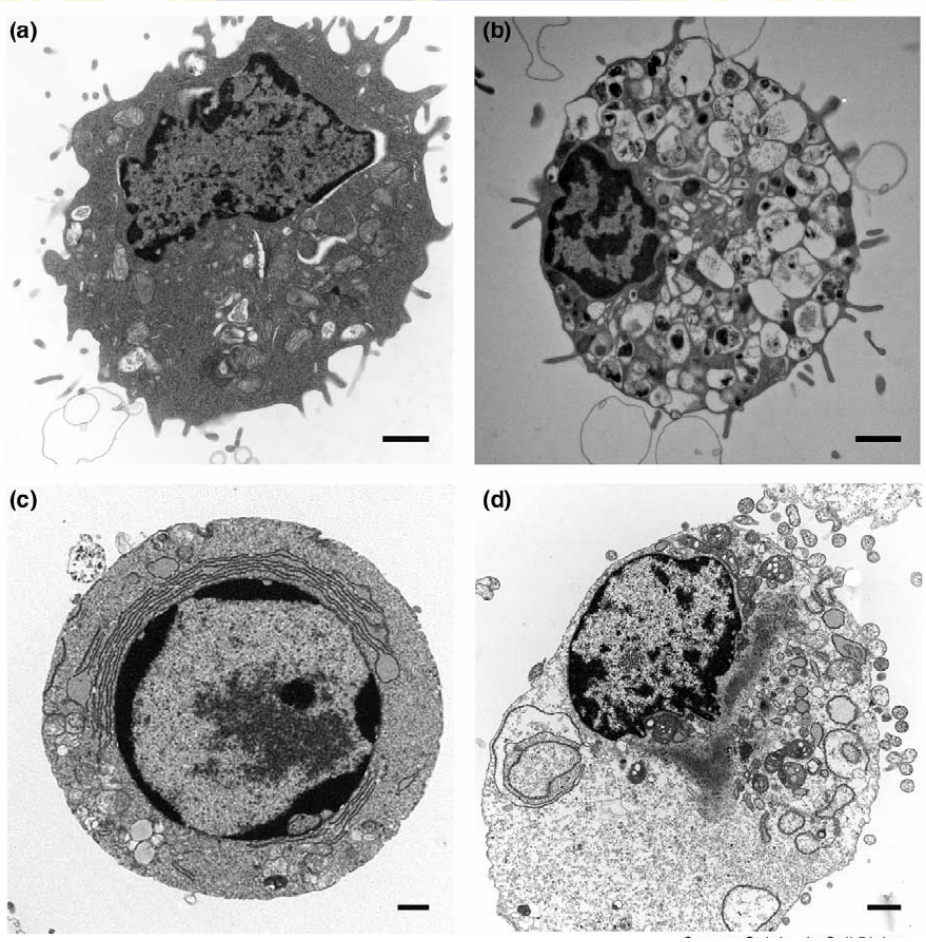

Figure 1. The morphological features of (a) normal, (b) autophagic, (c) apoptotic and (d) necrotic cells. The scale bar represents 1 μm (29).

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Apoptotic cell death is characterized by cell shrinkage, blebbing of plasma membrane, condensation of cytoplasm and chromatin, and DNA fragmentation. Subsequently, cell breaks up into membrane-enclosed vesicle known as apoptotic body that is then eliminated by phagocytosis without inflammatory response. Another type of cell death is autophagic cell death categorized by the accumulation of autophagic vacuoles in the cytoplasm of dying cell. This type of cell death is necessary when degradation of whole organ or tissue is needed and the activity of phagocytic cells is not sufficient to eliminate all of the death cell bodies (30). During necrosis, cell is insulted by physical damage as well as pathological stimuli leading to lose the control of its ionic balance. Consequently, cell becomes an irreversible swelling of cytoplasm and organelles and the disruption of plasma membrane. The release of cellular contents and proinflammatory molecules provokes the inflammation around the dying cell (29).

Apoptosis

Apoptosis is a programmed cell death which is regulated by a variety of cellular signaling pathways. Cell death by apoptosis is activated by many stimuli including intracellular stress and ligation to the death receptor. It is controlled by hundreds of genes which are responsible for different death signal transduction. The major factors that serve as the central components for apoptotic response such as caspases, Bcl-2 family, cytochrome c, and suppressor of apoptosis are described below.

Caspase, a central component of apoptotic response, is the cysteine protease that cleaves protein next to aspartic acid residue. According to their structures and functions, caspases can be divided into two classes; the initiator caspases including caspase-2,-8,-9, and-10 and effector caspases which are caspase-3,-6,-7 and-14 (31). The caspases are synthesized as catalytically inactive zymogen and undergo proteolytic activity during apoptosis (32). However, the caspase activities are also regulated by inhibitor of apoptosis proteins (IAPs). The IAP structure consists of caspase binding BIR domain and RING finger domain with ubiquitin ligase activity that binds and inhibits the initiator and effector caspases.

Bcl-2 family consists of approximately 20 homologues which play important roles in pro- and anti-apoptosis. According to the distinct structures that contain at

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least one in four conserve motifs called Bcl-2 homology domain (BH1-4), Bcl-2 family is classified into 3 major groups. The first group is Bcl-2 subfamily which acts as anti-apoptosis including Bcl-2, Bcl-X_L, Bcl-w, MCL1 and A1. In contrast to Bcl-2 subfamily, other groups which are Bax subfamily (Bax, Bak and Bok), and BH3 subfamily (Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL) act as pro-apoptosis that drives in the apoptotic signaling (33).

Cytochrome C is involved in the mitochondria mediated apoptotic pathway. The loss of mitochondrial membrane permeability due to the activation of pro-apoptotic molecules coupled with the inhibition of anti-apoptosis molecules causes the release of cytochrome C into cytosol. In cytosol, cytochrome C induces the oligomerization of apoptotic-protease-activating factor-1 (APAF1) resulting in apoptosome formation. This complex recruits and activates caspase-9 and other executioner caspases leading to apoptosis (34, 35).

Multiple apoptotic pathways

Once cell commits to die by apoptosis, it can drive into several signaling pathways depending on the stimuli. The molecular events of apoptosis initiate by apoptotic inducing agents, activate the signal transduction cascade, and cleave the cellular components by proteolytic enzyme. Typically, three major pathways are classified that are receptor mediated (extrinsic pathway), mitochondria mediated (intrinsic pathway), and endoplasmic reticulum (ER) stress pathway. Caspases generally regulate all types of death. However, some apoptotic cells may die without caspase activation. This type of death is called caspase-independent cell death (36).

Receptor mediated, extrinsic pathway

Ligation of death receptor with its ligand initiates the extrinsic pathway, which results in the formation of death-inducible signaling complex (DISC). This complex activates the pro-caspase-8. The activated caspase-8 can trigger the pro-caspase-3 and other effector caspases that result in some of the typical morphological changes of apoptotic cell such as DNA fragmentation, apoptotic body formation and ultimately lead to cell death.

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Mitochondria mediated, intrinsic pathway

The perturbation of mitochondrial membrane peameability as a result of the intracellular stress that stimulates the Bcl-2 family proteins causes the release of several proteins including cytochrome C from the mitochondrial intermembrane space into cytosol. In the cytosol, cytochrome C forms the apoptosome complex with APAF-1 in the presence of dATP. The apoptosome in turn activates caspase-9 and other caspases leading to apoptosis. Cross-talk between receptor mediated and mitochondria mediated pathway occurs when Bid, a Bcl-2 family protein, is cleaved by caspase-8 into truncated form (t-Bid). The active form of Bid induces the oligomerization of Bax and/or Bak which then translocate to the mitochondrial outer membrane (37).

Likewise, the intrinsic pathway can mediate caspase independent cell death which is the release of at least two proteins, apoptosis inducing factor (AIF) and endonuclease G (Endo G) from mitochondria. These proteins directly translocate into nucleus and might induce DNA degradation.

An example of extrinsic and intrinsic pathway is illustrated in figure 2. The cross-talk between these pathways is demonstrated at truncation of Bid.

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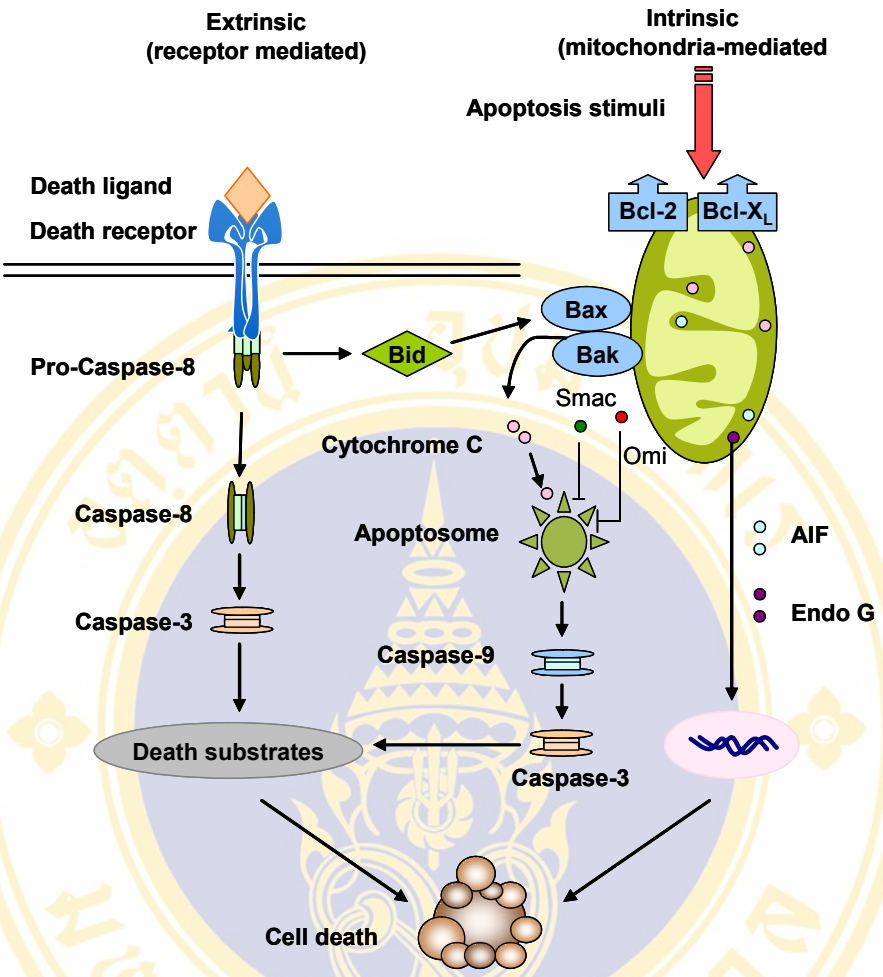

Figure 2. The receptor mediated and mitochondria mediated apoptosis pathways (37).

ER stress mediated apoptosis pathway

Typically, ER provides the protection of cell death from incorrectly folded protein, oxidative stress, calcium disturbances and hypoxia. However, the persistent and excessive unfold and misfold proteins trigger ER stress cell death. Cells respond to these stress stimuli via several signals including proteases, kinases, transcription factors, Bcl-2 family proteins and their modulators (figure 3).

Unfolded proteins binding to glucose regulated protein 78 (Grp78) also known as immunoglobulin heavy-chain binding protein (Bip) induce the release of Ire1α, a transmembrane serine/threonine (Ser/Thr) kinase, which affects gene regulation. Also, in the response of ER stress, PERK (PKR-like ER kinases) and ATF6 (activating transcription factor-6) are released from ER. Oligomerization and phosphorylation of

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PERK activate eIF2α that subsequently inhibit the general mRNA translation. Under this condition, however, activated eIF2α allows for preferential translation of selected mRNA including ATF4 encoding several small short upstream open reading frames (uORFs) bypass scanning system.(38, 39) The activation of transcription factors ATF4, ATF6 and XBP-1 induces the expression of C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage inducible gene 153 (GADD153), which is a member of C/EBP family of bZIP transcription factors. Overexpression of GADD153 induces apoptosis through inhibiting the anti-apoptosis Bcl-2 family.

Prolonged ER stress also stimulates the activation of pro-caspase-12 which is localized on the outer membrane of ER. Caspase-12 is activated by ER stress but not by receptor- or mitochondria- mediated apoptotic signals. Caspase-12 is stimulated either by complex of endoribonuclease Ire1α and adaptor protein TRAF-2 (tumour necrosis factor (TNF) receptor associated factor 2) or through cleavage by m-calpain (37). The Ire1α/TRAF-2 complex can activate apoptotic signaling kinase1 (ASK-1), which leads to activation of c-Jun N-terminal kinase (JNK) pathway and p38 MAPK. JNK-mediated phosphorylation activates the pro-apoptotic protein, Bim, while it decreases the anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. Both of JNK and p38 MAPK enhance the GADD153 activity by the phosphorylation at the serine 78 and 81 (40).

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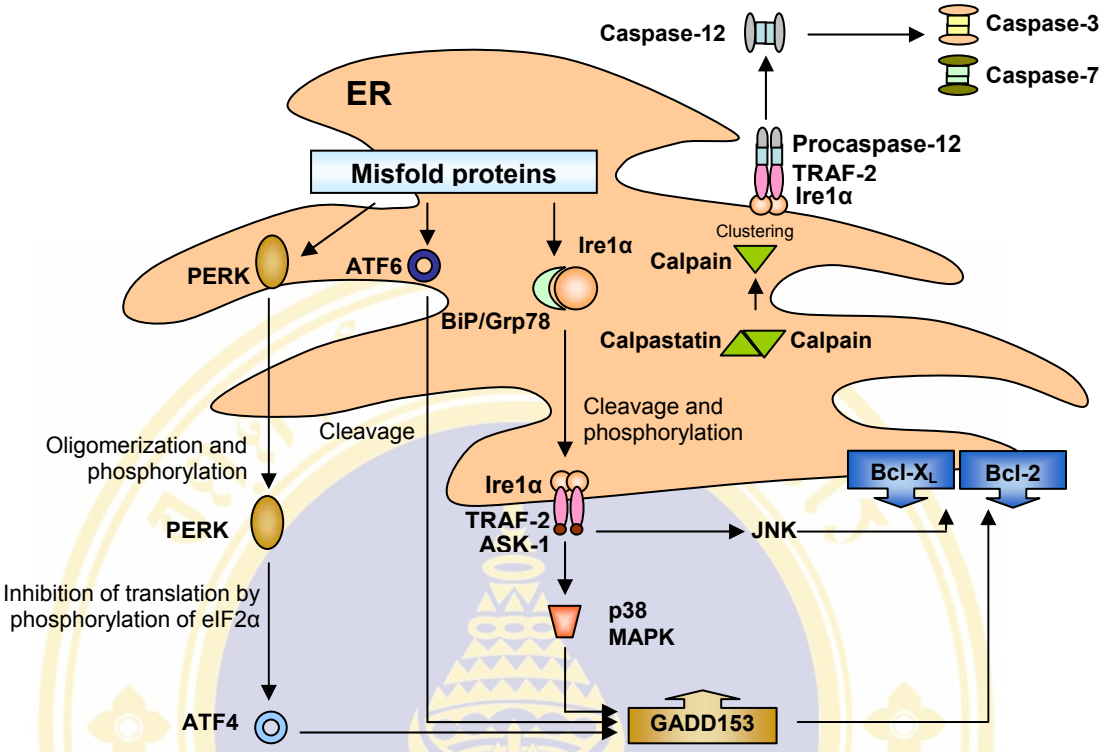

Figure 3. ER stress and consequences. The accumulation of misfold protein activates Ire1α, ATF6, and PERK. The activation of Ire1α and ATF6 induces the expression of ER stress responsive genes while activation of PERK inhibits the general mRNA translation. Moreover, caspase-12 activation occurs either by cleavage with m-calpain or formation complex of Ire1α with TRAF-2. Ire1α-TRAF-2 complex stimulates ASK-1 which in turn activates JNK pathway and p38 MAPK. The overexpression of GADD153 induced from ATF4, ATF6 and p38 down-regulates the anti-apoptotic molecules such as Bcl-2 and Bcl-X_L that subsequently transduce signal into mitochondria mediated death. (modified from (37, 40))

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Caspase-3 deficient breast cancer MCF-7 cell

The breast cancer MCF-7 cell line derived from a pleural effusion of a patient with metastatic breast cancer since 1970 (3). Due to a deletion in the *casp-3* gene, MCF-7 cell line lacks of the caspase-3 expression which is known as a cardinal caspase in the activation of both extrinsic and intrinsic signals (3). The loss of caspase-3 may protect cell against ionization radiation, and several chemotherapeutic agents (5).

VR-3848

The cycloheptapeptide named VR-3848 is extracted from Thai tropical plant in the family of *Euphobiaceae* by Prof. Vichai Reutrakul and colleagues. Local Thai people, in some region, use the root part as skin-whitening agent.

VR-3848 has ability to kill many types of cancerous cells. For instance, this compound has growth inhibitory effect against MCF-7 cell line at nanomolar concentration and 10-fold higher growth inhibitory effect than chemotherapeutic agent, vinblastine on LU-1. The cytotoxic effect of VR3848 is specific to tumor cell (2). In the preliminary studies, our laboratory used cDNA macroarray analysis for screening the genes that involve in VR-3848 induced breast cancer MCF-7 cell death. The results demonstrated that anti-cancer activity of VR-3848 induced death was mediated via at least three modes of apoptotic death, which were receptor mediated pathway, mitochondrial-dependent but caspase-3 independent pathway, and ER stress induced cell death pathway.

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DNA microarray

DNA microarray allows us to measure the expression levels of thousands of gene at the same time. The analysis of differentially expressed genes is widely used and applied in many fields such as medicine and the pharmaceutical, biotechnology, and toxicology. The basic principles involve extraction of RNA from biological samples in which two or more different conditions are compared. The RNA is then copied, and amplified, while it is labeled with either fluorescent nucleotides or a tag that is later stained with fluorescence. Next, labeled cRNA can be hybridized onto the separate arrays that contain thousands of short oligonucleotide probes specific to each gene for the one color system. Alternatively, the slide is hybridized simultaneously with Cy5 and Cy3 labeled samples for the two color system. Following stringency washes to remove nonspecific target, the intensities of each gene on the slides are detected by scanner (Figure 4). The acquired data will be further analyzed.

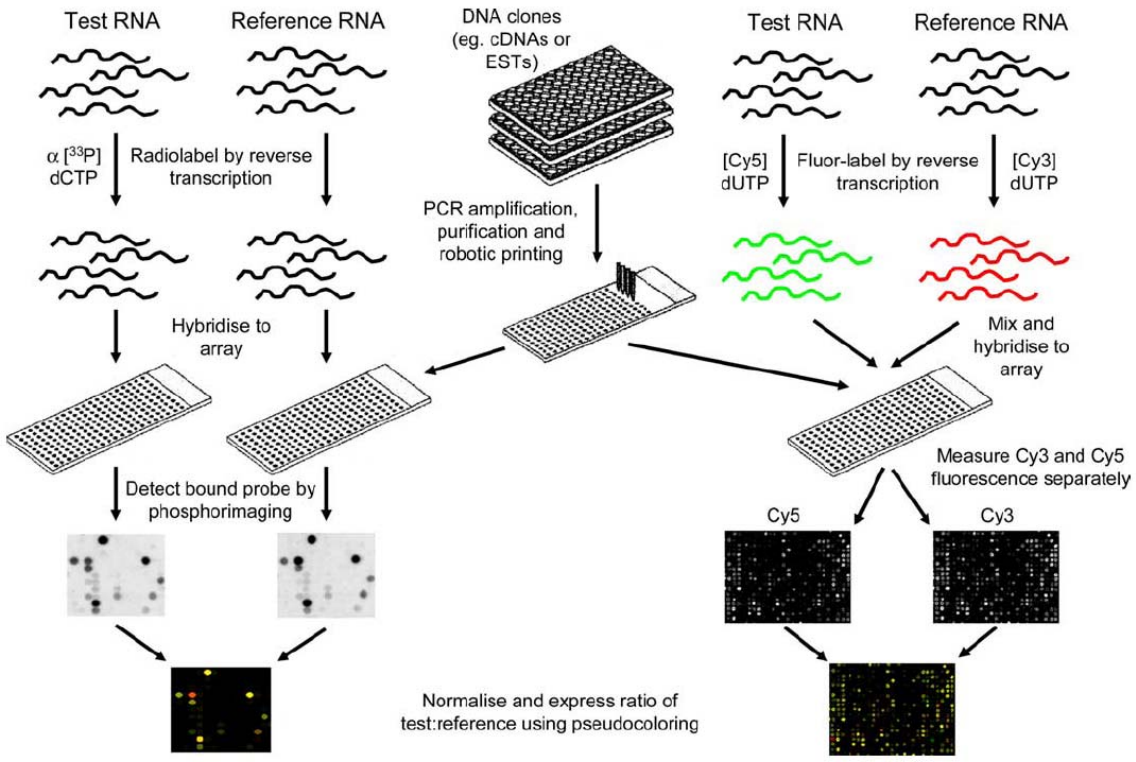

Figure 4. Schematic of various steps in microarray experiment (7).

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Microarray data analysis

The goals of microarray are to contribute the expression pattern and to identify the differential gene expression between two or more conditions (41). Currently, there are a large number of statistical and computational approaches available for analysis of microarray. These strategies can be broadly classified into two categories; supervised approach or analysis to determine genes for accurately discriminating groups of samples or diseases and unsupervised approach or analysis to characterize the data set without a priori input on genes (42).

Preprocessing

Since the expression data are measured from microarray scanner, preprocessing processes are needed to ensure that the data are high quality and suitable for analysis. These processes include filtering or removing the features that have some kinds of problems such as irregular shape or contaminated from dust, and manufacturer flagged feature. Moreover, the feature intensities are then subtracted with blackground signal to exclude the non-specific hybridization of labeled target to slide. The analysis works well when the feature intensity is higher than the background intensity (43).

In order to compare the intensity across the slides, the normalizations are needed to remove some of variations and to enable comparison of multiple arrays. There are several normalizing methods which adjust a set of microarray data, for examples, using median intensity level, using a nonlinear smoothing curve, using some controls or housekeeping genes that expect to have the same intensity level across all of the samples.

Dissimilarity measure

A dissimilarity measure indicates the degree of similarity between two genes. Cluster analysis constructs from dissimilarity measure to group the feature with similar pattern. A common such method is Euclidean distance that measures the straight-line distance between points in two or three dimensional spaces in which each axis is gene expression in separate biological sample. In two dimensions, Euclidean distance is calculated using Pythagorean theorem. Another dissimilarity measure is the Pearson correlation coefficient which is a measurement of the degree of fit to a linear

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regression line to data points. This measurement calculates as the average distance of points from the linear- regression line. The dissimilarity measures are shown in figure 5.

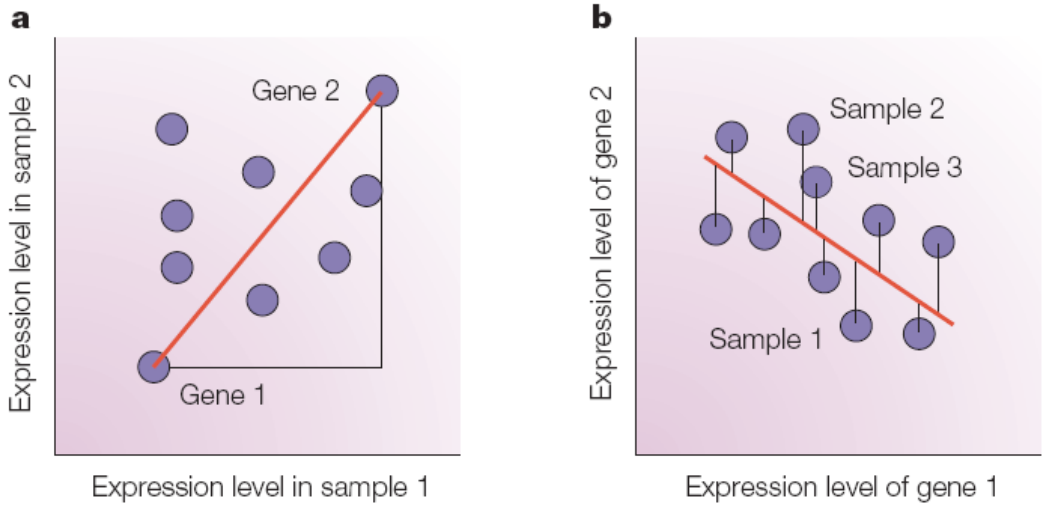

Figure 5. Dissimilarity measures (a) Euclidean distance (b) Pearson correlation coefficient (44).

Unsupervised methods

Unsupervised or clustering method tries to group genes in the data set with shown similar pattern. There are three classes of technique; feature determination or determining gene with interesting properties without specifically looking for a priori pattern such as principle component analysis; cluster determination or determining genes which have a similar pattern of gene expression such as hierarchical clustering, self organizing map (SOM), and K-mean clustering; and network determination or representing the gene-gene or gene-phenotype interactions such as Boolean networks, Bayesian network and relevance network.

The hierarchical clustering is commonly used unsupervised technique in which the most similar pattern of gene expressions is clustered together. Such clustering is done by iteratively grouped the highly correlated gene or set of genes from their expression measurement. Hierarchical clustering can visualize by dendrogram in which the similar gene pattern represents near each other. The length of branch is inversely proportional to degree of similarity (figure 6a).

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K-means clustering and SOM are similar to hierarchical clustering but the approach is quite different. Instead of hierarchical clustering, the number of clusters is set as an input parameter. The center of each cluster known as centroid is randomly assigned from the data set. Distance of each gene is calculated and is assigned to the closet centroid. This repeat until no gene is reassigned (45). (figure 6b)

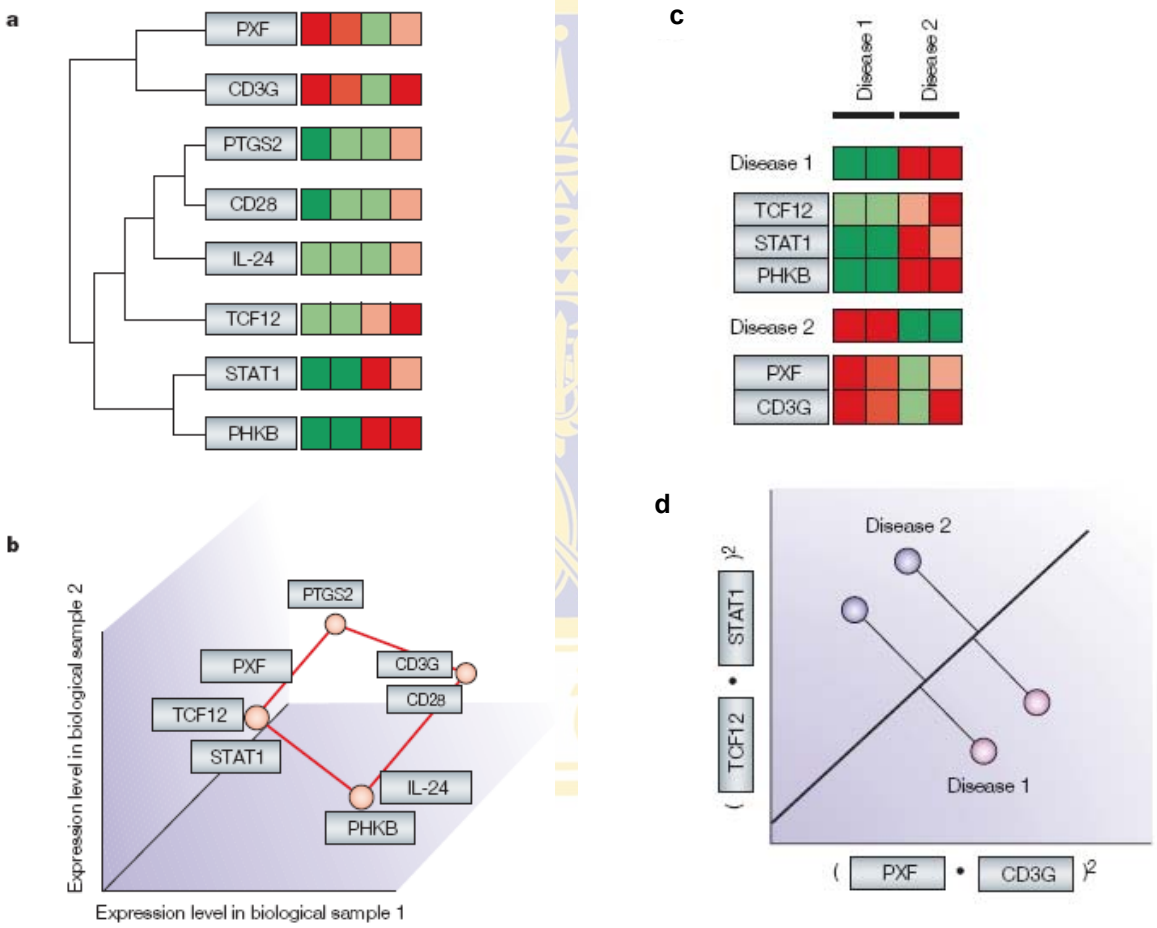

Figure 6. Clustering and network-determination used in microarray analysis. (a) Hierarchical clustering. (b) K-mean clustering and SOM. (c) The nearest-neighbour supervised method. (d) Support vector machine (44).

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Supervised methods

Supervised methods are used in order to identify the different expression of gene between groups of samples such as tumor and normal. In order to find the significantly different expressed genes, various algorithms are used including fold change, parametric test such as t-test, significant analysis of microarray (SAM) (46), and analysis of variance (ANOVA); non-parametric test such as Wilcoxon's 2-sample test that is also extensively used in microarray data.

T-statistics are typically used in statistical analysis to determine whether the expression of particular genes is significantly different between two sample conditions. However, the modified version of t-test, significant analysis of microarray (SAM), has some benefits to control the type I error in data analysis (47). SAM method assigns the change in gene expression relative to the standard deviation of repeated measurements (permutation). For genes with score higher than an adjustable threshold, SAM uses permutations to estimate the percentage of significant genes which are not truly different, the false discovery rate (FDR) (48).

Other algorithms including nearest neighbour approach for finding individual significant gene that best fits to desired patterns, for instance, a gene with high expression in disease 1 and low expression in disease 2 or vice versa (figure 6c); and support vector machines that uses the several mathematics in the combination of genes to find the best line that separates the group of genes and biological samples (figure 6d).

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Application of microarray on gene expression in cancer

The advent of microarray provides several applications on the basis of comparing the expression between at least two different conditions. For cancer studies, microarray technology has been used in order to investigate cancer stage classification, tumor specific molecular marker, resistance and sensitive to therapeutic drugs, and identification of potential targets for novel molecular therapeutics (7, 49). These advantages provide a better understanding of the basic biology, diagnosis and treatment of cancer. Four major fields of cancer and cancer treatment research are as following.

1. MOLECULAR CANCER CLASSIFICATION:

The challenge of cancer treatment is the specific types of cancer. For examples, the therapeutic agent against one cancer is frequently inactive in other cancers. Furthermore, the indistinguishable tumors by histological technique often show difference in clinical response to treatment with the same drug. In the cancer classification, Golub et al. demonstrated that the gene expression pattern analysis coupled with neighborhood analysis could be used to predict the discrimination between acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) (50). Likewise, other cancers were also used gene expression pattern in classifications such as breast cancer (51), cutaneous melanoma (52), prostate cancer (53), ovarian cancer (54), and cervical cancer (55). Therefore, these efficiencies of microarray analysis to classify types and stages of cancer have the benefits of better diagnosis for the suitable treatment.

2. THE SENSITIVITY AND RESISTANCE TO DRUG:

Resistance to chemotherapeutic drugs causes to limit the efficacy of cancer treatment and is a serious problem in cancer therapy. Microarray technique has a power to elucidate the molecular pathways implicated in drug resistant mechanism and in predicting drug sensitivity. The previous study has been investigated the molecular mechanism of acquired resistance to camptothecin, a topoisomerase I inhibitior, using in the treatment of lung, breast, ovarian and colon cancer. The camptothecin resistant prostate cancer cell line showed 181 significant alteration of gene expression by using

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a stratum-adjusted Kruskal-Wallis test (56). Moreover, another application of this technique is to predict the response to common drug. Chang J.C. et al investigated that the primary breast tumor treated with docetaxel showed 92 significantly different gene expressions between sensitive and resistant tumor (57). This investigation is further utilized to reduce unnecessary treatment in breast cancer. Recently, Villeneuve D.J. et. al. (58) studied the gene expression pattern using cDNA microarray analysis in the establishment of the resistant mechanisms against paclitaxel or doxorubicin by comparing the wide type MCF-7 cell line and isogenic paclitaxel-resistant or doxorubicin-resistant derivative cell line. The results found that the significant majority were drug specific and coded for proteins involved in drug transport, drug metabolism, growth, survival, and cell death control. Thus, the insights into the mechanism of drug action and the diverse pathways in drug sensitivity and resistance may be invaluable for design of more strategic treatments that are most appropriate for an individual tumor.

3. IDENTIFICATION OF TUMOR MARKER:

Several researches attempt to identify subsets of genes that show differential expression between healthy tissue or cell line and their tumor counterparts to identify molecular markers in several tumors. Nowadays, the molecular markers of many cancer types including ovarian carcinomas, oral cancer, melanoma, colorectoral cancer, prostate cancer and breast cancer have been identified (Table 5). Mimori K. et. al (59) demonstrated the differential gene expression profiling by using cDNA microarray between normal cell, primary carcinoma and metastatic carcinoma cell to identify the critical genes associated for progression of breast cancer. As a result, the motility related protein-1 (MRP-1/CD9), peripheral myelin protein-22 (PMP-22) and caspase 3 (CASP-3) were down-regulated in metastatic compared to primary breast carcinoma. This investigation demonstrates an application of microarray in the identification of metastatic marker genes that are utilized in clinical cancer diagnosis.

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Table 5. Cancer markers (modified form (60) and Spendlove Research Foundation http://www.spendloveresearch.org/ResearchPages/tumor&cancertypes.htm).

Types of cancer	Molecular markers
Lung	CAE, CA15-3, CA 19-9, CA125, HCG-β, ferritin, NSE
Pancrease	AFP, CEA, CA15-3, CA125, HCG-β
Kidney	AFP, CEA, HCG-β, IL-2, IL-6, B2M, Vimentin
Breast	CEA, CA15-3, CA125, IL-6, Collagen XXIII, MMP, MMP inhibitor, Her-2, PAI-1, PAI-2, Cathepsin B and L,
Ovarian	AFP, CA15-3, CA19-9, CA125, HCG-β, IL-6, B2M, ICTP, SELDI pattern
Cervical	AFP, CA125
Uterine	CEA, CA19-9, CA125, HCG-β
Testicular	AFP, PAP, HCG-β, ferritin
Prostate	PSA, PSAF, PSAC, PAP, B2M, A2M, antizyme, antizyme inhibitor, collagen XXIII, MMP, MMP inhibitor, EZH2
Liver	CEA, CA15-3, CA19-9, CA125, HCG-β, Myc and A1B1
Gastro	CEA, CA19-9, CA125, CASP3
Colorectal	CEA, CA19-9, CA125, PSAF, HCG-β
Bile Duct	CA19-9
Thyroid	CEA, Calcitonin, hTG, NSE
Wilms Tumor	hTG, NSE
Bladder	CEA, BTA, MMP22, HCG-β, Calreticulin, Survivin
Brain	HCG-β
Burkitts Lymphoma	Ferritin
Nueroblastoma	Ferritin, NSE
Leukemia	Ferritin, B2M
Multiple Myeloma	IL-2, IL-6, B2M
Lymphoma	B2M

A2M = alpha 2 macroglobulin
AFP = alpha fetoprotein
B2M = beta 2 microglobulin
BTA = Bladder tumor antigen
CEA = carcinogenic embryonic antigen
CA 15-3 = carbohydrate antigen 15-3
CA 19-9 = carbohydrate antigen 19-9
CA 125 = carbohydrate antigen 125
EZH2 = Enhancer of zeste homolog 2
hCGβ = human chorionic gonadotropin beta
hTG = human thyroglobulin
ICTP = Carboxy teminal telopeptide of type 1 collagen
IL-2 = interleukin 2
IL-6 = interleukin 6
MMP = Matrix metalloproteinases
NSE = neuron specific enolase
PSA = free prostate specific antigen + prostate specific antigen - alpha(1) antichymotrypsin complex
PSAF = free prostate specific antigen
PSAC = prostate specific antigen - alpha(1)antichymotrypsin complex
PAP = prostatic acid phosphatase
SELDI pattern = Surface-enhanced laser desorption ionization

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4. DRUG TARGET DISCOVERY:

Gene expression profiling from microarray analysis is used to identify the molecular target and to elucidate the cellular mechanisms of both novel and established drugs. A number of investigates demonstrates the gene expression responses following treatment with commonly used chemotherapeutic drug or irradiation. Li Y. et. al. measured the alteration of gene expression to identify novel molecular targets of prostate cancer cell lines that were responded to docetaxel and estramustine combined treatment. The differently expressed genes can utilize to generate the molecular mechanism in the inhibition of cell proliferation and to further optimize the therapeutic strategies in prostate cancer treatment (61).

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CHAPTER 3
MATERIALS AND METHODS

1. MCF-7 cell cultivation

Breast cancer MCF-7 cell line (ATCC number is HTB-22) was obtained from Assist. Professor Kulawee Sujarit (Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand). Cells were cultured in Minimum Essential Medium (MEM) (Invitrogen Corporation) containing Earle's salts, L-glutamine, non-essential amino acids and 1.5 g of sodium bicarbonate, 10% heat inactivated fetal bovine serum (FBS), 1% sodium pyruvate (Invitrogen Corporation), and 0.01 mg/ml Bovine insulin (Sigma). The MCF-7 medium was kept at -4ºC and pre-warmed at 37ºC before use. The confluent cells were passaged every 3 days with 0.05% typsin/EDTA.

2. VR-3848 drug treatment

VR-3848, a 7-mer cycloheptapeptide, was kindly provided by Professor Vichai Reutrakul (Department of Chemistry, Faculty of Sciences, Mahidol University, Thailand). MCF-7 cells were treated with either 0.1% of control Dimethylsulfoxide (DMSO) (Sigma) or 100 nM VR-3848 in 0.1% DMSO and were harvested at various time points. After drug treatment, both of detached and attached cells were collected by centrifugation and washed with phosphate buffer saline (PBS) pH 7.4. The cell pellets were kept at -80ºC.

3. Detection of apoptosis

Apoptotic cells can be detected using nuclear morphological changes such as DNA fragmentation and nuclear condensation as markers. These characteristics were visualized by using fluorescent dye, 4', 6-diamino-2-phenylindole (DAPI) staining and enzymatic labeling TdT-mediated dUTP nick end labeling (TUNEL) assay.

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3.1 DAPI staining

Cells with either control DMSO or VR-3848 drug treatment were collected at 1, 3, 6, 12, 18, and 24 hours. After centrifuged and washed with PBS, cells were fixed with 300 µl of ice cold absolute methanol. Fixed cells were then stained with 0.5 µg/ml of DAPI working solution at 37ºC for 20 minutes, washed twice with 300µl of absolute methanol and mounted onto a glass slide with a drop of 50% glycerol. The apoptotic cells with chromatin condensation and nuclear fragmentation can be detected under fluorescent microscope. At least 500 random cells were counted and apoptotic cells were presented in percentage.

3.2 TUNEL enzymatic assay

The detection of apoptosis was confirmed with TUNEL enzymatic labeling assay. Harvested cells were washed with PBS and immediately fixed with freshly prepared 0.4% paraformaldehyde at room temperature for 30 minutes. The fixed cells were loaded onto the slide chambers and centrifuged at 600 g at room temperature for 10 minutes and then washed with 200 µl of PBS containing 1% of bovine serum albumin (BSA). The 100 µl of permeabilization solution containing with 0.1% Triton X and 0.1% sodium citrate was added to fixed cells and incubated at room temperature for 10 minutes. Cells were washed twice and stained with 25µl of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (Boehringer Mannheim, Germany) at 37ºC for 1 hour, and washed once. Cells were added with 50µl of blocking reagent at room temperature for 30 minutes and subsequently incubated with 40 µl of antifluorescein antibody conjugated with alkaline phosphatase (Roche) at 37ºC for 1 hour. The substrates, which are 5-Bromo-4-chloro-3-indolyl-phosphate and 4-Nitroblue tetrazolium chloride (Boehringer Mannheim), were directly added to the cells and incubated for 20 minutes in the dark. The stained cells were washed twice and mounted with glycerol. The brown nuclei of apoptotic cells were detected under light microscope.

4. Microarray

In order to investigate the response of MCF-7 cells to VR-3848 stimulation, an alteration of MCF-7 gene profiling was studied using cDNA macroarray screening. In

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the present work, Codelink™ Whole Genome Bioarray (GE healthcare) with single color system was used. This gene chip is composed of 30 oligomer probes of 57,000 transcripts. Among these transcripts, 45,600 transcripts are well characterized according to NCBI UniGene build, RefSeq database, dbEST database and Incyte LifeSeq™ databases.

To perform a DNA microarray screening, RNA was extracted from cells treated with VR-3848 or with 0.1% DMSO as control compound at 1, 3, 6, 12, and 18 hours. The overview steps of microarray are shown in figure 7.

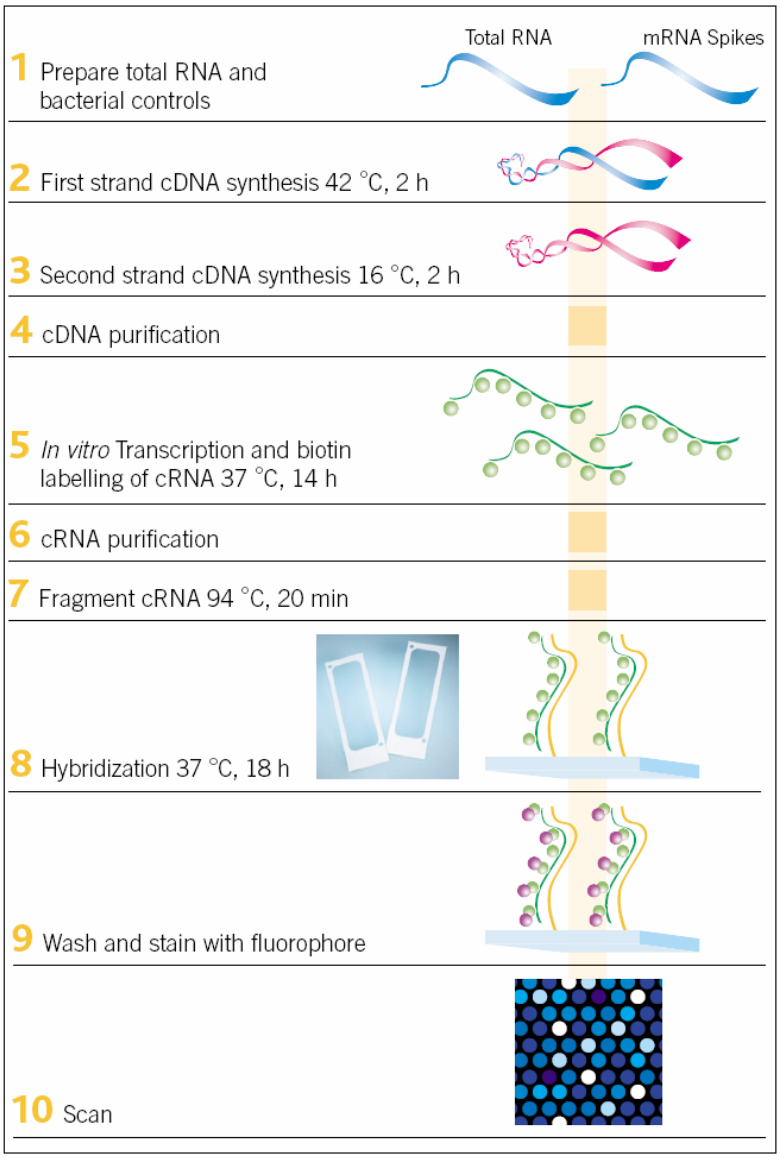

Figure 7. The overview of target preparation and hybridization steps of microarray.

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4.1 Total RNA extraction and purification

Total RNA were isolated and purified by Nucleospin RNA II Kit (Macherey-Nagel) as followed. Cells were lysed with 350 µl of lysis buffer containing 0.01% of β-mercaptoethanol. Then, the lysate was passed through Nucleospin Filter unit to reduce the viscosity and centrifuged at 11,000 g for 1 minute. The filter was removed and 350 µl of 70% ethanol was added to adjust RNA binding condition. The homogenized lysate was loaded into Nucleospin column and centrifuged for 30 second at 8,000 g. Membrane was washed with 350 µl of membrane desalting buffer and centrifuged at 11,000 g for 1 min to dry the membrane. To eliminate the DNA contamination, the 95 µl of DNase reaction mixture was added onto the silica membrane and incubated at room temperature for 15 minutes. Then, membrane was washed twice with washing buffers. Finally, the total purified RNA was eluted using 60 µl of nuclease free water.

The purity of RNA was quantified using UV spectrophotometry by the ratio of A_{260}: A_{280}. The high quality of RNA was required for microarray experiment which should have A_{260}: A_{280} ratio between 1.8 and 2.1. The total RNA concentration was calculated as equation below.

$$\text{Total RNA } (\mu g/ml) = A_{260} \text{ x dilution factor x } 40\ (\mu g/ml)$$

In addition, the integrity of extracted RNA was assessed by using agarose gel electrophoresis. The good integrity RNA should contain well defined 28S and 18S ribosomal RNA (rRNA) at approximately 4.5 and 1.9 kb and ratio of 28S: 18S band intensities equal to 2.

4.2 cDNA synthesis and purification

For each RNA sample, 2 µg of total RNA were used to synthesize the first strand cDNA. For accurate assessment of efficiency and sensitivity of assay, each RNA sample was added with 2 µg of bacterial control mRNA mixture. After incubation at 70ºC for 10 minutes with T7 Oligo (dT) primer, the RNA template was subjected to the first cDNA strand synthesis with the following reagents, 10x first strand buffer, 5mM dNTP mix, RNase inhibitor and reverse transcriptase and incubated at 42ºC for 2

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hours. Subsequently, the second strand cDNA synthesis was achieved by adding 10x second strand buffer, 5mM dNTP mix, DNA polymerase and RNase H. Double-stranded cDNA can be stored at -20ºC or preceded to the next step.

The purification of cDNA was performed using GFX PCR DNA purification kit (GE heathcare). Briefly, cDNA solution was added with 500 µl of capture buffer and then transferred to GFX column. The unwanted primers and impurities such as salts, enzymes, and unincorporated nucleotides do not bind to the glass fiber matrix, but flow through the column. The membrane was washed with 500 µl of wash buffer. The cDNA was eluted directly from the membrane with 30 µl of nuclease free water and repeated once to generate a total 60 µl of eluate. Then, cDNA solution was concentrated in a SpeedVac concentrator under medium heat to minimize the eluate volume to 9.5 µl.

4.3 In vitro transcription and biotin labeling of cRNA

The purified cDNA was then subjected to cRNA synthesis reaction containing 10xT7 Reaction Buffer, ATP, GTP, CTP, UTP, 10mM biotin-11-UTP, and 10xT7 enzyme mixture. The biotin was incorporated into cRNA which was amplified for 18 hours in an air incubator at 37ºC. The biotin labeled cRNA was recovered using Nucleospin RNA II Kit. The cRNA purity was measured by UV spectrophotometry at 260 nm and 280 nm. The A_{260}: A_{280} ratio should be between 1.8-2.1. The high quality of sample was required prior to hybridize onto microarray glass slide.

4.4 Hybridization

The amount of 10 µg of biotin labeled cRNA from each sample was incubated in fragmentation buffer at 94ºC for 20 minutes to produce cRNA fragments of length 35-200 nucleotides. Subsequently, the cRNA target was denatured at 90ºC for 5 minutes in denaturing buffer. The denatured cRNA target was loaded onto the microarray glass slide where it bound to specific cDNA probe. The hybridization was incubated for 18 hours at 37ºC with continuous shaking at 500 rpm. To remove unbound cRNA, the slide was washed in 0.75x TNT buffer (Tris-HCl pH 7.6, NaCl, and Tween 20) at 46ºC for exactly 1 hour. Next, the bound target was stained with Cy5-Steptavidin working solution and washed stringency with 1x TNT buffer four times and followed by 0.1x

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SSC/0.05% Tween 20 solution to remove excess hybridization solution and reduce cross-hybridization. Finally, the slide was dried by centrifugation at 644 g for 3 minutes. The slide was kept in the dry and a light-protected slide box until it was scanned. The slide was usually scanned immediately.

4.5 Microarray scanning

The image pattern of gene expression was measured by using Applied Precision's Array WoRx® Biochip reader. In this study, the Cy5 fluorescent hybridization from each spot on microarray slide was scanned and represented as the signal of gene expression. The image was saved as 16 bit gray scale TIFF image format (tiff file).

5 Microarray data analysis

5.1 Preprocessing and data filtering

The image from microarray slide was analyzed using Codelink Expression Analysis software version 4.1. The digital TIFF image file from each sample was converted to numerical measure of hybridization intensity. The ratio of the estimated signal intensity was calculated according to following equations.

$$\text{Signal to noise ratio (SNR)} = \frac{\text{Signal mean}}{\text{Background median} + C \times \text{Background standard deviation}}$$

Where default constant multiplier (C) = 1.5. The C value is to exclude the spot that is not reliable and low intensity.

After calculating, the only spot signals of which SNR was equal or larger than 1 was used for further analysis. In contrast, the low intensity spot where SNR was less than 1 and saturated spot where the intensity exceeds a saturation level that is the capability of recorded intensity by scanner were no longer analyzed. Moreover, the features that had an irregular shape, contaminated spot, and manufacture flagged feature were also discarded prior to data analysis.

To compare the data to the different slides, the data variation was reduced by using normalization method. The normalized intensity was calculated from signal intensity divided by median intensity from gene of interest probe.

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5.2 Statistical approach for selecting differentially expressed genes

The normalized intensity of each gene was subjected to The Institute for Genomic Research Multiple Experiment Viewer (TMEV) Version 3.1 (62). TMEV provides the viewing of microarray slide representation and the identification of genes and expression pattern of interest. The expression data from control and drug treatments were transformed to Log2 intensity and were recoded as TDMS Format (Tab Delimited Multiple Sample (.txt) Files). These data were loaded to TMEV programmed. The percentage cutoff filter was used to determine whether gene should be excluded. In this study, the percentage cutoff is set the value to 100. It indicates that every one of gene expression values must be valid.

The Significance Analysis of Microarray (SAM) is used to select significant gene based on differential expression sets of sample. SAM is the modified version of T-statistics that relative difference is equal to the difference of mean intensity divided by the adjusted standard variation to minimize the different in the coefficient of variation. The valuable feature of SAM is that it can estimate the value of the false discovery rate (FDR). In general, the number of gene expressions where FDR value is less than 5% is called significance. Moreover, two-fold change was also used to cutoff the significantly expressed genes from SAM at FDR = 5% for high stringency.

To analyze the gene expression pattern response to VR-3848 stimulation, the significant genes by SAM and two fold analysis then performed the k-means clustering. This algorithm tries to find genes with the most similar expression pattern to a random assigned partition. It starts by choosing a number of clusters. Then, each gene is reassigned to clusters based on the similar pattern of gene expression and cluster center. In the iterative manner, each cluster is updated with average expression pattern of genes assigned to that cluster. This process continues until all genes are mapped to each cluster (42, 63).

6 Validation of significant differentially expressed genes

6.1 Reverse transcription and polymerase chain reaction (RT-PCR)

The $3x10^6$ MCF-7 cells from control and VR-3848 drug treatments were extracted for total cellular RNA by using Nucleospin RNA II. The amount of total RNA in particular time points was quantified by UV spectrophotometry at wavelength

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of 260 nm. For the reverse transcription, 10 µl of total RNA was added to the following reagents including 5x AMV reverse transcriptase buffer, 0.1M dithiothreitol (DTT), 10mM dNTP mix, 200 µg/ml Oligo (dT) 15 primer, 200 µg/ml random primer, 40 Unit/µl RNase inhibitor and 10 Unit/µl AMV Reverse transcriptase enzyme. To complete the cDNA synthesis, the reaction mixture was incubated at 42ºC for 1 hour. The cDNA was diluted with 30 µl of distilled water and kept at -20ºC until used.

The genes of interest and internal control gene i.e. human β-actin were amplified by PCR. The reagent mixtures including 10x reaction buffer, 25mM $MgCl_2$, 20µM forward primer, 20µM reverse primer, 10mM dNTP and 5Unit/ml Taq DNA polymerase were added to 2.5 µl of cDNA. The primers of interested genes were designed by Primer 3 program (White Head Institute for Biomedical Research, Cambridge, MA, USA) and listed in table 6.

The PCR reactions were performed in the PCR Thermal Cycler machine. PCR conditions were used as following. After denaturing at 94ºC for 5 minutes, 30 cycles consisted of 94ºC for 30 seconds, followed by 30 seconds of annealing temperature depending on particular genes, and 72ºC, 30 seconds for extension. After complete amplification, cDNA was continue incubated 72ºC for 7 minutes and finally kept the PCR products at 4ºC or and -20ºC for semi quantitative experiment.

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Table 6. The list of primers was used for RT-PCR analysis for mRNA expression.

NCBI accession No.	Gene	Product (base pair)	Annealing Temp	Orientation	Sequence
NM_004574.2	ARTS	163	56	Sense	5'-TGTGAAGCTGAGGACAATGC-3'
				Antisense	5'-GGTACCACTTTCCCGAGTCA-3'
NM_001675.2	ATF4	227	56	Sense	5'-TCAAACCTCATGGGTTCTCC-3'
				Antisense	5'-AGTGTCATCCAACGTGGTCA-3'
NM_000104.2	CYP1B1	164	56	Sense	5'-CACCAAGGCTGAGACAGTGA-3'
				Antisense	5'-GATGACGACTGGGCCTACAT-3'
S62138.1	DDIT3	309	56	Sense	5'-GAAACGGAAACAGAGTGGTCATTCCCC-3’
				Antisense	5'-GTGGGATTGAGGGTCACATCATTGGCA-3’
NM_015675.1	GADD45β	213	56	Sense	5'-GAGACTGGAGCTGAGCGTCT-3'
				Antisense	5'-TATGCTTCCCATCTCGCTCT-3'
L19314.1	HES1	103	56	Sense	5'-AGGCGGACATTCTGGAAATG-3'
				Antisense	5'-CGGTACTTCCCCAGCACACTT-3'
NM_002165.2	ID1	212	56	Sense	5'-CGGATCTGAGGGAGAACAAG-3'
				Antisense	5'-CTGAGAAGCACCAAACGTGA-3'
NM_002192.1	INHBA	238	56	Sense	5'-CCTCGGAGATCATCACGTTT-3'
				Antisense	5'-CCCTTTAAGCCCACTTCCTC-3'
AW292830.1	KLF6	383	58	Sense	5'-ACCCGGCCCGACATGGACGTG-3'
				Antisense	5'-CAGGCTGTTGTTCTCTAAAGTT-3'
NM_001300.3	KLF10	281	58	Sense	5′-AAAGTTCCCATCTGAAGGCCCA-3'
				Antisense	5′-GGTTGGAGGTAGAGCAATGTCA-3'
NM_016084.3	RASD1	156	56	Sense	5'-AGCCGAGGGTGGATTTATCT-3'
				Antisense	5'-AACCCGGAATCACAGACAAG-3'
NM_030952.1	SNARK	208	64	Sense	5'-GACAGCTGCTTTTCCCTGAC-3'
				Antisense	5'-CAAACCCTCTCAGCCTTCTG-3'
AB002307.2	SRCAP	195	56	Sense	5'-CCTGGTCACCCTCTGTTGTT-3'
				Antisense	5'-GGTGGCTAGAGCCTGAGATG-3'
NM_016639.1	TNFRSF12A	161	56	Sense	5'-CTGGCTCCAGAACAGAAAGG-3'
				Antisense	5'-GAGTGGGGCCTAGTGTCAAG-3'

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6.2 Gel electrophoresis and semi-quantitative analysis of gene expression

The PCR products were detected by agarose gel electrophoresis. The 2% agarose gel was melted in 1xTBE electrophoresis buffer pH8.0 (Tris, Boric acid, and EDTA). The genes of interest and internal control (human β-actin) were run at 90 voltages. After electrophoresis, the gene expression was stained with ethidium bromide. Expression of each gene was performed in three independent experiments.

The intensity of gene expression was semi-quantified by densitometer (GE healthcare). The level of gene expression was calculated. The human β-actin gene expression was used as an internal control using the following equation.

$$\text{Level of gene expression} = \frac{\text{Intensity of each gene}}{\text{Intensity of β-actin}} \times 100$$

7. Statistics

The significant difference of gene expression between control and drug treatments was tested by SPSS 11.5 for window software. Significance was turned on when p-value <0.05 by using pair T-test.

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CHAPTER 4
RESULTS

1. VR-3848 induced apoptosis of MCF-7 cell line.

VR-3848 exerts a potent killing activity to breast cancer, MCF-7, cell. To investigate the killing mechanism, nuclear morphological changes of VR-3848 treated MCF-7 cells were studied using DAPI staining and TUNEL assay. The fluorescent dye, DAPI, detects the apoptotic characteristics (nuclear condensation and nuclear fragmentation) by binding to a DNA minor groove (64). In case of TUNEL assay, the DNA strand breaks generated during apoptosis are detected by labeling of free 3'OH termini with fluorescein conjugated nucleotide in the presence of terminal deoxynucleotidyl transferase and anti-fluorescein antibody conjugated with alkaline phosphatase. These apoptotic cells can be detected under microscope.

The nuclear morphological changes of VR-3848 treated MCF-7 cells were demonstrated in figure 8. The nuclei of MCF-7 cells treated with 0.1% DMSO, a control compound, showed homogeneous bright-blue staining nuclei while the nuclei of cells undergoing apoptosis, VR-3848 treated cells, were condensed or fragmented. For TUNEL assay, this technique demonstrates chromosomal DNA strand break, therefore, after enzyme-substrate reaction, cells with brown nuclei were illustrated under microscope. This data confirms that VR-3848 kills MCF-7 cells by apoptosis.

The kinetic of VR-3848 induced apoptosis was monitored. As shown in figure 9, MCF-7 cells were treated with 100 nM of VR-3848. The number of apoptotic cells was counted at various time points after DAPI staining. As expected, the longer time of treatment the higher number of apoptotic cells were detected. The percentage of apoptosis was 2.03, 3.21, 7.80, 16.73, 34.99, and 48.28 at 1, 3, 6, 12, 18, and 24 hours after treatment while the number of apoptotic cells was less than 5% in control experiment.

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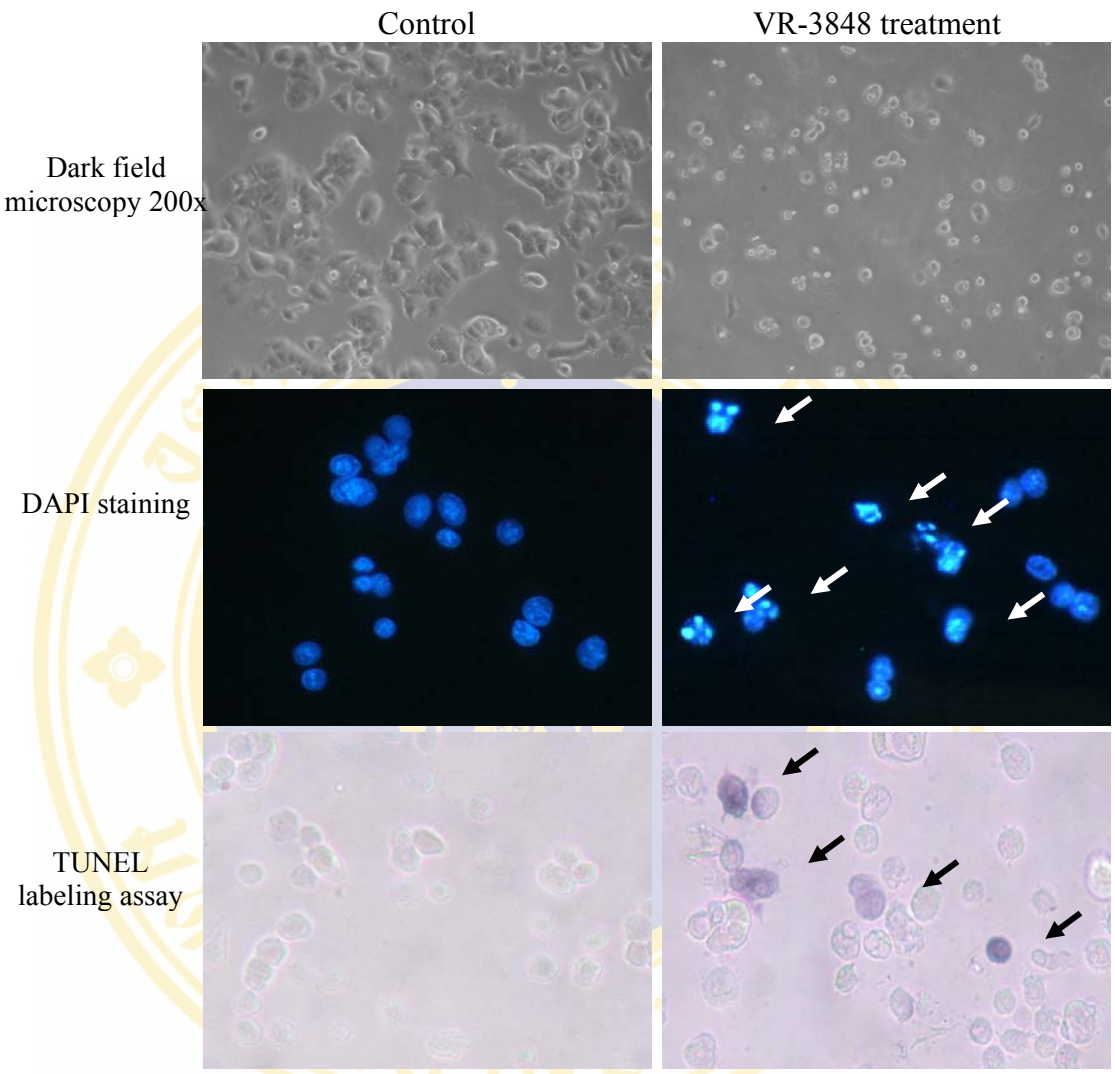

Figure 8. Detection of VR-3848 induced MCF-7 apoptosis using DAPI and TUNEL staining. The morphological changes of control cells and 100 nM VR-3848 treated cells at 24 hours were monitored under microscope. The apoptotic cells (arrow) with chromatin condensation and nuclear fragmentation were discriminated from normal cells by fluorescent DAPI staining and TUNEL labeling assay.

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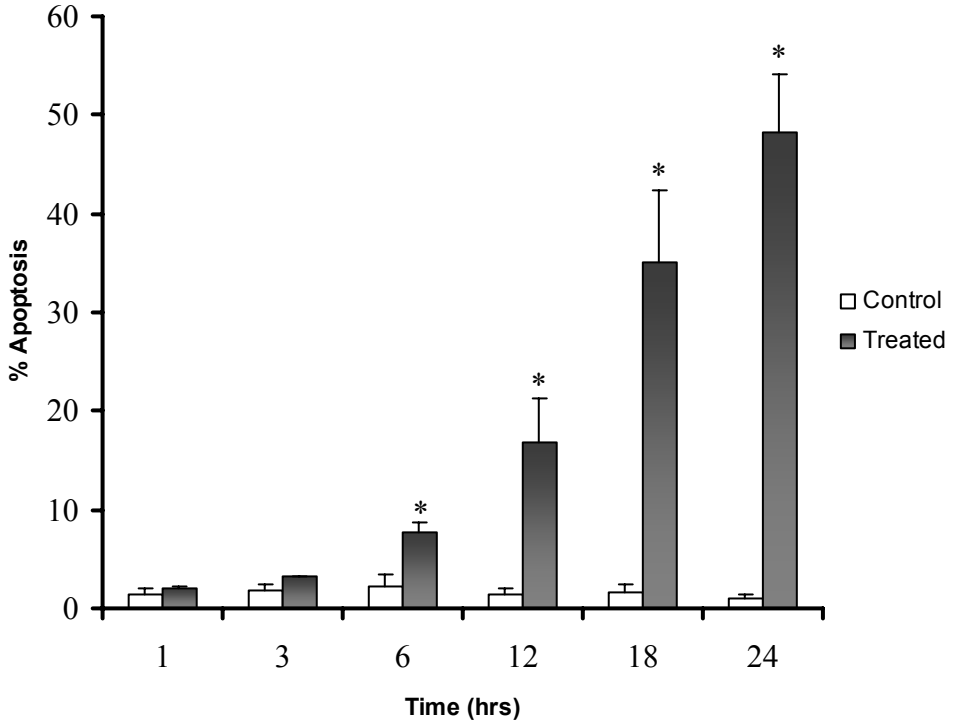

Figure 9. Determination of apoptotic cell numbers at various time points after VR-3848 treatment. MCF-7 cells were treated with either 100 nM of VR-3848 or with 0.1% DMSO, control compound. Results are mean ± standard error (SE) from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the control and treatment groups at specified time points using paired t-test.

2. Analysis of a global gene expression alteration in VR-3848 treated MCF-7 cells.

In order to understand molecular process of VR-3848- induced apoptosis, a gene expression profile at various time points of VR-3848 treatment was investigated using cDNA array screening. In this experiment, mRNA was extracted from VR-3848 treated cells at 1, 3, 6, 12 and 18 hours of treatment and from control cells at the matching time points. These mRNA were then amplified, transcribed into cRNA and labeled with biotin. The biotinylated cRNA were then hybridized to gene chips. The level of individual gene expression was analyzed as mentioned in the Material and Methods.

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2.1 Reproducibility of the microarray experiment

To assess the reliability of DNA microarray experiment performed in the present study, the reproducibility of the technique was tested. The single RNA sample was divided into two set of RNA and subjected into RNA labeling procedure. The two identical sets of labeled RNA were hybridized to two sets of gene array. The hybridization intensities were acquired and analyzed. The acquired data from these two hybridizations were plotted against each other and the correlation between the two sets of data was tested using Pearson correlation coefficient (R^2). The correlation coefficient was 0.983 at p-value <0.05 indicating the high reproducibility of this technique used in our study. (figure 10)

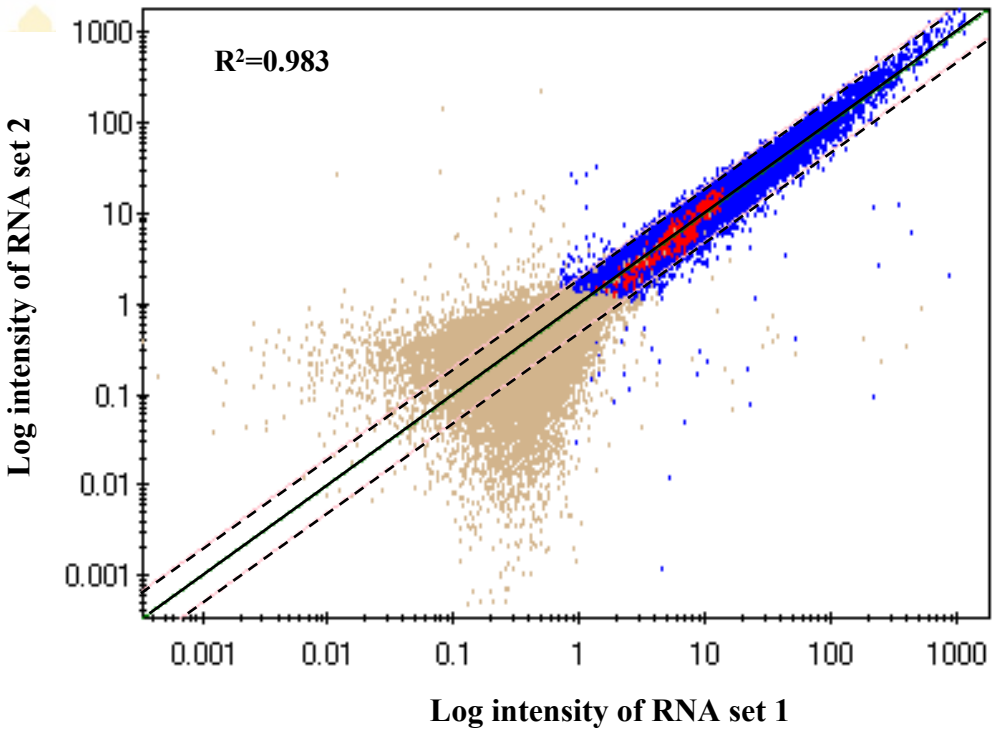

Figure 10. The reproducibility of microarray data. Each axis represented the logarithm intensity scale of normalized gene intensity from two sets of samples. The diagonal line represents the line of identity. The dash line represents two-fold differences between intensities from the two sets of arrays. Pearson's correlation coefficient (R^2) was 0.983 at p-value < 0.05.

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2.2 Screening of gene expression alteration at various time points after VR-3848 treatment.

To understand death response of MCF-7 cells stimulated by VR-3848, gene expression profiles were investigated at 1, 3, 6, 12, and 18 hours of treatment. The acquired hybridization intensities from VR-3848 treated and 0.1% DMSO treated at indicating time points were analyzed for the significantly altered signals using significance analysis of microarray (SAM) with the estimating false discovery rate (FDR) at 5%. These significant genes whose expression of VR-3848 treated was 2 fold higher or lower than that of 0.1% DMSO treated as reference were considered as up- or down-regulation. The 4,075 genes were significantly altered in response to VR-3848. The number of significant genes at 3, 6, 12, and 18 hours was summarized in table 7.

Table 7. A summary of number of gene whose expression was altered in response to VR-3848 stimulation.

Level of gene expression	Numbers of gene at specific time (hr) after treatment.			
	3	6	12	18
Up-regulation	85	593	1228	1684
Down-regulation	158	341	1491	2206
Total	243	934	2719	3890

Genes listed in table 7 were categorized and grouped according to biological function based on Gene Ontology. As shown in table 8 and 9, most of genes that are affected by VR-3848 treatment were involved in transcription, translation and signal transduction. The alteration of gene expressions in group of transcription and translation was 21, 105, 266, and 351 genes. Among these genes, 14, 87, 164, and 209 genes were up-regulated and 7, 18, 102, and 142 genes were down-regulated at 3, 6, 12, and 18 hours respectively. Approximately 50% of significant genes have functionally unknown.

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Table 8. The numbers of gene in each biological category that were up-regulated in response to VR-3848 treatment.

Biological functions	Numbers of up-regulated genes at specific time (hrs) after treatment			
	3	6	12	18
Signal transduction	12	82	133	187
Transcription and Translation	14	87	164	209
Cell growth and Development	12	44	81	114
Immune and inflammatory	5	29	50	52
Metabolism	1	18	41	57
Cell cycle	0	23	28	51
Transport	2	27	74	106
Response to Stimuli	2	13	26	26
Apoptosis	3	26	38	41
Phosphorylation and kinase	3	31	66	101
Unknown	43	288	623	850
Total of significant genes	85	593	1228	1684

Table 9. The numbers of gene in each biological category that were down-regulated in response to VR-3848 treatment.

Biological functions	Number of down-regulated genes at specific time (hrs) after treatment			
	3	6	12	18
Signal transduction	16	16	122	162
Transcription and Translation	7	18	102	142
Cell growth and Development	9	12	53	78
Immune and inflammatory	1	2	20	27
Metabolism	0	5	111	166
Cell cycle	1	4	39	62
Transport	4	10	97	165
Response to Stimuli	0	2	18	27
Apoptosis	0	5	35	46
Phosphorylation and kinase	1	6	48	76
Unknown	117	269	814	1152
Total of significant genes	158	341	1491	2206

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To analyze the gene expression pattern during VR-3848 stimulation, the 4,075 significantly altered genes were classified by using k-means clustering. This clustering starts with a random initial partition and groups genes based on similarity between expression pattern and center of partition. As illustrated in figure 11, k-means cluster analysis demonstrates the dynamic expression pattern during VR-3848 stimulation.

Cluster1 is the group of genes, 477 genes, which expression was greatly suppressed during VR-3848 treatment. The second cluster is composed of 1,264 genes whose expression was slightly down-regulated during treatment. The third and fourth cluster contains 575 genes and 228 genes respectively. Expression of genes of the third group was transiently increased at early phase but was decreased at the later phase of treatment while expressions of gene in 4th cluster were dramatically increased during treatment. The 5th cluster contains 244 genes which their expression patterns were only transiently altered at early phase. The last cluster is group of 1,287 genes which their expressions were gradually increased at the late phase of treatment.

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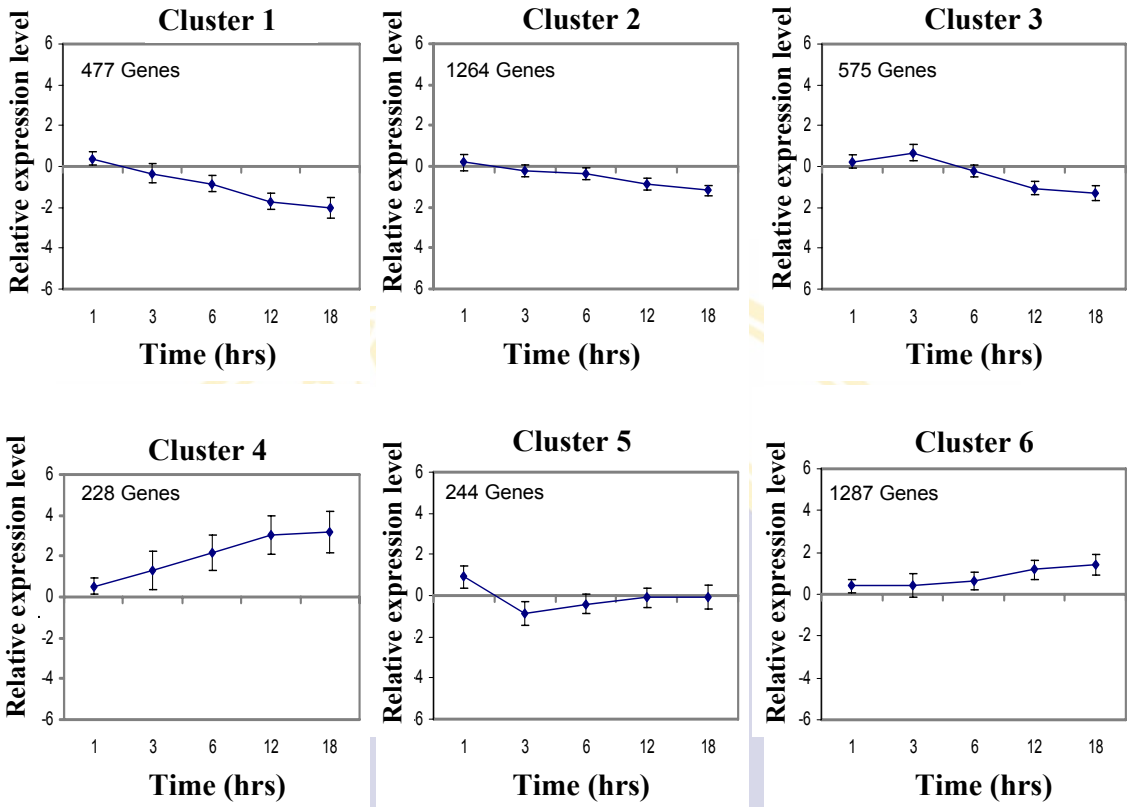

Figure 11. K-means clustering analysis represented the gene expression pattern during VR-3848 stimulation. This clustering grouped 4,075 expression-altered genes into 6 clusters based on their similarity of expression pattern at 5 time points. Each cluster represents the average ± standard deviation of gene expression pattern within the cluster. The y-axis represents the relative expression level which is log2 ratios of VR-3848 treated to control intensities and x-axis represents treatment time.

From the k-means analysis results, the cluster 1 and 4 showed strongly alteration with progressive decrease and increase of gene expression level during VR-3848 stimulation respectively. We therefore first considered genes belonging to these clusters that may play important roles in VR-3848 induced cell death. The cluster 1 and 4 with 169 and 114 functionally known genes were categorized and listed in table 10 and 11 while the 308 and 114 genes with unknown function were listed in table 17 and 18. (see Appendix C)

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Table10. The list of functionally known genes in cluster 1

NCBI Accession No.	Genes		Functional category
NM_018571.4	ALS2CR2	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2	Anti-apoptosis
NM_004281.2	BAG3	BCL2-associated athanogene 3	Anti-apoptosis
AK125533.1	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	Anti-apoptosis
NM_006378.2	SEMA4D	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	Anti-apoptosis
NM_019003.1	SPIN2	spindlin family, member 2	Anti-apoptosis
NM_001188.2	BAK1	BCL2-antagonist/killer 1	Apoptosis related
NM_004324.3	BAX	BCL2-associated X protein	Apoptosis related
NM_032992.1	CASP6	caspase 6, apoptosis-related cysteine protease	Apoptosis related
NM_022073.2	EGLN3	egl nine homolog 3 (C elegans)	Apoptosis related
NM_024712.2	ELMO3	engulfment and cell motility 3 (ced-12 homolog, C elegans)	Apoptosis related
BX648349.1	ELMOD2	ELMO/CED-12 domain containing 2	Apoptosis related
W38580.1	HRMT1L1	HMT1 hnRNP methyltransferase-like 1 (S cerevisiae)	Apoptosis related
NM_014061.3	MAGEH1	melanoma antigen, family H, 1	Apoptosis related
NM_015375.1	RIPK5	receptor interacting protein kinase 5	Apoptosis related
NM_000407.3	GP1BB	glycoprotein Ib (platelet), beta polypeptide	Cell adhesion
H15096.1	PCDH7	protocadherin 7	Cell adhesion
NM_018936.2	PCDHB2	protocadherin beta 2	Cell adhesion
NM_018937.2	PCDHB3	protocadherin beta 3	Cell adhesion
NM_007280.1	OIP5	Opa-interacting protein 5	Cell communication
NM_198287.1	ING4	inhibitor of growth family, member 4	Cell cycle arrest
BC033173.1	ANAPC11	APC11 anaphase promoting complex subunit 11 homolog (yeast)	Cell cycle related
BC003122.1	CABLES2	Cdk5 and Abl enzyme substrate 2	Cell cycle related
AK092726.1	CHC1	regulator of chromosome condensation 1	Cell cycle related
NM_006037.2	HDAC4	histone deacetylase 4 (HDAC4)	Cell cycle related
NM_016596.2	HDAC7A	histone deacetylase 7A	Cell cycle related
NM_006739.2	MCM5	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S cerevisiae)	Cell cycle related
NM_016095.1	PSF2	DNA replication complex GINS protein PSF2	Cell cycle related
NM_006306.2	SMC1L1	SMC1 structural maintenance of chromosomes 1-like 1 (yeast)	Cell cycle related
BG354579.1	CBX2	chromobox homolog 2 (Pc class homolog, Drosophila)	chromosome organization
NM_003077.2	SMARCD2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	chromosome organization
NM_001202.2	BMP4	bone morphogenetic protein 4	Development
NM_004395.2	DBN1	drebrin 1	Development
NM_023031.1	FGFR2	fibroblast growth factor receptor 2	Development
NM_002573.2	PAFAH1B3	platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	Development

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Table10. The list of functionally known genes in cluster 1 (continued)

NCBI Accession No.	Genes		Functional category
NM_020796.2	SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	Development
NM_000231.1	SGCG	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	Development
NM_005668.3	SIAT8D	sialyltransferase 8D (alpha-2, 8-polysialyltransferase)	Development
NM_006943.2	SOX12	SRY (sex determining region Y)-box 12	Development
NM_005634.2	SOX3	SRY (sex determining region Y)-box 3	Development
NM_012391.1	SPDEF	SAM pointed domain containing ets transcription factor	Development
BX641075.1	TPM3	tropomyosin 3	Development
NM_005148.2	UNC119	unc-119 homolog (C elegans)	Development
NM_201599.1	ZNF261	zinc finger protein 261	Development
M27333.1	TRG@	T cell receptor gamma-chain processed pseudogene mRNA	Immune response
NM_021116.1	ADCY1	adenylate cyclase 1 (brain)	Metabolism
NM_001116.1	ADCY9	adenylate cyclase 9	Metabolism
AK026966.1	AK3	adenylate kinase 3	Metabolism
NM_000692.3	ALDH1B1	aldehyde dehydrogenase 1 family, member B1	Metabolism
NM_004037.5	AMPD2	adenosine monophosphate deaminase 2 (isoform L)	Metabolism
NM_021184.1	APOM	apolipoprotein M	Metabolism
NM_030765.2	B3GNT4	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4	Metabolism
AK000158.1	CA12	carbonic anhydrase XII	Metabolism
NM_001757.1	CBR1	carbonyl reductase 1	Metabolism
NM_000071.1	CBS	cystathionine-beta-synthase	Metabolism
NM_006012.1	CLPP	ClpP caseinolytic protease, ATP-dependent, proteolytic subunit homolog (E coli)	Metabolism
NM_004393.1	DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	Metabolism
NM_004446.1	EPRS	glutamyl-prolyl-tRNA synthetase	Metabolism
NM_024306.2	FA2H	fatty acid 2-hydroxylase	Metabolism
NM_173540.1	FUT11	fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	Metabolism
NM_133443.1	GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2	Metabolism
NM_004832.1	GSTW1	glutathione S-transferase omega 1	Metabolism
NM_183239.1	GSTW2	glutathione S-transferase omega 2	Metabolism
AW104464.1	HIBCH	3-hydroxyisobutyryl-Coenzyme A hydrolase	Metabolism
NM_000190.2	HMBS	hydroxymethylbilane synthase	Metabolism
NM_006597.3	HSPA8	heat shock 70kDa protein 8	Metabolism
NM_014214.1	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	Metabolism
NM_000883.2	IMPDH1	inosine monophosphate dehydrogenase 1	Metabolism
NM_000221.1	KHK	ketohexokinase (fructokinase)	Metabolism
NM_000235.1	LIPA	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	Metabolism

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Table10. The list of functionally known genes in cluster 1 (continued)

NCBI Accession No.	Genes		Functional category
NM_153613.1	LOC254531	PlSC domain containing hypothetical protein	Metabolism
NM_033300.1	LRP8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor (LRP8), transcript variant 1, 2, 3	Metabolism
NM_005911.4	MAT2A	methionine adenosyltransferase II, alpha	Metabolism
NM_002949.2	MRPL12	mitochondrial ribosomal protein L12	Metabolism
NM_017840.2	MRPL16	mitochondrial ribosomal protein L16	Metabolism
NM_014763.2	MRPL19	mitochondrial ribosomal protein L19	Metabolism
NM_023937.2	MRPL34	mitochondrial ribosomal protein L34	Metabolism
NM_005467.2	NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	Metabolism
NM_016256.1	NAGPA	N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase	Metabolism
AB033052.2	NLN	neurolysin (metallopeptidase M3 family)	Metabolism
NM_024663.2	NPEPL1	aminopeptidase-like 1	Metabolism
NM_015922.1	NSDHL	NAD(P) dependent steroid dehydrogenase-like	Metabolism
NM_006117.2	PECI	peroxisomal D3,D2-enoyl-CoA isomerase	Metabolism
NM_018441.2	PECR	peroxisomal trans-2-enoyl-CoA reductase	Metabolism
D31765.1	POP1	processing of precursor 1, ribonuclease P/MRP subunit (S. cerevisiae)	Metabolism
NM_005051.1	QARS	glutaminyl-tRNA synthetase	Metabolism
NM_005063.3	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	Metabolism
NM_024330.1	SLC27A3	solute carrier family 27 (fatty acid transporter), member 3	Metabolism
NM_024838.3	THNSL1	threonine synthase-like 1 (bacterial)	Metabolism
NM_003312.4	TST	thiosulfate sulfurtransferase (rhodanese)	Metabolism
NM_003362.2	UNG	uracil-DNA glycosylase	Metabolism
H15407.1	VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	Metabolism
AB033058.1	DLG3	discs, large homolog 3 (neuroendocrine-dlg, Drosophila)	Negative regulation of cell proliferation
NM_130469.2	JDP2	jun dimerization protein 2	Negative regulation of cell proliferation
NM_000251.1	MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E coli)	Negative regulation of cell proliferation
NM_022477.2	NDRG3	NDRG family member 3	Negative regulation of cell proliferation
NM_006545.3	TUSC4	tumor suppressor candidate 4	Negative regulation of cell proliferation
U83115.1	AIM1	non-lens beta gamma-crystallin like protein	Cell proliferation
NM_000714.3	BZRP	benzodiazapine receptor (peripheral)	Cell proliferation
W93682.1	CIP29	cytokine induced protein 29 kDa	Cell proliferation
W86753.1	CXCL16	chemokine (C-X-C motif) ligand 16	Cell proliferation
NM_032638.3	GATA2	GATA binding protein 2	Cell proliferation
NM_001505.1	GPR30	G protein-coupled receptor 30	Cell proliferation
NM_002083.2	GPX2	glutathione peroxidase 2 (gastrointestinal)	Cell proliferation

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Table10. The list of functionally known genes in cluster 1 (continued)

NCBI Accession No.	Genes		Functional category
NM_005567.2	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	Cell proliferation
NM_022368.2	PJA1	praja 1	Cell proliferation
NM_005982.1	SIX1	sine oculis homeobox homolog 1 (Drosophila)	Cell proliferation
L40522.1	SMA3	survival of motor neuron 1, telomeric	Cell proliferation
BX538220.1	TRIM25	tripartite motif-containing 25	Cell proliferation
NM_014112.1	TRPS1	trichorhinophalangeal syndrome I	Cell proliferation
W63635.1	UBE2I	ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	Cell proliferation
NM_003942.1	RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4	Prosphorylation
NM_013332.1	HIG2	hypoxia-inducible protein 2	Response to stimuli
NM_002434.1	MPG	N-methylpurine-DNA glycosylase	Response to stimuli
NM_001235.2	SERPINH1	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	Response to stimuli
NM_021158.3	TRIB3	tribbles homolog 3 (Drosophila)	Response to stimuli
BU945292.1	XBP1	X-box binding protein 1	Response to stimuli
NM_019037.1	EXOSC4	exosome component 4	RNA processing
NM_024775.9	GEMIN6	gem (nuclear organelle) associated protein 6	RNA processing
NM_032620.1	GTPBP3	GTP binding protein 3 (mitochondrial)	RNA processing
NM_024075.1	LENG5	leukocyte receptor cluster (LRC) member 5	RNA processing
NM_032881.1	LSM10	LSM10, U7 small nuclear RNA associated	RNA processing
NM_020230.4	PPAN	peter pan homolog (Drosophila)	RNA processing
BX648288.1	RBM17	RNA binding motif protein 17	RNA processing
NM_006638.1	RPP40	ribonuclease P 40kDa subunit	RNA processing
NM_025265.2	SEN2L	tRNA splicing endonuclease 2 homolog (S. cerevisiae)	RNA processing
NM_015242.2	CENTD2	centaurin, delta 2	Signal transduction
BX649076.1	CNIH4	cornichon homolog 4 (Drosophila)	Signal transduction
NM_003481.1	GNB3	ubiquitin specific protease 5 (isopeptidase T)	Signal transduction
NM_016194.2	GNB5	guanine nucleotide binding protein (G protein), beta 5	Signal transduction
NM_006301.2	MAP3K12	mitogen-activated protein kinase kinase kinase 12	Signal transduction
NM_004579.2	MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)	Signal transduction
NM_021005.2	NR2F2	nuclear receptor subfamily 2, group F, member 2	Signal transduction
NM_002646.2	PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	Signal transduction
NM_014353.4	RAB26	RAB26, member RAS oncogene family	Signal transduction
NM_003475.2	RASSF7	Ras association (RalGDS/AF-6) domain family 7	Signal transduction
NM_005873.1	RGS19	regulator of G-protein signalling 19	Signal transduction
NM_002957.3	RXRA	retinoid X receptor, alpha	Signal transduction
NM_004619.2	TRAF5	TNF receptor-associated factor 5	Signal transduction
NM_021643.1	TRIB2	tribbles homolog 2 (Drosophila)	Signal transduction

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Table10. The list of functionally known genes in cluster 1 (continued)

NCBI Accession No.	Genes		Functional category
NM_003796.2	C19orf2	chromosome 19 open reading frame 2	Transcription and Translation
NM_004364.2	CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	Transcription and Translation
NM_004095.2	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	Transcription and Translation
NM_005836.1	HRSP12	heat-responsive protein 12	Transcription and Translation
AK025941.1	KLF16	Kruppel-like factor 16	Transcription and Translation
BU734212.1	LHX1	LIM homeobox 1	Transcription and Translation
NM_004789.3	LHX2	LIM homeobox 2	Transcription and Translation
NM_019014.3	POLR1B	polymerase (RNA) I polypeptide B, 128kDa	Transcription and Translation
NM_007062.1	PWP1	nuclear phosphoprotein similar to S cerevisiae PWP1	Transcription and Translation
NM_004237.2	TRIP13	thyroid hormone receptor interactor 13	Transcription and Translation
NM_005726.2	TSFM	Ts translation elongation factor, mitochondrial	Transcription and Translation
NM_003409.2	ZFP161	zinc finger protein 161 homolog (mouse)	Transcription and Translation
NM_014519.2	ZNF232	zinc finger protein 232	Transcription and Translation
NM_006973.1	ZNF32	zinc finger protein 32	Transcription and Translation
NM_032792.2	ZNF499	zinc finger protein 499	Transcription and Translation
NM_001089.1	ABCA3	ATP-binding cassette, sub-family A (ABC1), member 3	Transport
NM_022341.1	COG8	component of oligomeric golgi complex 8	Transport
AA379810.1	COX5B	cytochrome c oxidase subunit Vb	Transport
NM_006855.2	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	Transport
BI715175.1	MRS2L	MRS2-like, magnesium homeostasis factor (S. cerevisiae)	Transport
NM_006822.1	RAB40B	RAB40B, member RAS oncogene family	Transport
NM_002871.3	RABIF	RAB interacting factor	Transport
NM_016524.2	SYT17	synaptotagmin XVII	Transport
NM_178014.2	TUBB	tubulin, beta	Transport
NM_032525.1	TUBB6	tubulin, beta 6	Transport
NM_052875.2	VPS26B	vacuolar protein sorting 26 homolog B (S. cerevisiae)	Transport
NM_007235.3	XPOT	exportin, tRNA (nuclear export receptor for tRNAs)	Transport

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Table10. The list of functionally known genes in cluster 1 (continued)

NCBI Accession No.	Genes		Functional category
NM_002797.2	PSMB5	proteasome (prosome, macropain) subunit, beta type, 5	Ubiquitin
NM_015983.2	UBE2D4	ubiquitin-conjugating enzyme E2D 4 (putative)	Ubiquitin
NM_198183.1	UBE2L6	ubiquitin-conjugating enzyme E2L 6	Ubiquitin

Table 11. The list of functionally known genes in cluster 4

NCBI Accession No.	Genes		Functional category
NM_000675.3	ADORA2A	adenosine A2a receptor	Apoptosis related
NM_001175.1	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	Apoptosis related
NM_033027.2	AXUD1	AXIN1 up-regulated 1	Apoptosis related
NM_002982.2	CCL2	chemokine (C-C motif) ligand 2	Apoptosis related
NM_001964.2	EGR1	early growth response 1	Apoptosis related
NM_005253.3	FOSL2	FOS-like antigen 2	Apoptosis related
NM_001924.2	GADD45A	growth arrest and DNA-damage-inducible, alpha	Apoptosis related
NM_015675.1	GADD45B	growth arrest and DNA-damage-inducible, beta	Apoptosis related
NM_002192.1	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	Apoptosis related
NM_012329.1	MMD	monocyte to macrophage differentiation-associated	Apoptosis related
NM_006154.1	NEDD4	neural precursor cell expressed, developmentally down-regulated 4	Apoptosis related
NM_015277.2	NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	Apoptosis related
NM_006403.2	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	Apoptosis related
NM_003998.2	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Apoptosis related
NM_020529.1	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Apoptosis related
NM_003821.4	RIPK2	receptor-interacting serine-threonine kinase 2	Apoptosis related
NM_003264.2	TLR2	toll-like receptor 2	Apoptosis related
NM_003842.3	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	Apoptosis related
NM_182919.1	TRIF	TIR domain containing adaptor inducing interferon-beta	Apoptosis related
NM_006398.2	UBD	ubiquitin D	Apoptosis related
NM_021101.3	CLDN1	claudin 1	Cell adhesion
NM_017556.1	FBLP-1	filamin-binding LIM protein-1	Cell adhesion
NM_000201.1	ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Cell adhesion
NM_002205.1	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Cell adhesion
NM_005562.1	LAMC2	laminin, gamma 2	Cell adhesion

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Table 11. The list of functionally known genes in cluster 4 (continued)

NCBI Accession No.	Genes		Functional category
S62138.1	DDIT3	DNA-damage-inducible transcript 3	Cell cycle arrest
AB024402.1	ING1	p47	Cell cycle arrest
NM_002198.1	IRF1	interferon regulatory factor 1	Cell cycle arrest
NM_001300.3	KLF6	core promoter element binding protein	Cell cycle arrest
NM_004073.2	PLK3	polo-like kinase 3 (Drosophila)	Cell cycle arrest
NM_004071.1	CLK1	CDC-like kinase 1	Cell cycle related
NM_005178.2	BCL3	B-cell CLL/lymphoma 3	Cell growth and development
NM_001554.3	CYR61	cysteine-rich, angiogenic inducer, 61	Cell growth and development
NM_012081.3	ELL2	elongation factor, RNA polymerase II, 2	Cell growth and development
NM_005860.1	FSTL3	follistatin-like 3 (secreted glycoprotein)	Cell growth and development
NM_002658.1	PLAU	plasminogen activator, urokinase	Cell growth and development
NM_020127.1	TUFT1	tuftelin 1	Cell growth and development
NM_001657.2	AREG	amphiregulin (schwannoma-derived growth factor)	Cell proliferation
NM_005746.1	PBEF1	pre-B-cell colony enhancing factor 1	Cell proliferation
AK128645.1	SDCBP	syndecan binding protein (syntenin)	Cell proliferation
NM_013376.1	SERTAD1	SERTA domain containing 1	Cell proliferation
NM_014755.1	SERTAD2	SERTA domain containing 2	Cell proliferation
NM_013448.2	BAZ1A	bromodomain adjacent to zinc finger domain, 1A	Chromosome organization
AW364689.1	CHD2	chromodomain helicase DNA binding protein 2	Chromosome organization
NM_003522.3	HIST1H2BG	histone 1, H2bf	Chromosome organization
NM_021065.2	HIST1H3D	histone 1, H2ad (HIST1H2AD), H3d	Chromosome organization
NM_003831.2	RIOK3	RIO kinase 3 (yeast)	Chromosome organization
NM_001710.3	BF	B-factor, properdin	Immune response related
NM_000064.1	C3	complement component 3	Immune response related
NM_002987.2	CCL17	chemokine (C-C motif) ligand 17	Immune response related
NM_002090.1	CXCL3	chemokine (C-X-C motif) ligand 3	Immune response related
NM_014002.1	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	Immune response related
NM_000593.4	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Immune response related
NM_000499.2	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	Metabolism

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Table 11. The list of functionally known genes in cluster 4 (continued)

NCBI Accession No.	Genes		Functional category
NM_000104.2	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	Metabolism
NM_001976.2	ENO3	enolase 3, (beta, muscle)	Metabolism
NM_005110.1	GFPT2	glutamine-fructose-6-phosphate transaminase 2	Metabolism
NM_003364.2	UPP1	uridine phosphorylase 1	Metabolism
NM_004024.2	ATF3	activating transcription factor 3	Negative regulation of cell proliferation
NM_006806.3	BTG3	BTG family, member 3	Negative regulation of cell proliferation
NM_001901.1	CTGF	connective tissue growth factor	Negative regulation of cell proliferation
NM_012141.1	DDX26	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	Negative regulation of cell proliferation
NM_182643.1	DLC1	deleted in liver cancer 1	Negative regulation of cell proliferation
NM_016084.3	RASD1	RAS, dexamethasone-induced 1	Negative regulation of cell proliferation
NM_000636.1	SOD2	superoxide dismutase 2, mitochondrial	Negative regulation of cell proliferation
NM_005655.1	TIEG	TGFB inducible early growth response	Negative regulation of cell proliferation
NM_000623.2	BDKRB2	bradykinin receptor B2	Negative regulation of cell proliferation
NM_001511.1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Negative regulation of cell proliferation
NM_004428.2	EFNA1	ephrin-A1	Negative regulation of cell proliferation
NM_005524.2	HES1	hairy and enhancer of split 1, (Drosophila)	Negative regulation of cell proliferation
NM_000600.1	IL6	interleukin 6 (interferon, beta 2)	Negative regulation of cell proliferation
NM_004235.3	KLF4	Kruppel-like factor 4 (gut)	Negative regulation of cell proliferation
NM_006164.2	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	Negative regulation of cell proliferation
NM_004221.3	NK4	natural killer cell transcript 4	Negative regulation of cell proliferation
NM_006167.2	NKX3-1	NK3 transcription factor related, locus 1 (Drosophila)	Negative regulation of cell proliferation
NM_002616.1	PER1	period homolog 1 (Drosophila)	Negative regulation of cell proliferation
NM_002885.1	RAP1GA1	RAP1, GTPase activating protein 1	Negative regulation of cell proliferation
NM_031437.1	RASSF5	Ras association (RalGDS/AF-6) domain family 5	Negative regulation of cell proliferation

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Table 11. The list of functionally known genes in cluster 4 (continued)

NCBI Accession No.	Genes		Functional category
NM_006528.2	TFPI2	tissue factor pathway inhibitor 2	Negative regulation of cell proliferation
NM_020666.2	CLK4	CDC-like kinase 4	Phosphorylation
NM_198393.1	TEX14	testis expressed sequence 14	Phosphorylation
NM_005490.1	SH2D3A	SH2 domain containing 3A	Response to stimuli
NM_030952.1	SNARK	likely ortholog of rat SNF1/AMP-activated protein kinase	Response to stimuli
AL832599.2	STARD4	START domain containing 4, sterol regulated	Response to stimuli
NM_178815.2	ARL8	ADP-ribosylation factor-like 8	Signal transduction
NM_018659.2	C17	cytokine-like protein C17	Signal transduction
NM_014395.1	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	Signal transduction
NM_004431.2	EPHA2	ephrin receptor A2	Signal transduction
NM_005534.2	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	Signal transduction
NM_013278.3	IL17C	interleukin 17C	Signal transduction
NM_145242.1	OR7E91P	olfactory receptor, family 7, subfamily E, member 91 pseudogene	Signal transduction
NM_181523.1	PIK3R1	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	Signal transduction
NM_130781.1	RAB24	RAB24, member RAS oncogene family	Signal transduction
NM_006834.2	RAB32	RAB32, member RAS oncogene family	Signal transduction
NM_031934.3	RAB34	RAB34, member RAS oncogene family	Signal transduction
NM_004251.3	RAB9A	RAB9A, member RAS oncogene family	Signal transduction
NM_000376.1	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	Signal transduction
NM_004433.3	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	Transcription
NM_005239.3	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	Transcription
NM_006734.2	HIVEP2	immunodeficiency virus type I enhancer binding protein 2	Transcription
NM_005848.1	MYCPBP	c-myc promoter-binding protein	Transcription
NM_002503.3	NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	Transcription
NM_004556.1	NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Transcription
NM_005126.2	NR1D2	nuclear receptor subfamily 1, group D, member 2	Transcription
NM_002908.1	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	Transcription
NM_006509.2	RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	Transcription
AB002307.2	SRCAP	Snf2-related CBP activator protein	Transcription
NM_003407.1	ZKSCAN1	zinc finger protein 36, C3H type, homolog (mouse)	Transcription

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Table 11. The list of functionally known genes in cluster 4 (continued)

NCBI Accession No.	Genes		Functional category
NM_003440.2	ZNF140	zinc finger protein 140	Transcription
AK027128.1	ZNF277	zinc finger protein 277	Transcription
NM_032148.2	SLC41A2	solute carrier family 41, member 2	Transport
BX537539.1	TRPV3	transient receptor potential cation channel, subfamily V, member 3	Transport
NM_183419.1	RNF19	ring finger protein 19	Ubiquitin
NM_018700.2	TRIM36	tripartite motif-containing 36	Ubiquitin

Table 10 demonstrated that most of down-regulated genes in the cluster 1 are functions in metabolism, cell proliferation, development, regulation of cell cycle. In contrast, table 11 showed that most of up-regulated genes in the cluster 4 are apoptosis related gene, negative regulation of cell proliferation, cell cycle arrest, and transcription and translation.

2.3 Identification of early gene response to VR-3848 stimulation.

To identify the molecular targets of VR-3848 induced cell death, the alteration of gene expressions at 1 hr after treatment was analyzed. The significant differences in an alteration of expression level were analyzed by using SAM analysis at 5% of FDR and two-fold change analysis. The result showed that expressions of 155 genes, at 1 hr after treatment, were significantly altered. Fifty two out of 155 genes were functionally known genes while 103 genes have an unknown function (see table 19 in Appendix D). Fifty two known genes were listed in table 12.

Table 12. The list of known genes response to VR-3848 stimulation at 1 hr of treatment

Genes		Fold change	FDR
Transcription and translation			
CBFB	Core-binding factor, beta subunit	2.37 ± 0.75	1.20
EGR4	Early growth response 4	2.94 ± 0.09	0.00
FOXP1	Forkhead box P1	2.81 ± 0.37	0.00
HES1	Hairy and enhancer of split 1, (Drosophila)	2.52 ± 0.09	0.00
HES4	Hairy and enhancer of split 4 (Drosophila)	2.17 ± 0.20	1.59
MLLT10	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10	2.15 ± 0.03	0.82

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Table 12. The list of known genes response to VR-3848 stimulation at 1 hr of treatment (continued)

Genes		Fold change	FDR
SERTAD3	SERTA domain containing 3	2.52 ± 0.61	1.59
SOX2	SRY (sex determining region Y)-box 2	2.20 ± 0.34	1.83
SRCAP	Snf2-related CBP activator protein	2.38 ± 0.22	0.92
SSA2	Sjogren syndrome antigen A2 (60kDa, ribonucleoprotein autoantigen SS-A/Ro)	2.50 ± 0.36	0.92
ZNF24	Zinc finger protein 24	2.72 ± 0.04	0.00
ZNF37A	Zinc finger protein 37A	2.39 ± 0.17	1.01
ZNF131	Zinc finger protein 131	2.04 ± 0.16	1.61
ZNF281	Zinc finger protein 281	3.03 ± 0.89	0.00
Signal transduction and cell-cell signaling			
EPS15	Epidermal growth factor receptor pathway substrate 15	2.08 ± 0.19	1.61
KLF10	Kruppel-like factor 10	2.25 ± 0.29	1.59
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	2.06 ± 0.12	1.44
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA)	2.38 ± 0.67	1.40
PBEF1	Pre-B-cell colony enhancing factor 1	3.11 ± 0.23	0.00
RASD1	RAS, dexamethasone-induced 1	3.41 ± 0.15	0.00
SH3BP5	SH3-domain binding protein 5 (BTK-associated)	2.08 ± 0.13	1.48
SSTR1	Somatostatin receptor 1	2.96 ± 0.49	0.00
Cell growth and development			
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.16 ± 0.13	0.69
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	2.69 ± 0.11	0.00
JARID2	Jumonji, AT rich interactive domain 2	2.15 ± 0.03	0.82
KLF6	Kruppel-like factor 6	2.67 ± 0.22	0.00
KRT9	Keratin 9 (epidermolytic palmoplantar keratoderma)	2.80 ± 0.02	0.00
SIX1	Sine oculis homeobox homolog 1 (Drosophila)	3.07 ± 0.64	0.00
Transport			
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	2.44 ± 0.34	0.82
NPC1	Niemann-Pick disease, type C1	2.28 ± 0.03	1.11
STX17	Syntaxin 17	2.24 ± 0.01	1.01
TOM1L2	Target of myb1-like 2 (chicken)	2.03 ± 0.18	1.40
Stress response			
DDIT3	DNA-damage-inducible transcript 3	2.56 ± 0.26	0.00
SNARK	SNF1/AMP-activated protein kinase	2.57 ± 0.46	0.89

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Table 12. The list of known genes response to VR-3848 stimulation at 1 hr of treatment (continued)

Genes		Fold change	FDR
Apoptosis			
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	2.62 ± 0.40	1.01
Other functions			
ACN9	ACN9 homolog (S cerevisiae)	2.54 ± 0.20	0.00
ANKIB1	Ankyrin repeat and IBR domain containing 1	2.81 ± 1.57	2.58
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	3.10 ± 1.22	0.74
BRD2	Bromodomain containing 2	2.02 ± 0.14	1.61
CD46	CD46 molecule, complement regulatory protein	2.15 ± 0.60	2.17
FUSIP1	FUS interacting protein (serine-arginine rich) 1	2.09 ± 0.28	1.40
H2AFJ	H2A histone family, member J	2.54 ± 1.16	2.17
MPP6	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	2.50 ± 0.93	1.20
MT1X	Metallothionein 1X	2.67 ± 1.01	1.40
PSMC2	Proteasome (prosome, macropain) 26S subunit, ATPase, 2	2.33 ± 0.17	0.92
STRN3	Striatin, calmodulin binding protein 3	2.26 ± 0.14	0.82
PLK3	Polo-like kinase 3 (Drosophila)	2.05 ± 0.14	1.87
PRSS16	Protease, serine, 16 (thymus)	2.53 ± 0.02	0.00
TOB2	Transducer of ERBB2, 2	2.57 ± 0.44	0.91
TRIP12	Thyroid hormone receptor interactor 12	2.46 ± 0.12	0.00
UBE2E1	Ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	3.32 ± 0.70	0.00
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	2.42 ± 0.56	1.48

At early phase of treatment, several cellular functions were affected. These genes included transcription and translation, signal transduction, cell growth and development, transport, cell cycle, stress response, apoptosis, and phosphorylation. Majority of affected genes were genes involved in transcription and translation. The first stimulated apoptotic gene was death receptor gene, the TNF-receptor superfamily gene. To confirm the gene alteration at 1 hour of treatment, approximately 20 % of genes that are well characterized in biological functions were selected for validation at genomic level using RT-PCR method. The validation was performed using RNA extracted from both 30 min and 1 hour of treatment. Result at 30 minutes of treatment

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may give us the earliest gene or a group of genes that respond to VR-3848 treatment. Types of genes that were selected for validation are described as follows.

Transcription and translation genes.

From microarray analysis at 1 hour of treatment, several transcription factor families were up-regulated such as zinc finger transcription factors (EGR4, MLLT10, SSA2, ZNF24, 37A, 131, and 281), forkhead box family (FOXP1), and basic helix-loop-helix family (HES1, and HES4). Moreover, the transcriptional coactivators such as SERTAD3 and SRCAP were also responded to VR-3848 treatment. To confirm the microarray results, the expression of HES1 and SRCAP at 30 and 60 min were monitored using RT-PCR as demonstrated in figure 12. The result showed that the level of SRCAP gene expression was significantly stimulated at 30 and 60 minutes of treatment. The HES1 gene expression was strongly up-regulated at 60 minutes but not at 30 minutes.

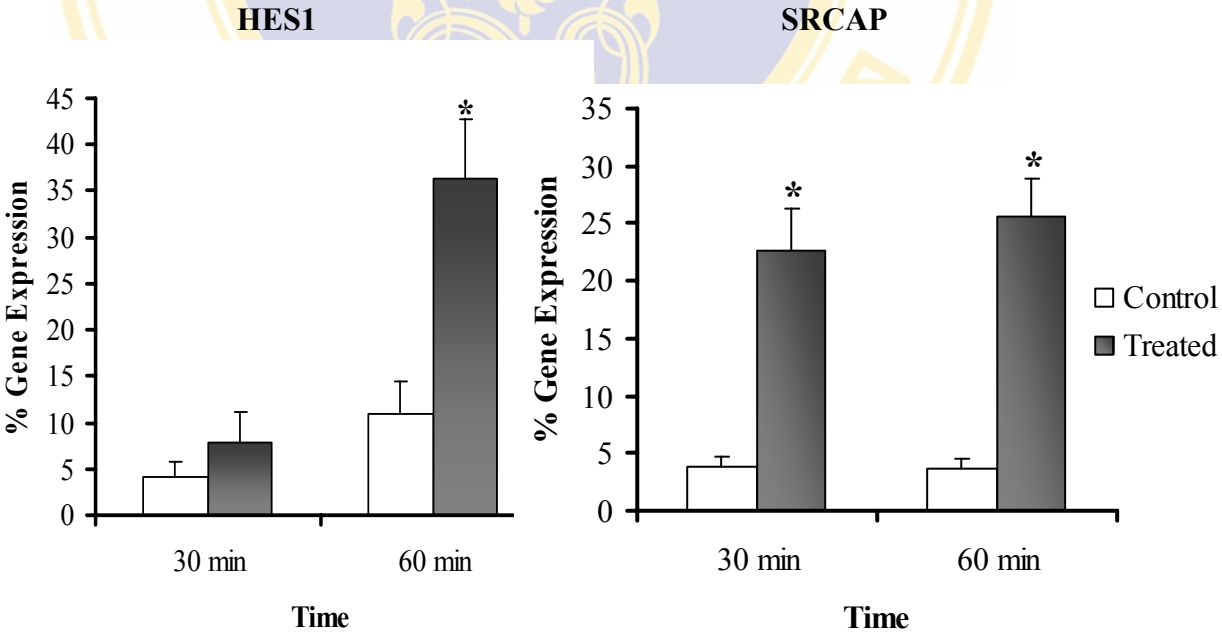

Figure 12. The upregulation of HES1 and SRCAP gene expression using RT-PCR at indicating time after VR-3848 treatment. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

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Cellular signaling and signal transduction genes

The expression of EPS15, KLF10, MIF, NFKBIA, RASD1, PBEF1, SH3BP5 and SSTR1 genes that implicate in signal transduction were increased during the early time point. These genes are responsible for distinct signaling pathways. While KLF10 gene, a member of zinc finger transcriptional factor, involves in transforming growth factor signaling pathway, RASD1 and SSTR1 genes implicate in G-protein couple signaling pathway. Among these genes as illustrated in figure 13, the expressions of KLF10 and RASD1 were further analyzed using RT-PCR. The results showed that the expression levels of KLF10 and RASD1 were significantly altered at 60 minutes but not at 30 minutes of treatment.

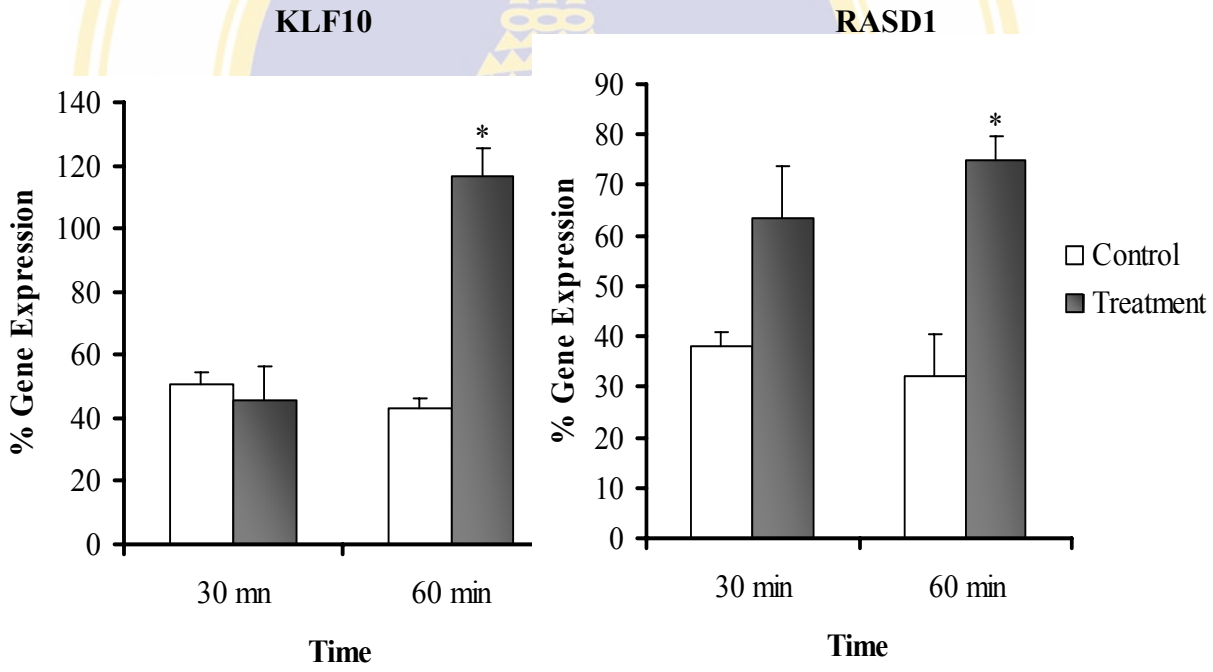

Figure 13. The up-regulation of KLF10 and RASD1 gene expression to VR-3848 stimulation was detected by RT-PCR. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

Cell growth and development genes

During the VR-3848 treatment, genes that regulate the cell growth, proliferation and development such as ID1, ID3, JARID2, KLF6, SIX1 and KRT9 were up-

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regulated in the early phase. Among these genes, the expressions of ID1 and ID3 belonging to the member of helix-loop-helix proteins that inhibit the transcription by forming heterodimer with specific transcription factors were studied using RT-PCR. We also investigated the expression of KLF6 gene whose function is the regulation of transcription in several pathways. As illustrated in figure 14, the level of KLF6, and ID1 gene expression was significantly altered at 60 minutes but not at 30 minutes after VR-3848 drug treatment.

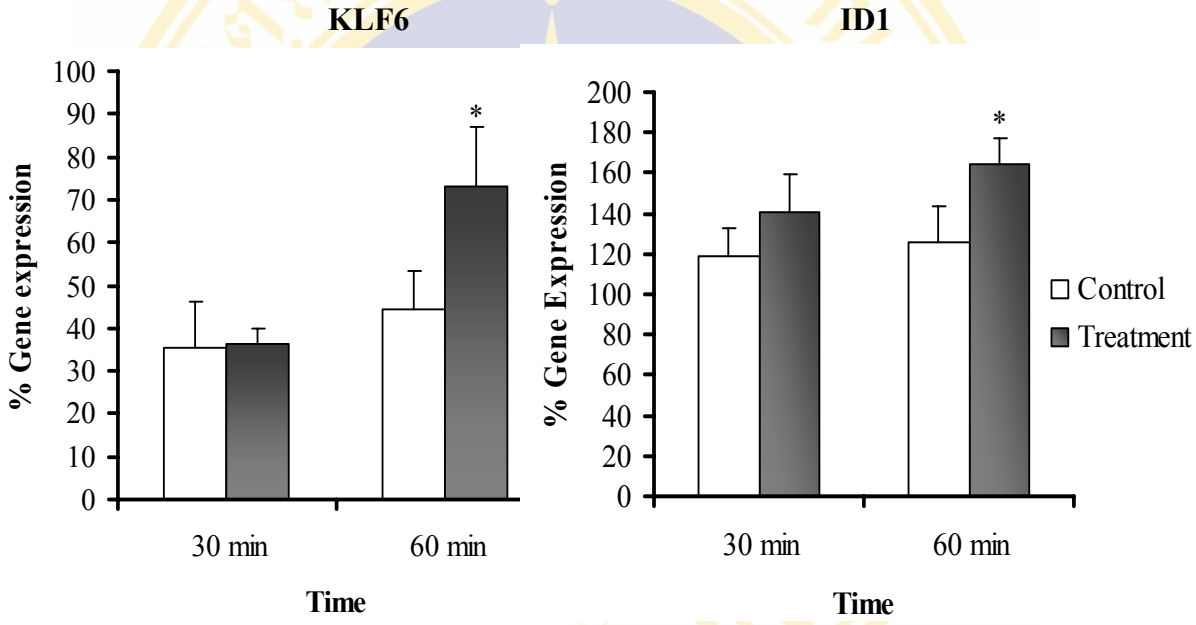

Figure 14. The up-regulation of KLF6 and ID1expressions was detected by using RT-PCR. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

Transport genes

The expression of four genes involves in cellular transportation, electron transport gene such as CYP1B1, cholesterol transport gene such as NPC1 and intracellular membrane trafficking genes such as STX17 and TOM1L2 were upregulated. Among these genes, expression of CYP1B1 was confirmed by RT-PCR method. As demonstrated in figure 15, the level of CYP1B1 gene expression was strongly increased at 60 minutes after treatment.

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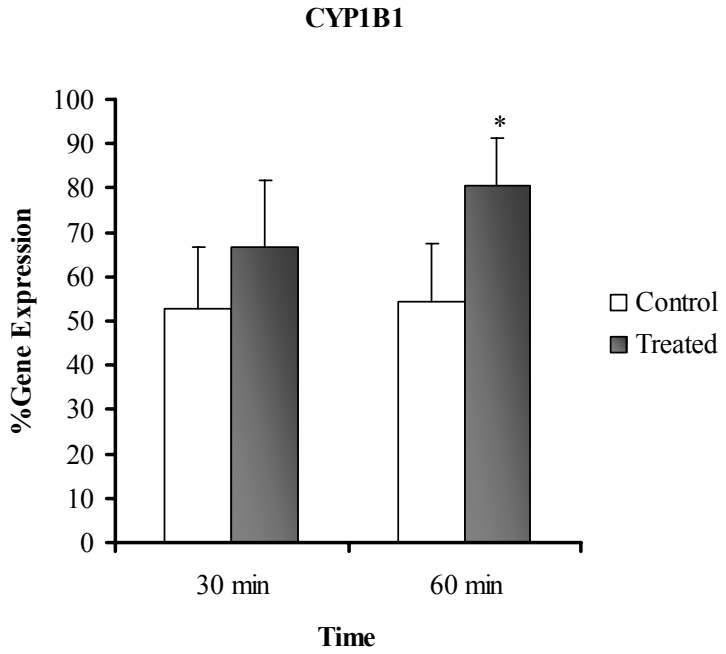

Figure 15. The up-regulation of CYP1B1 gene expression was monitored by RT-PCR. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

Apoptosis gene

TNFRSF12A belonging to a member of tumor necrosis factor family involved in receptor mediated apoptosis pathway was up-regulated during VR-3848 treatment at 60 minutes. As shown in figure 16, the significant up-regulation of TNFRSF12A gene was demonstrated at 1 hour but not 30 minutes of treatment using RT-PCR method.

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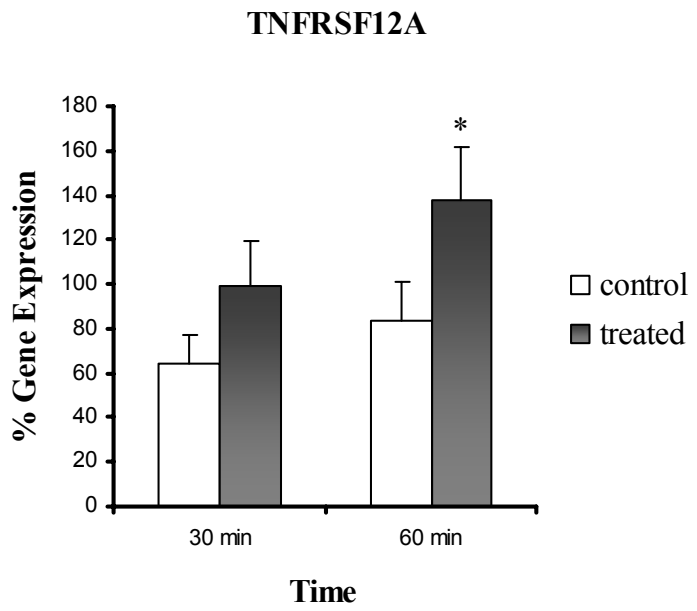

Figure 16. The up-regulation of TNFRSF12A gene expression was monitored by RT-PCR. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

Stress response genes

During early treatment, the expression of stress responsive genes such as DDIT3 which is also known as GADD153 and SNARK were increased. GADD153 has been known to be up-regulated during ER-stress response. It mediates the apoptosis pathway by inhibiting the anti-apoptosis genes i.e. Bcl-2 and Bcl-X_L. The expression of SNARK gene is generally regulated by ER and cellular stress pathway. To confirm the up-regulation of these genes, expression levels of GADD153 and SNARK were studied using RT-PCR as shown in figure 17. The result showed that the stress response genes, GADD153 and SNARK were significantly stimulated at 1 hour after treatment.

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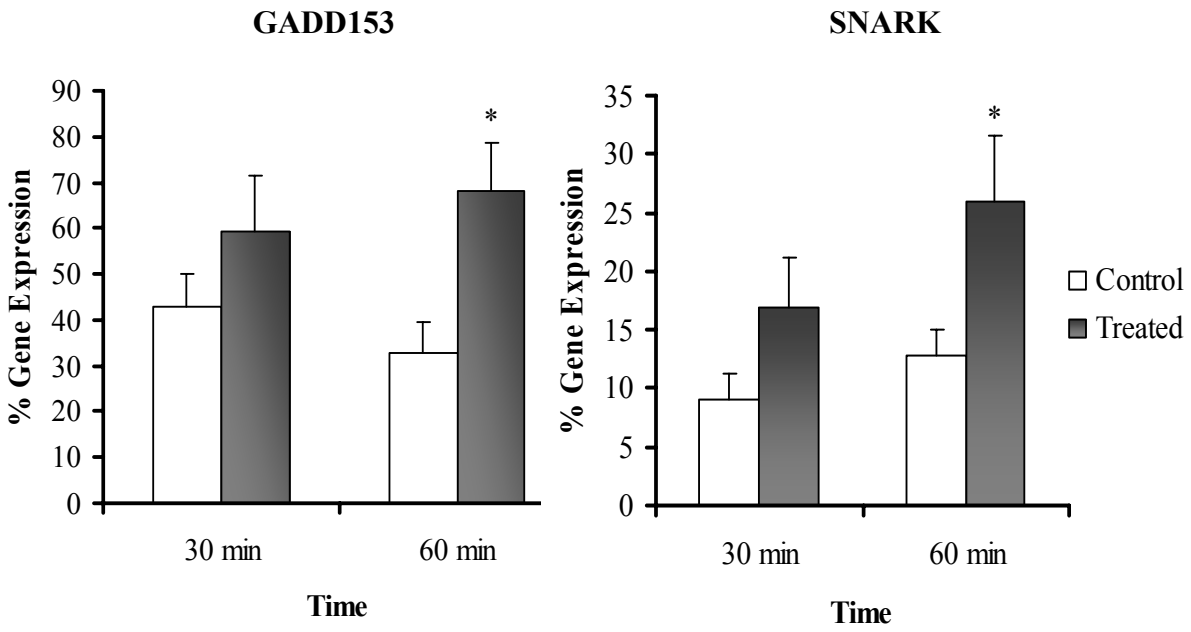

Figure 17. The up-regulation of GADD153 and SNARK gene expressions was measured by RT-PCR. Results are mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the VR-3848 and untreated groups of indicating time points using paired t-test.

3. Validation of global gene expression profile using RT-PCR.

As demonstrated in figure 8 and 9, VR-3848 induced apoptosis in MCF-7 cells. Up-regulated apoptotic genes and suppressed cell cycle genes were shown in table 13-16 suggested that various apoptotic pathways were stimulated during VR-3848 treatment. The examples of these pathways are

1. The receptor-ligand mediated apoptosis pathway, in which VR-3848 stimulated up-regulation of death ligands, receptors and their cascade genes such as TNFRSF6, 9, 10B, 12A, INHBA, ADORA2A, and CASP9 and down-regulation of apoptotic inhibitor such as API5 and AVEN.

2. Mitochondria mediated apoptosis pathway, in which VR-3848 stimulated up-regulation of pro-apoptotic genes such as BCL2L13 and BIK and down-regulation of anti-apoptotic genes such as BAG3 and 5.

3. ER-stress mediated pathway, in which stress responsive genes such as AHR, GADD45A, B, and G, ATF3 and 4, and PERK were up-regulated during VR-3848 treatment.

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4. Cell cycle arrest, in which VR-3848 inhibited cell cycle genes such as ANAPC2, 5, and 11, CDK4, and 5, MCM5, and 6, and activated cell cycle inhibitor genes such as CDKN1B (p27, Kip1), 1C (p57, Kip2), and 2D (p19).

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Table 13. List of receptor mediated and apoptosis mediator genes at indicating time points after VR-3848 treatment. The difference of gene expressions are shown in log 2 scale

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
ADORA2A	Adenosine A2a receptor	-	-	2.43 ± 0.04	3.43 ± 0.06	3.78 ± 0.09
API5	Apoptosis inhibitor 5	-	-	-	-	-1.09 ± 0.08
AVEN	Apoptosis, caspase activation inhibitor	-	-	-	-	-1.62 ± 0.10
CASP6	Caspase 6, apoptosis-related cysteine protease	-	-	-	-2.19 ± 0.41	-2.65 ± 0.36
CASP9	Caspase 9, apoptosis-related cysteine protease	-	-	1.58 ± 0.00	1.65 ± 0.23	-
CCL2	Chemokine (C-C motif) ligand 2	-	3.67 ± 0.09	4.40 ± 0.36	4.17 ± 0.09	5.11 ± 0.07
CFLAR	CASP8 and FADD-like apoptosis regulator	-	-	1.08 ± 0.13	2.11 ± 0.35	1.98 ± 0.02
CGREF1	Cell growth regulator with EF hand domain 1	-	-	-	-1.15 ± 0.18	-
CIDEC	Cell death activator CIDE-3	-	-	-	-	1.26 ± 0.03
CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	-	-	-	1.06 ± 0.55	1.14 ± 0.05
CTNNBL1	Catenin, beta like 1	-	-	-	-1.22 ± 0.12	-1.19 ± 0.16
DAPK2	Death-associated protein kinase 2	-	-	-	-	1.89 ± 0.09
DAPK3	Death-associated protein kinase 3	-	-	-	1.08 ± 0.20	1.12 ± 0.08
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	-	-	-	-	-1.38 ± 0.23
DUSP1	Dual specificity phosphatase 1	-	-	-	2.05 ± 0.60	1.42 ± 0.04
EGLN3	Egl nine homolog 3 (C elegans)	-	-	-	-1.33 ± 0.17	-1.56 ± 0.28
ELMO3	Engulfment and cell motility 3 (ced-12 homolog, C elegans)	-	-	-	-1.68 ± 0.12	-2.22 ± 0.03
EP300	E1A binding protein p300	-	-	-	1.38 ± 0.31	1.34 ± 0.14

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Table 13. List of receptor mediated and apoptosis mediator genes at indicating time points after VR-3848 treatment. The difference of gene expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment					
		1hr	3hr	6hr	12hr	18hr	
FADD	Fas (TNFRSF6)-associated via death domain	-	-	-	-1.39 ± 0.26	-1.20 ± 0.17	
FAIM	Fas apoptotic inhibitory molecule	-	-	-	-1.26 ± 0.10	-1.23 ± 0.31	
HD	Huntingtin (Huntington disease)	-	-	-	-1.19 ± 0.19	-1.12 ± 0.30	
IER3	Immediate early response 3	-	-	1.54 ± 0.06	1.62 ± 0.11	1.08 ± 0.03	
IGF1R	Insulin-like growth factor 1 receptor	-	-	-	-	-1.47 ± 0.13	
INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	-	1.56 ± 0.40	2.68 ± 0.07	4.01 ± 0.14	3.28 ± 0.31	
MAP2K5	Mitogen-activated protein kinase kinase 5	-	-	-	-1.18 ± 0.16	-1.02 ± 0.55	
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	-	1.64 ± 0.76	1.99 ± 0.24	2.93 ± 0.23	2.76 ± 0.04	
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.22 ± 0.41	2.33 ± 0.31	2.22 ± 0.30	2.30 ± 0.31	2.40 ± 0.15	
NOTCH2	Notch homolog 2 (Drosophila)	-	-	-	1.08 ± 0.28	1.91 ± 0.10	
PAWR	PRKC, apoptosis, WT1, regulator	-	-	-	1.23 ± 0.61	1.26 ± 0.08	
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	-	-	-	1.33 ± 0.03	1.81 ± 0.35	
PRKR	Protein kinase, interferon-inducible double stranded RNA dependent	-	-	-	-	-1.10 ± 0.10	
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	-	-	-	-1.27 ± 0.34	-1.31 ± 0.44	
RIPK2	Receptor-interacting serine-threonine kinase 2	-	-	1.96 ± 0.13	2.65 ± 0.21	2.90 ± 0.32	
RRAGC	Ras-related GTP binding C	-	-	-	1.05 ± 0.41	1.29 ± 0.53	

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Table 13. List of receptor mediated and apoptosis mediator genes at indicating time points after VR-3848 treatment. The difference of gene expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
SULF1	Sulfatase 1 (SULF1)	-	-	1.26 ± 0.28	1.35 ± 0.32	1.04 ± 0.47
TNF	Tumor necrosis factor (TNF superfamily, member 2)	-	-	-	-	1.25 ± 0.81
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	-	-	1.90 ± 0.10	1.90 ± 0.48	2.31 ± 0.36
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	1.38 ± 0.22	-	1.20 ± 0.16	1.86 ± 0.05	2.03 ± 0.16
TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	-	-	-	-	1.03 ± 0.10
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	-	-	-	1.50 ± 0.53	2.04 ± 0.03
TRAF1	TNF receptor-associated factor 1	-	-	2.10 ± 0.22	3.87 ± 0.55	-
TRAF2	TNF receptor-associated factor 2	1.07 ± 0.85	-	-	-	1.34 ± 0.22
TRAF3	TNF receptor-associated factor 3	-0.23 ± 0.12	-	1.41 ± 0.07	1.76 ± 0.33	2.21 ± 0.44
TRAF4	TNF receptor-associated factor 4	-	-	-	-	1.10 ± 0.16
TRAF5	TNF receptor-associated factor 5	-	-	-	-1.66 ± 0.06	-1.78 ± 0.11
TRIB3	tribbles homolog 3 (Drosophila)	-	-	-1.08 ± 0.04	-2.17 ± 0.39	-1.99 ± 0.08

(-) means no alteration of gene expression, (- x) means gene expression was suppressed.

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Table 14. List of mitochondrial- mediated apoptosis at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
BAG3	BCL2-associated athanogene 3	-	-	-	-1.61 ± 0.33	-1.76 ± 0.06
BAG5	BCL2-associated athanogene 5	-	-	-	-1.31 ± 0.09	-1.54 ± 0.07
BAK1	BCL2-antagonist/killer 1	-	-	-	-1.53 ± 0.51	-1.55 ± 0.13
BAX	BCL2-associated X protein	-	-	-	-1.58 ± 0.26	-1.90 ± 0.35
BCL2L13	BCL2-like 13 (apoptosis facilitator)	-	-	-	-	1.05 ± 0.02
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	-	-	-	-1.77 ± 0.18	-1.54 ± 0.21
FOXO1A	forkhead box O1A (rhabdomyosarcoma)	-	-	-	1.37 ± 0.86	1.52 ± 0.64
FOXO3A	forkhead box O3A	-	-	1.09 ± 0.82	-	1.41 ± 0.66
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	-	1.30 ± 0.40	2.44 ± 0.13	2.48 ± 0.30	-

(-) means no alteration of gene expression, (- x) means gene expression was suppressed.

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Table 15. List of cellular and ER stress related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale

GENE		The fold change difference in each time points after VR-3848 treatment					
		1hr	3hr	6hr	12hr	18hr	
AHR	Aryl hydrocarbon receptor	-	-	1.19 ± 0.17	-	1.35 ± 0.21	
ATF3	Activating transcription factor 3	1.07 ± 0.44	-	5.15 ± 0.23	6.03 ± 0.77	5.47 ± 0.15	
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)	-	1.00 ± 1.11	-	1.21 ± 0.01	1.56 ± 0.03	
ERN1	ER to nucleus signalling 1	-	-	-	-	1.33 ± 0.08	
GADD45A	Growth arrest and DNA-damage-inducible, alpha	-	1.89 ± 1.34	2.30 ± 0.11	2.85 ± 0.29	2.32 ± 0.02	
GADD45B	Growth arrest and DNA-damage-inducible, beta	-	1.84 ± 0.72	2.13 ± 0.05	2.98 ± 0.42	2.79 ± 0.09	
GADD45G	Growth arrest and DNA-damage-inducible, gamma	-	-	-	1.61 ± 0.59	2.56 ± 0.05	
GSTA4	Glutathione S-transferase A4	-	-	-	-1.20 ± 0.26	-	
HIG2	Hypoxia-inducible protein 2	-	-	-1.13 ± 0.06	-1.89 ± 0.05	-1.71 ± 0.06	
HRI	Heme-regulated initiation factor 2-alpha kinase	-	-	-	-1.24 ± 0.01	-1.60 ± 0.18	
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	-	-0.17 ± 0.08	-	1.26 ± 0.28	1.48 ± 0.21	
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3	-	-	-	-1.38 ± 0.05	-1.24 ± 0.10	
MICA	MHC class I polypeptide-related sequence A	-	-	-	-1.14 ± 0.06	-1.50 ± 0.07	
MIG-6	Mitogen-inducible gene 6	-	-	1.37 ± 0.22	1.45 ± 0.42	-	

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Table 15. List of cellular and ER stress related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3	-	1.08 ± 0.73	1.35 ± 0.43	1.04 ± 0.56	1.15 ± 0.08
SERPINH1	Serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	-	-	-	-1.77 ± 0.24	-2.34 ± 0.21
SGK	Serum/glucocorticoid regulated kinase	-	-	-	1.79 ± 0.64	-
SUI1	Putative translation initiation factor	-	-	-	1.09 ± 0.10	1.24 ± 0.25
TAOK2	TAO kinase 2	-	-	-	-1.20 ± 0.13	-1.06 ± 0.23

(-) means no alteration of gene expression, (- x) means gene expression was suppressed.

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Table 16. List of cell cycle related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale

GENE		The fold change difference in each time points after VR-3848 treatment					
		1hr	3hr	6hr	12hr	18hr	
ACPP	Acid phosphatase, prostate	-	-	1.16 ± 0.02	1.56 ± 0.11	1.19 ± 0.31	
ANAPC11	Anaphase promoting complex subunit 11	-	-	-1.53 ± 0.38	-2.84 ± 0.01	-2.99 ± 0.20	
ANAPC2	Anaphase promoting complex subunit 2	-	-	-	-1.24 ± 0.17	-1.21 ± 0.36	
ANAPC5	Anaphase promoting complex subunit 5	-	-	-	-	-1.27 ± 0.03	
ATR	Ataxia telangiectasia and Rad3 related	-	-	-	-1.11 ± 0.05	-1.00 ± 0.17	
BCL3	B-cell CLL/lymphoma 3		1.32 ± 0.35	2.25 ± 0.09	2.49 ± 0.20	2.92 ± 0.13	
BTG3	BTG family, member 3	-	1.46 ± 0.65	1.46 ± 0.12	2.49 ± 0.39	2.25 ± 0.37	
CABLES2	Cdk5 and Abl enzyme substrate 2		-	-	-2.21 ± 0.12	-2.49 ± 0.08	
CCNG2	cyclin G2	-	-	-	0.31 ± 0.96	1.13 ± 0.08	
CCNT2	cyclin T2		1.16 ± 0.81	1.45 ± 0.51	1.43 ± 0.51	2.10 ± 0.27	
CDC2	cell division cycle 2, G1 to S and G2 to M	-	-	-	-	1.02 ± 0.15	
CDC25B	cell division cycle 25B	-	-	-	-	1.03 ± 0.24	
CDC45L	CDC45 cell division cycle 45-like (S cerevisiae)	-	-	-	-	-1.20 ± 0.18	
CDK2AP1	CDK2-associated protein 1	-	-	-	-1.32 ± 0.39	-1.21 ± 0.01	
CDK4	cyclin-dependent kinase 4	-	-	-	-1.02 ± 0.31	-1.28 ± 0.20	
CDK5	cyclin-dependent kinase 5	-	-	-	-	-1.47 ± 0.04	
CDK6	cyclin-dependent kinase 6	-	-	-	1.21 ± 0.10	1.41 ± 0.05	
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-	-	-	-	1.20 ± 0.05	
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	-	-	-	1.79 ± 0.02	1.27 ± 0.07	
CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	-	-	-	-	1.43 ± 0.08	
CHAF1A	chromatin assembly factor 1, subunit A (p150)	-	-	-	-	-1.34 ± 0.01	

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Table 16. List of cell cycle related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
CLK1	CDC-like kinase 1	-	1.62 ± 0.94	1.64 ± 0.35	2.36 ± 0.49	2.53 ± 0.24
CNAP1	chromosome condensation-related SMC-associated protein 1	-	-	-	-	1.23 ± 0.05
CUL4A	cullin 4A	-	-	-	-1.57 ± 0.12	-1.71 ± 0.06
DKC1	dyskeratosis congenita 1, dyskerin	-	-	-	-1.13 ± 0.02	-1.68 ± 0.30
ESPL1	extra spindle poles like 1 (S cerevisiae)	-	1.40 ± 1.71			1.22 ± 0.36
FRK	fyn-related kinase	-	0.23 ± 0.30	-	-	1.97 ± 0.11
FZR1	fizzy/cell division cycle 20 related 1 (Drosophila)	-	-	-	-	-1.26 ± 0.04
GPS1	G protein pathway suppressor 1	-	-	-	-1.20 ± 0.57	-1.64 ± 0.03
GSPT1	G1 to S phase transition 1	-	-	-	-1.33 ± 0.23	-1.54 ± 0.03
HCAP-G	chromosome condensation protein G	-	1.25 ± 1.25	1.11 ± 0.01	1.66 ± 0.37	2.41 ± 0.03
HDAC4	histone deacetylase 4	-	-	-	-1.62 ± 0.29	-1.43 ± 0.24
HDAC5	histone deacetylase 5	-	-	-		1.02 ± 0.09
HDAC7A	histone deacetylase 7A	-	-	-	-1.57 ± 0.27	-1.57 ± 0.04
IFITM1	interferon induced transmembrane protein 1 (9-27)	-	-	-	-1.14 ± 0.20	-1.27 ± 0.08
ING1	Inhibitor of growth family, member1	-	1.07 ± 0.12	1.47 ± 0.12	2.36 ± 0.18	1.85 ± 0.13
JAG2	jagged 2	-	-	-	-1.95 ± 0.30	-1.69 ± 0.21
MACF1	microtubule-actin crosslinking factor 1	-	-	-	-1.22 ± 0.30	-1.39 ± 0.01
MAPK13	mitogen-activated protein kinase 13	-	-	-	-1.03 ± 0.04	-1.18 ± 0.15
MAPK3	mitogen-activated protein kinase 3	-	-	-	-1.15 ± 0.48	-1.41 ± 0.08
MAPK6	mitogen-activated protein kinase 6	-	-	-	-	1.00 ± 0.08
MATK	megakaryocyte-associated tyrosine kinase	-	-	-	-1.79 ± 0.83	-1.83 ± 0.03

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Table 16. List of cell cycle related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment					
		1hr	3hr	6hr	12hr	18hr	
MCM5	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S cerevisiae)	-	-	-	-1.59 ± 0.08	-2.25 ± 0.12	
MCM6	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S pombe) (S. cerevisiae)	-	-	-	-1.07 ± 0.40	-1.60 ± 0.25	
MIS12	log of yeast Mis12	-	-	-	1.04 ± 0.34		
MRPL41	mitochondrial ribosomal protein L41	-	-	-		-1.07 ± 0.03	
MSF	MLL septin-like fusion	-	-	-	-1.39 ± 0.16	-1.60 ± 0.01	
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E coli)	-	-	-	-2.07 ± 0.15	-2.60 ± 0.25	
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	-	-	1.12 ± 0.27	1.09 ± 0.46	-	
NDN	necdin homolog (mouse)	-	-	-	-	-1.30 ± 0.19	
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	-	1.57 ± 1.42	2.52 ± 0.09	-	1.70 ± 0.13	
NEK9	NIMA (never in mitosis gene a)-related kinase 9 [	-	-	-	-	1.34 ± 0.01	
NUDC	nuclear distribution gene C homolog (A nidulans)	-	-	-	-	-1.09 ± 0.11	
NUDT6	nudix (nucleoside diphosphate linked moiety X)-type motif 6	-	-	-	-	-1.33 ± 0.28	
PCBP4	poly(rC) binding protein 4	-	-	-	-1.33 ± 0.30	-1.66 ± 0.10	
PCNA	proliferating cell nuclear antigen	-	1.09 ± 0.96		-1.03 ± 0.29	-1.29 ± 0.10	
PLK3	polo-like kinase 3 (Drosophila)	1.04 ± 0.10	1.03 ± 0.00	1.60 ± 0.23	2.32 ± 0.10	2.53 ± 0.17	
PPM1D	protein phosphatase 1D magnesium-dependent, delta isoform	-	1.18 ± 0.68	1.12 ± 0.35	1.17 ± 0.50	1.58 ± 0.58	

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Table 16. List of cell cycle related genes at indicating time points after VR-3848 treatment. The different expressions are shown in log 2 scale (continued)

GENE		The fold change difference in each time points after VR-3848 treatment				
		1hr	3hr	6hr	12hr	18hr
PR48	protein phosphatase 2A 48 kDa regulatory subunit	-	-	-	-1.32 ± 0.29	-1.73 ± 0.09
PRKCA	protein kinase C, alpha	-	-	-	-1.59 ± 0.19	-
RASSF1	Ras association (RalGDS/AF-6) domain family 1	-	-	1.25 ± 0.29	1.77 ± 0.48	-
RBM5	RNA binding motif protein 5	-	-	-	-	1.55 ± 0.21
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	-	-1.16 ± 0.40	-	-	-1.23 ± 0.05
S100A6	S100 calcium binding protein A6 (calcyclin)	-	-	-	-	1.21 ± 0.04
SFN	stratifin	-	-	-	-1.64 ± 0.41	-1.71 ± 0.13
SIPA1	signal-induced proliferation-associated gene 1	-	-	-	-1.47 ± 0.07	-1.06 ± 0.06
SMC1L1	SMC1 structural maintenance of chromosomes 1-like 1 (yeast)	-	-	-	-1.32 ± 0.49	-1.95 ± 0.15
SPIN2	spindlin family, member 2	-	-	-1.10 ± 0.27	-1.95 ± 0.05	-2.15 ± 0.11
STAT1	signal transducer and activator of transcription 1, 91kDa	-	-	-	-	1.70 ± 0.26
STRN3	striatin, calmodulin binding protein 3	1.18 ± 0.09	-	-	1.24 ± 0.06	1.18 ± 0.03
TFDP1	transcription factor Dp-1					-1.23 ± 0.01
TLK1	tousled-like kinase 1	-	-1.31 ± 1.31	-	-	1.13 ± 0.59
TLK2	tousled-like kinase 2					1.53 ± 0.16
TUSC4	tumor suppressor candidate 4	-	-	-1.07 ± 0.14	-2.44 ± 0.26	-2.45 ± 0.13
VEGF	vascular endothelial growth factor	-	-	-	-	1.11 ± 0.00
VEGFB	vascular endothelial growth factor B	-	-	-	-	-1.39 ± 0.20
VEGFC	vascular endothelial growth factor C	-	-	-	2.00 ± 0.40	2.57 ± 0.02
WEE1	WEE1 homolog (S pombe)	-	-	1.25 ± 0.31	1.12 ± 0.49	1.25 ± 0.12

(-) means no alteration of gene expression, (- x) means gene expression was suppressed.

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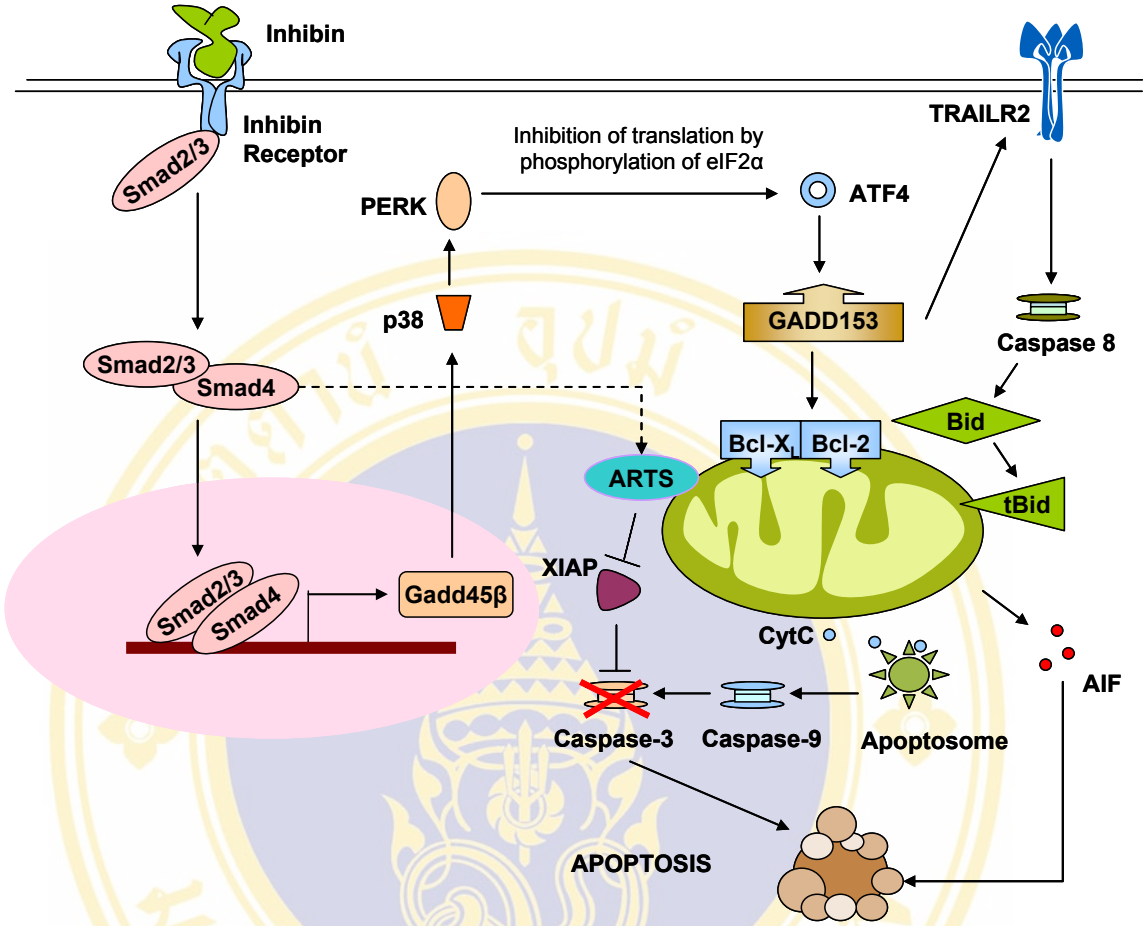

Figure 18. The inhibin-mediated apoptosis pathway modified from Sanchez-Capelo et. al (65). The arrow line indicates the stimulatory signaling, T-shape line indicates the inhibitory signaling, and dash line indicates tentative stimulatory signaling.

We then selected one of these pathways which is inhibin mediated apoptosis pathway for validation. This pathway was drawn in diagram as shown in figure 18. This diagram is modified from the pathway reported by Sanchez-Capelo et. al (65). Inhibin is a member of transforming growth factor beta family (TGF-β). It acts on the target via autocrine or paracrine by binding to specific receptor that shares homology with the TGF-β superfamily receptor. This binding triggers a signaling cascade mediated by a complex of intracellular Smad 2 and 3 proteins which then bind to Smad 4 and translocate into nucleus. This complex stimulates the expression of inhibin responsive genes such as GADD45β. The phosphorylation of p38 as a consequence of GADD45β stimulation induces the oligomerization and

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phosphorylation of PERK and subsequently transcription of ATF4 gene. ATF4 gene expression triggers the up-regulation of GADD153 resulting in activation of extrinsic and intrinsic apoptosis pathway. For extrinsic pathway, GADD153 triggers the TRAILR2 (also called TNFRSF10B) expression (66-68). The activation of TRAILR2 subsequently stimulated the caspase-8 and other caspases resulting in cell death. For intrinsic pathway, GADD153 causes down-regulation of anti-apoptotic molecules, Bcl-2 and Bcl-X_L that ultimately leads to cell death.

The pro-apoptotic protein, ARTS, is stimulated by TGF-β signaling. During the activation, ARTS releases from mitochondia and suppresses the level of inhibitor of apoptosis (XIAP) thereby promoting caspase-3 activation and apoptosis (69, 70). Since the MCF-7 cell is caspase-3 deficiency, the induction of apoptosis via caspase-3 dependent pathway could be inhibited.

To verify that this pathway is one of apoptotic mechanism induced by VR-3848, the expression level of the following genes, ATF4, ARTS, GADD45β, and INHBA were detected by RT-PCR.

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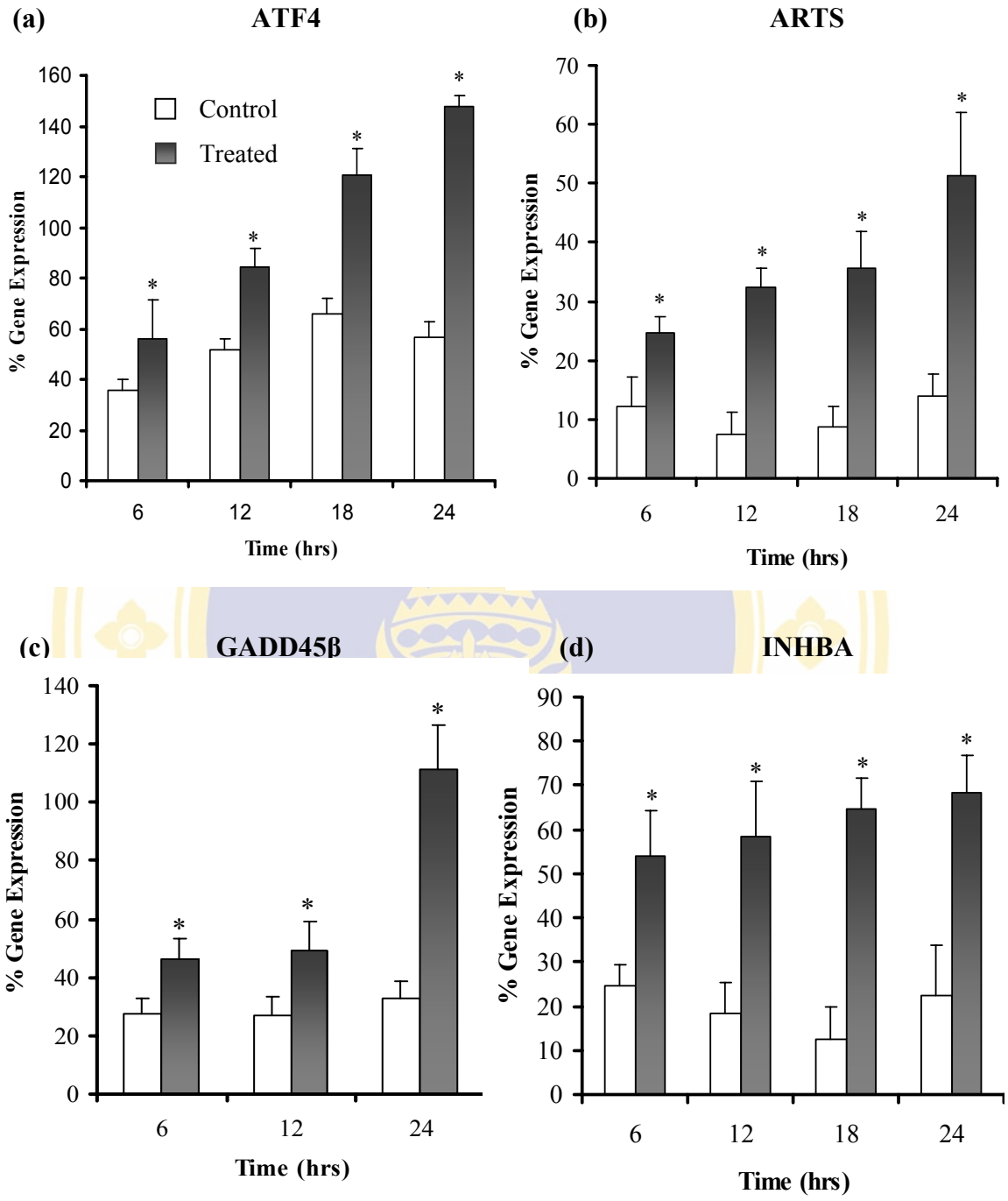

Figure 19. The expression levels of ER stress responsive genes which were (a) ATF4, (b) ARTS, (c) GADD45β and (d) INHBA during the VR-3848 treatment were detected by RT-PCR. Results were mean ± SE from three independent experiments. (*) demonstrates the significant difference at p-value <0.05 between the control and treatment groups in particular time points using paired t-test.

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As illustrated in figure 19, ARTS, AFT4, GADD45β, and INHBA were strongly stimulated in response to VR-3848 stimulation. The data confirm DNA array screening and indicated that the inhibin signaling may be one of the mechanisms that VR-3848 induced apoptosis in MCF-7 cells.

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CHAPTER 5
DISCUSSION

VR-3848, a 7-mer cycloheptapeptide, is novel compound extracted from tropical Thai plant *Euphobiaceae.* This compound exerts the growth inhibitory in caspase-3 deficient breast cancer MCF-7 cell line. The presence of apoptotic characteristics with chromatin condensation and nuclear fragmentation suggested that the VR-3848 treated MCF-7 cell dies by apoptosis. The previous studies demonstrated that VR-3848 kills the cancerous cells at nanomolar concentration depending on cell type. VR-3848 has a 10-fold higher efficiency on non small lung cancer cell LU-1 while it has 5-fold less active in breast cancer MCF-7 cell line than vinblastine (1, 6). This result suggested that the target of VR-3848 and vinblastine may be difference. From these efficiency and novelty, it is worth finding the target and anticancer mechanism of VR-3848.

The partial mechanism of VR-3848 induced MCF-7 cell death was first elucidated by Jarin Kramyu, a Master degree student in our group. Jarin screened apoptotic gene expression during VR-3848 treatment using cDNA macroarray analysis. His investigation suggested that at least three modes of apoptosis which are receptor-mediated death, mitochondrial mediated but caspase-independent death, and ER-stress induced death involve in VR-3848 stimulated MCF-7 cell death (6). However, the apoptotic pathway mentioned above may be a late stage in response; therefore, the VR-3848 responsive genes are remained to be investigated.

From DNA microarray analysis, we found that the molecular response to VR-3848 treatment was complex and seemed to be mediated by various pathways. The dynamic expression pattern of 4,075 significant altered genes was analyzed by using k-means clustering. This result revealed 6 groups based on similarity of expression pattern which were (1) greatly down-regulation, (2) gradually down-regulation, (3) transient up-regulation at the early phase and then down-regulation afterward, (4) greatly up-regulation, (5) transiently down-regulation at the early phase of treatment, and (6) gradually up-regulation. We first considered the alteration of genes in cluster 1

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and 4 since they seem to be dramatically significant alteration during VR-3848 treatment. For down-regulated genes in the cluster 1, VR-3848 suppresses the expression of genes that function in metabolism such as adenylate cyclase genes (ADCY1 and 9), mitochondrial ribosomal protein (MRPL12, 16, 19, and 34), regulation of cell proliferation genes such as CXCL16, BZRP, CIP29, GATA2, PJA1, and SMA3, anti-apoptosis genes such as ALS2CR2, BNIP2, BAG3, SEMA4D, and SPIN2, development genes such as BMP4, FGFR2, PAFAH1B3, SPDEF, and also transcription and translation genes such as EIF4EBP1, HRSP12, LHX1 and 2. Among these genes, we found that VR-3848 have an impact on metabolism genes the most. In contrast, genes that were activated the most in cluster 4 were genes involving in apoptosis, cell cycle arrest, negative regulation of cell proliferation, and signal transduction and transcription and translation. These data indicate that VR-3848 induced the cell apoptosis by down-regulated genes that control the normal cellular machinery together with up-regulated genes that play a negative role in cellular proliferation.

At the early stage of treatment, VR-3848 up-regulated several biological functions including transcription and translation, signal transduction, cell growth and development, cell cycle, stress response, apoptosis, and phosphorylation. For a large number of genes reported in this screening, their relevance to VR-3848 induced cell death is unclear. However, the altered expression of several genes with known functions and their associated biological processes are conspicuous. Since apoptosis is a key phenomenon in our system, therefore, genes from each group that are involved in apoptosis are selected for discussion.

Transcription and translation genes are major groups that early responds to VR-3848. Among these genes, only SRCAP gene is significantly increased at 30 minutes after treatment, the earliest time point of our study. This gene serves as the transcriptional coactivator for several transcriptional factors interacting with CBP (cAMP responsive element binding protein) in steroid mediated pathway (71, 72). The structure of SRCAP is homology to EP400 that can form large complexes to NCOR1, SMARCA4, SMARCC1, SMARCC2, SMARCB1 and cyclin dependent kinase inhibitor 3 (CDKN3) (73). This observation suggested that the VR-3848 inhibited MCF-7 cell proliferation may involve SRCAP mediated signaling pathway. To prove

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this notion, blocking of SRCAP during VR-3848 treatment would be required. VR-3848 is a cyclic compound, whether it can interact to steroid superfamily receptor and mediates death through this pathway would be investigated.

Likewise, at the early stage of treatment, VR-3848 stimulated the expression of other transcription factors. Most of these have been reported in the anti-proliferative activity. The bHLH transcription factor superfamily, HES1, is known to inhibit the proliferation of neuronal cell (74) and MCF-7 breast cancer cell (75). Similarly, ID1 and ID3 function as a negative regulator for transcription in wide range of cellular processes. The interaction of ID proteins to several bHLH transcription factors prevents the transcriptional activity (76). In addition, Krupple like factors, KLF10 and KLF6 belonging to zinc finger transcription induce apoptosis in various cancerous cells. (77-79). KLF10 which is also called TIEG (TGF-β inducible early gene) mediates apoptotic death via transforming growth factor (TGF-β) and Smad signaling (56, 80). Also, KLF6 suppresses cell growth through the inhibition of cyclin D1/Cdk4 during G1 phase of cell cycle (81). Other genes such as JARID2, ZFP36 and EGR4 also participate in negative regulation of cell proliferation (82-85). These data suggested that the anti-proliferation mechanism of VR-3848 involves the activation of genes which block the process of transcription and translation.

Additionally, VR-3848 also stimulated the up-regulation of RASD1 and SSTR1 involving in G-protein coupled receptor signaling. The Ras related protein, RASD1, and the somatostatin receptor, SSTR1 have been studied as anti-growth in breast cancer cells (86, 87). The results suggested that these genes may regulate the anti-proliferative effects in MCF-7 cell during VR-3848 treatment.

VR-3848 stimulates the cytochrome P450 1B1 (CYP1B1) at the early time point. The CYP1B1, a heme-containing monoxygenase enzyme, functions not only in electron transferring but also as a xenobiotic metabolizing enzyme including carcinogen, plant toxin and a variety of anti-cancer drugs. Some of anti-cancer drugs such as Reveratrol, Aryl oxime prodrug and Phortress require the CYP1B1 activation that converts these prodrugs to anti-cancer metabolite or nitric oxide production leading to induce cell death (88). The significantly increasing expression of CYP1B1 suggested that the anti-cancer activity of VR-3848 may be regulated by this drug metabolizing enzyme.

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Stress responsive genes including DDIT3 (also known as GADD153 or CHOP) and SNARK, a member of the AMPK subfamily of serine/threonine protein kinases were activated at the early stage of treatment. The induction of these genes proposed that the VR-3848 treatment may mediate the MCF-7 cell death through ER-stress signaling pathway. The activation of GADD153 induced cell death has been reported in other compounds such as Celecoxib, a selective cyclooxygenase2 inhibitor, and Dihydroxyphenylethanol, a phenol antioxidant derived from olive oil (89, 90).

TNFRSF12A, a member of tumor necrosis factor (TNF) receptor superfamily was stimulated at the early response to VR-3848. TNFRSF12A which is also known as fibroblast growth factor inducible 14 (Fn14) is responsible for various cellular functions including cell proliferation, differentiation and death (91). TNFRSF12A mediated the multiple death pathways including caspase dependent and indirect effected via endogenous TNF production in the TWEAK, the TNFRSF12A ligand, treatment (92). In this study, the increasing level of TNFRSF12A expression at 1 hr of treatment suggested that the receptor mediated apoptosis pathway might be the early signaling of VR-3848 induced cell death.

At the early stage of treatment, VR-3848 stimulates gene involved in other functions such as cytoskeleton, ubiquitination, mRNA stability, and protein binding. These genes are ACN9, ANK1B1, ANK3, BRD2, CD46, FUSIP1, H2AFJ, MPP6, MT1X, PLK3, PRSS16, PSMC2, STRN3, TOB2, TRIP12, UBE2E1, and ZFP36. However, the exact functions of these genes in breast cancer are still unclear. Therefore, the activity of these genes in this system needs to be further explored.

From the previous study, cDNA macroarray analysis reveals that three possible mechanisms of VR-3848 induced cell death are receptor mediated cell death, mitochondrial mediated but caspase independent cell death and ER stress mediated cell death. From microarray data analysis, several receptor-mediated apoptotic genes which were ADORA2A, inhibin beta A, RIPK2, TNFRSF10B (TRAILR2), and TNFRSF12A were up-regulated during VR-3848 treatment. Among these genes, TNF receptors are widely studied in regulation of apoptotic pathway. In general, these receptors interact with their specific ligand. This complex then recruits and activates the initiator caspase-8 and -10. These caspases directly activate downstream caspases or induce the mitochondria pathway leading to death of cell.

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Inhibin, a member of transforming growth factor beta (TGF-β) superfamily, has diverse functions which are differentiation and death. Inhibin acts on it target cells via autocrine and paracrine. By binding to specific receptor, inhibin activates the intracellular Smad proteins. These molecules recruit co-smad protein and translocate into nucleus leading to induce the inhibin responsive gene expressions. These responsive genes mediate death signaling via caspase dependent and ER stress induced cell death pathways. It has been studied that inhibin is able to suppress breast cancer cell proliferation (93, 94). The strong expression of inhibin in VR-3848 treated cell suggested that inhibin may play an important role in VR-3848 stimulated MCF-7 cell death. Several apoptotic mediator genes such as DAPK2 and -3, SULF1, TRAF1-4, PDCD4 and PAWR were up-regulated suggested that these genes may facilitate the MCF-7 cell death.

Several mitochondrial mediated apoptotic genes were altered during VR-3848 treatment. The pro-apoptotic molecules which were BCL2L13 and BIK were slightly increased during VR-3848 treatment. Other compounds which are the proteosome inhibitor bortezomib and doxycilin treatments stimulate accumulation of BIK which serves as mediator for induced cell death (95, 96). VR-3848 treatment suppresses the anti-apoptosis molecules such as BAG 3, and 5. Surprisingly, proapoptotic molecules, BAK1 and BAX, were down-regulation after VR-3848 treatment. These data suggested that other pro-apoptotic molecules may compensate for function of such genes. MCL-1, a member of Bcl-2 family, has been described as anti-apoptotic molecule. However, the alternative splicing variant MCL-1 gene exerts pro-apoptotic function (97, 98). In our experiment, VR-3848 induced strong up-regulation of MCL-1. Therefore, the responsibility of MCL-1 gene in this situation requires further investigation.

ARTS, the mitochondrial protein, functions as pro-apoptotic molecule by suppressing XIAP function (69, 70). TGF-β triggers the release of ARTS from mitochondria. In the presence of ARTS, XIAP function will be blocked, thus caspase-3, -7, and -9 will be fully activated (99). In VR-3848 treatment, the strong up-regulation of ARTS was found. Because of the lack of the target of XIAP, caspase-3 function in MCF-7 cell, the induction of apoptosis via caspase-3 dependent pathway would not take place.

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The forkhead box transcription factors, FOXO1A and 3A were stimulated during VR-3848 treatment. It has been reported that these molecules mediate apoptosis through mitochondrial pathway by stimulating the expression of BIM, pro-apoptotic molecule in paclitaxel treated MCF-7 cell (100). Furthermore, FOXOs can inhibit cell cycle progression by increasing cyclin dependent kinase inhibitor, p27 and inhibiting the CDK4 during G1/S phase (101). The up-regulation of these genes may facilitate apoptosis as well as cell cycle arrest in VR-3848 treated cell.

The cellular and ER stress mediated cell death as the results of perturbation by stress stimuli from misfold protein, oxidative stress, calcium disturbances and some chemotherapeutic compounds (102). During ER stress, PERK and ATF6 are released from ER. The activated PERK phosphorylates eIF2α that subsequently inhibits the general transcription. However, activated eIF2α has been shown to selectively promote translation of ATF4 (38, 103). The transcription factors, ATF4, together with ATF6 and XBP-1 induce the expression of GADD153. GADD153 mediated cell death through receptor and mitochondria dependent pathway. In this experiment, the strong up-regulation of INHBA, ATF4, PERK and GADD153 in VR-3848 treated cell indicated that the ER stress induced death pathway is involved in MCF-7 cell death.

Aryl hydrocarbon receptor, AHR, has diverse roles in cellular functions including stress response, cell cycle arrest and apoptosis (104). When AHR interacts with its ligand, this complex translocates to nucleus and then induces the expression of target genes such as cytochrome P450 family (105, 106). The increasing level of AHR expression in VR-3848 stimulation suggested that this gene involves in induction of cell death which is similar to aminoflavone and benzothiazole which activate the AHR expression and exert the anti-growth in MCF-7 cell (107-109).

By alteration of gene expression, we demonstrated, to a greater extent, the MCF-7 gene expression in response to VR-3848 stimulation. To extensively explore the pathways in VR-3848 induced cell death, one of these pathways which is inhibin mediated death were selected. As demonstrated in figure 18 and gene list in table 13-16, INHBA, GADD45β, PERK, ATF4, GADD153, TRAILR2 (TNFRSF10B) and ARTS were up-regulated in our gene screening and in RT-PCR validation. This data supported our notion that inhibin stimulated pathway is one among various death pathways stimulated by VR-3848.

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The results from microarray showed that another group of genes affected from VR-3848 treatment was cell cycle machinery. The mammalian cell cycle typically involves a sequential stage including G1, S, G2 and M phases: G1 phase which corresponds to the gap between mitosis and initiation of DNA replication, S phase where DNA replication occurs, G2 phase where cell growth continues and protein synthesis prepares for mitosis, and M phase of cell cycle which corresponds to mitosis and usually follows by cell cytokinesis. The distinct phases of cell cycle are regulated by different types of regulatory molecules, cyclins and cyclin-dependent kinases (CDK). The different members of CDK family associating with specific cyclins regulates in each stages of cell cycle. The transition from G1 to S phase is regulated by the complex of CDK4, 6 interacting with Cyclin D and later formation of CDK2 and Cyclin E complex. Complexes of CDK2 and Cyclin E are responsible during DNA synthesis in S phase. The transition of G2 to M phase is regulated by complex of CDC2 and Cyclin B. The activation of CDKs for cell cycle progression is controlled by phosphorylation at activating site by CAK (CDK-activating enzyme) whereas the inhibition of CDK is regulated by inhibitory phosphorylation at inhibitory site.

From the alteration of gene expression listed in table 16, we proposed that VR-3848 treatment affected to cell cycle regulatory molecules by suppressing the cell cycle promoting genes and induction of the inhibitory gene expression. An alteration of following genes expression was used to compose our proposal. Cell cycle related genes such as KLF6, p57, p19, p27, Culin4A, AHR, PLK3, GADD45, WEE1 were up-regulated while CDK4, CDC45, MCM, and APCs were down-regulated. How do these genes participate in MCF-7 cell cycle arrest? As presented in figure 20, we proposed that VR-3848 treatment affected to all stages of cell cycle. During G1 phase, CDK4 was down-regulated after treatment. The CDK4 suppression may due to the stimulation of CDK inhibitors (p19 and p57). In addition, the stimulation of KLF6 as already discussed above may disrupt the complex of CDK4 and Cyclin D1 (81). At the transition G1/S phase, the activity of Cyclin E and CDK2 complex may be inhibited by CDK inhibitors p57 and p27. The induction of p27 expression may be induced from suppressing Culin4A (110) which is essential protein in cancer progression and activating AHR signaling (111). AHR functions not only the induction of the p27 expression but also repression of Cyclin E (106). During S phase of cell division, the

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unwinding of the DNA double strand by CDC45L and association of MCM proteins to origin replication complex initiate the DNA replication (112). The down-regulation of CDC45L and MCM proteins may result in suppression of cell cycle progression. The G2 to M phase transition was inhibited by inhibitory phosphorylation of CDC2 by WEE1 (113). In addition, GADD45 can arrest cell cycle by dissociating CDC2 and Cyclin B complex (114). Polo-like kinase 3 (PLK3) also regulated the cell cycle through stimulation of CHK2 which ultimately suppresses the CDC2 activity (115). The strongly up-regulation of PLK3, GADD45 and WEE1 suggested that VR-3848 arrests cell cycle progression at G2 to M phase transition. VR-3848 stimulation also suppressed the expression of APCs (anaphase promoting complex) that are essential for exiting from mitosis. These results indicated that VR-3848 treatment caused cell cycle arrest in MCF-7 cell.

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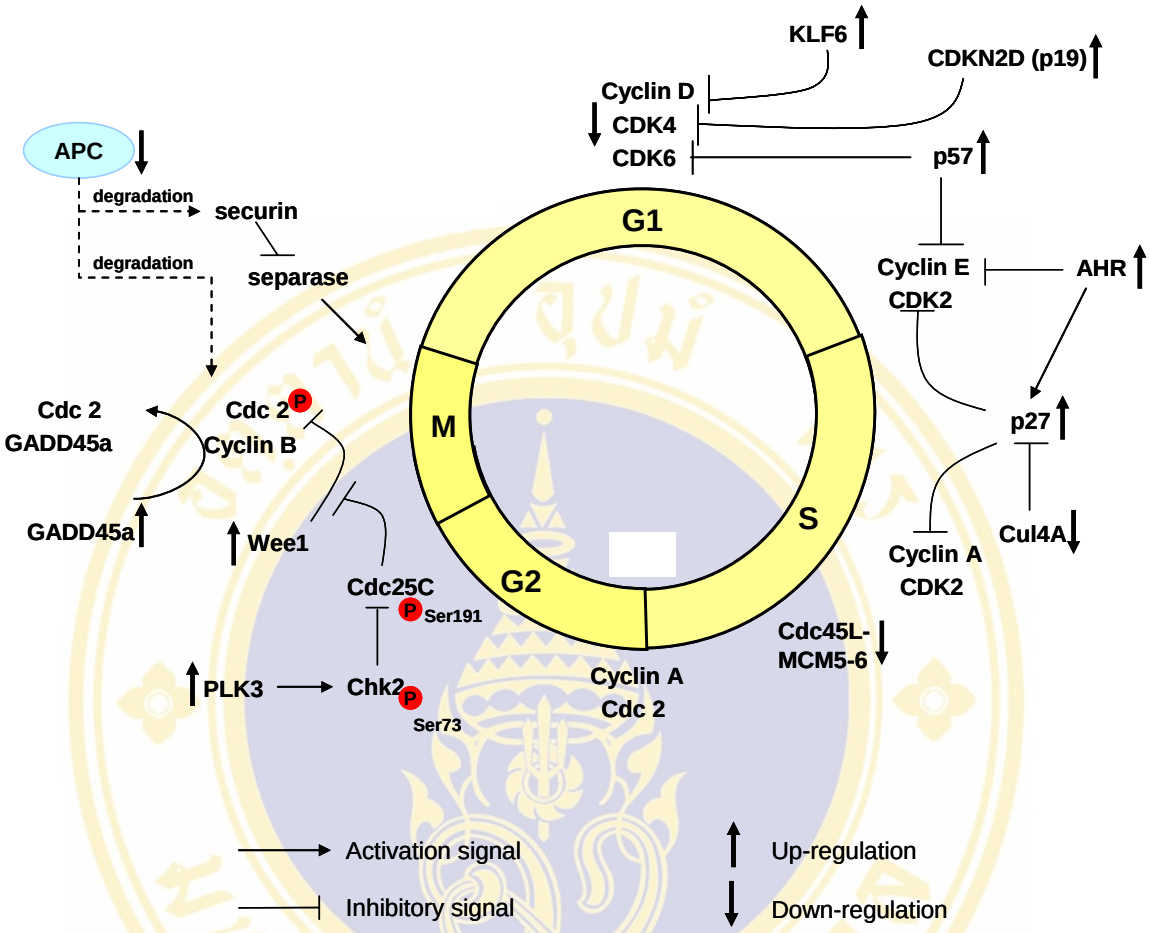

Figure 20. The possible mechanisms of VR-3848 stimulation in cell cycle arrest of MCF-7 cell.

Taken together, VR-3848, a cytoheptapeptide, induces breast cancer, MCF-7, cell death by apoptosis. The screening of gene alteration by using microarray analysis indicated that the anti-cancer activity of this compound may mediate through multiple mechanisms. The rapid increasing level of SRCAP gene expression, a coactivator for steroid receptor dependent transcription, helps us to hypothesize an important role of SRCAP in the killing mechanism. However, such SRCAP function has not been elucidated yet. For further study, the functional analysis such as siRNA is needed to identify the SRCAP function in VR-3848 treatment. Moreover, approximately 66% of gene expression at 1 hr is uncharacterized function. Therefore, it is suggested that VR-3848 may stimulate unexplored pathway.

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At the late time point, VR-3848 stimulates the alteration of genes involved in apoptosis and cell cycle. These data indicated that the anti-cancer activity of this compound is regulated not only at the stimulation of apoptotic pathways but also at the inhibition of cell cycle progression. The possible anti-cancer mechanisms of VR-3848 in MCF-7 are summarized in figure 21.

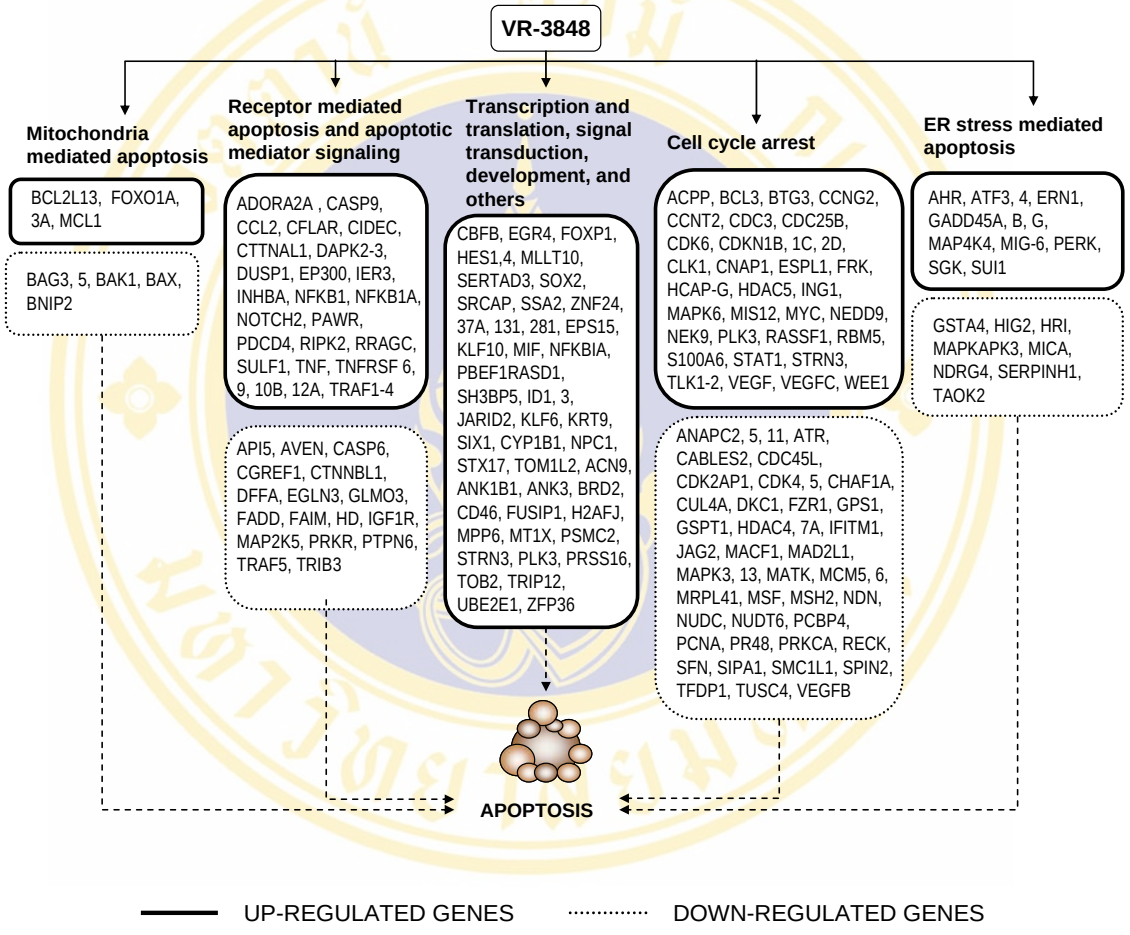

Figure 21. VR-3848 induced MCF-7 cell death was regulated by genes which involve in cell cycle inhibition and receptor-, mitochondria-, and ER stress-mediated apoptosis.

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CHAPTER 6
CONCLUSION

From our investigation, we concluded that VR-3848 stimulated MCF-7 cell death by apoptosis. The apparently apoptotic characteristics with nuclear condensation and nuclear fragmentation were confirmed by DAPI staining and TUNEL enzymatic staining assay.

To identify the possible target of VR-3848, the global gene expression profiling from DNA microarray analysis at 1 hour after treatment revealed that most of significantly altered genes were transcription and translation genes. We attempted to investigate the earliest gene in the response to VR-3848 stimulation. At the present result, SRCAP was significantly increased at 30 minutes after treatment. This data suggested that anti-cancer activity may be regulated by SRCAP signaling. However, the function of this gene in aspect of VR-3848 induced cell death requires further studied.

To elucidate, a greater extent, the downstream of anti-cancer mechanism, we found that VR-3848 stimulated genes related in cell cycle inhibition and three major apoptosis pathways which are receptor ligand mediated apoptosis, mitochondria mediated apoptosis, and ER-stress mediated apoptosis. The possible pathway which is inhibin-mediated apoptosis was demonstrated. Therefore, we proposed that anti-cancer activity of VR-3848 not only induces apoptosis but also arrests cell cycle progression in MCF-7 cell.

This finding demonstrated that VR-3848, a cycloheptapeptide derived from typical Thai plant, exerts a potent anti-proliferative activity at nanomolar concentration against breast cancer, MCF-7, cell. Therefore, this compound deserves to be developed into a promising anti-cancer agent.

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APPENDIX

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APPENDIX A
MEDIUM FOR MCF-7 CULTIVATION

1. MEM (Minimum essential medium) containing sodium bicarbonate solution

MEM with Earle's salt, 2 mM L-glutamine and		
0.1 mM non-essential amino acids (Invitrogen)	9.6	g
$NaHCO_3$	1.5	g
Nanopure distilled water up to	1,000	ml

2. MEM medium for MCF-7 cell cultivation

Heat inactivated fetal bovine serum	10	ml
100 mM sodium pyruvate (Invitrogen)	10	ml
0.01 mg/ml bovine insulin	1	ml
MEM containing sodium bicarbonate solution up to	100	ml

3. Phosphate buffer saline (PBS), Mg2+ and Ca2+ free, pH 7.4 (10x PBS)

NaCl (Merck)	80	g
KCl (Merck)	2	g
$Na_2HPO_4H_2O$ (Merck)	14.42	g
KH_2PO_4 (Merck)	2.40	g
Deionized distilled water up to	1,000	ml

4. 0.05% Trypsin solution

0.25 % Trypsin EDTA	10	ml
1x PBS containing 0.2% EDTA	40	ml

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APPENDIX B
DNA MICROARRAY

1. Working bacterial control mRNA mixture dilution methods.

1. Prepare 16.7 ng/µl combined bacterial control stock solution as follows:

5 µl	0.1 µg/µl Bacterial control mRNA *araB*
5 µl	0.1 µg/µl Bacterial control mRNA *ent F*
5 µl	0.1 µg/µl Bacterial control mRNA *fix B*
5 µl	0.1 µg/µl Bacterial control mRNA *gnd*
5 µl	0.1 µg/µl Bacterial control mRNA *his B*
5 µl	0.1 µg/µl Bacterial control mRNA *leu B*
30 µl	Final volume

2. Prepare a dilution of bacterial control mRNA for a final concentration of 50.2 pg/µl *araB, entF, fix B, gnd, hisB, and leuB.*

3 µl	16.7 ng/µl combined bacterial control stock
997 µl	Nuclease-free water
1000 µl	Final volume

3. Prepare a working solution of bacterial control mRNA

2 µl	50.2 pg/µl combined bacterial control stock
998 µl	Nuclease –free water
1000 µl	Final Volume

4. Add 1.0 µl of working solution of bacterial control mRNA mixture (0.1 pg/µl *araB, entF, fix B, gnd, hisB, and leuB*) for every 1.0 µg of total RNA.

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2. Washing solutions

1x TNT buffer (1 liter)

1. Add the reagents following:

100 ml	1 M Tris-HCl, pH 7.6 (Merck)
30 ml	5 M NaCl (Merck)
0.5 ml	Tween 20 (Amresco)
870 ml	Deionized water

2. Filter 1xTNT through a 0.2 µm filter and transfer to isopropanol-rinsed reagent container. This solution is stored up to two weeks at ambient temperature.

0.75x TNT buffer

1. Dilute 1x TNT buffer by adding 25 ml of deionized water to 75 ml of 1x TNT buffer per 100 ml of buffer required.

2. Filter 1xTNT through a 0.2 µm filter and transfer to isopropanol-rinsed reagent container. This solution is stored up to two weeks at ambient temperature.

TNB buffer stock solution

1. Add the reagents as following:

52.2 ml	Nuclease free water
6 ml	1 M Tris-HCl, pH 7.6 (Merck)
1.8 ml	5 M NaCl (Merck)

2. Slowly add 0.3 g of ECL blocking reagent (GE Healthcare) on a stir plate heated to 60 ºC.

3. Turn off heat and continue mixing for 30 minutes. Filter through a 0.88 µm filter while the solution is still warm.

4. TNB buffer is stored for 12 weeks at -20 ºC. Thaw TNB overnight at 4 ºC before use. Thawed TNB buffer can be used at 4 ºC up to one week.

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Cy5-Streptavidin stock solution

1. Add 1 ml of nuclease free water to the 1 mg of lyophilized Cy5-Streptavidin.
2. Mix solution by pipetting up and down. Place on ice for 5 minutes and repeat the mixing three additional times, placing on ice between mixing. Protect this solution from light at all times.
3. Aliquot the Cy5-streptavidin solution for the number of microarray. This solution is stored up to six months (or until expiry date) at -20 ºC. Avoid multiple freeze-thaw cycles.

1: 500 Cy5-streptavidin working solution

1. Add 6.8 µl of the Cy5-Streptavidin stock solution to 3,393.2 µl of filtered TNB buffer for each slide.
2. Mix gently by inversion. Use this working solution within 15 minutes of preparation.

0.1x Standard saline citrate (SSC)/ 0.05% Tween 20 (500 ml)

1. Prepare 20x SSC by adding reagent as following:

175.3 g	NaCl (Merck)
88.2 g	$Na_3Citrate.2H_2O$ (Sigma)
1000 ml	Deionized distilled water

2. Prepare 0.1x SSC/0.05% Tween 20, add the following:

2.5 ml	20 x SSC buffer
250 µl	Tween 20
497.25 ml	Deionized distilled water

3. Mix well by swirling. Filter SSC solution through a 0.2 µm filter and transfer to isopropanol-rinsed reagent container. This solution is stored up to two weeks at ambient temperature.

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APPENDIX C

THE SIGNIFICANT GENES WITH UNKNOWN FUNCTION BY K-MEANS CLUSTERING ANALYSIS

Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis.

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment																	
	1 hr			3 hr			6 hr			12 hr			18 hr					
AA010870.1	-			-			-			-			0.28	±	0.06			
AA156360.1	-			-			-			0.38	±	0.09	0.29	±	0.06			
AA194820.1	-			-			-			0.35	±	0.03	0.24	±	0.01			
AA234690.1	-			-			-			0.37	±	0.05	0.30	±	0.01			
AA302833.1	-			-			-			0.36	±	0.04	0.33	±	0.02			
AA370555.1	-			-			-			0.31	±	0.05	0.30	±	0.01			
AA406499.1	-			0.39	±	0.18	0.31	±	0.01	0.24	±	0.03	0.40	±	0.01			
AA419568.1	-			-			-			0.35	±	0.01	0.34	±	0.00			
AA420438.1	-			-			0.46	±	0.05	0.30	±	0.12	-					
AA442210.1	-			-			0.25	±	0.02	0.12	±	0.02	0.10	±	0.01			
AA524295.1	-			-			-			0.33	±	0.04	0.28	±	0.00			
AA527854.1	-			-			-			0.29	±	0.00	0.20	±	0.03			
AA602964.1	2.50	±	0.84	0.44	±	0.21	0.43	±	0.00	-			0.38	±	0.01			
AA613572.1	-			-			0.43	±	0.03	0.17	±	0.03	0.12	±	0.01			
AA769043.1	-			-			0.48	±	0.12	0.42	±	0.02	-					
AB007969.1	-			-			-			0.29	±	0.11	0.26	±	0.01			
AB028949.1	-			-			-			0.35	±	0.01	0.41	±	0.04			
AB029017.1	-			-			-			0.23	±	0.02	0.20	±	0.00			
AB041269.1	-			-			-			0.35	±	0.01	0.25	±	0.03			
AB046799.1	-			-			0.44	±	0.03	0.37	±	0.07	0.37	±	0.04			
AF034176.1	-			-			-			0.36	±	0.05	0.31	±	0.01			
AF034176.1	-			-			-			0.35	±	0.06	0.28	±	0.03			
AF138859.1	-			-			-			0.47	±	0.09	-					
AI086336.1	-			-			0.42	±	0.02	0.42	±	0.02	-					
AI126289.1	-			-			0.47	±	0.03	-			0.31	±	0.07			
AI218517.1	2.32	±	0.47	-			0.43	±	0.08	0.26	±	0.00	0.24	±	0.07			
AI241504.1	-			-			-			0.42	±	0.02	0.32	±	0.01			
AI356405.1	-			0.42	±	0.04	0.38	±	0.03	0.32	±	0.03	0.35	±	0.05			
AI424832.1	-			-			-			0.36	±	0.05	0.36	±	0.01			
AI621093.1	-			-			0.47	±	0.03	0.29	±	0.04	0.26	±	0.02			
AI637910.1	-			-			-			0.37	±	0.07	0.30	±	0.07			
AI718818.1	2.54	±	0.63	0.50	±	0.20	0.32	±	0.04	0.23	±	0.08	-					
AI823969.1	-			-			0.26	±	0.12	0.23	±	0.00	0.22	±	0.00			
AI873847.1	-			-			-			0.34	±	0.01	0.23	±	0.02			
AJ420529.1	-			-			-			0.36	±	0.04	0.30	±	0.06			
AK001094.1	-			0.41	±	0.17	0.27	±	0.03	0.36	±	0.01	0.36	±	0.03			
AK021523.1	-			0.37	±	0.13	0.39	±	0.04	-			-					

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment					
	1 hr	3 hr	6 hr	12 hr	18 hr	
AK021954.1	-	-	0.33 ± 0.06	0.20 ± 0.03	0.16 ± 0.01	
AK022479.1	-	-	0.45 ± 0.09	0.28 ± 0.01	0.28 ± 0.03	
AK022703.1	-	-	-	0.33 ± 0.01	0.22 ± 0.03	
AK022936.1	-	-	-	0.35 ± 0.03	0.23 ± 0.01	
AK023036.1	-	-	-	0.32 ± 0.06	0.38 ± 0.04	
AK023647.1	-	-	-	0.29 ± 0.03	0.23 ± 0.00	
AK024204.1	-	-	0.42 ± 0.07	0.28 ± 0.00	0.29 ± 0.10	
AK024294.1	-	-	0.48 ± 0.04	0.28 ± 0.07	0.16 ± 0.02	
AK024865.1	-	-	-	0.36 ± 0.05	0.32 ± 0.01	
AK025203.1	-	-	-	0.19 ± 0.01	0.15 ± 0.00	
AK025687.1	-	-	-	0.21 ± 0.04	0.20 ± 0.03	
AK025766.1	-	-	0.40 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	
AK027767.1	-	-	-	0.28 ± 0.06	0.24 ± 0.03	
AK056982.1	-	-	-	0.35 ± 0.01	0.32 ± 0.02	
AK092442.1	-	-	-	0.26 ± 0.04	0.17 ± 0.01	
AK093346.1	-	-	-	0.32 ± 0.02	0.33 ± 0.03	
AK093406.1	3.36 ± 0.54	-	0.46 ± 0.07	0.43 ± 0.03	0.40 ± 0.07	
AK095831.1	-	-	-	0.35 ± 0.01	0.33 ± 0.11	
AK096154.1	-	-	-	0.37 ± 0.03	0.29 ± 0.01	
AK096415.1	-	-	0.48 ± 0.01	0.31 ± 0.02	0.36 ± 0.07	
AK096975.1	-	-	-	0.34 ± 0.03	0.32 ± 0.03	
AK097239.1	-	-	-	0.33 ± 0.00	-	
AK098016.1	-	-	0.35 ± 0.09	0.27 ± 0.08	0.22 ± 0.01	
AK098252.1	-	-	-	0.36 ± 0.01	0.30 ± 0.02	
AK123427.1	-	-	-	0.35 ± 0.03	0.18 ± 0.00	
AK124539.1	-	-	-	0.25 ± 0.23	-	
AK124968.1	-	-	-	0.49 ± 0.07	0.21 ± 0.01	
AK125482.1	-	-	-	0.34 ± 0.04	0.31 ± 0.05	
AK125879.1	-	-	-	0.23 ± 0.01	0.18 ± 0.02	
AK131381.1	-	-	-	0.31 ± 0.00	0.34 ± 0.04	
AK131382.1	-	-	-	0.43 ± 0.12	0.27 ± 0.02	
AL040498.1	-	-	-	0.36 ± 0.10	0.35 ± 0.06	
AL157484.1	-	-	0.44 ± 0.00	0.29 ± 0.07	0.45 ± 0.04	
AL360140.1	2.40 ± 0.50	-	0.46 ± 0.09	0.31 ± 0.04	0.30 ± 0.05	
AL390134.1	-	-	-	0.35 ± 0.03	0.29 ± 0.03	
AL831837.1	-	-	-	0.31 ± 0.02	0.38 ± 0.05	
AL833462.1	-	0.36 ± 0.00	0.26 ± 0.03	0.15 ± 0.02	0.22 ± 0.10	
AL833859.1	-	-	-	0.40 ± 0.04	0.31 ± 0.07	
AV658299.1	-	0.44 ± 0.21	0.18 ± 0.01	-	0.32 ± 0.02	
AW021725.1	2.30 ± 0.74	0.30 ± 0.06	0.46 ± 0.10	0.26 ± 0.05	0.22 ± 0.00	
AW023005.1	-	-	-	0.34 ± 0.02	0.36 ± 0.02	
AW118601.1	-	-	-	0.33 ± 0.05	0.33 ± 0.14	
AW139066.1	-	-	0.46 ± 0.00	0.26 ± 0.05	0.22 ± 0.06	
AW241731.1	-	-	0.45 ± 0.01	0.43 ± 0.00	-	
AW276726.1	2.13 ± 0.86	-	0.50 ± 0.03	0.44 ± 0.01	-	
AW291146.1	-	-	0.46 ± 0.04	0.29 ± 0.02	-	
AW292657.1	2.06 ± 0.11	0.44 ± 0.11	0.40 ± 0.02	0.26 ± 0.03	0.22 ± 0.00	
AW385968.1	-	-	-	0.26 ± 0.02	0.16 ± 0.00	
AW445209.1	-	-	-	0.35 ± 0.02	0.33 ± 0.03	

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment														
	1 hr			3 hr			6 hr			12 hr			18 hr		
AW629179.1	-			-			0.37	±	0.04	0.31	±	0.03	0.30	±	0.10
AW946373.1	-			-			-			0.23	±	0.04	0.30	±	0.20
AW958932.1	-			-			-			0.35	±	0.04	0.41	±	0.01
AW970240.1	2.23	±	0.11	0.36	±	0.06	0.30	±	0.08	0.33	±	0.06	-		
AW974076.1	-			-			0.33	±	0.04	0.34	±	0.10	0.38	±	0.08
AW975281.1	-			-			0.26	±	0.04	0.16	±	0.01	0.23	±	0.01
BC002330.2	-			-			-			0.22	±	0.02	0.22	±	0.03
BC004287.1	-			-			0.46	±	0.03	0.20	±	0.02	0.15	±	0.01
BC016138.1	-			-			-			0.31	±	0.05	0.27	±	0.02
BC017737.1	2.04	±	0.14	0.43	±	0.12	0.39	±	0.01	-			0.34	±	0.06
BC034587.1	-			-			-			0.36	±	0.07	0.37	±	0.06
BC041380.1	-			-			-			0.35	±	0.08	0.39	±	0.01
BC044311.1	-			-			0.41	±	0.02	0.43	±	0.07	0.36	±	0.04
BC060805.1	-			-			-			0.41	±	0.03	0.23	±	0.01
BC067892.1	-			-			-			0.31	±	0.06	0.31	±	0.01
BC071568.1	2.16	±	0.37	0.42	±	0.09	0.42	±	0.06	0.44	±	0.05	-		
BE407125.1	-			0.48	±	0.08	0.39	±	0.08	0.20	±	0.03	0.19	±	0.00
BE697175.1	-			-			-			0.38	±	0.01	0.26	±	0.06
BE719128.1	-			-			-			-			0.34	±	0.13
BF055490.1	2.53	±	0.19	-			0.41	±	0.02	0.24	±	0.07	0.19	±	0.00
BF477507.1	-			-			-			0.33	±	0.09	0.28	±	0.02
BF510074.1	-			0.25	±	0.12	0.32	±	0.18	0.42	±	0.02	0.35	±	0.01
BF966833.1	-			-			0.41	±	0.08	0.13	±	0.02	-		
BG029962.1	-			-			-			0.32	±	0.04	0.29	±	0.05
BG117761.1	-			-			-			0.29	±	0.01	0.32	±	0.05
BG957974.1	-			-			-			0.28	±	0.05	0.26	±	0.03
BG988807.1	-			-			0.49	±	0.02	0.42	±	0.11	0.36	±	0.02
BI493492.1	-			-			0.48	±	0.03	0.37	±	0.08	-		
BI494495.1	-			0.42	±	0.13	0.30	±	0.05	0.17	±	0.02	0.10	±	0.00
BI494557.1	-			0.40	±	0.02	0.42	±	0.05	0.32	±	0.04	0.48	±	0.03
BI494600.1	2.88	±	0.27	0.46	±	0.14	0.29	±	0.01	0.21	±	0.01	-		
BM128032.1	-			-			-			0.30	±	0.09	0.23	±	0.06
BM666683.1	-			-			-			0.33	±	0.07	-		
BM678148.1	-			0.45	±	0.03	0.35	±	0.05	0.29	±	0.05	0.30	±	0.01
BM684035.1	-			-			0.47	±	0.01	0.41	±	0.30	-		
BM685802.1	-			-			-			0.30	±	0.02	0.33	±	0.02
BM695576.1	-			-			0.49	±	0.02	0.30	±	0.00	0.25	±	0.01
BM701419.1	-			-			0.50	±	0.00	0.36	±	0.13	0.42	±	0.08
BM715449.1	-			-			0.40	±	0.01	0.26	±	0.02	0.28	±	0.04
BM727182.1	-			-			0.46	±	0.08	0.27	±	0.04	-		
BM929333.1	-			-			0.47	±	0.05	0.42	±	0.08	0.28	±	0.00
BM979825.1	-			-			-			0.39	±	0.05	0.25	±	0.02
BQ012774.1	-			-			-			0.31	±	0.03	0.29	±	0.04
BQ013066.1	3.38	±	0.10	0.40	±	0.13	0.44	±	0.01	0.42	±	0.04	0.39	±	0.04
BQ024204.1	-			-			0.32	±	0.05	0.36	±	0.03	0.42	±	0.05
BQ025233.1	2.78	±	0.31	0.40	±	0.10	0.28	±	0.06	0.32	±	0.02	0.30	±	0.01
BQ025812.1	-			-			0.47	±	0.02	0.24	±	0.04	0.22	±	0.02
BQ028406.1	-			-			0.45	±	0.03	0.23	±	0.04	-		
BQ424847.1	-			0.46	±	0.05	-			0.24	±	0.07	0.23	±	0.06

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment					
	1 hr	3 hr	6 hr	12 hr	18 hr	
BQ439091.1	-	0.48 ± 0.08	-	0.25 ± 0.09	0.37 ± 0.03	
BQ771515.1	-	0.39 ± 0.03	0.34 ± 0.06	0.22 ± 0.04	0.20 ± 0.09	
BU429796.1	-	-	-	0.24 ± 0.02	0.29 ± 0.02	
BU608039.1	-	-	0.39 ± 0.05	0.37 ± 0.06	0.33 ± 0.04	
BU624020.1	-	-	-	0.30 ± 0.12	0.23 ± 0.02	
BU633018.1	-	-	0.46 ± 0.05	-	0.20 ± 0.02	
BU684170.1	-	0.39 ± 0.04	0.28 ± 0.02	0.10 ± 0.02	0.13 ± 0.04	
BU737447.1	-	-	-	0.39 ± 0.08	0.29 ± 0.05	
BX089753.1	-	-	0.31 ± 0.01	0.34 ± 0.02	-	
BX100863.1	-	-	0.40 ± 0.03	0.18 ± 0.00	-	
BX101711.1	-	-	-	0.24 ± 0.03	0.08 ± 0.01	
BX104870.1	-	-	0.41 ± 0.02	0.28 ± 0.00	0.30 ± 0.04	
BX538248.1	-	-	0.49 ± 0.06	0.28 ± 0.04	0.33 ± 0.01	
BX647147.1	-	-	-	0.36 ± 0.07	0.27 ± 0.01	
BX648343.1	-	-	-	0.37 ± 0.03	0.37 ± 0.01	
BX648396.1	-	-	-	0.35 ± 0.04	0.36 ± 0.02	
BX648468.1	-	-	0.37 ± 0.03	0.17 ± 0.04	0.16 ± 0.03	
BX648964.1	-	-	-	0.23 ± 0.02	0.17 ± 0.01	
BX649033.1	2.38 ± 0.62	-	0.35 ± 0.00	0.26 ± 0.01	-	
CA417101.1	-	-	-	0.32 ± 0.03	-	
CA848700.1	-	-	0.48 ± 0.10	0.21 ± 0.07	-	
CB068328.1	-	0.38 ± 0.13	0.39 ± 0.02	0.32 ± 0.03	0.32 ± 0.03	
CF123899.1	-	0.42 ± 0.03	0.47 ± 0.02	0.37 ± 0.10	0.31 ± 0.15	
CN305388.1	-	-	0.40 ± 0.10	0.44 ± 0.09	-	
D55056.1	-	-	-	0.42 ± 0.02	0.24 ± 0.01	
F34272.1	-	-	-	0.29 ± 0.02	0.26 ± 0.04	
H30200.1	-	-	-	0.36 ± 0.07	0.29 ± 0.00	
H51256.1	-	-	0.47 ± 0.01	0.31 ± 0.03	0.41 ± 0.04	
H65460.1	-	-	0.35 ± 0.05	0.30 ± 0.05	-	
H93600.1	-	-	-	0.31 ± 0.01	0.32 ± 0.04	
N26608.1	-	0.39 ± 0.21	0.22 ± 0.01	0.43 ± 0.25	0.37 ± 0.03	
N47708.1	-	-	-	0.30 ± 0.01	0.33 ± 0.06	
N51335.1	-	-	-	0.31 ± 0.05	0.25 ± 0.00	
N51982.1	-	-	-	0.32 ± 0.02	0.31 ± 0.01	
N62595.1	-	-	0.42 ± 0.03	0.18 ± 0.00	0.18 ± 0.02	
N69189.1	-	-	-	0.38 ± 0.05	0.30 ± 0.06	
N90917.1	-	-	-	0.38 ± 0.08	0.25 ± 0.08	
NM_001306.2	2.10 ± 0.39	-	-	0.39 ± 0.08	0.32 ± 0.06	
NM_002652.2	-	-	-	0.32 ± 0.03	0.23 ± 0.04	
NM_002816.2	-	-	-	0.39 ± 0.00	0.27 ± 0.06	
NM_003634.1	-	-	-	0.39 ± 0.02	0.18 ± 0.04	
NM_003681.3	-	-	-	0.35 ± 0.09	0.19 ± 0.01	
NM_003928.1	-	-	-	0.22 ± 0.01	0.15 ± 0.01	
NM_004175.3	-	-	-	0.36 ± 0.13	0.34 ± 0.09	
NM_004726.1	-	-	-	0.35 ± 0.05	0.28 ± 0.01	
NM_005111.5	-	-	-	0.32 ± 0.05	0.24 ± 0.02	
NM_006589.2	-	-	-	0.26 ± 0.06	0.17 ± 0.00	
NM_007210.2	-	-	0.45 ± 0.05	0.31 ± 0.05	0.39 ± 0.20	
NM_012151.2	-	-	0.43 ± 0.09	0.19 ± 0.00	0.19 ± 0.04	

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment					
	1 hr	3 hr	6 hr	12 hr	18 hr	
NM_013299.1	-	-	-	0.29 ± 0.10	0.30 ± 0.06	
NM_013402.3	-	-	-	0.41 ± 0.06	0.25 ± 0.00	
NM_014186.1	-	-	-	0.34 ± 0.03	0.26 ± 0.08	
NM_014367.3	-	-	-	0.41 ± 0.01	0.25 ± 0.04	
NM_014412.1	-	-	-	0.33 ± 0.01	0.18 ± 0.00	
NM_014730.2	-	-	-	0.29 ± 0.05	0.25 ± 0.00	
NM_014909.2	-	-	0.35 ± 0.06	0.11 ± 0.03	0.09 ± 0.03	
NM_015558.3	-	-	-	0.32 ± 0.07	0.34 ± 0.09	
NM_015582.1	-	-	-	0.30 ± 0.07	0.18 ± 0.01	
NM_015595.2	-	-	-	0.30 ± 0.03	0.34 ± 0.01	
NM_015621.2	-	-	-	0.38 ± 0.06	0.34 ± 0.05	
NM_015710.2	-	-	-	0.37 ± 0.08	0.29 ± 0.02	
NM_015916.3	-	-	-	0.27 ± 0.03	0.47 ± 0.06	
NM_016133.2	-	-	0.49 ± 0.06	0.27 ± 0.05	0.33 ± 0.05	
NM_016172.1	-	-	-	0.25 ± 0.04	0.21 ± 0.03	
NM_016466.4	-	-	-	0.37 ± 0.02	0.34 ± 0.05	
NM_016466.4	-	-	-	0.41 ± 0.06	0.30 ± 0.06	
NM_016534.2	-	-	0.43 ± 0.01	0.24 ± 0.03	0.24 ± 0.00	
NM_016535.2	-	-	-	0.23 ± 0.04	0.30 ± 0.07	
NM_016535.2	-	-	-	0.24 ± 0.06	0.30 ± 0.01	
NM_016608.1	-	-	0.26 ± 0.00	0.27 ± 0.02	0.32 ± 0.05	
NM_017693.2	-	-	-	0.32 ± 0.03	-	
NM_017701.2	-	-	-	0.33 ± 0.01	0.32 ± 0.02	
NM_017793.1	-	-	0.48 ± 0.07	0.25 ± 0.02	0.29 ± 0.01	
NM_017947.1	-	-	-	0.29 ± 0.06	0.15 ± 0.00	
NM_017983.3	-	-	0.50 ± 0.03	0.21 ± 0.03	0.14 ± 0.00	
NM_018044.2	-	-	-	0.29 ± 0.05	0.27 ± 0.04	
NM_018170.2	-	-	-	0.30 ± 0.10	0.23 ± 0.04	
NM_018285.1	-	-	-	0.31 ± 0.15	0.32 ± 0.02	
NM_018418.1	-	-	0.46 ± 0.00	0.28 ± 0.03	0.31 ± 0.00	
NM_019008.4	-	-	-	0.39 ± 0.06	0.40 ± 0.04	
NM_019008.4	-	-	-	0.31 ± 0.07	0.31 ± 0.00	
NM_019015.1	-	-	-	0.28 ± 0.01	0.37 ± 0.02	
NM_020119.3	-	0.41 ± 0.08	0.24 ± 0.03	0.28 ± 0.02	-	
NM_020223.1	-	-	-	0.29 ± 0.00	0.35 ± 0.05	
NM_020247.3	-	-	-	0.33 ± 0.02	0.22 ± 0.02	
NM_020457.2	-	-	0.38 ± 0.03	-	0.22 ± 0.01	
NM_020655.2	-	-	-	0.34 ± 0.08	0.23 ± 0.02	
NM_021932.4	-	-	-	0.37 ± 0.05	0.30 ± 0.04	
NM_021933.1	-	-	0.48 ± 0.03	0.23 ± 0.01	0.17 ± 0.01	
NM_021941.1	-	-	-	0.28 ± 0.08	0.26 ± 0.01	
NM_022149.3	-	-	-	0.24 ± 0.05	0.19 ± 0.02	
NM_022163.2	-	-	-	0.31 ± 0.04	0.21 ± 0.02	
NM_022727.3	-	-	-	0.38 ± 0.05	0.30 ± 0.03	
NM_023936.1	-	-	-	0.47 ± 0.05	0.25 ± 0.03	
NM_024043.2	-	-	-	0.32 ± 0.08	0.24 ± 0.03	
NM_024054.1	-	-	0.46 ± 0.04	0.33 ± 0.01	0.34 ± 0.02	
NM_024084.1	-	-	-	0.37 ± 0.05	0.34 ± 0.09	
NM_024094.1	-	-	-	0.36 ± 0.08	0.18 ± 0.01	

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment				
	1 hr	3 hr	6 hr	12 hr	18 hr
NM_024096.1	-	-	-	0.35 ± 0.05	0.21 ± 0.00
NM_024111.2	-	0.28 ± 0.04	0.42 ± 0.10	0.41 ± 0.06	0.31 ± 0.01
NM_024320.2	-	-	0.40 ± 0.03	0.17 ± 0.02	0.11 ± 0.00
NM_024333.1	-	-	-	0.33 ± 0.03	0.18 ± 0.02
NM_024509.1	-	-	-	0.33 ± 0.03	0.41 ± 0.08
NM_024632.2	-	-	0.43 ± 0.01	0.30 ± 0.02	0.37 ± 0.01
NM_024661.2	-	-	-	0.23 ± 0.02	0.17 ± 0.00
NM_024747.4	-	-	-	0.30 ± 0.14	0.21 ± 0.06
NM_024871.1	-	-	0.42 ± 0.04	0.15 ± 0.02	0.13 ± 0.00
NM_024923.2	-	-	-	0.37 ± 0.03	0.39 ± 0.01
NM_025073.1	-	-	-	0.39 ± 0.07	0.31 ± 0.01
NM_025108.1	-	-	-	0.35 ± 0.07	0.32 ± 0.07
NM_030573.1	-	-	0.45 ± 0.19	0.26 ± 0.04	0.19 ± 0.00
NM_030577.1	-	-	-	0.24 ± 0.02	0.20 ± 0.02
NM_030583.1	-	-	-	0.33 ± 0.03	0.26 ± 0.04
NM_030801.2	-	-	-	0.34 ± 0.00	0.38 ± 0.01
NM_030806.2	-	-	-	0.40 ± 0.08	0.23 ± 0.03
NM_030811.3	-	-	-	0.39 ± 0.11	0.17 ± 0.02
NM_031425.1	-	-	0.43 ± 0.04	0.18 ± 0.05	0.15 ± 0.01
NM_031426.2	-	-	-	0.32 ± 0.02	0.12 ± 0.01
NM_031443.2	-	-	-	0.32 ± 0.07	0.31 ± 0.02
NM_031469.1	-	-	-	0.35 ± 0.03	0.36 ± 0.04
NM_032042.3	-	-	-	0.33 ± 0.03	0.40 ± 0.11
NM_032296.1	-	-	-	0.35 ± 0.08	0.39 ± 0.02
NM_032313.2	-	-	-	0.31 ± 0.04	0.31 ± 0.06
NM_032344.1	-	-	-	0.37 ± 0.00	0.32 ± 0.00
NM_032429.1	-	-	-	0.18 ± 0.02	0.29 ± 0.13
NM_032627.1	-	-	-	0.38 ± 0.08	0.26 ± 0.01
NM_032631.2	-	-	-	0.26 ± 0.01	0.26 ± 0.04
NM_032795.1	-	-	-	0.39 ± 0.06	0.26 ± 0.00
NM_032806.4	-	-	0.49 ± 0.01	0.30 ± 0.09	0.20 ± 0.01
NM_032853.2	-	-	0.40 ± 0.09	0.27 ± 0.01	0.18 ± 0.01
NM_033271.1	-	-	-	0.33 ± 0.05	0.27 ± 0.01
NM_053004.1	-	-	-	0.18 ± 0.01	0.20 ± 0.01
NM_058187.3	-	-	-	0.22 ± 0.02	0.21 ± 0.02
NM_058190.1	-	-	-	0.36 ± 0.13	0.24 ± 0.04
NM_058192.2	-	-	-	0.39 ± 0.17	0.33 ± 0.05
NM_080821.1	-	-	-	0.26 ± 0.02	0.15 ± 0.04
NM_130809.2	-	-	0.50 ± 0.03	0.24 ± 0.06	0.20 ± 0.04
NM_133375.2	-	-	-	0.35 ± 0.03	0.35 ± 0.02
NM_133444.1	-	-	-	0.36 ± 0.08	0.33 ± 0.01
NM_138389.1	-	-	0.41 ± 0.06	0.26 ± 0.07	0.20 ± 0.01
NM_138399.2	-	-	-	0.26 ± 0.01	0.23 ± 0.01
NM_138431.1	-	-	-	0.26 ± 0.05	0.19 ± 0.05
NM_138443.2	-	-	-	0.45 ± 0.19	0.20 ± 0.02
NM_138446.1	-	-	-	0.35 ± 0.02	0.35 ± 0.07
NM_138462.2	-	-	-	0.30 ± 0.07	0.33 ± 0.03
NM_138959.1	-	-	0.35 ± 0.05	0.26 ± 0.04	0.16 ± 0.01
NM_144581.1	-	-	-	0.39 ± 0.03	0.30 ± 0.01

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Table17. The list of functionally unknown genes was down-regulation in the cluster 1 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment					
	1 hr	3 hr	6 hr	12 hr	18 hr	
NM_144584.1	-	-	-	0.37 ± 0.09	0.27 ± 0.04	
NM_144626.1	-	-	-	0.21 ± 0.01	0.21 ± 0.00	
NM_144707.1	-	-	-	0.35 ± 0.00	0.43 ± 0.01	
NM_152260.1	-	-	0.48 ± 0.09	0.26 ± 0.00	0.25 ± 0.01	
NM_152285.1	-	-	-	0.40 ± 0.04	0.26 ± 0.02	
NM_152465.1	-	-	0.49 ± 0.05	0.37 ± 0.03	0.29 ± 0.00	
NM_152692.2	-	-	-	0.30 ± 0.09	0.24 ± 0.03	
NM_152832.1	-	-	-	0.39 ± 0.07	0.34 ± 0.00	
NM_153213.2	-	2.71 ± 2.08	0.44 ± 0.01	0.21 ± 0.08	0.16 ± 0.02	
NM_153712.3	-	-	-	0.34 ± 0.08	0.36 ± 0.04	
NM_173514.1	-	-	-	0.32 ± 0.02	0.34 ± 0.04	
NM_177926.1	-	-	-	0.30 ± 0.02	0.32 ± 0.01	
NM_181785.1	-	-	-	0.25 ± 0.06	0.25 ± 0.04	
NM_181843.1	-	-	-	0.34 ± 0.02	0.22 ± 0.01	
NM_198519.1	-	-	-	0.36 ± 0.02	0.23 ± 0.02	
NM_212551.1	-	-	-	0.37 ± 0.07	0.30 ± 0.01	
R16740.1	-	-	0.41 ± 0.03	0.37 ± 0.03	0.32 ± 0.03	
R28101.1	-	-	-	0.24 ± 0.02	0.21 ± 0.03	
R60691.1	-	-	-	0.31 ± 0.06	0.39 ± 0.04	
R80806.1	-	-	-	0.32 ± 0.03	0.22 ± 0.02	
R98102.1	-	-	-	0.36 ± 0.13	0.38 ± 0.06	
U90729.1	-	-	0.27 ± 0.03	0.33 ± 0.08	0.31 ± 0.01	
W02629.1	-	-	-	0.25 ± 0.04	0.17 ± 0.00	
W51965.1	-	-	0.38 ± 0.04	0.24 ± 0.03	0.27 ± 0.01	
W67981.1	-	-	0.44 ± 0.01	0.24 ± 0.04	0.39 ± 0.01	
W73998.1	-	-	-	0.24 ± 0.04	0.21 ± 0.04	

(-) means no alteration of gene expression

Table18. The list of functionally unknown genes was up-regulation in cluster 4 from k-means clustering analysis

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment					
	1 hr	3 hr	6 hr	12 hr	18 hr	
AA234276.1	-	-	2.88 ± 0.50	8.20 ± 0.11	-	
AA411574.1	-	-	2.50 ± 0.15	7.96 ± 2.48	11.81 ± 2.27	
AA432383.1	-	2.87 ± 1.62	3.25 ± 0.87	7.02 ± 1.53	8.88 ± 0.10	
AA702785.1	-	3.03 ± 1.58	4.91 ± 1.31	6.92 ± 0.62	6.24 ± 2.44	
AB002344.2	-	-	-	5.97 ± 2.39	5.90 ± 0.37	
AB028952.1	-	-	2.31 ± 0.40	4.85 ± 1.55	6.31 ± 1.62	
AI634809.1	-	-	2.79 ± 0.39	5.62 ± 0.07	6.73 ± 1.41	
AI927713.1	-	-	-	5.44 ± 1.05	11.00 ± 1.22	
AK022550.1	-	2.79 ± 1.81	2.82 ± 0.35	5.00 ± 0.22	3.73 ± 0.21	
AK023614.1	-	-	3.06 ± 0.49	5.24 ± 0.87	7.27 ± 0.54	
AK024582.1	-	2.82 ± 1.49	10.49 ± 1.44	-	30.56 ± 7.35	
AK056401.1	-	-	4.83 ± 0.35	13.26 ± 1.52	14.23 ± 1.67	
AK091810.1	-	-	2.37 ± 0.59	5.59 ± 1.79	6.68 ± 0.00	

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Table18. The list of functionally unknown genes was up-regulation in cluster 4 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment														
	1 hr			3 hr			6 hr			12 hr			18 hr		
AK095308.1	-			-			3.36	±	0.23	5.50	±	0.69	4.25	±	0.19
AK123369.1	-			-			3.36	±	0.40	4.69	±	0.47	6.03	±	0.48
AK128346.1	-			-			6.13	±	1.91	16.58	±	4.32		-	
AK129550.1	-			-			2.65	±	1.06	6.52	±	0.48	9.96	±	3.32
AL117595.1	-			4.13	±	1.98	5.46	±	0.59	5.35	±	0.81	5.43	±	0.69
AL713660.1	-			2.18	±	0.45	4.21	±	0.58	4.08	±	0.30	3.20	±	0.34
AW025751.1	-			-			6.82	±	1.72	22.51	±	2.79	20.80	±	4.75
AW291049.1	-			-			5.02	±	0.65	16.92	±	2.08	17.19	±	2.48
AW292830.1	2.25	±	0.29	5.65	±	1.18	3.11	±	0.29	5.47	±	0.97	5.54	±	0.24
AW299264.1	-			-			3.67	±	1.15	9.10	±	1.73	15.00	±	2.60
AW450103.1	-			-			3.48	±	0.47	5.70	±	0.24	4.43	±	0.54
AW593242.1	-			-			5.01	±	0.40	15.41	±	2.19	19.05	±	5.21
BC006273.2	-			3.19	±	0.11	7.55	±	1.46	9.34	±	1.42	8.91	±	0.76
BC006384.1	-			-			3.31	±	0.20	10.08	±	0.90	6.71	±	0.43
BC007386.2	-			-			2.68	±	0.55	3.74	±	0.10	6.02	±	0.47
BC037804.1	-			3.55	±	1.02	9.02	±	1.14	18.76	±	7.75	16.34	±	3.14
BC042140.1	-			2.63	±	0.16	3.13	±	0.10	7.48	±	0.83	7.46	±	0.26
BC042589.1	-			3.01	±	0.76	3.27	±	1.18	4.43	±	0.92	5.97	±	1.15
BC052239.1	-			-			3.12	±	0.24	6.13	±	1.83	5.82	±	0.94
BE326984.1	-			-			3.21	±	0.56	7.71	±	1.33	8.24	±	4.08
BF509764.1	-			-			2.88	±	0.49	5.92	±	2.73	7.10	±	2.25
BF511844.1	-			3.20	±	0.77	10.91	±	1.10	15.95	±	0.44	12.80	±	0.37
BF594491.1	-			-			3.93	±	0.72	5.99	±	0.19	5.29	±	1.22
BI497415.1	-			-			4.77	±	0.21	5.76	±	0.49	3.39	±	0.46
BI828078.1	-			-			2.94	±	0.36	8.23	±	0.12	10.03	±	0.73
BM451023.1	-			-			3.44	±	0.00	7.16	±	1.27	6.58	±	0.69
BM684070.1	-			-			3.33	±	0.10	5.58	±	2.23	5.19	±	0.49
BM929354.1	-			12.99	±	0.69	34.06	±	1.88	66.88	±	8.76	62.11	±	0.04
BM973460.1	-			-			2.57	±	0.26	6.95	±	3.82	8.43	±	2.92
BQ188618.1	-			-			3.04	±	0.08	15.91	±	1.89	13.08	±	0.32
BQ189193.1	-			2.07	±	0.42	4.32	±	0.69	6.62	±	1.26	7.61	±	1.68
BU624191.1	-			-			3.50	±	0.26	8.00	±	0.10	5.91	±	1.17
BU681282.1	-			-			2.57	±	0.60	7.52	±	0.48	8.16	±	1.59
BU754054.1	-			-			4.32	±	0.28	6.84	±	0.09	5.24	±	0.32
BX103846.1	-			-			5.35	±	0.30	6.68	±	1.43	3.83	±	0.90
BX111290.1	-			-			6.51	±	0.59	14.51	±	1.75	11.62	±	0.45
BX538329.1	-			-			4.71	±	0.35	17.56	±	1.69	24.52	±	4.43
BX648785.1	-			-			2.85	±	0.22	5.84	±	1.66	7.85	±	1.12
BX649153.1	2.90	±	0.31	3.08	±	0.08	4.66	±	0.27	5.20	±	0.80	4.14	±	0.31
CA306881.1	-			-			2.92	±	0.23	6.26	±	1.06	10.02	±	1.78
CB529394.1	-			2.04	±	0.22	2.61	±	0.10	4.50	±	0.56	5.76	±	1.70
H25689.1	-			3.19	±	0.20	13.30	±	1.56	19.39	±	9.31	16.29	±	5.34
INCYTE UNIQUE	2.12	±	0.06	4.72	±	0.06	3.62	±	0.23	6.25	±	1.85	4.85	±	0.96
INCYTE UNIQUE	-			-			2.64	±	0.28	4.50	±	0.01	5.37	±	0.56
INCYTE UNIQUE	3.00	±	0.08	6.54	±	0.44	10.13	±	0.78	16.44	±	2.41	13.12	±	3.26

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Table18. The list of functionally unknown genes was up-regulation in cluster 4 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment														
	1 hr			3 hr			6 hr			12 hr			18 hr		
L19314.1	2.52	±	0.09	6.94	±	1.54	12.52	±	3.47	8.01	±	1.14	12.20	±	1.70
N29918.1		-			-		3.83	±	0.28	12.76	±	2.32	17.14	±	2.53
N32748.1		-		3.36	±	1.01	3.08	±	0.45	7.08	±	1.73	7.75	±	1.48
NM_001731.1		-		2.58	±	0.57	2.63	±	0.49	3.73	±	0.90	5.01	±	1.06
NM_002999.2		-		2.36	±	1.36	4.77	±	0.87	8.41	±	2.39	8.82	±	0.62
NM_003514.2		-			-		2.48	±	0.35	5.01	±	1.66	5.28	±	0.82
NM_003530.3	2.14	±	0.20	4.50	±	2.12	3.73	±	0.71	5.70	±	0.62	4.75	±	0.28
NM_003542.3	2.83	±	1.61		-		2.22	±	0.69	5.59	±	0.52	4.64	±	0.17
NM_003543.3		-		5.71	±	1.21	4.55	±	0.76	13.93	±	1.43	16.70	±	3.43
NM_003548.2	2.23	±	0.75	3.77	±	0.52	6.34	±	0.93	14.22	±	2.49	15.37	±	2.27
NM_003706.1		-		2.63	±	0.78	6.87	±	1.90	18.05	±	0.93	17.83	±	3.48
NM_004148.2		-		3.79	±	3.23	2.89	±	0.39	4.05	±	1.24	3.62	±	0.36
NM_006457.1		-			-		3.48	±	0.43	5.94	±	0.27	4.87	±	1.04
NM_006472.1	3.57	±	2.27	10.50	±	4.56	16.11	±	0.43	18.32	±	2.74	14.27	±	1.93
NM_006813.1		-		6.17	±	4.36	4.47	±	0.14	4.08	±	0.61	4.04	±	0.00
NM_013269.1		-			-		2.57	±	0.07	9.30	±	1.69	12.48	±	2.83
NM_014344.2		-		2.21	±	1.39	4.92	±	0.63	6.45	±	1.49	5.93	±	0.26
NM_014957.2		-		3.16	±	1.54	2.35	±	0.17	4.23	±	0.88	5.60	±	0.05
NM_015266.1		-			-		2.49	±	0.24	3.85	±	0.42	7.01	±	1.48
NM_016081.2		-			-		2.89	±	0.32	6.96	±	1.16	4.78	±	0.14
NM_016321.1		-		2.25	±	0.30		-		41.38	±	9.25	23.05	±	3.40
NM_016545.3		-			-		3.06	±	0.18	5.03	±	0.54	6.67	±	1.64
NM_017899.1		-			-		4.43	±	0.77	8.04	±	1.40	7.45	±	0.08
NM_018282.1		-			-		3.15	±	0.52	5.87	±	1.64	7.20	±	1.87
NM_018370.1		-			-		2.98	±	0.20	6.99	±	2.12	6.26	±	0.61
NM_018672.2		-			-		3.19	±	0.14	6.16	±	1.53	9.45	±	1.66
NM_018842.3		-		3.22	±	2.72	3.55	±	0.40	4.65	±	0.99	5.67	±	0.01
NM_020130.2		-		3.42	±	1.58	10.85	±	2.09	10.29	±	4.23	12.48	±	2.07
NM_020307.2		-		5.87	±	3.46	6.55	±	0.20	10.41	±	2.46	13.01	±	0.60
NM_021059.2		-		5.52	±	0.94	17.37	±	3.26	39.62	±	7.80	36.97	±	2.06
NM_021127.1		-		2.90	±	1.48	5.36	±	0.14	9.64	±	1.79	8.72	±	1.22
NM_022351.2		-			-		3.10	±	0.38	12.34	±	3.63	7.71	±	3.16
NM_025079.1		-		3.72	±	0.35	5.11	±	0.46	6.96	±	1.00	7.49	±	0.36
NM_031412.1		-			-		3.04	±	0.54	6.09	±	1.27	6.12	±	1.89
NM_031419.1		-		3.34	±	2.12	8.77	±	3.80	9.09	±	4.30	8.23	±	1.02
NM_032130.1		-			-		3.24	±	0.19	10.89	±	1.01	8.84	±	0.87
NM_032871.3		-			-		3.18	±	0.01	4.22	±	0.18	4.93	±	1.76
NM_052966.1		-			-			-		9.66	±	3.24	9.92	±	0.08
NM_080574.2		-			-		2.48	±	0.41	4.89	±	0.61	10.47	±	1.20
NM_133636.1		-		3.03	±	1.76	2.82	±	0.69	4.14	±	0.23	4.53	±	0.05
NM_133639.2		-		2.61	±	2.20	3.66	±	0.44	4.66	±	1.14	5.95	±	0.82
NM_145804.1		-		2.28	±	0.77	3.44	±	0.14	4.93	±	0.59	4.26	±	0.25
NM_147189.1		-		2.50	±	1.09		-		5.52	±	0.16	4.94	±	0.35
NM_152281.1		-		2.57	±	0.91	2.95	±	0.27	5.06	±	1.82	7.33	±	1.95
NM_172138.1		-			-			-		6.40	±	1.93	12.04	±	3.70
NM_172140.1		-			-		2.19	±	0.43	8.70	±	2.85	17.23	±	13.20

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Table18. The list of functionally unknown genes was up-regulation in cluster 4 from k-means clustering analysis (continued).

NCBI Accession No.	The fold change difference in each time points after VR-3848 treatment											
	1 hr	3 hr			6 hr			12 hr			18 hr		
NM_177424.1	-	2.33	±	1.17	3.57	±	0.23	4.83	±	1.22	4.54	±	1.99
NM_181531.1	-	2.41	±	0.73	4.15	±	0.35	10.66	±	0.73	17.35	±	3.08
NM_183376.1	-	9.08	±	5.10	9.14	±	0.02	15.71	±	2.09	16.60	±	2.06
NM_197955.1	-	3.15	±	0.72	5.43	±	0.81	11.06	±	0.15	21.00	±	2.02
NM_199349.1	-		-		2.57	±	0.10	7.18	±	0.44	12.18	±	3.31
NM_201557.1	-		-		3.04	±	0.46	7.74	±	0.82	8.24	±	0.43
NM_203411.1	-	5.70	±	0.03	7.58	±	0.74	10.33	±	1.88	9.25	±	0.20
R72835.1	-		-		2.32	±	0.64	4.80	±	0.22	7.22	±	0.83
U60873.1	-		-			-		6.77	±	0.36	5.47	±	0.25
U63828.1	-		-			-		4.08	±	2.19	8.53	±	0.34

(-) mean no alteration of gene expression.

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APPENDIX D

THE FUNCTIONALLY UNKNOWN GENES IN THE EARLY RESPONSE TO VR-3848

Table19. The list of functionally unknown genes response to VR-3848 stimulation at 1 hr of treatment.

NCBI Accession number	Fold change	FDR
AA214042.1	2.79 ± 0.56	1.11
AA486515.1	2.33 ± 0.31	0.70
AA558704.1	4.07 ± 2.49	0.67
AA568123.1	2.52 ± 0.42	0.88
AA678039.1	2.16 ± 0.21	1.59
AA705451.1	2.26 ± 0.00	1.11
AA807209.1	2.26 ± 0.00	0.00
AA889260.1	2.93 ± 0.31	0.00
AI051235.1	2.42 ± 0.33	0.82
AI051962.1	2.98 ± 0.10	0.00
AI207469.1	2.30 ± 0.06	1.01
AI332638.1	2.50 ± 0.13	0.00
AI498508.1	2.11 ± 0.06	0.70
AI672357.1	2.12 ± 0.14	1.59
AI718818.1	2.54 ± 0.63	1.89
AI733652.1	2.10 ± 0.03	0.74
AI765125.1	2.25 ± 0.23	0.69
AI792721.1	4.19 ± 0.14	0.00
AI798609.1	2.17 ± 0.21	1.59
AI889352.1	3.88 ± 0.16	0.00
AI907551.1	2.71 ± 0.49	1.01
AI925036.1	2.40 ± 0.06	0.00
AI954275.1	3.69 ± 0.89	0.00
AI972618.1	3.07 ± 0.22	0.00
AK000296.1	2.86 ± 0.02	0.00
AK002088.1	3.34 ± 1.49	0.77
AK056213.1	2.12 ± 0.07	0.73
AK074440.1	2.56 ± 0.70	1.59
AK093406.1	3.36 ± 0.54	0.00
AK094156.1	2.74 ± 0.41	0.00
AK095902.1	2.08 ± 0.09	1.59
AK097966.1	2.03 ± 0.03	1.89
AK098572.1	2.59 ± 0.62	0.70
AK125133.1	4.60 ± 0.74	0.00
AK130672.1	3.79 ± 0.02	0.00
AL359599.1	3.79 ± 0.02	0.00
AL360140.1	3.79 ± 0.02	1.59

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Table19. The list of functionally unknown genes response to VR-3848 stimulation at 1 hr of treatment (continued).

NCBI Accession number	Fold change	FDR
AL602447.1	2.49 ± 0.61	1.59
AV737724.1	2.49 ± 0.61	0.77
AW025427.1	2.73 ± 0.36	0.00
AW074626.1	2.21 ± 0.09	0.82
AW102654.1	3.80 ± 1.36	0.00
AW105170.1	2.10 ± 0.06	0.69
AW134473.1	2.65 ± 0.34	0.00
AW157431.1	2.11 ± 0.15	1.59
AW293452.1	2.69 ± 0.90	1.48
AW294696.1	2.55 ± 0.43	0.90
AW296107.1	2.67 ± 0.25	0.00
AW296419.1	2.33 ± 0.41	1.59
AW296451.1	3.03 ± 0.04	0.00
AW673901.1	2.61 ± 0.38	1.01
AW970116.1	3.09 ± 1.52	1.61
AW970240.1	2.23 ± 0.11	0.82
AW971070.1	2.20 ± 0.02	0.96
AY037867.1	2.58 ± 0.22	0.00
BC010534.1	2.51 ± 0.25	1.19
BC041658.1	2.48 ± 0.53	0.66
BC042989.1	2.05 ± 0.03	1.95
BE070450.1	2.16 ± 0.19	1.59
BF439738.1	3.23 ± 0.30	0.00
BF508226.1	2.38 ± 0.06	0.00
BF508301.1	2.84 ± 0.05	0.00
BF508499.1	2.41 ± 0.05	0.00
BF513066.1	4.76 ± 0.50	0.00
BG615758.1	2.70 ± 0.78	0.67
BG944372.1	2.64 ± 0.59	0.80
BI494600.1	2.88 ± 0.27	0.00
BM677556.1	2.42 ± 0.16	1.11
BM700222.1	2.12 ± 0.01	0.81
BM983364.1	3.06 ± 0.39	0.00
BQ006286.1	2.62 ± 0.23	0.00
BQ013066.1	3.38 ± 0.10	0.00
BQ025233.1	2.78 ± 0.31	0.00
BQ025484.1	2.09 ± 0.09	1.89
BQ028015.1	2.44 ± 0.08	0.00
BU754159.1	2.63 ± 0.03	0.00
BX089756.1	3.04 ± 1.13	0.74
BX094605.1	2.68 ± 0.40	0.00
BX100091.1	2.70 ± 0.36	0.00
BX108121.1	3.64 ± 0.10	0.00
BX111550.1	2.23 ± 0.24	1.95
BX113590.1	2.44 ± 0.47	0.67
BX353482.2	2.25 ± 0.31	1.59
BX648297.1	2.67 ± 0.22	0.00
BX649153.1	2.90 ± 0.31	0.00
CA775800.1	2.67 ± 0.32	0.00
CB852608.1	2.45 ± 0.60	1.48

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Table19. The list of functionally unknown genes response to VR-3848 stimulation at 1 hr of treatment (continued).

NCBI Accession number	Fold change	FDR
CB997196.1	2.30 ± 0.05	1.11
CD699610.1	2.12 ± 0.13	1.59
CF887351.1	2.16 ± 0.06	0.79
N25754.1	2.94 ± 0.38	0.00
NM_003530.3	2.14 ± 0.20	1.59
NM_004765.2	2.31 ± 0.01	0.00
NM_013275.3	2.67 ± 0.06	0.00
NM_016628.2	2.14 ± 0.18	1.59
NM_017641.2	2.38 ± 0.31	0.78
NM_017742.3	2.33 ± 0.09	1.01
NM_020193.2	2.47 ± 0.10	0.00
NM_020803.3	2.29 ± 0.07	0.99
NM_022763.2	2.64 ± 0.36	0.00
NM_024319.2	2.55 ± 0.76	1.88
NM_025095.1	2.60 ± 0.02	0.00
W84667.1	2.07 ± 0.02	0.70

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BIOGRAPHY

NAME	Promsin Masrinoul
DATE OF BIRTH	April 13, 1982
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2000-2004: Bachelor of Science (Biology) Mahidol University, 2004-2006: Master of Science (Microbiology)
SCHOLARSHIP	Development and Promotion of Science and technology Talents project (DPST)
E-MAIL ADDRESS	promsinyor@yahoo.com
HOME ADDRESS	9 village No. 8 Nongkhaem sub-district, Nongkhaem district, Bangkok 10160, Thailand

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