APOPTOSIS INDUCTION BY VR-3848 ISOLATED FROM *EUPHOBIACEAE* IN HUMAN LUNG CANCER CELL LINE

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It has been demonstrated that a variety of anticancer drugs inhibit the growth of carcinoma cells by induction of apoptosis. VR-3848 is a potent-cytotoxic-unknown compound purified from *Euphobiaceae,* a tropical Thai plant. The concentration of VR-3848 that corresponds to half of the viability (GI_{50}) was 10 nM. The ability of VR-3848 to initiate apoptosis was investigated in a human lung (LU-1) cancer cell line. Treatment of cells with VR-3848, GI_{50} (10 nM), 10x GI_{50} (100 nM), and 20x GI_{50} (200 nM) or vinblastine (a positive control), 2xGI_{50} (200 nM) between 3 and 48 hours caused morphological changes consistent with the induction of apoptosis. Apoptosis induced by VR-3848 detected by 4′, 6-Diamino-2-phenylindole (DAPI) staining and visualized by a fluorescence microscope was time- and --dose dependent. The peaks of apoptosis were seen at 48 hours incubation period up to 30% and 40% in cells treated with VR-3848, 100 nM and 200 nM, respectively. In addition, studies by agarose gel electrophoresis, a characteristic ladder of DNA fragments in multiples of 180-200 base pairs, was observed in DNA extracted from cells treated with VR-3848, 200 nM for 48 hours. The results were similar in cells treated with vinblastine 200 nM for 48 hours. To examine whether the cysteine protease, CPP32 (caspase-3), contributes to the VR-3848-induced apoptosis, the expression of mRNA for CPP32 in LU-1 cells was performed by RT-PCR. The results revealed that both control and treated LU-1 cells expressed the almost same levels of mRNA for CPP32. Importantly, at the apoptosis-inducing concentration, VR-3848 also induced the activation of caspase-3 between 3 and 48 hours. Furthermore, 5 μM of a specific inhibitor of caspase-3-like protease, Ac-DEVD-CHO, significantly blocked CPP32 activity. In summary, the findings demonstrate that VR-3848 may induce cell death in LU-1 cell lines via apoptosis which is mediated by the activation of caspase-3.

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รุ่งกานต์ ภู่ตระกรณ์ชัย: ศึกษากลไกการตายของเซลล์มะเร็งปอดของคน โดยสารบริสุทธิ์ (วีอาร์-3848) ที่สกัดจากต้นยูโฟบีเอซี (APOPTOSIS INDUCTION BY VR-3848 ISOLATED FROM *EUPHOBIACEAE* IN HUMAN LUNG CANCER CELL LINE) คณะกรรมการควบคุมวิทยานิพนธ์: กุลวีณ์ สุจริต, Ph.D., ภาวิณี ปียะจตุรวัฒน์, ปร.ด., วิชัย ริ้วตระกูล, Ph.D., ทรงศักดิ์ เพ็ชรมิตร, ปร.ด. 122 หน้า ISBN 974-664-014-3

ได้มีรายงานว่า ยาต้านมะเร็งหลายชนิดออกฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์มะเร็งโดยผ่านทางกลไกการตายแบบเอปอบโตซิส วีอาร์-3848 เป็นสารบริสุทธิ์ที่สกัดจากพืชป่าเขตร้อนต้นยูโฟบีเอซี เป็นสารที่ยังไม่ทราบโครงสร้างที่แน่นอน แต่พบว่ามีฤทธิ์ฆ่าเซลล์มะเร็งที่เพาะเลี้ยงได้ ความเข้มข้นของวีอาร์-3848 ที่ยับยั้งการเจริญเติบโตของเซลล์ได้ครึ่งหนึ่งของจำนวนเซลล์ทั้งหมด มีค่าเท่ากับ 10 นาโนโมลาร์ ได้ทำการศึกษาผลของวีอาร์-3848 ในการชักนำให้เกิดเอปอบโตซิสในเซลล์มะเร็งปอดของคนระหว่างเวลา 3 และ 48 ชั่วโมงที่ความเข้มข้น 10, 100 และ 200 นาโนโมลาร์ และใช้วินบลาสตินที่ความเข้มข้น 200 นาโนโมลาร์เป็นตัวควบคุมเชิงบวก เซลล์ที่ได้รับสารที่ความเข้มข้นเหล่านี้ จะมีการเปลี่ยนแปลงรูปร่าง การตายเนื่อง จากวีอาร์-3848 ถูกทดสอบโดยใช้วิธีย้อมสีนิวเคลียสของเซลล์ด้วยสี DAPI (4,3-Diamino-2-phenylindole) และพบว่าการตายนี้ขึ้นกับเวลาและความเข้มข้นของสารที่ใช้ ค่าเปอร์เซ็นต์สูงสุดของการตายของสารวีอาร์-3848 ที่ 100 นาโนโมลาร์ และ 200 นาโนโมลาร์ หลังจากสัมผัสกับยาเป็นเวลานาน 48 ชั่วโมงอยู่ที่ประมาณ 30% และ 40% นอกจากนี้ยังทำการศึกษาการเปลี่ยนแปลงทางชีวเคมีของนิวเคลียสของเซลล์ พบลักษณะการเรียงตัวเป็นขั้นบันไดของดีเอ็นเอบนอกาโรสเจล ในเซลล์ที่ได้รับสารวีอาร์-3848 และวินบลาสตินที่ความเข้มข้น 200 นาโนโมลาร์ หลังจากใส่ยาเป็นเวลานาน 48 ชั่วโมง เพื่อศึกษาว่าสารวีอาร์-3848 ทำให้เซลล์ตายชนิดเอปอปโตซิสนั้นเกิดจากการทำงานของเอ็นไซม์คาสเพส-3 ได้ทำการศึกษาการแสดงออกของยีนคาสเพส-3 ที่มาควบคุม โดยวิธีอาร์ที-พีซีอาร์ พบว่าสารที่ความเข้มข้นที่ทำให้เซลล์ตายแบบเอปอปโตซิส ให้ผลในการแสดงออกของยีนไม่แตกต่างจากกลุ่มเซลล์ที่ไม่ได้รับสารวีอาร์-3848 อย่างไรก็ตามจากการวัดระดับของเอ็นไซม์คาสเพส-3 พบว่าระดับของคาสเพส-3สูงขึ้นในช่วงเวลาที่เกิดเอปอปโตซิส และถูกยับยั้งได้โดยตัวยับยั้งที่เฉพาะเจาะจงต่อคาสเพส-3 ดังนั้นจากผลการทดลองทั้งหมดสรุปได้ว่า สารวีอาร์-3848 ฆ่าเซลล์โดยผ่านกลไกการตายแบบเอปอบโตซิส ซึ่งอาจเป็นผลจากการทำงานของคาสเพส-3 ซึ่งกลไกการทำงานที่แน่นอนของวีอาร์-3848ต้องทำการศึกษาโดยละเอียดต่อไป

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LIST OF ABBREVIATIONS

Asp	=	Aspartic acid
bp	=	base pair
BCS	=	bovine calf serum
BSA	=	bovine serum albumin
$^{\circ}C$	=	degree celcius
cDNA	=	complementary deoxyribonucleic acid
cm^2	=	square centimeter
cm^3	=	cubic centimeter
CO_2	=	carbondioxide
dATP	=	deoxy adenosine triphosphate
dCTP	=	deoxy cytosine triphosphate
dGTP	=	deoxy guanosine triphosphate
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxy nucleotide triphosphate
dTTP	=	deoxy thymidine triphosphate
DTT	=	dithiothreitol
EDTA	=	ethylenediamine
e.g.	=	for example
g	=	gram
Glu	=	Glutamic acid

GI_{50}	=	concentration that inhibits 50% cell proliferation
ml	=	milliliter
mm	=	millimeter
mm^3	=	cubicmillimeter
mM	=	millimolar
N_2	=	nitrogen
nm	=	namometer
nM	=	nanomolar
OD	=	optical density
OD260	=	optical density at 260 nm
OD280	=	optical density at 280 nm
OD515	=	optical density at 515 nm
PBS	=	phosphate buffered saline
RNA	=	ribonucleic acid
RNase	=	Ribonuclease
rpm	=	revolution per minute
RT-PCR	=	reverse transcriptase polymerase chain reaction
SDW	=	sterile distilled water
SRB	=	sulforhodamine B
TBE	=	Tris-borate-EDTA
TCA	=	Trichloroacetic acid
TE	=	Tris-EDTA
Tris-HCl	=	Tris-hydrogenchloride

U	=	unit
UV	=	ultraviolet
Val	=	Valine
w/v	=	weight per volume
µg	=	microgram
µl	=	microliter
%	=	percent

CHAPTER I

INTRODUCTION

Cancer is the third cause of death in Thai population, next to cardiovascular disease and accident, respectively. Among those types of cancer, lung cancer is one of the most common prevalent cancers in Thai population and in the developing countries of the world. In 1995, there was a report from the National Cancer Institutes, Ministry of Public Health of Thailand (1) that the incidence of lung cancer in male was highest accounting for about 18%. In female, lung cancer was the fourth rank after cervix uteri, breast and oral cavity, respectively. These data are similar to the report in 1997 from the USA which showed an increase in the incidence rate (17% in men and 12% in women) and mortality rate (34% in men and 12% in women) of lung cancer in both sexes (2). Thus, at present, it seems to be that lung cancer is a leading cause of cancer deaths.

To date, a variety of anticancer drugs have been demonstrated to inhibit the growth of carcinoma cell by induction of apoptotic cell death. Apoptosis or programmed cell death is an important process in a wide variety of different biological systems including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and also in chemical-induced cell death. The dysregulation of apoptosis pathway can play a significant role in the growth and therapeutic responsiveness of cancer cells. Apoptosis requires tightly related death pathways, including activation of cysteine proteases of the caspase

family. These are characterized by the cleavage of specific substrates after an aspartic residue and considered to be essential in the execution stage of the apoptotic process. Among the caspase family, caspase-3/CPP32-like proteases appears to be one of the most important caspase in apoptosis of mammalian cells.

As a part of an ongoing project to discover natural product cancer chemotherapeutic agents, VR-3848, an unknown natural compound, was purified from *Euphobiaceae.* It was found to inhibit cell proliferation against various human cultured cancer cell lines, i.e., lung (LU-1), breast (BCA-1), colon (COL-1), epidermoid carcinoma in the mouth (KB), and also mouse lymphoid neoplasm (P_{388}). Further studies using ASK assay (Sujarit *et al.,* 1998) (3) demonstrated that the mechanism of action of VR-3848 on cell growth inhibition could not be due to interfering with the function of microtubules. However, at present, the mechanism by which VR-3848 inhibits the growth of human cultured cancer cell is still unknown.

CHAPTER II

OBJECTIVES

Thus, the purposes of the present study were as follows:

1. To examine whether VR-3848 isolated from *Euphobiaceae* inhibits the growth of human lung (LU-1) cancer cell line via apoptotic cell death.

1.1 Dose-response study of VR-3848 and vinblastine (a positive control) on the growth of LU-1 cell

1.2 Induction of apoptosis by VR-3848 and vinblastine, apoptotic cell death was detected by phase-contrast microscope and 4′,6-Diamino-2-phenylindole (DAPI) staining and agarose gel electrophoresis.

2. To further investigate the possible role of caspase-3 (CPP32) on the VR-3848-induced apoptosis in LU-1 cell line.

2.1 Expression of caspase-3 gene in LU-1-induced apoptosis by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.2 Evaluation of the caspase 3 activity using a fluorogenic assay.

Vinblastine was used as a positive control in the present work because it has been reported by Tashiro *et al.* (1998) (4) that vinblastine induced apoptotic cell death in human small cell lung carcinoma, Ms-1 cells, by activation of caspase-3. In addition, LU-1 cell was used as a model in this study since it has been shown that lung cancer is a leading cause of cancer deaths in the developed nations of the world and also in Thailand. Furthermore, one major problem in treatment of cancer by chemotherapy

approaches is drug resistance, therefore, development of new anticancer compounds capable of circumventing the drug resistance is necessary.

In this study, it was aimed to gain more information on the mechanism of action of the unknown purified compound, VR-3848, on the inhibitory effect of cancer cell growth. In addition, the work provides a basis for further investigations of the role of caspases in the regulation of cell death, a better understanding of the role of gene that regulates apoptosis. Consequently, the information obtained from this work may eventually lead to the development of a new antitumor drug from Thai tropical plants and help in the treatment of cancer by using gene therapy to induce tumor cell death by apoptosis in the future.

CHAPTER III

LITERATURE REVIEWS

Cancer cells are defined by two heritable properties: they and their progeny first, reproduce in defiance of the normal restrains and second, invade and colonize teritories normally reserved for other cells. It is the combination of these features that makes cancers peculiarly dangerous (5). Other environmental and genetic factors are important in the etiology of cancer, and it seems highly probable that derangements of the mechanisms that control cell replication and in the synthesis of nucleotides and proteins are common features of malignant transformation. There is increasing evidence to implicate the insertion of foreign nucleic acid sequences in the host's genome (so-called oncogenes) in the genesis of cancer. It may be that more than one defect is required to produce (6)

3.1 LUNG CANCER

3.1.1 Incidence and Epidemiology

Examination of the variation in mortality and incidence of certain cancers over time reveals the importance of environmental factors in cancer etiology. Mortality rates for a number of cancers in the United States have changed substantially over the past 60 years whereas the rates for several other cancers have remained relatively stable. Increasing long-term trends of lung cancer and decreasing long-term trends of stomach cancer in developed countries during most of this century have led to hypotheses implicating tobacco smoking and food preservation modalities,

respectively, in their causations. Specifically, the rapid rise of lung cancer mortality among men during the first half of the century and the much slower rise of the corresponding mortality among women during the same period pointed to a strong carcinogen that was widespread among men, but much less so among women during period (7). In 1995, The National Cancer Institute, Department of Medical Service, Ministry of Public Health of Thailand reported the 10 leading sites of cancer in male and female which is summarized in Table 3.1 (1).

Table 3.1 The 10 leading sites of cancer in Thai population

Male	**% incidence**	**Female**	**% incidence**
Lung	17.8	Cervix uteri	33.1
Liver	14.0	Breast	31.5
Oral cavity	10.5	Oral cavity	4.0
Colorectum	6.6	Lung	3.3
Nasopharyx	4.9	Ovary	3.0
Brain	4.6	Colorectum	2.6
Lymphoma	4.4	Liver	2.5
Skin	4.1	Brain	2.2
Esophagus	3.5	Corpus uteri	2.1
Larynx	2.9	Lymphoma	1.9

3.1.2 Etiology and Risk factor

The specialized epithelial cells at all levels of the respiratory tract are thought to arise from primordial undifferentiated cells of endodermal origin (8). These cells form a diverticulum which further divided to form the trachea and primary bronchi which then further branch to give rise to the remainder of the bronchial tree.

It is clinically useful to broadly categorize bronchogenic cancers into two groups which reflect their biology and management: small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC).

3.1.2.1 Small cell lung cancers

SCLCs are relatively sensitive to cytotoxic chemotherapy and radiation therapy. They account for about 25% of lung tumors and are usually centrally located but occasionally arise peripheral. Clinically, they are characterized by a more rapid growth rate as well as early metastasis dissemination. Before the advent of systemic therapy, local surgical or radiation therapy alone resulted in very poor median survivals, ranging from 8 to 17 weeks and 5 year survivals of less than 1% (9). Effective chemotherapy has allowed the control of disseminated disease and improved the median survival of patients to 1 year or more and increasing the number of long-terms disease-free survivors to between 5 to 10% (10).

Most patients will die early from recurrence of the initial tumor; among those patients who survive longer, there is a high incidence of second primary cancers (chiefly non-small cell lung cancer) and other tobacco-related malignancies (11).

3.1.2.2 Non-small cell lung cancers

This is a morphologically diverse group which includes squamous (epidermoid) carcinoma, adenocarcinoma, and large cell carcinoma.

The squamous phenotype used to be the predominant form of lung cancer worldwide, however, its relative and absolute incidence in the United States (and other parts of the world such as East Asia) has dramatically decreased within the last two decades. Epidemiologically, it is strongly associated with cigarette smoking and this explains its frequent association with metaplastic and dysplastic changes in adjacent epithelium.

Adenocarcinomas have become the most common form of lung cancer in the United States. In general, they tend to arise in the peripheral airways and may possess distinctive intracellular mucus granules as part of their acinar/glandular differentiation. While glandular formation and mucin production are hallmarks of adenocarcinomas express these features.

The large cell carcinoma is the least common of all NSCLC tumors. It is undifferentiated tumors which do not fall into the small cell category, and which lack squamous cell or glandular differentiation at the light microscope level (12).

3.1.3 Principle of therapy

3.1.3.1 Small - cell lung carcinoma

SCLCs are relative sensitive to cytotoxic chemotherapy and radiation therapy (13). Combination chemotherapy is indicated for patients with both limited and extensive disease. Radiation to the primary site and mediastinum improves local control and survival in patients with limited disease. Patients who achieve a complete

remission are considered for prophylactic cranial irradiation. Surgical resection is rarely required.

3.1.3.2 Non - small cell lung cancer

Surgical resection is the preferred treatment for clinical stage I or stage II disease. Curative radiotherapy is given to patients with early - stage disease in whom resection is impossible. Adjuvant radiotherapy in completely resected stage II and stage III squamous - cell carcinoma may decrease local recurrence but does not affect survival. Stage III lung cancer is treated with radiotherapy in an attempt to either delay the onset of symptomatic disease or improve functional status. Within stage III, N2 nodal disease indicates a particularly poor prognosis. Preoperative radiotherapy and neoadjuvant chemotherapeutics regimens have not improved survival.

Locally symptomatic components of stage IV disease may be treated with palliative radiotheraypy. Single - agent and combination chemotherapy produce response rates of 10% to 20%. Performance status is the most important prognostic factor for chemotherapy. Encouraging response rates are being obtained with taxol and the topoisomerase inhibitor topotecan.

Table 3.2 Summary properties of SCLC and NSCLC (14-16)

Features	**SCLC**	**NSCLC**		
		Squamous cell carcinoma (epidermoid)	**adenocarcinoma**	**Large cell carcinoma**
1. Location	- Intramucosal - Bronchial mucosa	- Proximal segmental bronchi	- Alveolar surface epithelium - bronchial mucosal glands (peripheral part of lung)	- Peripheral part of lung
2. Histology	- Prominent clusters of anaplastic large cells	- Sheets of epithelial cells	- Tumors from glands and produce mucin	- Similar adenocarcinoma
3. Incidence	25%	30%	40%	10-20%
4. Active drugs	- Paclitaxel, Docetaxel, Irinotecan	Vinorelbine, Paclitaxel,	Vinblastine, Edatrexate,	Irinotecan, Docetaxel

3.2 ANTICANCER AGENTS (17, 18)

At present, chemotherapy provides palliative rather than curative therapy for many other forms of disseminated cancer. Anticancer agents can be divided into 7 groups.

3.2.1 Alkylating agents

Alkylating agents appear to exert their cytotoxic effects by interactions of their electrophilic functional (chloroethyl) groups with nucleoplilic cellular targets, such as the 7-nitrogen of quanine in DNA or RNA. Cross-linking of DNA appears to be of major importance to the cytotoxic action of alkylating agents, replicating cells are most

susceptible to these drugs. The example of alkylating agents are alkyl sulfonates, cisplatin, nitrogen mustards, nitiosoureas and triazenes. The important toxic effect of therapeutic doses of all the alkylating drugs is depression of bone marrow and subsequent leukopenia and thrombocytopenia.

3.2.2 Antimetabolites

Antimetabolites are designed as competitive inhibitors of enzymatic reactions of intermediary metabolism or inactive analogs of reaction substrates. Antimetabolites are not commonly given as single agents because they are phase specific and affect macromolecular syntheses at multiple points. The major classes of antimetabolites are folic acid analogue (e.g., methotrexate), purine antagonists (e.g., mercaptopurine), and pyrimidine antagonists (e.g., fluorouracil, 5-FU). Toxic effects are obserbed in the bone marrow, skin, and gastrointestinal mucosa.

3.2.3 Mitotic Inhibitors

Microtubules are integral components of the mitotic spindle, which can be disrupted by both vinca alkaloid, such as vinblastine and vincristine, and taxanes, resulting in metaphase arrest in dividing cells. Therefore, antimicrotubule agents may affect both neoplastic and nonmalignant cells in interphase, in addition to the mitotic phases of cell cycle. Its major toxicities are bone marrow suppresion, especially in patients with pre-existing hepatic impairment.

3.2.4 DNA Topoisomerase II Inhibitors

DNA Topoisomerase II is a homodimeric protein (molecular weight = 170,000 daltons) and major component of the nuclear matrix. It may have important roles in DNA packaging (i.e., higher order structure of chromatin), replication, and

transcription. Because the normal regulation of topoisomerase II inhibitor is probably linked to the ability of a cell to enter a G_0 period, malignant cells, which do not accumulate in G_0 during quiesence, fail to have diminished topoisomerase II activity and consequently may be more sensitive to drugs that interact with this enzyme. It appears that topoisomerase II inhibitor is epipodophyllotoxins (e.g., etoposide and teniposide had a major role in the treatment of small cell lung cancer and lymphoma) which can *in vitro* and *in vivo* stimulate topoisomerae II to produce site-specific double-strand breaks in the DNA helix.

3.2.5 Antitumor antibiotics

Many of these antibiotics bind to DNA through intercalation between specific bases and block the synthesis of new RNA or DNA (or both), cause DNA strand scission, and interfere with cell replication. All of the clinically useful anticancer antibiotics now available are products of various strains of the soil fungus *Streptomyces.* These include the anthracyclines, actinomycin, bleomycin, mitomycin, and plicamycin. Bone marrow depression is the major toxicity of this agents.

3.2.6 Hormone Therapy

Hormone therapy relies on the presence of receptors for endogenous hormones required for cell proliferation. Unlike agents in the other classes of antineoplastic drugs, members of this class generally do not cause severe toxicity. Sex hormones or their antagonists are most effective in tumors arising from cells that are normally hormones dependent, namely breast and prostate. There are several ways in which hormones can affect malignant cells:

A. a hormone may have a direct cytotoxic action on the malignant cell.

B. a hormone may suppress production of other hormones by a feedback mechanism.

3.2.7 Miscellaneous agents

1. Amsacrine

   Mechanism : Chromosome breakage

   Toxicity : Hematologic toxicity

2. Asparaginal

   Mechanism : Inhibition of protein synthesis

   Toxicity : Normal cells are less susceptible

3. Hydroxyurea

   Mechanism : Inhibition DNA synthesis, enzyme ribonucleotide reductase

   Toxicity : Bone marrow depression

4. Mitoxantrone

   Mechanism : DNA strand breakage, inhibit both DNA and RNA synthesis

   Toxicity : Cardiac toxicity

5. Retinoic acid derivatives

   Mechanism : Disrupt gene for nuclear receptor -α for retinoic acid

   Toxicity : Mucocutaneous, skeletal, liver, and teratogenic effects.

3.3 APOPTOSIS

There are at least two types of cell death, apoptosis and necrosis. To date, there has been much evidence to show that most anticancer drugs induce cell death by apoptosis.

3.3.1 Apoptosis and necrosis

Apoptosis, also called programmed cell death, is one of the two common forms of cell death described in higher eukaryotes, including plants, slime mold, insects and vertebrates (19). Kerr *et al.* 1972 (20) named this form of cell death from the ancient Greek word for the "falling off" of petals from flowers or leaves from trees. In contrast to the injury-induced death or necrosis, apoptosis is observed in both heathly and neoplastic of adult and embryonic tissue (20). Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and also in chemical-induced cell death. The dysregulation of apoptotic pathway can play a significant role in the growth and therapeutic responsiveness of cancer cells.

3.3.1.1 Morphological changes of cells during apoptosis

The morphological changes of apoptosis can be divided into three phases (21).

Phase I. There is reduction in nuclear size with condensation of chromatin into crescentic caps at the periphery of the nucleus.

Phase II. There is blebbing at the cell surface and crenation of the nuclear outline, leading to controlled fragmentation of both nucleus and cytoplasm

which subsequently split up into particles of various size called "apoptotic bodies". Some apoptotic bodies contain variably-sized, spherical, nuclear fragments of condensed chromatin. These bodies phagocytosed by neighbouring variable tumor cells or macrophage.

Phase III. The residual nuclear and cytoplasmic structures undergo progressive degradation. Subsequently, cells develop, "secondary necrosis", which apoptotic body membranes disappear and the appearance is that of a residual lysosomal body (22).

The proposed stages in apoptosis is shown in Figure 3.1.

On the other hand, necrosis is pathological cell death which occurs during the causes of cell injury and repair. This type of cell death occurs in response to harmful condition, such as toxic substances, metabolic poison, hyperthermia, hypoxia, ischemia, and direct cell trauma (19). In necrosis, there are early change in mitochondial shape and function, the cell becomes unable to maintain homeostasis. Cells undergoing necrosis loss plasma membrane integrity and can not regulate osmotic pressure. Therefore, cell will rupture and spill out the cellular content into surrounding tissue space which can provoke an inflammation (23). The difference in cellular changes during cell undergoing apoptosis and necrosis is shown in Table 3.3.

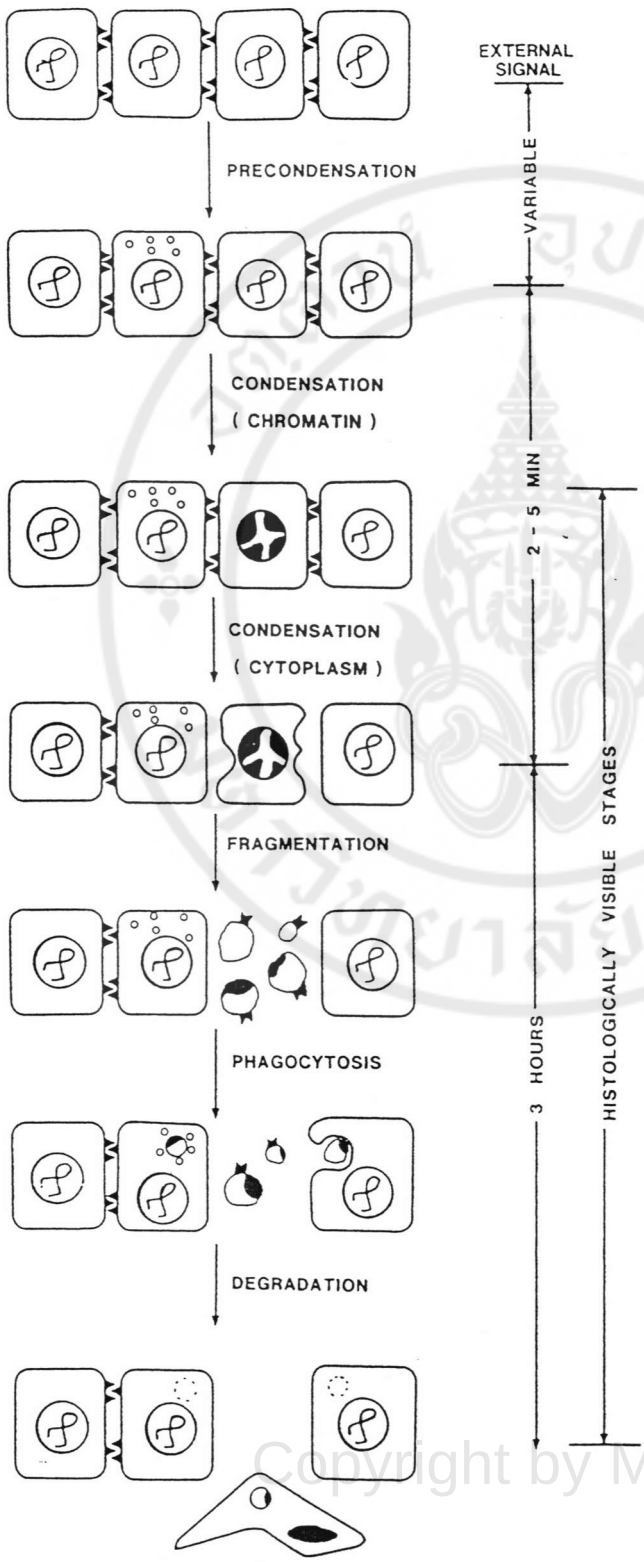

Figure 3.1 Proposed stages in apoptosis (23). The condensation of chromatin in the nucleus of the affected cell and the breakdown of cell-cell interactions as the cell undergoing apoptosis is isolated from its neighbors. The condensation phase has been broken down into 2 phases, chromatin condensation and cytoplasmic condensation. The next stage, apoptotic cell may be fragmented into a number of small "apoptotic bodies", some of which contain portions of the fragmented nucleus. New epitopes on the surface of the apoptotic bodies induced recognition by the neighboring cells and macrophages. The final stages of the process are the phagocytosis of apoptotic bodies by these cells and the subsequent degradation of apoptotic bodies by the lysosomes of the recipient cells (24).

Table 3.3 Comparison of cellular alterations in apoptosis to those in necrosis [from Webb *et al.,* 1997 (25)].

Cellular changes	**Apoptosis**	**Necrosis**
DNA	Internucleosomal cleavage (Ladder)	Degradation (no ladder)
Nucleus	Chromatin margination	Pyknosis
Membrane integrity	Persists until late	Comprised early
Mitochondria	Appear normal ultrastructurally	Swelling, Ca^{2+} uptake
Inflammatory changes	No	Yes
Pattern affected	Individual cells affected	Foci of multiple cells
Cell volume	become small	become bigger
Cell fragmentation	yes (Apoptotic bodies)	no (Cell lysis)

3.3.2 Mechanism of apoptosis

Most anticancer drugs activate the apoptotic pathway, a genetically regulated form of cell death (20, 26). This is an active process-requiring the interaction and cooperation of a large variety of different genes, many of which are also involved in permitting a cell to enter cell cycle. There is a common set of mechanisms which cells utilize in order to initiate the complex series of events leading to their elimination without provoking a pathological response from neighboring cells. It starts with a **signaling** event, and follows by a **response** reaction that finally leads to **execution** of the apoptotic machinery. Triggers of apoptosis include deprivation of growth factors, presence of receptor-ligand complexes on the cell surface, toxins, hyperthermia,

viruses, free radicals, irradiation and chemotherapeutic drugs. These signals can be divided as being external and internal.

A. External signal

External signal is the presence of a family of receptors belonging to the tumor necrosis factor (TNF) receptor superfamily and CD95 (also call Fas or Apo-1), characterized by the presence of extracellular cysteine-rich domain and a cytoplasmic domain termed death domain (DD) which tranduces the death signal. Binding of ligand to the death receptors on the cell surface causes them to trimerize which presumably results in conformational changes to the cytoplasmic portions of the receptor molecules which allows the binding of a family of signaling or adapter molecules that also contain DD to form a death-inducing signaling complex (DISC) (Table 3.4). In addition to DD, some receptors may also contain a domain known as death effector domain (DED) which enables them to recruit other molecules containing DED. For example, procaspase-8 (also known as FLICE/MACH/Mch5) has DED at its N-terminal region and binds to DISC composed of CD95/FAS/APO-1 and FADD/MORT1 where it is autoactived (presumably due to oligomerization) and released into the cytoplasm to initiate the caspase pathway.

B. Internal signal

Internal signaling of apoptosis (due to oxidative stress, irradiation or presence of virus, toxin, and chemotherapy drug) surprisingly occurs through the release of cytochrome c from the mitochondrion. Along with ATP, cytochrome c causes Apaf-1 to oligomerize and recruit procaspase-9. Apaf-1 binds to procaspase-9 through a domain called CARD (caspase recruitment domain) which is similar on both

molecules. Under normal conditions, CARD in Apaf-1 is not available for interaction with procaspase-9, until Apaf-1 is bound with cytochrome c and ATP (27, 28). The result is activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells.

An example of the internal and external signal pathways is shown in Figure 3.2.

Table 3.4 Death receptors and their adapters (DISC)

Receptor (death domain)	Adapter (death effector domain)
CD95/APO-1/FAS	FADD/MORT-1
DR3/TRAMP/wsl-1/APO-3	MADD
DR4/APO-4/TRAIL-R	RIP
LARD	RAIDD/CRADD
TNF-R1	TRAD

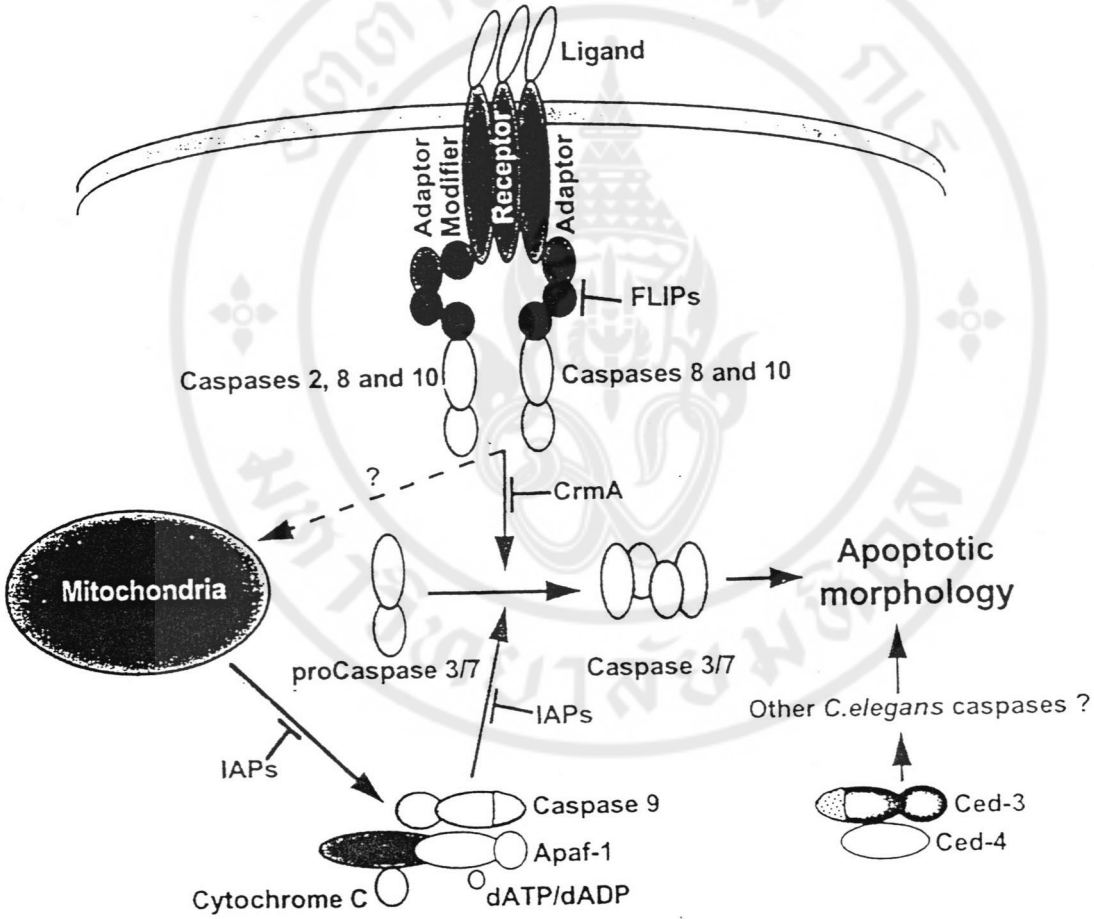

Figure 3.2 Role of caspases in induction of apoptosis mediated by internal and external signal pathways (29).

3.3.3 Genetics and Biochemistry of Apoptosis

3.3.3.1 Apoptosis in *Caenorhabditis elegans*

The nematode *C. elegans* is a particularly useful model for the study of genetic regulation of apoptosis. In the worm three genes namely, *ced-3, ced-4*, and *ced-9* are directly involved in the decision to enter the death pathway, executions of the death pathway, engulfment of the dying cell, and degradation of the cellular remnants upon engulfment. Both *ced-3* and *ced-4* are killer genes, required for the cell to die and are thought to encode the final effectors of the death pathway. On the other hand, *ced-9*, is a survival gene required for suppressing death in cells programmed to survive through regulation of *ced-3* and *ced-4* activity (30).

3.3.3.2 Mammalian homologs of CED-9

The mechanism of cell death in the worm appears to be conserved, at least in part, in vertebrates. The first evidence for this came with the discovery that *bcl-2*, a gene that is a negative regulator of apoptosis in mammals, prevented cell death when expressed in *C. elegans* (31). The anti-apoptosis protein CED-9 is homologous to *bcl-2*, a mammalian oncogene product (32), and other Bcl-2 family members, such as Bcl-x_L. Bcl-2 was first identified as an oncogene involved in B-cell lymphomas. Expression of Bcl-2 is altered by a chromosomal translocation, t (14;18), placing the gene close to the enhancer sequence of the immunoglobulin heavy chain locus at 14p32. There are at least 10 human proteins belonging to the Bcl-2/CED-9 family: five are anti-apoptotic and the other five are pro-apoptotic proteins (Table 3.5). These proteins form homo- and heterodimers, and the composition of these dimers determines the apoptotic outcome. Association of a pro-apoptotic factor (Bad or Bax)

with an anti-apoptotic factor (Bcl-2 or Bcl-x_L) results in apoptosis, whereas dissociation of these complexes leads to protection against apoptosis.

Table 3.5 Bcl-2/CED-9 protein family

Inhibitors	Promotors
Bcl-2	Bad
Bcl-x_L	Bak
A1	Bax
Mcl-1	Bid
Bcl-w	Bik/Nbk

3.3.3.3 Mammalian homologs of CED-3

It was reported that *ced-3* is related to the gene encoding mammalian interleukin 1β-converting enzyme (ICE) (33). These findings strongly suggested that proteases, in particular cysteine proteases of the ICE/CED-3 family, play a principle role in the biochemical events governing apoptosis in both nematodes and mammals.

In addition, the human homologue of CED-4 is known as **Apaf-1** (**ap**optosis-**a**ctivating **f**actor 1) which is required for activation of caspase-3.

Since the recognition of the similarity between CED-3 and ICE in 1993, a further nine related ICE-like proteases have been identified. The trivial name proposed for all family members is caspase, the "c" denoting a **cysteine** protease and the "aspase" referring to the ability of the these enzymes to cleave after an **aspartic** acid residue. The cysteine protease or caspase family has been demonstrated to induce

apoptosis when it is over expressed. Suggesting that caspase enzyme is important in apoptosis mechanism. ICE, the first family member, is caspase-1.

3.4 CASPASES

The maintenance of homeostasis in organisms requires a combination of many different pathway that relay specific post-translation through recognition of endogenous proteins, and usually these modifications alter the properties of the protein targets telling them where to go and what to do. The advantage of utilizing proteases this purpose, rather than conventional signal transduction proteins, such as protein kinases and phosphatases, is that they produce irrevesible events and commit pathways of no return. Perhaps the best described example of proteolytic signaling is the blood coagulation cascade, which is transmitted through sequential activation of serine proteases with a dominant specificity for cleaving after Arg residues. An analogous proteolytic signaling system that operates inside cells is the caspase network, which has recently received a lot of attention because of its control role in promoting apoptotic cell death.

3.4.1 Caspase family

A growing number of ICE-like cysteine proteases have been isolated and characterized, at least ten caspases have been identified as shown in Table 3.6.

Caspases share the following characteristics: they are homologous cysteine proteases belonging to the family C14 in the Barrett and Rawlings classification (34). The known ones all cleave preferentially after Asp residues in a peptide substrate, a specificity very rare among proteolytic enzymes. This specificity allows the caspases to perform highly selective proteolytic events in the cytosol of animal cells.

Table 3.6 Members of caspase family (35)

Name	Other names	Peptide, Chemical inhibitors	Substrate
Caspase-1 (36)	ICE, CED-3	zVAD, YVAD, DEVD, NO	pIL-1β, pYAMA, PARP, pNEDD2, pICE, actin, PITSLRE
Caspase-2 (37, 38)	NEDD-2/ICH-1	zVAD	PARP, pNEDD2
Caspase-3 (39, 40)	CPP32/YAMA/ Apopain	DEVD-CHO, zVAD, TPCK, NO	pCPP32, PARP, PKCδ, PITSLRE, DNA-PK, SREBP, pRB, rho-GDI
Caspase-4 (41-43)	TX/Ich-2/ICE_{rel} II	zVAD, YVAD, DEVD	pICE, pTX, PARP
Caspase-5 (43)	ICE_{rel} III		
Caspase-6 (44)	Mch-2	zVAD, TLCK	PARP, lamins
Caspase-7 (45-47)	Mch-3/CMH-1/ICE-LAP3	EVD, DEVD-CHO	PARP, pcaspase-6
Caspase-8 (48-50)	FLICE/MACH/Mch-	zVAD, DEVD, IETD	pICE, pCPP32-like
Caspase-9 (51, 52)	ICE-LAP6/Mch-6		PARP
Caspase-10 (50)	Mch-4	DEVD	PARP, pICE-like, pCPP32-like

Though the caspases are not the only enzyme system that participate in apoptosis, their role is essential. This conclusion is based largely on the following observations: (1) caspase zymogens are seen to be processed during apoptosis, or *in vitro* models of apoptosis, (2) at least *in vitro*, they cut proteins whose cleavage is

associated with apoptotic cell death, (3) specific caspase inhibitors prevent the developement of apoptosis.

One can recognize distinct groups of caspases from their domain structure, substrate specificity and sequence relatedness. A useful distinction between the caspases is based on their position in the cytokine activation or apoptotic signaling pathways. Figure 3.3 summarizes the distinctions, with a view to classifying their functions into cytokine activators, apoptotic initiators and executioners. From the perspective of apoptosis it is apparent that several distinct triggers converge on the activation of executioner by first activating initiator caspases. Thus, a hierarchical relation is postulated to exist between the initiators and the executioner, with the former containing large N-terminal extensions necessary for the initiators phase. The executioners activate pro-apoptotic factors and cleave key proteins required for the maintenance of homeostasis (51, 52).

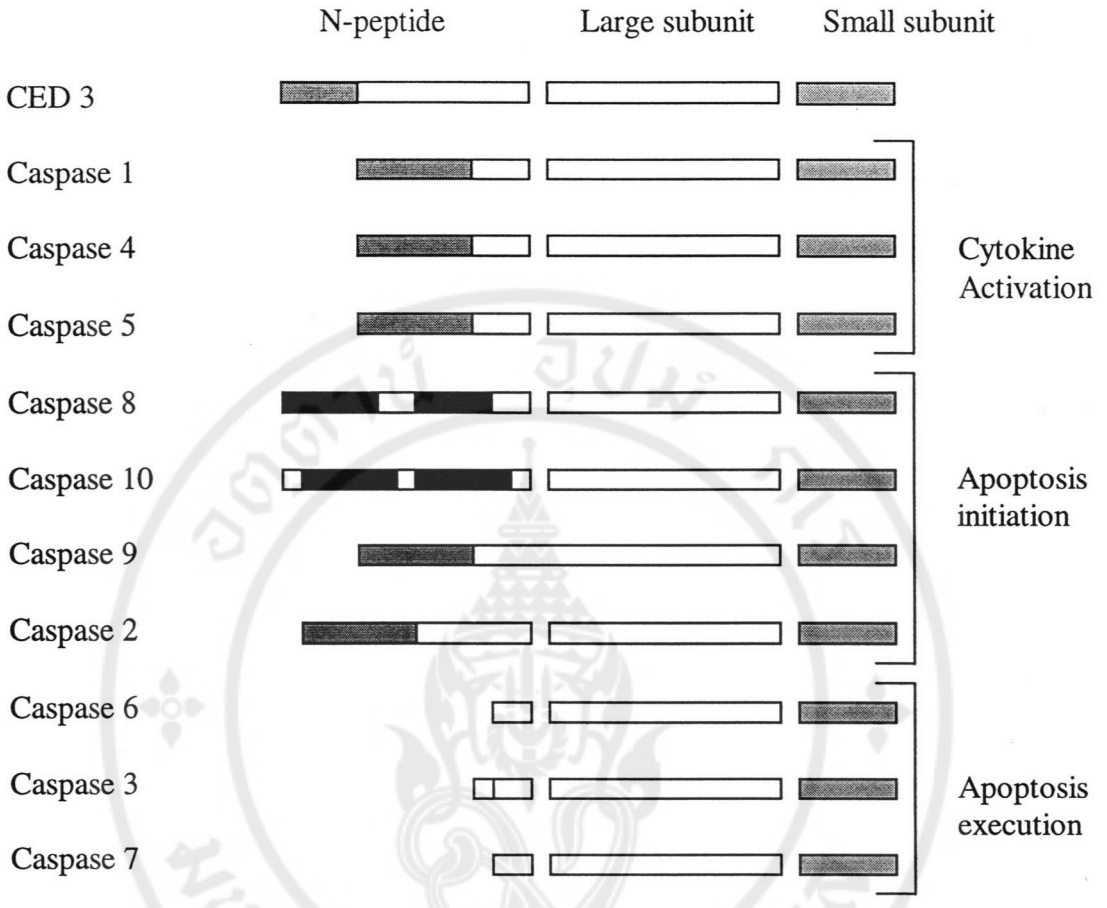

Figure 3.3 Human caspase family relationships. The caspases can be divided into three groups based on their deduced biologic functions, i.e. cytokine activation, apoptosis initiation and apoptosis execution, as review in (51-53). The length of the N-peptides varies from 22 amino acid residues for caspases 3 and 6 to over 200 in caspases 8 and 10, whereas the mature enzymes show high sequence identity and little variation in length. Thus, caspases 8 and 10 contain tandem death effector domain (DED domains) (in black) required for binding to adapter proteins during death receptor ligation. Caspase 1, 2, 4, 5 and 9 as well as *ced-3* contain a CARD domain (in gray) which is recognized by sequence similarity and postulated to be another protein interaction domain required for assembly of activation complexes (29).

3.4.2 Mechanism of caspase activation and their functions

Caspases share similarities in amino acid sequence, structure, and substrate specificity. They are all expressed as proenzymes (30 to 50 kDa) that contain three domains: an NH_2-terminal domain, a large subunit (≈ 20 kDa), and a small subunit (≈ 10 kDa) (Figure 3.4). In common with other protease zymogens, generation of the active form requires limited proteolysis (54). In contrast to most other protease zymogens, where removal of an N-terminal pro-peptide is the activating event, caspase activation results from cleavage in an interdomain linker segment to give a heterodimeric enzyme, with both chains containing essential components of the catalytic machinery (Figure 3.5). Many systems have been shown to activate caspase cascades, and caspases are capable of producing some of the biochemical markers of apoptosis.

Once activated, some of the caspases cleave other specific protein in the cell to help in killing the cell quickly and neatly (54). For example, they cleave proteins supporting the nuclear membrane, thereby helping to dismantle the nucleus; they cleave a protein that normally holds a DNA-degrading enzyme (a DNase) in an inactive form, freeing the DNase to cut up the DNA in the cell's nucleus (55); they cleave protein constituents of the cell's skeleton and other proteins involved in the attachment of cells to their neighbour, thereby helping the dying cell to detach and round up, making it easy to ingest; and so on.

One of the earliest changes in apoptosis is the movement of a negatively charged phospholipid molecule (phosphatidylserine) from the inner to the outer surface of the cell membrane where it helps mark the cell surface so that the dying cell is

quickly recognized and eaten by a neighbouring cell (56). It is still uncertain how caspase activation leads to this lipid rearrangement. Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer. Crystal structures caspases have been determined in cases, two heterodimer associate to a form a tetramer, with two catalytic sites that appear to function independently (57-59). Within each catalytic domain, the large and small subunits are intimately associated, with both contributing residues necessary for substrate binding and catalysis.

Two features of the proenzyme structure are central to the mechanism of activation of these enzymes. First, the NH_2- terminal domain, which is highly variable in sequence and length, is involved in regulation of activation. Second, all domains are derived from the proenzyme by cleavage at caspase consensus sites, implying that these enzymes can be activated either autocatalytically or in a cascade by enzymes with similar specificity.

Caspases are among the most specific of proteases, with an unusual and absolute requirement for cleavage after aspartic acid. Recognition of at least four amino acids NH_2-terminal to the cleavage site is also a necessary requirement for efficient catalysis. The preferred tetrapeptide recognition motif differs significantly among caspases and explains the diversity of their biological functions (60).

Their specificity is even more stringent: not all proteins that contain the optimal tetrapeptide sequence are cleaved, implying that tertiary structural elements may influence substrate recognition. Cleavage of proteins by caspases is not only specific, but also highly efficient (Kcat/Km > 10^6 $M^{-1}s^{-1}$). The strict specificity of

caspases is consistent with the observation that apoptosis is not accompanied by indiscriminate manner, usually at a single site, resulting a loss or change in function.

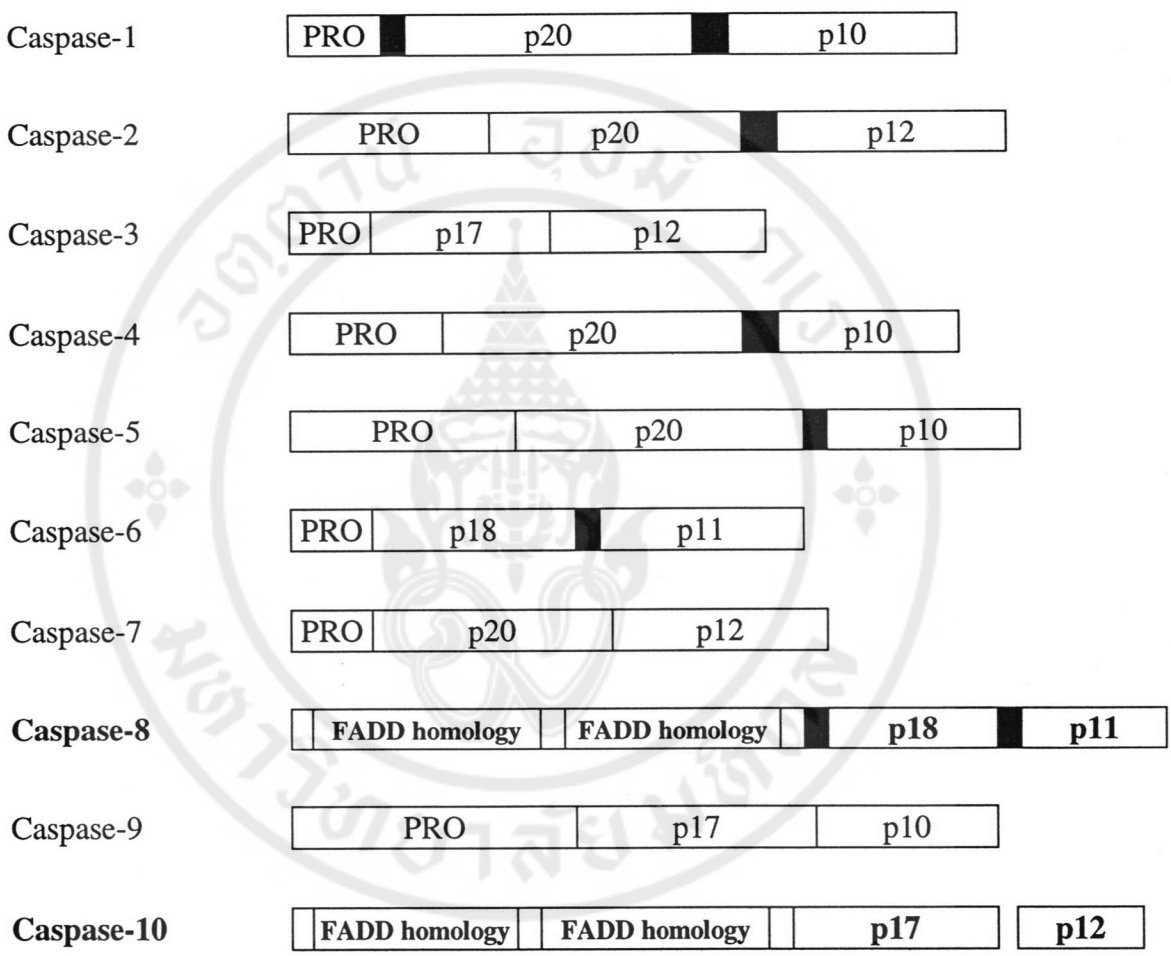

Figure 3.4 Caspases are synthesized as proenzymes, with a N-terminal peptide or prodomain (PRO), and two subunits sometimes separated by a linker peptide (black box). Based on caspase-1 and caspase-3, active enzymes are heterotetramers of two large (≈ 20 kDa: p20) and two small (≈ 10 kDa: p10) subunits (53).

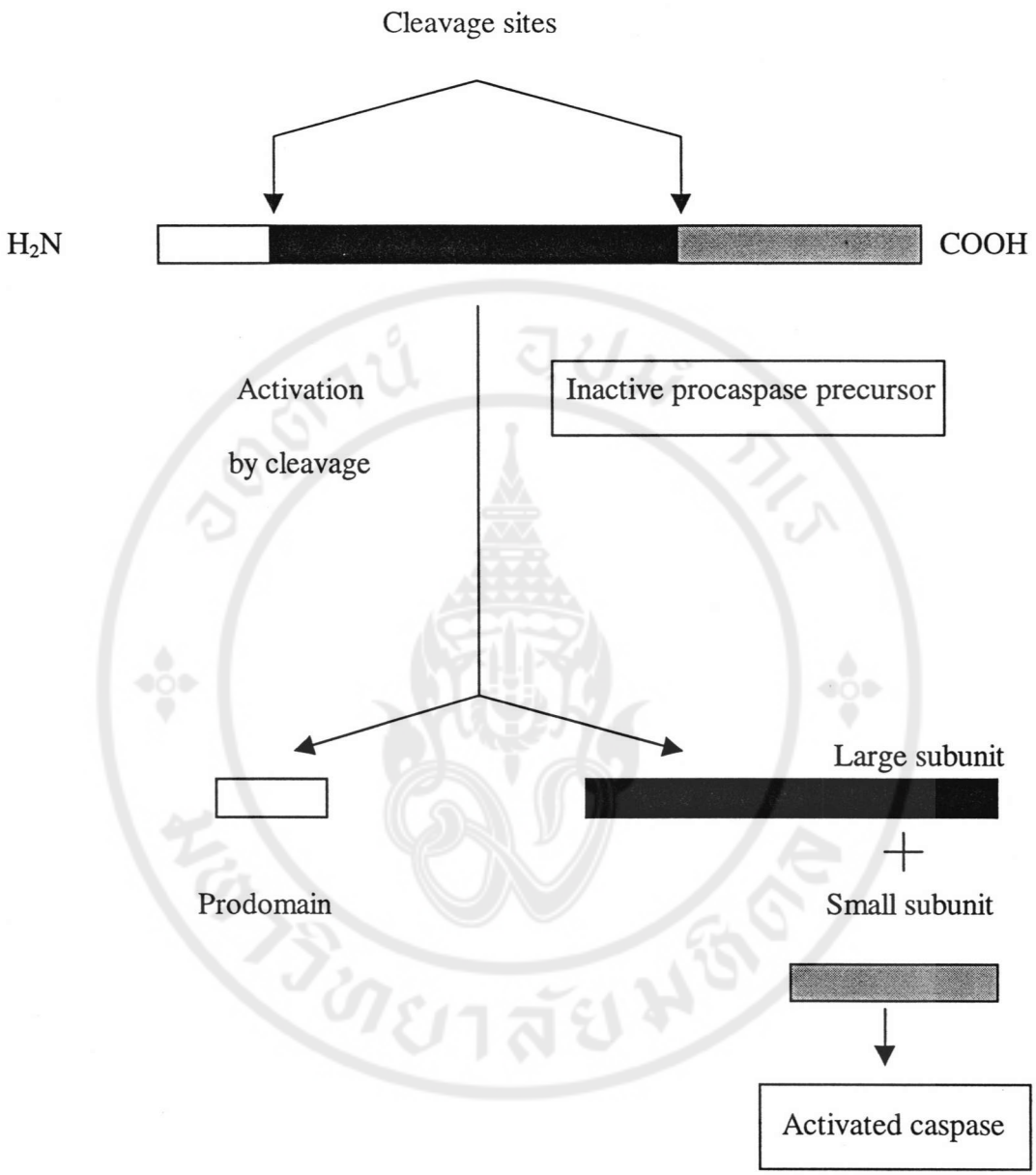

Figure 3.5 Activation of a caspase. The protein is made as a large inactive precursor (a procaspase), which is activated by cleavage at two aspartic acids. The "prodomain" is discarded, and the large and small subunits form the active enzyme. It is though that there are two large and two small subunits in an activated caspase (not shown). The activating cleavages are usually catalyzed by caspase or procaspase molecule themselves (61).

3.4.3 Inhibitors of caspases

Several macromolecular and peptide-based inhibitors of caspases have been identified and used to secure a role for at least some of these enzyme in apoptosis. The inhibitors of caspases can be divided into three groups.

3.4.3.1 Cytokine response modifier A (Crm A) is a 38 kDa serpin from cowpox virus that appears to facilitate infection through both inhibition of the host inflammatory response, and inhibition of apoptosis (62). Crm A has been evaluated as an inhibitor of both caspase-1 and caspase-3, and found to exhibit very different potencies against the two enzyme (63, 64). It is a potent inhibitor of caspase-1 and a weak caspase-3 inhibitor.

3.4.3.2 Another viral gene product, a 35 kDa protein from baculovirus (p35), also appears to attenuate apoptosis through inhibition of the caspases. Expression of p35 has been shown to prevent cell death in insect cells, *C. elegans*, and in mammalian systems of apoptosis (65). It has been shown to be an irreversible inhibitor of several family members, including caspases 1, 2, 3, 4 and CED-3 (65, 66). The ability of this protein to inhibit cell death induced by a variety of stimuli in phylogenetically diverse organisms is further evidence for an evolutionarily conserved role for the caspases.

3.4.3.3 Small molecule inhibitors, several classes of reversible and irreversible peptide-based inhibitors have been designed using strategies that have proven successful for inhibition of other cysteine proteases (67). Reversible inhibitors include aldehydes, nitriles and ketones. Irreversible inhibitors are of the general structure. Peptide-CO-CH_2-X, where X is a halide ion (chloromethyl-ketones,

fluoromethyketones), - N_2 (diazomethyketones), -OCOR [(acyloxy) methyketones], or -OR [a-(pyrazoloxy) methyketones and (phosphinyloxy) methyketones].

The peptide moiety of these inhibitors determines their selectivity for particular caspases. For example, the tetrapeptide aldehyde based on the pro-IL-1β cleavage site, Ac-YVAD-CHO, is a potent inhibitor of caspase-1, and a weak inhibitor of caspase-3. In contrast, the aldehyde containing the PARP cleavage site, Ac-DEVD-CHO, is a relatively potent inhibitor of both caspase-1 and caspase-3, with a 49-fold preference for caspase-3 (68).

3.4.4 Caspase-3/YAMA/APOPAIN/CPP32

3.4.4.1 Significance of caspase 3

Among the caspase family, caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which are cleaved in many different systems during apoptosis. Using the DNA sequence encoding the active site of caspase-1 and CED-3 to search an expressed sequence tag database, a human sequence was identified, cloned and shown to encode a 32 kDa cysteine protease, called CPP32 (39). Independently, two other groups identified caspase-3, one naming it YAMA (the Hindu god of death) (40) and the other apopain (63). CPP32, interleukin-1β-converting enzyme (ICE) - like cysteine protease, has been implicated in the pathway of apoptosis in mammalian cells based on several observations.

Firstly, CPP32 is closely related to an apoptosis promoting gene (*ced-3 of Caenorhabditis elegans*) in terms of both sequence similarity and substrate

specificity (39, 66). Secondly, CPP32 activity markedly elevated in cells undergoing apoptosis induced by a variety of reagents (40, 63, 69). Finally, a tetrapeptide aldehyde inhibitor that specificity inhibits CPP32 activity also blocks the ability of cytosol from apoptotic cells to induce apoptosis-like changes in normal nuclei *in vitro*.

CPP32 normally exists in the cytosol as a 32 kDa inactive precursor and only becomes activated when cells are undergoing apoptosis. The immature 32-kDa caspase-3 (pro-caspase-3) is cleaved into a 12 kDa fragment and 17 kDa biologically active caspase-3. The active enzyme derived from the precursor protein by cleavage at Asp- 28- Ser- 29 and Asp-175- Ser-176 (63). Activated caspase-3 cleaves poly (ADP-ribose) polymerase (PARP), an enzyme that responds to DNA damage by polyribosylation of itself and of other substrates (40, 63, 70). The pathway of signaling, the majority of responses occurs through activation of caspase-3. Procaspase-3 can be activated by caspase-8 (external signal pathway) or by caspase-9 (internal signal pathway). In turn caspase-3 can activate other caspases (caspase-6, caspase-9). The ability of caspases to activate one another potentially creates an amplification circuit.

Other substrates of caspases include both nuclear factors degrades are retinoblastoma protein (RB1) and MDM2, an inhibitor of p53 tumor suppressor. Loss of these two proteins leads to activation of E2F and p53, which are known to be activators of apoptosis.

3.4.4.2 Structure of caspase-3

CPP32 is both structurally and functionally similar to ICE (71). The three-dimensional structures have thus far been determined for caspase-3 (59, 72). With the important exception of specificity determining surface loops, the published structures superimpose well, all demonstrating a molecule composed of two large and two small subunits. Caspase-3 structure shows the caspase existing as tetramers consisting of two large and two small subunits giving rise to a two fold symmetry of the dimer as illustrated in Figure 3.6.

3.4.4.3 Function of caspase-3

The execution phase of apoptosis is poorly understood at the molecular level. There is involvement of endonucleases to degrade chromosomal DNA. The presence of DNase called CAD (caspase-activated DNase) appears through the degradation of its inhibitor (ICAD) by caspase-3 (73). Proteolysis of fodrin and actin can account for changes to the cell outer surface (blebbing) and lamins are cleaved at a single site by caspases, causing lamina to collapse and contributing to chromatin condensation. Caspases also reorganize cell structures indirectly by cleaving several proteins involved in cytoskeleton regulation, including gelsolin, focal adhesion kinase (FAK), and p21-activated kinase 2 (PAK 2). Cleavage of these proteins results in deregulation of their activity. For example, in the case of gelsolin (a protein that severs actin filaments in a regulated manner), caspase cleavage generates a fragment that is instead constitutively active (73). Appearance of phosphatidylserine on the outer leaflet of the plasma membrane has been ascribed to loss of in ATP-dependent aminophospholipid translocase activity or activation of a scramblase (which exchanges

phospholipids across the bilayer). Therefore, caspase-3 is required for certain distinctive biochemical and morphological changes during apoptosis (74).

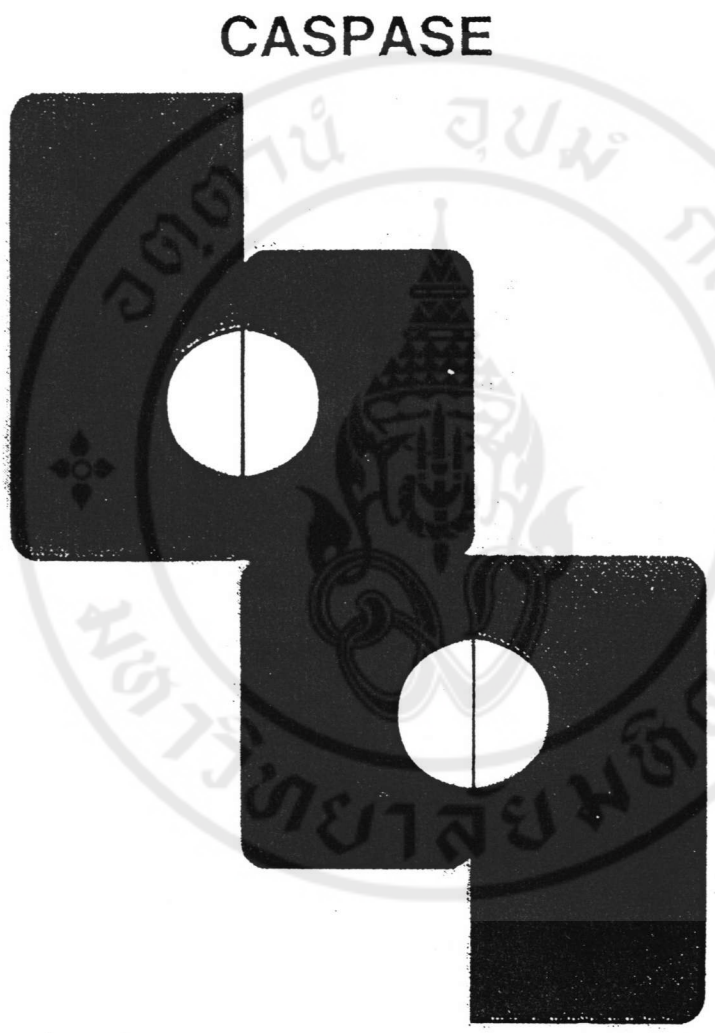

Figure 3.6 The diagram showing the tetrameric structure of caspase-3 consisting of two large (~ 20 kD) and two small (~10 kD) subunits (73).

3.5 ACTIVATION OF CASPASE BY SMALL PEPTIDE COMPOUNDS

One of the major problems in cancer chemotherapy is drug resistance. Some tumor types, e.g., non-small cell lung cancer and colon cancer, exhibit *primary* resistance, i.e., absence of response on the first exposure, to currently available standard agents. *Acquired* resistance develops in a number of drug-sensitive tumor types. Experimentally, drug resistance can be highly specific to a single drug and usually is based on a change in the tumor cells'genetic apparatus with *amplification* or increased expression of one or more specific genes. Therefore, a major effort to develope anticancer drugs through both empirical screening and rational design of new compounds has now been under way for three decades. This leads to the discovery of some small peptide compounds.

3.5.1. Didemnin B (DB) isolated from the *Tridemnum* genus of marine tunicates. It is one member of a class of natural cyclic depsipeptides. Li *et al.* 1984 (75) found that DB inhibits protein synthesis in cell culture, which has been suggested to be the mechanism leading to cell death. Additional activities of DB have been shown by Grubb *et al.* (1995) (76) that DB is a rapid and potent inducer of apoptosis in HL-60 cells. Furthermore, treatment MCF-7 cells with DB at the IC_{50} dose, 12 nM, has demonstrated that DB induces apoptotic cell death via caspase activation (Beidler, *et al.*, 1999) (77).

3.5.2 Jasplakinolide is a cyclodepsipeptide isolated from the marine sponge, *Jaspis johnstoni*, originally found to be an effective antifungal agent at micromolar concentrations (78) and later found to have antiproliferative activity in the nanomolar

range in acute myeloid leukemia and prostate carcinoma cells (IC_{50}= 34 nM) (79). Subsequently, Jasplakinolide has been shown to induce actin polymerization (80-83) Posey *et al.* 1999 (83) have reported that this compound induced apoptosis in IL-2-dependent murine cell line in a time- and concentration-dependent manner via the induction of caspase-3 like activity. This could be attenuated by the over expression of the anti-apoptotic protein Bcl-x_L.

3.6 VR-3848

As part of ongoing project to find natural product cancer chemotherapeutic compounds from Thai tropical plants, VR-3848 was discovered. It was purified from *Euphobiaceae* known in Thai name as **Takhe khumwang** (ตะเข้คุมวัง), and **Tao tua mia** (เต่าตัวเมีย) (84). Local people use the root part for face whitening .

Currently, the structure of VR-3848 is being investigated by Prof. Vichai Reutrakul's group, Dept. of Chemistry, Fac. of Science, Mahidol University. VR-3848 was a potent cytotoxic compound, it inhibited the growth of several human cultured cancer cell lines. Preliminary studies in our laboratory, it was found that the concentration of GI_{50} for VR-3848 against epidermoid carcinoma in the mouth (KB), lung (LU-1), COL (COL-1), breast (BCA-1) human cell lines and mouse lymphoid neoplasm (P_{388}) cell line were 1, 3, 3, 5, and 1 nM, respectively. The inhibitory effect on cancer cell growth could not be due to induction of mitotic arrest since studies using ASK assay (3) failed to detect the antimitotic action of this compound. Therefore, at present, how VR-3848 induced cell death in cultured cancer cell line is still not known. Since it has a potent cytotoxic effect on various human cultured

cancer cells, it was thought that VR-3848 may inhibit cell growth via apoptosis mediated by caspase activation.

CHAPTER IV

MATERIALS AND METHODS

4.1 MATERIALS

VR-3848 was kindly provided by Professor Vichai Reutrakul (Department of Chemistry, Faculty of Science, Mahidol University, Thailand). Briefly, it was extracted and purified from *Euphobiaceae*. Using bioassay-directed fractionation, the cytotoxic activity against LU-1 was found in methanol fraction. Minimum essential medium (MEM), non-essential amino acids (NAA), penicillin, streptomycin, fungizone and trypsin were obtained from GIBCO BRL (Grand Island, NY, USA). Bovine calf serum (BCS) was from Atlanta Biological (Norcross, GA, USA). Sulforhodamine B and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ciprofloxacin hydrochloride was from Pentex, Miles Inc. (Kankakee, IL, USA).

4.2 CELL CULTURE PROCEDURES

4.2.1 Cell line and cell maintenance

Human lung (LU-1) cancer cell line used in this study was kindly given by Professor John M. Pezzuto (Department of School Pharmacy, University of Illinois at Chicago, Chicago, USA). Cells were grown as monolayer in Minimum Essential Medium with Earle's salts and L-glutamine containg 5% BCS, 10% non-essential amino acids, 100 U/ml of penicillin, 100 µg/ml streptomycin and 1% fungizone

(250 μg/ml) (see Appendix I) in a humidified 5% CO_2 incubator at 37°C. The confluent cells in a 25 cm^2 flask were subcultured every 5 days with 0.05% trypsin/EDTA solution. To avoid mycoplasma contamination, cells were treated with 10 μg/ml of ciprofloxacin, a 4-fluoroquinolone antibiotic drug, for 15 days (85) before they were further used in the experiments. In addition, in order to have active and more homogenous cells, only cells at passages 7 to 20 after thawing which are in the exponential phase of cell growth were used in the study.

4.2.2 Determination of viable cell numbers

4.2.2.1 Total cell counting

During cell passage total cell count was performed by using a hemocytometer slide (Improved Neubauer 0.1 mm deep, BOECO, Germany) as described in Appendix II.

4.2.2.2 Sulforhodamine B assay

In addition, in some experiments, the number of viable cells was quantitated by determination of cellular protein content with the sulforhodamine B (SRB) assay as previously described by Skehan *et al.* (1990) (86) with slight modification.

ASSAY PRINCIPLE

SRB, a bright pink aminoxanthene dye with 2 sulphonic groups, binds electrostatically to basic amino acid residues of protein under mildly acidic condition in only the viable cells, but not in non-viable cells in which all proteins can be lysed by endogenous proteolytic enzymes. The dye can be quantitatively extracted from cells

and solubilized by a weak base for optical density measurement. This assay is simple, rapid, sensitive and results from this assay have been shown to be linear with the amount of protein (86, 87) (see Appendix III).

4.2.3 Preparation of cells for freezing and thawing

In order to have continuous cell supply, some LU-1 cells in exponential phase were collected and preserved in liquid nitrogen as cell stock. The healthy and non-contaminated cells were trypsinized, washed with growth medium and counted using a hemocytometer. Subsequently, 1 ml of $1x10^6$ cells in growth medium containing 10% DMSO was transfferred into a 2 ml cryotube. Then, the cryotubes were gradually reduced the temperature by placing in a chamber containing isopropyl alcohol. The chamber was kept at -80°C overnight before the cells were rapidly transferred into liquid nitrogen for storage. When required, thaw cells rapidly by immersing a frozen cryotube in a humidified 5% CO_2 incubator at 37°C. Subsequently, cell suspension was added into a 25 cm^2 culture flask containing growth medium and maintained at 37°C in a 5% CO_2 incubator.

4.2.4 Cytotoxicity assay (determination of GI_{50})

The cytotoxic effect of vinblastine and VR-3848 on human lung (LU-1) cancer cells in culture was determined by measuring total cellular protein using the SRB assay based on the modified method of Skehan *et al.* (1990) (86). The SRB assay was used to estimate the cell number in the cytotoxicity study because absorbance for the SRB assay has been shown to have a linear correlation with the number of viable cells and the determination of cell numbers by this method is rapid, sensitive and inexpensive (86, 87).

Briefly, LU-1 cells were maintained in a 96-well tissue culture microtiter plate and exposed to test compounds over a range of concentrations. After 48 hours of incubation, cell numbers were estimated using SRB staining. The degree of inhibition of growth, related to the concentration of the test compound, provides an indication of toxicity. The number of cells in the presence of test chemicals was comapred with that observed in control cultures and the percentage of cell survival was calculated from the ratio: (mean OD_{515} treated cells - mean OD_{515} Do) / (mean OD_{515} untreated with DMSO cells - mean OD_{515} Do) x 100 based on the method of Monks *et al.* (1991) (88) and plotted against various log concentrations of the test substances studied using GraphPad Prism, Non linear regression, sigmoid dose-response curve. Subsequently, the GI_{50}s (i.e., the concentration of vinblastine and VR-3848 producing 50% inhibition of growth) were determined and expressed as nmol/L and were used in the apoptotic study (see Appendix IV).

The average OD of control blank was approximately 0.04 whilst the concentration of DMSO (0.05-0.1%) used in the present study had no effect on LU-1 cells.

4.3 DETECTION OF APOPTOSIS

4.3.1 Analysis of morphological changes

4.3.1.1 Phase contrast microscope

4.3.1.2 4',6-Diamino-2-phenylindole (DAPI) staining

LU-1 cells were maintained in culture and exposed to the indicated concentrations of test compounds. Following 3, 6, 12, 24, and 48 hours of treatment, the morphological characteristics of apoptotic cells were examined under a phase

contrast microscope by a visual examination and some were photographed. They were also analyzed by staining nuclei with DNA binding dye [4',6-Diamino-2-phenylindole (DAPI)] as described previously (89).

In brief, the floating cells and the cells on the surface of the flask detached from their neighbours by trypsinization were pooled into a 15 centrifuged tube. For morphological analysis, after centrifugating at 3,000 rpm 4°C for 15 minutes (Sorvall RT 6000 D) approximately $1x10^6$ of treated cells per treatment were washed with PBS, fixed with ice-cold absolute methanol and stained with 1 μg/ml DAPI (Boehringer Mannheim) diluted in methanol at 37°C for 15 minutes. After washing with ice-cold absolute methanol and centrifugation at 6,000 rpm for 5 minutes, the cell pellets were resuspended in methanol to get an appropriate concentration. The cell suspension, 10-20 μl, was spreaded on a glass slide and the specimen was covered with an antifading agent, Vectashield (Vector Laboratories, Burlingame, CA). They were subsequently observed under a fluorescence microscope (Optiphot Labophot Episcopic-Fluorescence attachment EF-D, Nikon, Japan) for morphological features of apoptotic cells. At least five hundred cells per treatment were randomly counted and scored for the incidence of apoptotic cells at the magnification of 500x. The percentage of cell in apoptosis was calculated as the ratio of cells with apoptotic characteristic features, such as chromatin condensation and DNA disintegration (20) to the total number of cell count (500 cells) x 100. This protocol, only dead cells were shown significant staining of DNA with DAPI. Then, the percentage of apoptosis of LU-1 cells after treatment with VR-3848 or vinblastine from at least three experiments were averaged and plotted versus incubation time.

4.3.2 Analysis of DNA degradation

As one of the biochemical hallmarks of apoptosis is the cleavage of chromatin into nucleosomal fragments, a DNA degradation assay was conducted to detect genome digestion.

To detect DNA degradation, total genomic DNA was quantitatively isolated from untreated and treated cells after treatment by the modified method as previously described by Kaufman *et al.* (1995) (90) using a phenol-chloroform method. Then, the DNA was electrophoresed in a 1.8% (w/v) horizontal agarose gel and visualized on a UV transilluminator (wavelength 354 nm) after staining gel in 1 μg/ml of ethidium bromide solution. If cells were undergone apoptosis, the fragmented DNA observed under UV light would be seen as a "DNA ladder" in the gel and photographed using Kodak T400CN film (see Appendix V).

4.4 DETECTION OF CASPASE-3 GENE EXPRESSION BY RT-PCR

It is well established that caspases, protease enzymes, play an important role in apoptotic pathway. Among the caspase family, caspase 3 has been reported to be one of the key executioners of apoptosis in mammalian cells. Thus, the upregulation of caspase-3 gene during the VR-3848-induced apoptosis was investigated according to the instruciton of GIBCO and Perkin-Elemer using β-actin gene as an internal control.

Cells were exposed to the concentration of VR-3848 which induces apoptosis between 3 and 48 hours. They were subsequently extracted for total RNA. The RNA was reverse transcripted by Superscript neverse transcriptase with the specific caspase-

3 primer (39) to synthesize cDNA which was subsequently amplified by PCR. The amplified DNA was then observed in a 1% agarose gel on an UV transilluminator after staining in ethidium bromide solution. The PCR products were then photographed (see Appendix VI).

4.5 MEASUREMENT OF CASPASE-3 (CPP32) ACTIVITY

In order to investigate whether activation of caspase-3 contributes to the VR-3848-induced apoptosis in LU-1 cells, the proteolytic enzyme activity of caspase-3 was measured during apoptosis using a fluorogenic assay according to the modified method of Lei *et al.* (1998) (91).

ASSAY PRINCIPLE

The fluorogenic substrate for CPP32/caspase-3 (Ac-DEVD-MCA) is labeled with the fluorochrome 4-methyl-Coumaryl-7-Amide (MCA). The substrate produces a blue fluorescence that can be detected by exposure to UV light at 360 nm. MCA is released from this substrate due to cleavage by CPP32-like enzyme. Free MCA produces a yellow-green fluorescence that is monitored by a fluorometer at 460 nm. The amount of yellow-green fluorescence produced upon cleavage is proportional to the amount of CPP32 activity present in the sample (92).

Briefly, cells were received the same treatment as described in Experiment 4.3 except that only VR-3848, $20xGI_{50}$ and vinblastine, $2xGI_{50}$ were used in the assay. After havesting the cells, they were lysed in lysis buffer by subjecting them to four cycles of freezing and thawing based on the Promega's instruction. The concentration of protein in the lysate was determined by Bincinchoninic acid (BCA) assay based on the modified method of Smith *et al.*, (1985) (93) using bovine serum albumin as a

standard. Subsequently, caspase-3 activity in cell extracts was measured in the presence and absence of the specific caspase-3 inhibitor, Ac-Asp-Glu-Val-Asp-CHO or Ac-DEVD-CHO and then they were compared. The result was expressed as the fold induction of caspase-3 as seen in Appendix VII. If caspase-3 is involved in apoptosis, the activity of CPP32-like enzyme should be decreased after an addition of the specific inhibitor.

4.6 EXPERIMENTAL PROTOCOLS

PART I. TO EXAMINE WHETHER VR-3848 ISOLATED FROM *EUPHOBIACEAE* INHIBITS THE GROWTH OF HUMAN LUNG (LU-1) CANCER CELL LINE VIA APOPTOSIS

1.1 Cytotoxicity assay

This experiment was undertaken to see the cytotoxic effect of VR-3848 and vinblastine on the growth of LU-1 and also to determine the GI_{50} concentrations of these compounds. The cytotoxic effect of the chemicals on LU-1 cells in culture was measured by total cell protein using the SRB assay based on the modified method of Skehan *et al.* (1990) (86).

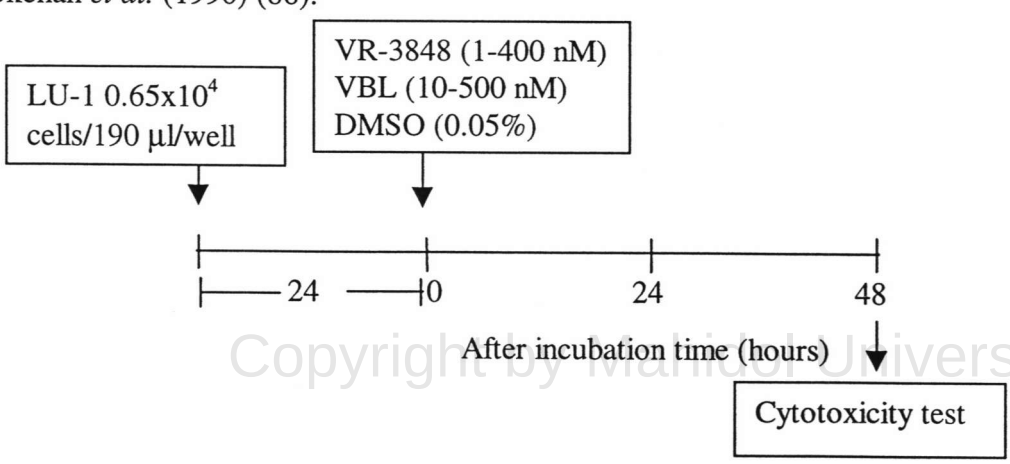

Figure 4.1 Schematic diagram showing the cytotoxicity assay protocol.

Briefly, LU-1 cell line was grown in a 96-well tissue culture microtiter plate at a density of $0.65x10^4$ cells/190 µl/well overnight. On the following morning, fresh medium was exchanged and cells were exposed to either various final concentrations of VR-3848 (1-400 nM) and vinblastine (VBL, 10-500 nM) dissolved in DMSO or 0.05% DMSO a further for 48 hours as shown in the protocol assay. Subsequently, the number of cells was quantitated using the SRB assay and the percentage inhibition of growth was calculated as previously described in the cytotoxicity assay Section 4.2.4. The percentage of growth inhibition was then plotted against time after incubation to obtain typical dose-respose curves which were used to determine the GI_{50} concentrations of the compounds. Consequently, three concentrations of VR-3848, GI_{50}, $10xGI_{50}$, and $20xGI_{50}$, and vinblastine at $2xGI_{50}$, were used in the apoptotic study.

1.2 Induction of apoptosis by VR-3848 and vinblastine in LU-1 cell line

The studies were designed to examine whether cell death effect of VR-3848 and vinblastine on LU-1 cells is resulted from apoptosis and whether apoptosis induced by VR-3848 is dose-and-time dependent manners.

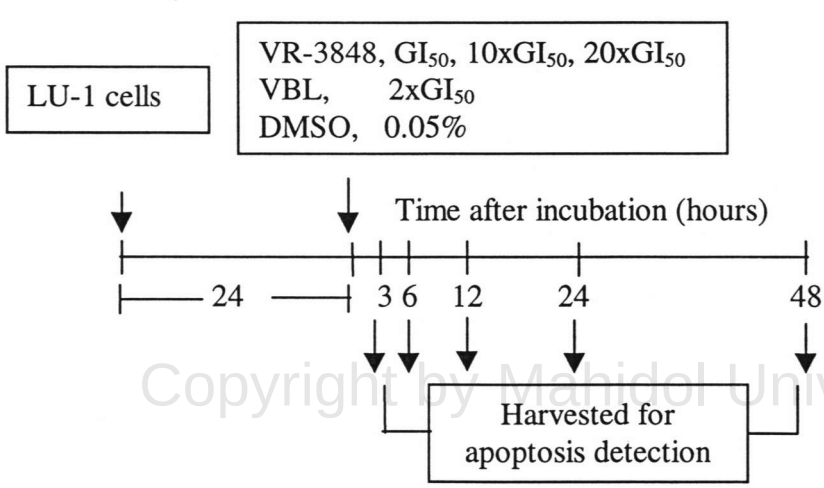

Figure 4.2 Diagram showing the apoptosis study protocol.

In order to demonstrate that VR-3848 and vinblastine have abilities to initiate apoptosis in LU-1 cell line, cells were treated with three concentrations of VR-3848, GI_{50} (10 nM), $10xGI_{50}$ (100 nM) and $20xGI_{50}$ (200 nM), and vinblastine, $2xGI_{50}$ (200 nM). Following incubation for 3, 6, 12, 24, and 48 hours, the treated cells were harvested at indicated times for detection of apoptotic features. The apoptotic cells are characterized as chromatin and cytoplasmic condensation, DNA fragmentation and apoptotic bodies formation which they can be revealed by phase contrast microscope and DAPI staining for morphological changes, and by agarose gel electrophoresis for DNA fragmentation. The procedures that were used to induce apoptosis in cell cultures and the detection of apoptosis for both the morphological and biochemical changes were already described in Section 4.3, Detection of apoptosis.

VR-3848, $10xGI_{50}$ and $20xGI_{50}$ and vinblastine, $2xGI_{50}$ were used in this study since, after treatment with those concentrations, DNA extracted from those cells demonstrated DNA laddering in a agarose gel. This is resulted from internucleosomal DNA fragmentation and is used as a biochemical hallmarks of apoptosis.

The results obtained in this study would indicate whether apoptosis is occurred in LU-1 cells after treatment with VR-3848 and vinblastine and whether the apoptosis induced by VR-3848 varies with dose and time.

PART II. TO FURTHER INVESTIGATE WHETHER CASPASE-3 IS INVOLVED IN THE VR-3848-INDUCED APOPTOSIS

To date, there has been much evidence to show that caspase-3 (CPP32) which is one of the aspartic acid-specific cysteine proteases family plays a key role in drug-induced apoptotic cell death in mammalian cells (40, 50, 63, 94). Therefore, the

following experiments were conducted in order to assess the contribution of CPP32-like enzyme in the VR-3848-induced apoptosis.

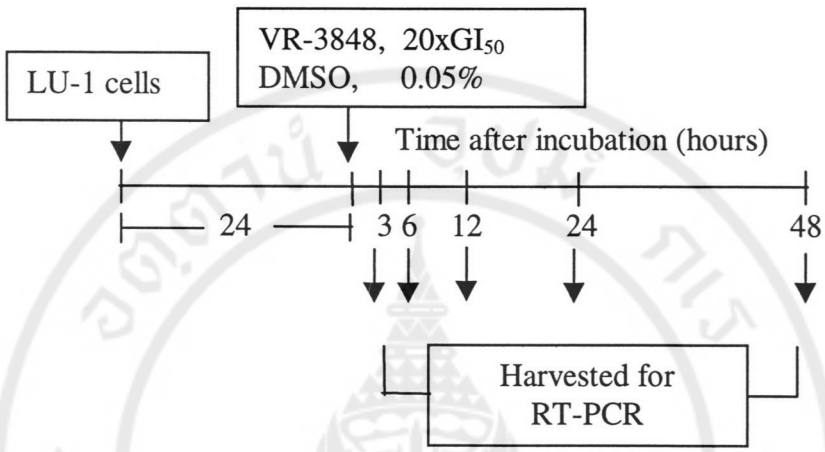

Figure 4.3 Schematic diagram showing the protocol for RT-PCR assay.

2.1 Expression of caspase-3 gene in LU-1 cells treated with VR-3848

This study was performed in order to analyse the expression of caspase-3 gene in VR-3848-treated LU-1 cells using RT-PCR based on the modified method of the instruction of GIBCO and Perkin-Elemer.

The same procedure was performed as in Experiment 1.2 except that LU-1 cells were exposed to only VR-3848 at $20xGI_{50}$ dose. Following incubation periods as indicated in Figure 4.3, cells were collected and subjected for total RNA extraction. RNA was reverse transcribed by Superscript reverse transcriptase with the specific caspase-3 primer to synthesize cDNA which was subsequently amplified by PCR. The amplified DNA was visualized in 1% agarose gels on an UV transilluminator after staining with ethidium bromide. The PCR products were then photographed. The detailed protocol for RT-PCR was already described in Appendix VI. All results were

compared with the expression of β-actin gene which was used as an internal control. The upregulation of caspase-3 gene in the VR-3848 treated cells during apoptosis would suggest that CPP32-like protease may regulate apoptosis at the mRNA level.

2.2 Activation of CPP32-like enzyme activity

In order to investigate whether CPP32 is activated during VR-3848-induced apoptosis, a fluorogenic assay was set up based on the method of Lei *et al.* (1998) (91) with slight modification. The assay was measured the CPP32-like enzyme activity during apoptosis in the presence and absence of a specific inhibitor for CPP32 according to the procedure mentioned in Section 4.5. Briefly, LU-1 cells were received the same conditions which induced apoptosis by VR-3848, $20xGI_{50}$ and vinblastine, $2xGI_{50}$ as described earlier in Experiment 1.2. Except that, after 3, 6, 12, 24, and 48 hours of treatment, cells were collected for protein extracts instead of preparation for detection of apoptosis. Following determination of protein concentration using BCA assay, crude cell extracts were measured for CPP32 activity. The activity of CPP32 in VR-3848- and vinblastine treated cells was compared with that observed in the control cells and the fold induction of caspase-3 activity was calculated as mentioned earlier. Further, the difference between the substrate cleavage activity levels in the presence and absence of inhibitor reflects the contribution of CPP32-like protease activity on apoptosis.

4.7 STATISTICAL ANALYSES

The statistical software package, SPSS for window version 7.5, was used for all statistical analyses. The results are expressed as the means and the standard error of

means ($\overline{X} \pm$ S.E.M). Student's paired t-test was used for the comparison between the control and treatment groups. Statistical significance of difference between groups was accepted taken at p-value less than 0.05 or exceeding the 95% critical value.

CHAPTER V

RESULTS

PART I. TO EXAMINE WHETHER THE INHIBITORY EFFECT OF VR-3848 ON THE GROWTH OF HUMAN LUNG (LU-1) CANCER CELL LINE IS CAUSED BY APOPTOTIC CELL DEATH

1.1 Growth inhibition assay

The microscopic pictures of normal human lung cancer cells grown in MEM supplemented with 5% BCS is shown in Figure 5.1. Since they have prominent nucleoli and big squamous-like morphology, LU-1 cell is classified as non-small cell lung cancer (personal communication).

Exposing LU-1 cells to various concentrations of vinblastine and VR-3848 for 48 hours produced a significant inhibition effect on cell proliferation. Typical dose-response curves for vinblastine and VR-3848 are shown in Figures 5.2 and 5.3, respectively. The GI_{50} values for vinblastine and the unknown compound were 100 nM and 10 nM, respectively. The result indicates that VR-3848 is more potent than vinblastine 10 fold.

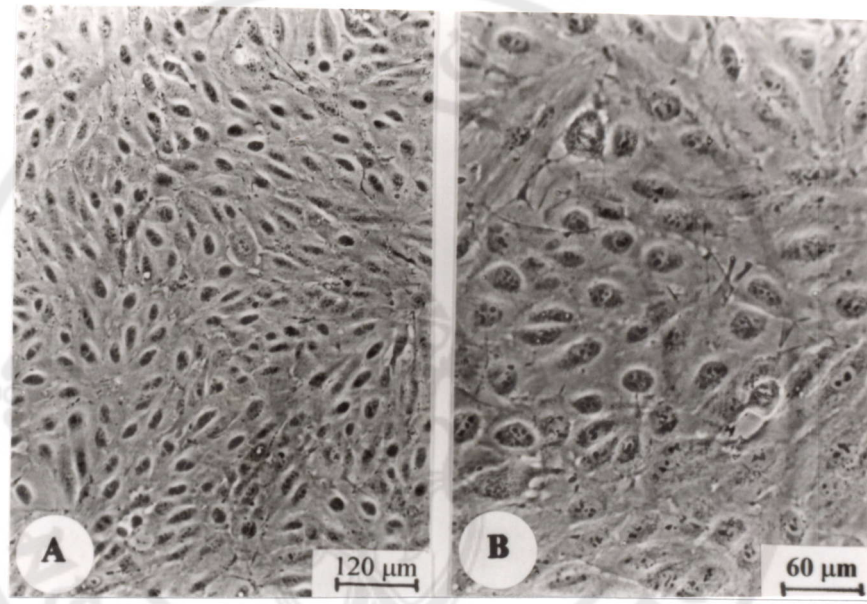

Figure 5.1 Phase-contrast microscopic pictures of human lung cancer cells grown in MEM supplemented with 5% BCS showing an epithelial-like morphology. Magnification 82.5x (A) and magnificantion 165x (B).

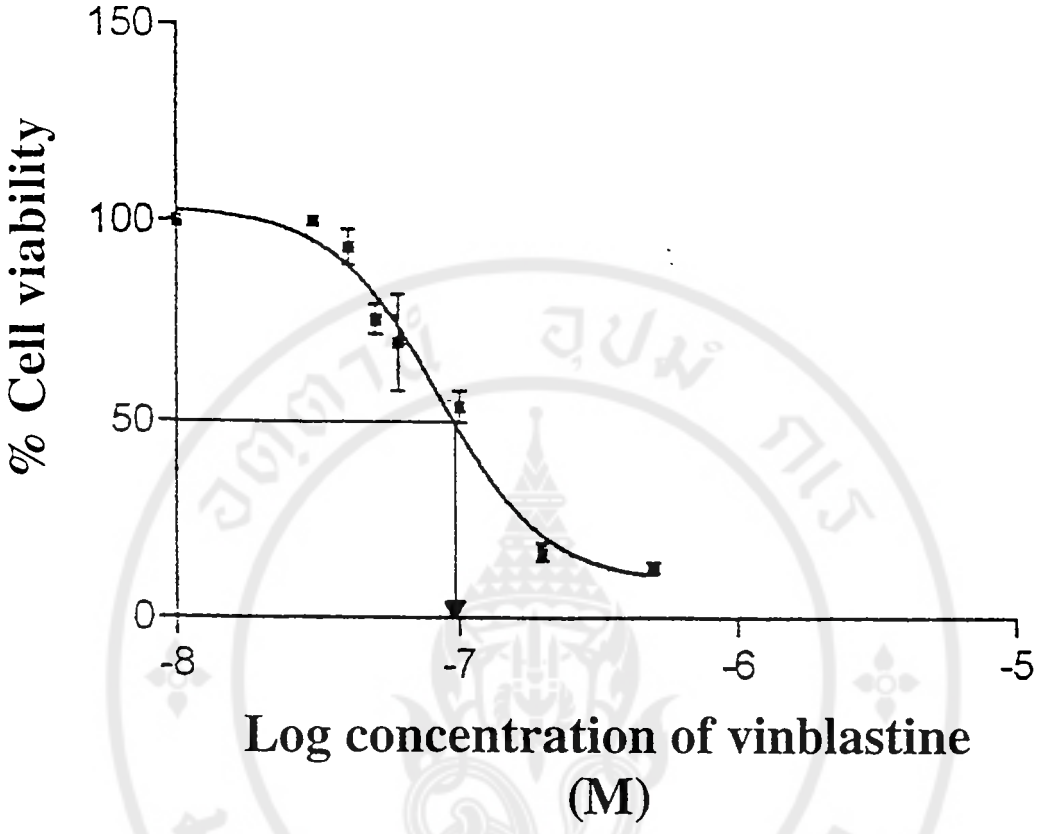

Figure 5.2. Dose-response effect of vinblastine on human lung cancer cell line after incubation for 48 hours. The x-axis represents log concentrations of vinblastine (M) and the y-axis is % cell viability. The GI_{50} (concentration of vinblastine which inhibits 50% of cell proliferation) for vinblastine was approximately 100 nM as indicated in the graph. Results are means ± S.E.M. from three independent experiments.

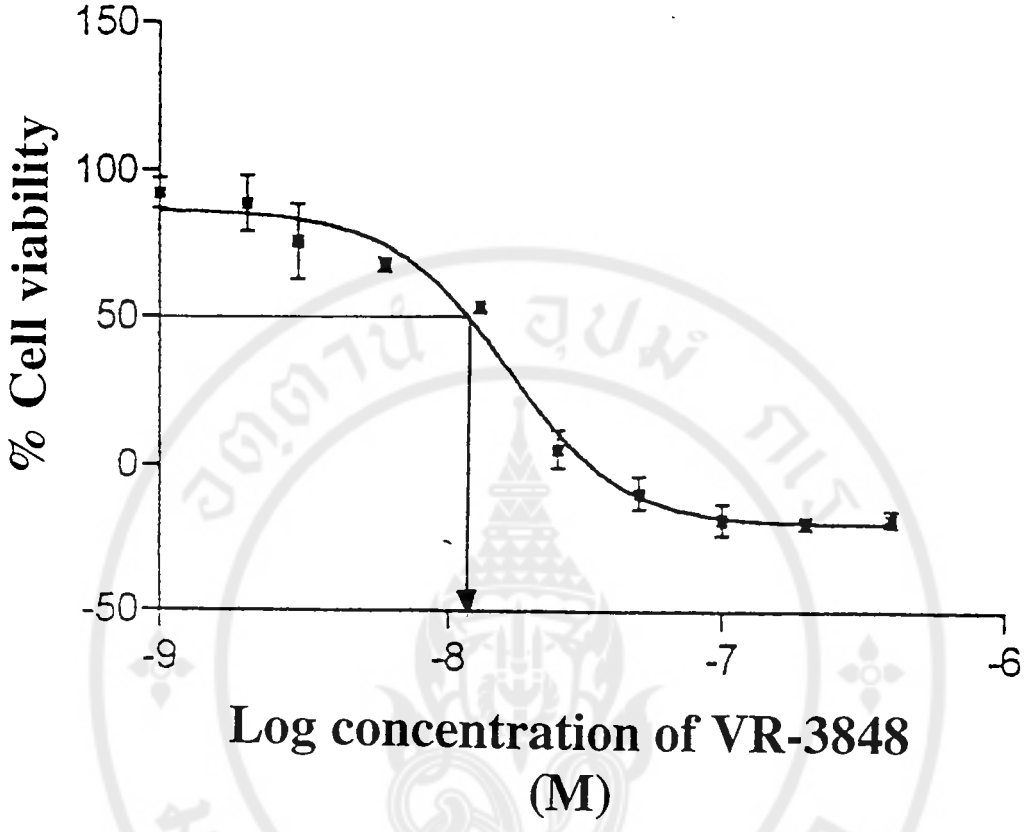

Figure 5.3. Dose-response effect of VR-3848 on human lung cancer cell line after incubation for 48 hours. The x-axis represents log concentration of VR-3848 (M) and the y-axis is % cell viability. The GI_{50} (concentration of VR-3848 which inhibits 50% of cell proliferation) for VR-3848 was approximately 10 nM as indicated in the graph. Results are means ± S.E.M. from at least three independent experiments.

1.2 Induction of apoptosis by VR-3848 and vinblastine in LU-1

To elucidate whether VR-3848 inhibits cancer cell growth by inducing apoptosis, LU-1 cells were treated with three doses of VR-3848, GI_{50} (10 nM), $10xGI_{50}$ (100 nM), and $20xGI_{50}$ (200 nM), vinblastine, $2xGI_{50}$ (200 nM) for 3-48 hours and they were subjected to morphological and biochemical analysis for apoptotic cell death.

1.2.1 Analysis of morphological features of apoptosis

Cell undergoing apoptosis can be recognized by specific changes in cell morphology including membrane blebbing, cell shrinkage, chromatin condensation, cytoplasmic condensation, DNA fragmentation, and the formation of apoptotic bodies (28). Thus, the morphology of apoptotic cell was evaluated in the present studies by observing under a phase contrast microscope and a fluorescence microscope.

1.2.1.1 Phase contrast microscope

The effects of vinblastine and VR-3848 on morphological changes in LU-1 cell were examined by a visual examination using phase contrast microscopy (Figures 5.4-5.5). Both vinblastine, $2xGI_{50}$, and VR-3848, $20xGI_{50}$ caused cell death suggested by apoptosis as seen in Figures 5.4 and 5.5, respectively. Apoptotic cells detached themselves from their viable neighbours, and along with the loss of contact regions, underwent compaction of nuclear chromatin and cytoplasmic condensation which resulted in cell shrinkage followed by apoptotic bodies formation. After an addition of vinblastine and VR-3848, these morphological changes were hardly observed between 3 and 12 hours of treatment (picture not shown). However, they were obviously seen at 24 hours and progressed until cells lost viability. It was

noted that at 24 hour after treatment more effect was observed in cells treated with vinblastine than that found in the VR-3848-treated cells.

Lesser effects were found in cells treated with lower doses of VR-3848, GI_{50}, and $10xGI_{50}$ (picture not shown).

1.2.1.2 4′, 6-Diamino-2-phenylindole (DAPI) staining

In addition, the changes in nuclei of LU-1 cells after exposure to the test substances were examined by staining nuclei with DNA binding dye (DAPI). Examination of DAPI-stained nuclei under the fluorescence microscope revealed that vinblastine and VR-3848 could induce apoptosis in LU-1 cells. Also, the induction of apoptosis by VR-3848 was time- and - dose dependent. LU-1 cells treated with vinblastine, $2xGI_{50}$ and VR-3848, $20xGI_{50}$ (200 nM) showed some characteristics of apoptotic cells as described previously. The nuclei of the treated cells were much smaller than those seen in the untreated cells. It was hardly to detect those changes between 3-12 hours after treatments. However, once apoptosis occurred after 24 hours DNA fragmentation and the formation of apoptotic bodies were much pronounced whereas the number of condensed cells decreased with incubation time (Figures 5.7 and 5.9). In contrast, cells treated with 0.05% DMSO were revealed as the intact round shape of nuclei containing very euchromatin throughout the whole experiments.

Further, when expressing cell death as the percentage value, it was shown that the percentage of apoptosis of LU-1 cell treated with vinblastine rapidly increased and approached the plateau value 34% at 24 hours ($p<0.001$), after that the number of apoptotic cell remained relatively constant until 48 hours after treatment

(Table 5.1 and Figures 5.10 and 5.11). On the other hand, the percentage of apoptosis found in LU-1 cells treated with high doses of VR-3848 treatment, $10xGI_{50}$ and $20xGI_{50}$, for 48 hours followed the same pattern, i.e., increased slowly from 3-12 hours and increased rapidly afterwards. The maximal values seen at 48 hours were approximately 31.2±1.0 ($p<0.001$) and 40.5±2.1 ($p<0.001$), respectively (Table 5.1 and Figures 5.12-5.13). The results were different from the data obtained from cells treated with VR-3848 at the concentration of the GI_{50} dose where the apoptotic cell number detected was low, the percentage of apoptosis varied between 1 and 9 % throughout the experiment (Table 5.1 and Figures 5.12-5.13).

These morphological changes after VR-3848 treatment observed by DAPI staining were in agreement with those seen by phase contrast microscope.

1.2.2 Analysis of biochemical feature of apoptosis

Further, since apoptosis can be biochemically demonstrated as a ladder characteristic of DNA fragments in multiples of 180-200 base pairs (25), the effect of vinblastine and VR-3848 on DNA fragmentation was also evaluated by agarose gel electrophoresis. As shown in Figures 5.14 and 5.15, a DNA ladder of 180-200 base pairs was induced by exposing LU-1 cells to vinblastine at a concentration of $2xGI_{50}$ for 48 hours. However, exposure LU-1 cells with the same dose of vinblastine for 24 hours or at lower doses (20 nM and 100 nM) of vinblastine for 48 hours did not demonstrate DNA fragmentation (data not shown). However, apoptosis induced by VR-3848 was shown to be time- and -dose dependent. This internucleosomal DNA fragmentation was first observed after 24 hours of incubation and more extensive ladder formation was clearly demonstrated after 48 hours of exposure to VR-3848,

$20xGI_{50}$ (Figure 5.14). Prolonged exposure LU-1 cells to the same dose of VR-3848 up to 72 hours produced a smear DNA pattern suggesting the development of secondary necrosis (data not shown). Further, the ladder formation could also be seen when treating cells with the $10xGI_{50}$ of VR-3848 for 48 hours, but not with the GI_{50} of the test substance (Figure 5.15).

By using three methods described above to in detection of apoptosis, i.e., phase contrast microscopy, DAPI-nuclei staining and agarose gel eletrophoresis, it can be concluded that VR-3848 may inhibit the growth of LU-1 cell line by induction of apoptosis.

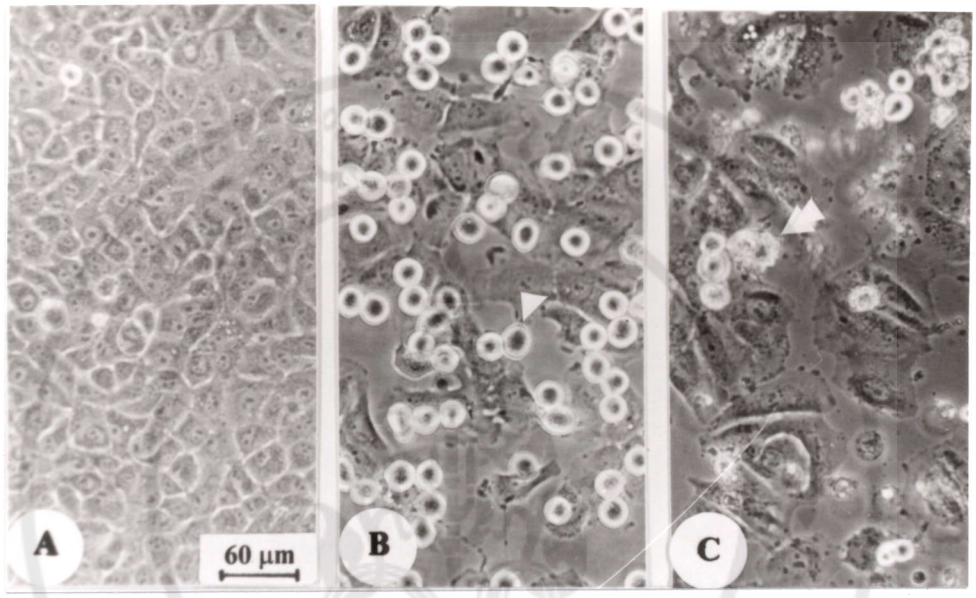

Figure 5.4 Effects of vehicle and vinblastine, $2xGI_{50}$, on LU-1 cell line detected under a phase contrast microscope. Cells were treated with 0.05% DMSO for 48 hours (A); or vinblastine $2xGI_{50}$ for 24 (B), and 48 (C) hours, respectively. Chromatin condensation (single arrow) and DNA fragmentation (double arrows) are shown. Magnification 165x .

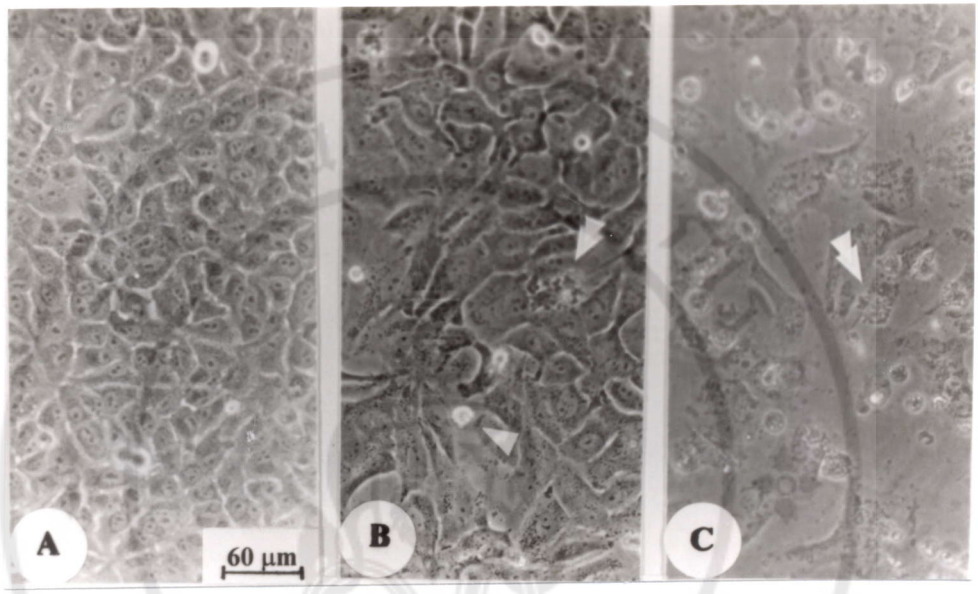

Figure 5.5 Effect of vehicle and VR-3848, 20xGI_{50} on LU-1 cell line detected under phase contrast micrscope. Cells treated with 0.05% DMSO for 48 hours (A); or VR-3848, 20xGI_{50}, for 24 (B), and 48 (C) hours, respectively. Chromatin condensation (single arrow) and DNA fragmentation (double arrows) are shown. Magnification 165x .

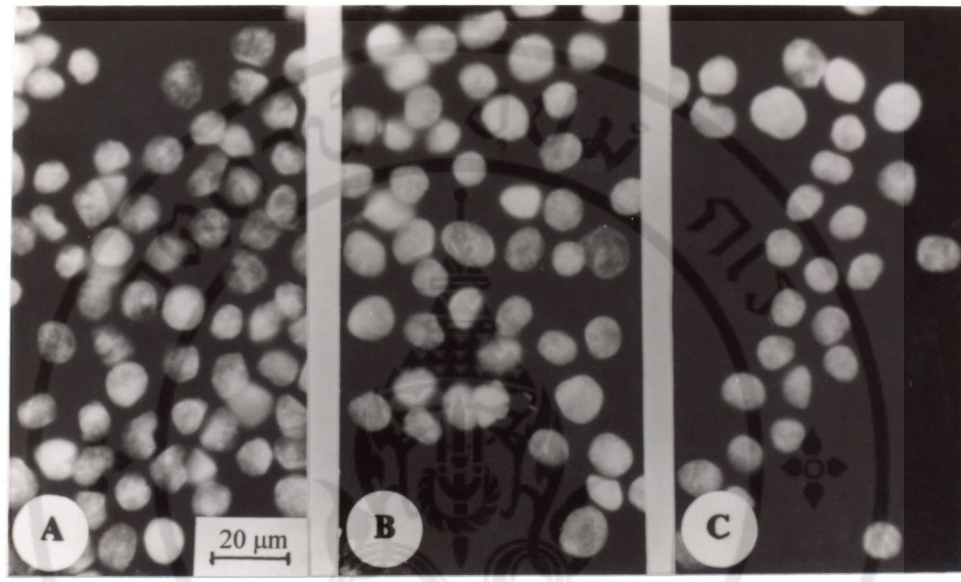

Figure 5.6 Effects of vehicle and vinblastine on LU-1 cells detected by DAPI staining. LU-1 cells were treated with 0.05% DMSO for 48 hours (A); or vinblastine, $2xGI_{50}$, for 3 (B), 6 (C) hours, respectively, and stained with DNA binding dye, DAPI (4′,6-Diamino-2-phenylindole) and photographed under a fluorescence microscope. Magnification 500x .

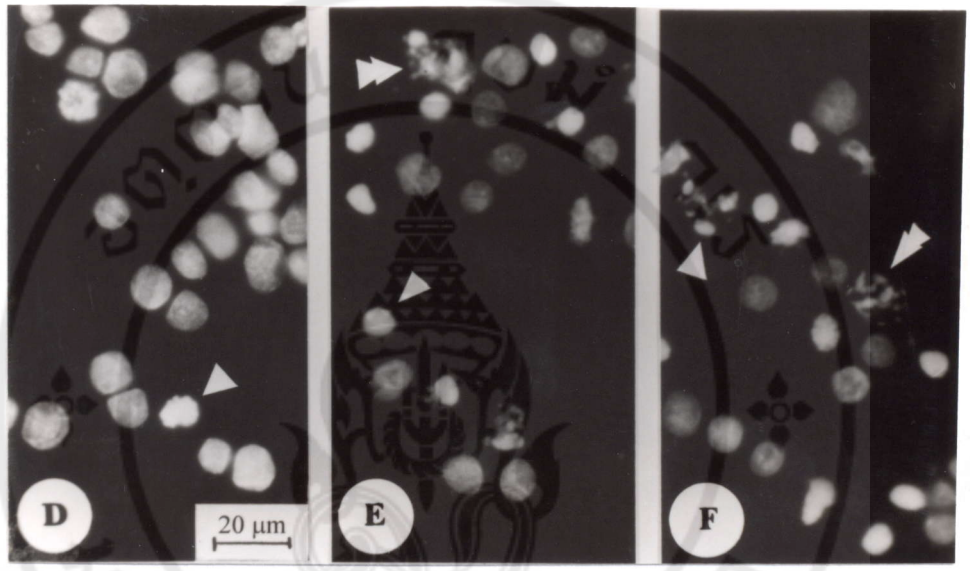

Figure 5.7 Effects of vinblastine on LU-1 cells detected by DAPI staining. LU-1 cells were treated with vinblastine, $2xGI_{50}$, for 12 (D), 24 (E), and 48 (F) hours, respectively, stained with DNA binding dye, DAPI (4′,6-Diamino-2-phenylindole) and photographed under a fluorescence microscope. Apoptotic nuclei are shown as chromatin condensation (single arrow) and DNA fragmentation (double arrow). Magnification 500x .

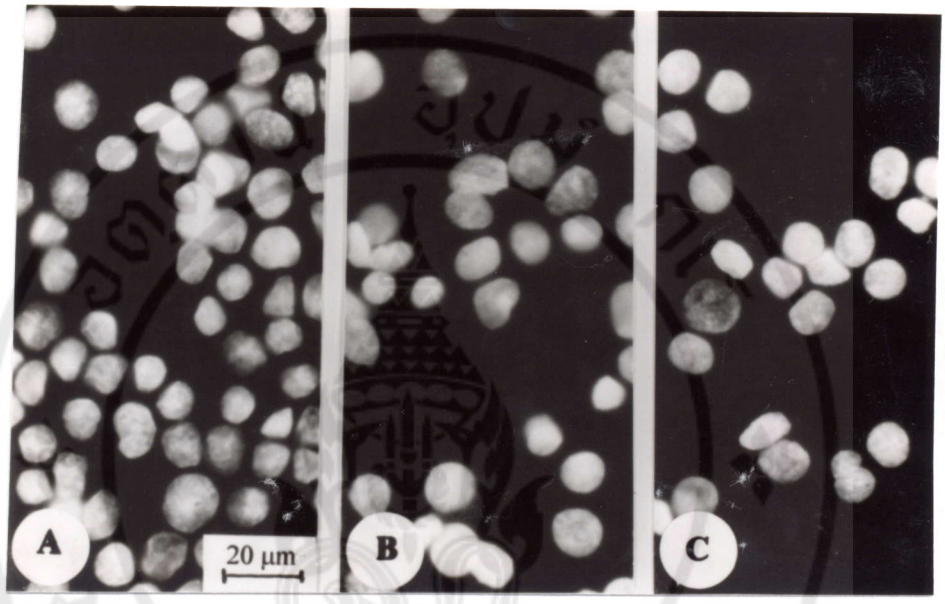

Figure 5.8 Effects of vehicle and VR-3848 on LU-1 cells detected by DAPI staining. LU-1 cells were treated with 0.05% DMSO for 48 hours (A); or VR-3848, 20xGI_{50}, for 3 (B), and 6 (C) hours, respectively, stained with DNA binding dye, DAPI (4′,6-Diamino-2-phenylindole) and photographed under a fluorescence microscope. Magnification 500x .

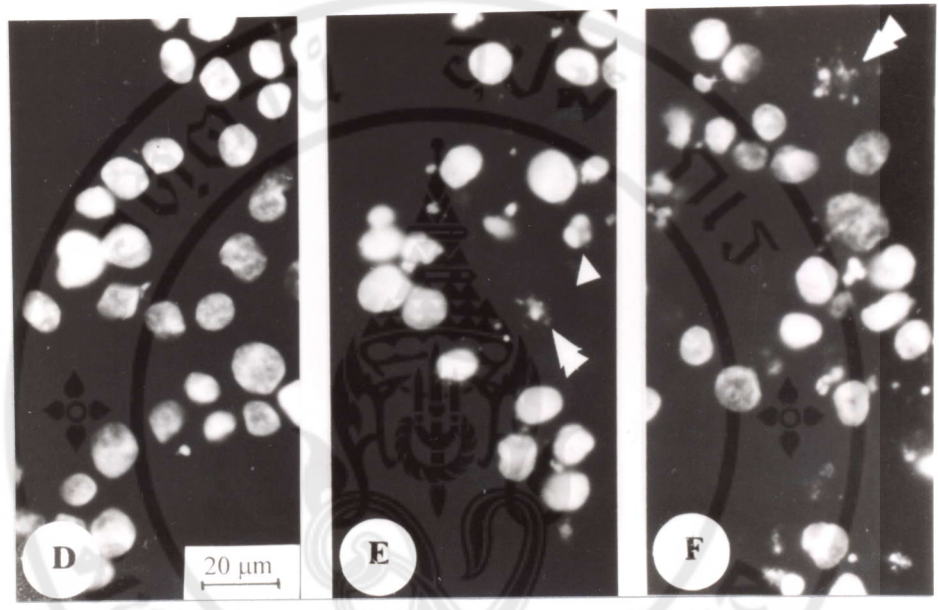

Figure 5.9 Effects of VR-3848 on LU-1 cells detected by DAPI staining. LU-1 cells were treated with BT-3848, $2xGI_{50}$, for 12 (D), 24 (E), and 48 (F) hours, respectively, stained with DNA binding dye, DAPI (4′,6-Diamino-2-phenylindole) and photographed under a fluorescence microscope. Apoptotic nuclei are shown as chromatin condensation (single arrow) and DNA fragmentation (double arrow). Magnification 500x .

Table 5.1 The percentage of apoptotic cells in human lung (LU-1) cancer cell line induced by vinblastine (VBL) and VR-3848. LU-1 cells were treated with vinblastine $2xGI_{50}$ (200 nM) and VR-3848 GI_{50} (10 nM), $10xGI_{50}$ (100 nM), and $20xGI_{50}$ (200 nM). Treated cells were fixed in cold methanol and stained with DNA binding dye, 4′, 6-Diamino-2-phenylindole (DAPI) for 15 minutes at 37°C. Results are means ± S.E.M. from three independent experiments.

Treatment	% Apoptotic cells Incubation time (hours)				
	3	**6**	**12**	**24**	**48**
DMSO	1±0.1	1.5±0.2	1.3±0	1.4±0.3	1.4±0.2
VBL, $2xGI_{50}$	$9.4±1.2^{***}$	$15.1±0.3^{***}$	$21.2±0.9^{***}$	$34.2±1.6^{***}$	$35.9±3.0^{***}$
VR-3848, GI_{50}	1.4±0.2	2.1±0.3	$3±0.1^{*}$	$6.7±1.0^{*}$	$7.9±0.5^{*}$
VR-3848, $10xGI_{50}$	1.7±0.5	2.4±0.2	$3.7±0.3^{*}$	$11.1±1.1^{***}$	$31.2±1.0^{***}$
VR-3848, $20xGI_{50}$	2.1±0.3	$3±0.5^{**}$	$5.7±0.7^{***}$	$14.8±0.8^{***}$	$40.5±2.1^{***}$

* $p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$; significantly different from the control group at the corresponding time (Student's paired t-test).

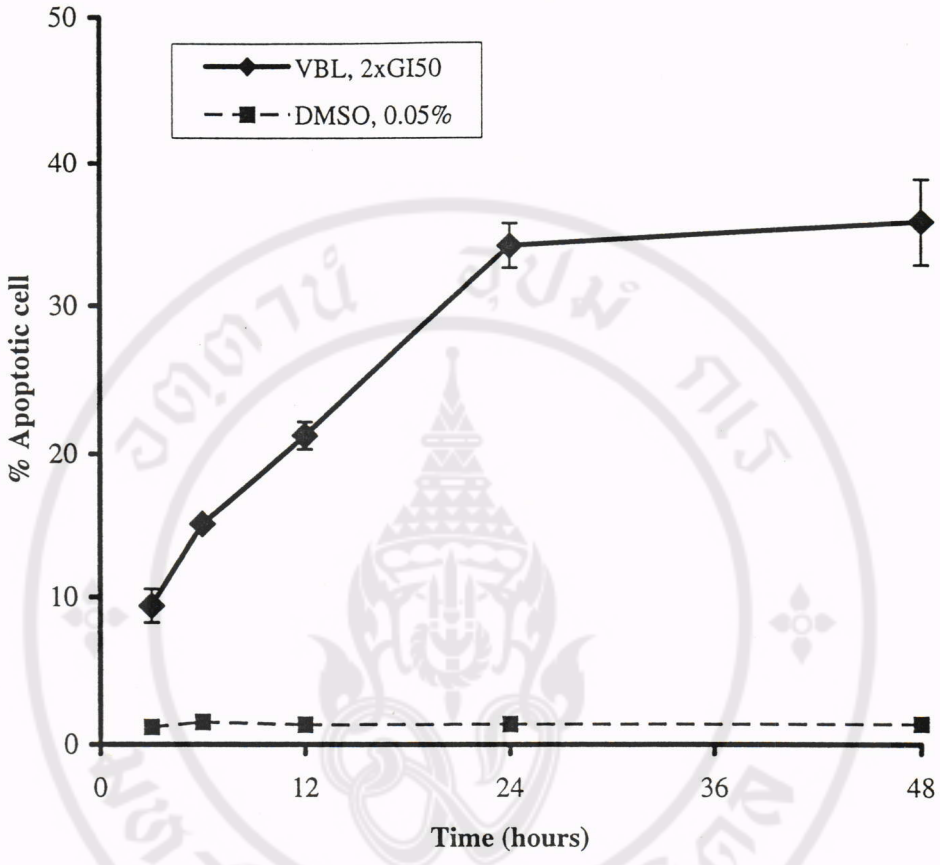

Figure 5.10 Kinetics of apoptosis of LU-1 cells after treatment with either DMSO or vinblastine, $2xGI_{50}$ from 3-48 hours. Cells were exposed to 0.05% DMSO or vinblastine for 3, 6, 12, 24, and 48 hours, then they were fixed with methanol and stained with 4′, 6-Diamino-2-phenylindole (DAPI). Apoptosis was determined at the indicated times. Data are expressed as the percentage of apoptotic cell at various times after incubation. Each point is means ± S.E.M. from three independent experiments.

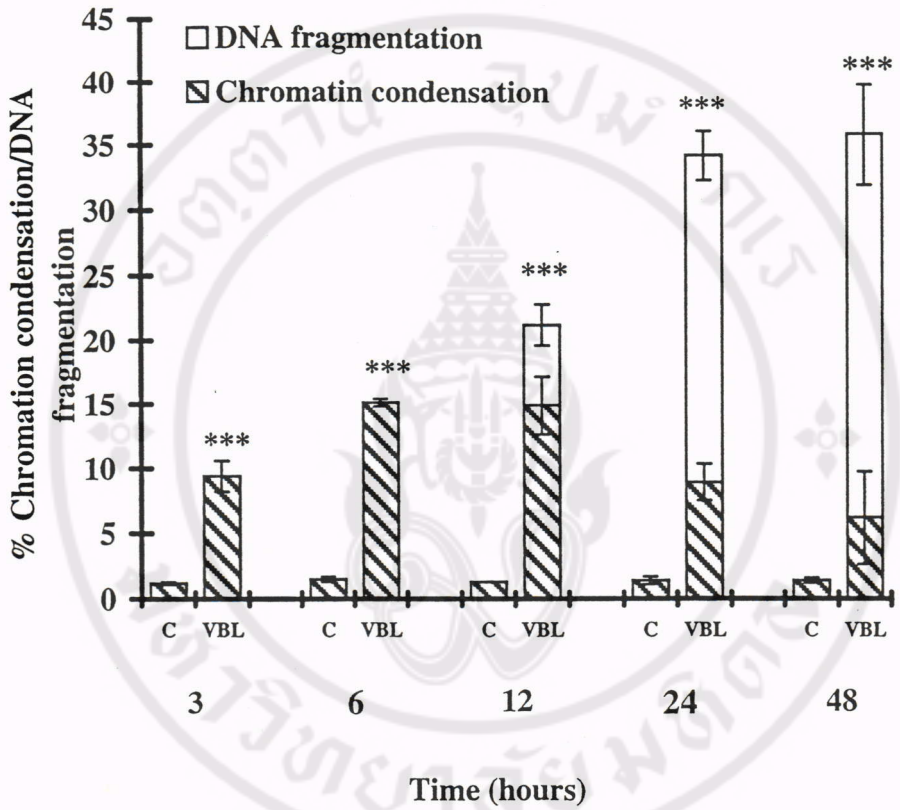

Figure 5.11 Apoptosis is shown as the percentage of chromatin condensation (▨) and DNA fragmentation (□) in LU-1 cells treated with DMSO (C) or vinblastine (VBL) at the indicated times. Results are means ± S.E.M. from three independent experiments.

*p<0.05, ** p<0.01, *** p<0.001, significantly different from the control group at the corresponding time.

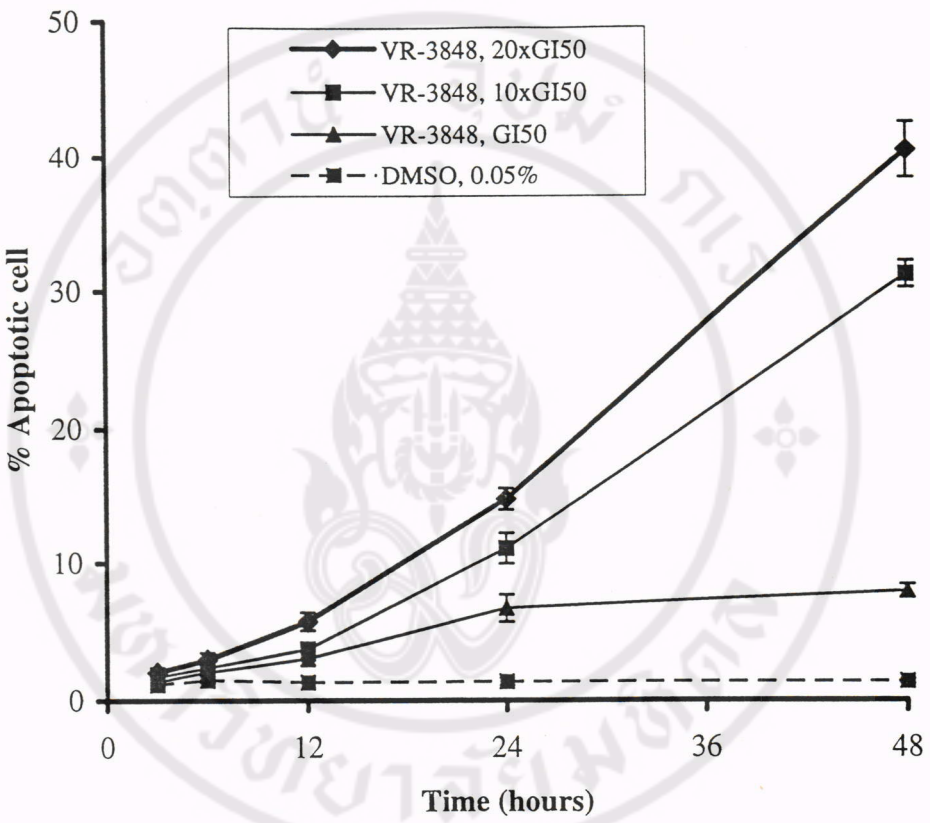

Figure 5.12 Kinetics of apoptosis of LU-1 cells after treatment with either DMSO or various doses of VR-3848 from 3-48 hours. Cells were exposed to 0.05% DMSO or VR-3848, GI_{50}, $10xGI_{50}$, and $20xGI_{50}$ for 3, 6, 12, 24, and 48 hours, then they were fixed with methanol and stained with 4′,6-Diamino-2-phenylindole (DAPI). Apoptosis was determined at the indicated times. Data are expressed as the percentage of apoptotic cell at various times after incubation. Each point is means ± S.E.M. from three independent experiments.

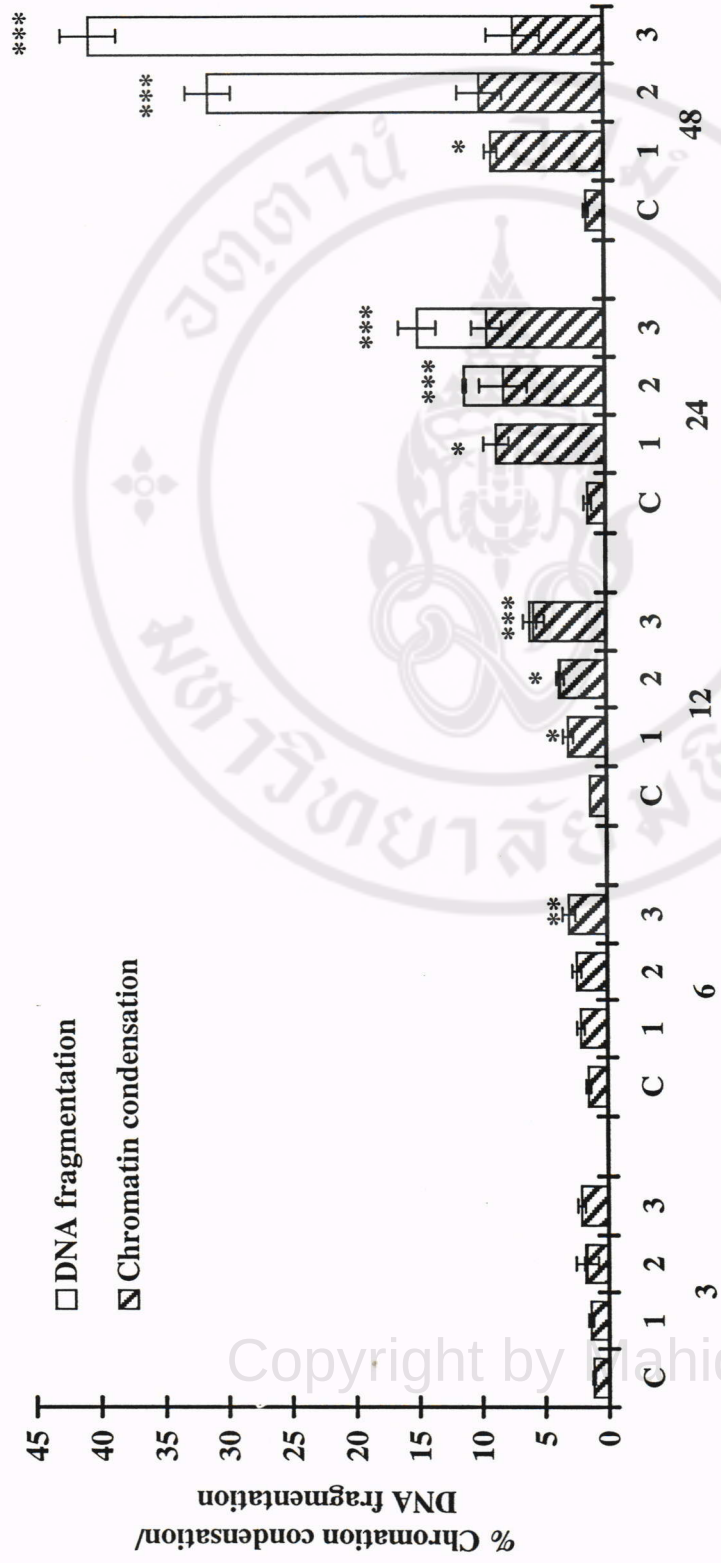

Figure 5.13. Apoptosis is shown as the percentage of chromatin condensation (▨) and DNA fragmentation (□) in LU-1 cell treated with DMSO (C) and various concentrations of VR-3848 as the indicated times. Results are means ± S.E.M. from three in dependent experiments. 1 = GI_{50}, 2 = 10xGI_{50}, 3 = 20xGI_{50} of VR-3848

*p<0.05, **p<0.01, ***p<0.001, significantly different from control group at the corresponding time.

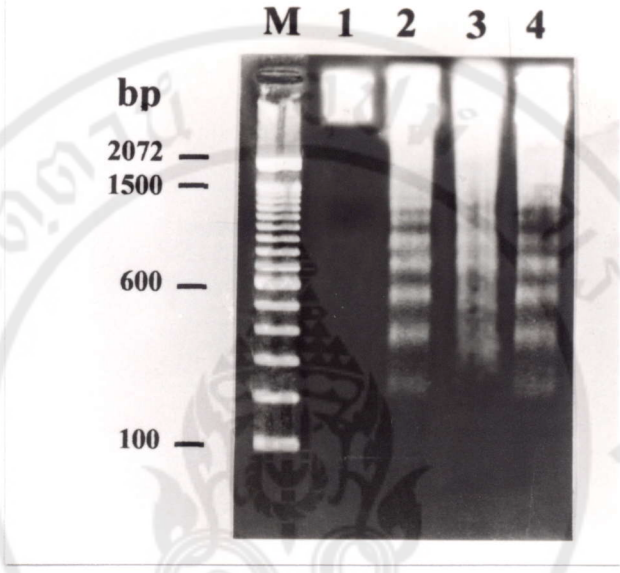

Figure 5.14 Time-dependent effects of VR-3848 on internucleosomal DNA cleavage in LU-1 cells. Cells were treated for 48 hours with either 0.05% DMSO (lane 1) or vinblastine, $2xGI_{50}$ (a positive control, lane 2) and VR-3848, $20xGI_{50}$, for 24 and 48 hours (lanes 3 and 4, respectively). Subsequently, the genomic DNA was extracted and electrophoresed in a 1.8% agarose gel. The ladder formations of multiples 180-200 bp fragment was observed in cells treated with vinblastine (lane 2) and VR-3848 for 24 and 48 hours (lanes 3 and 4, respectively). M is the 100 bp marker.

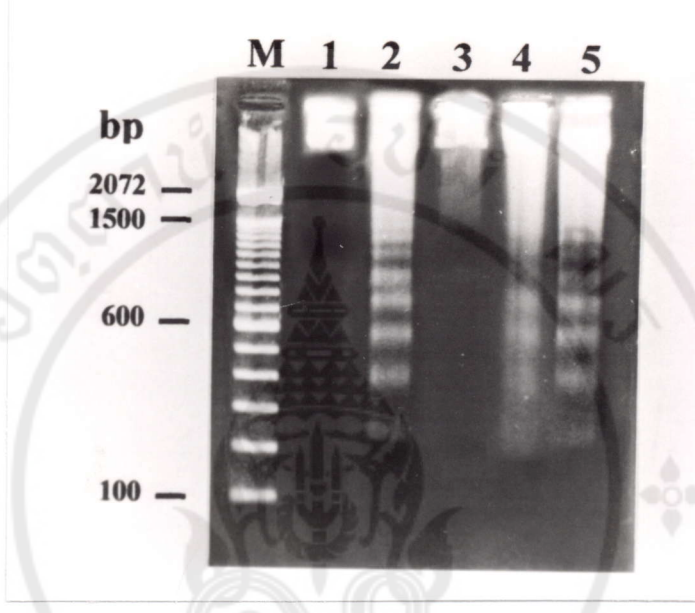

Figure 5.15 Dose-dependent effects of VR-3848 on internucleosomal DNA cleavage in LU-1 cells. Cells were treated with either 0.05% DMSO (lane 1) or vinblastine, $2xGI_{50}$ (a positive control, lane 2) and different doses of VR-3848, GI_{50}, $10xGI_{50}$, and $20xGI_{50}$ (lanes 3, 4, and 5, respectively). Subsequently, the genomic DNA was extracted and electrophoresed in a 1.8% agarose gel. The ladder formations of multiples 180-200 bp fragment was observed in cells treated with vinblastine (lane 2) and VR-3848, $10xGI_{50}$, and $20xGI_{50}$ for 48 h. (lanes 3, 4, and 5, respectively). M is the 100 bp marker.

PART II. TO FURTHER INVESTIGATE THE POSSIBLE ROLE OF CASPASE-3 (CPP32) IN THE VR-3848-INDUCED APOPTOSIS

2.1 Expression of caspase-3 gene in LU-1 cells treated with VR-3848

To determine whether CPP32 is involved in the VR-3848-induced apoptosis, LU-1 cells were treated with VR-3848, 20xGI_{50}, for 3-48 hours and the expression of CPP32 mRNA was detected using reverse transcriptase-polymerase chain reaction (RT-PCR). The results showed that caspase 3 gene is normally expressed in LU-1 cell line. As shown in Figure 5.16 the PCR products of the predicted sizes for CPP32 mRNA (543 bp) and β-actin mRNA (362 bp) used as the internal control gene were detected in untreated LU-1 carcinoma cell (lanes 2 and 1, respectively). However, unexpected results were obtained in VR-3848-treated cells to see that there was no significantly different in the levels of CPP32 mRNA expressed in untreated and treated cells throughout the experiment studied. In addition, the same result was seen in vinblastine-treated cells under the same conditions that induced apoptosis (data not shown).

Thus, the data suggest that apoptosis induced by VR-3848 in LU-1 cells may not be regulated by the expression of caspase 3 gene.

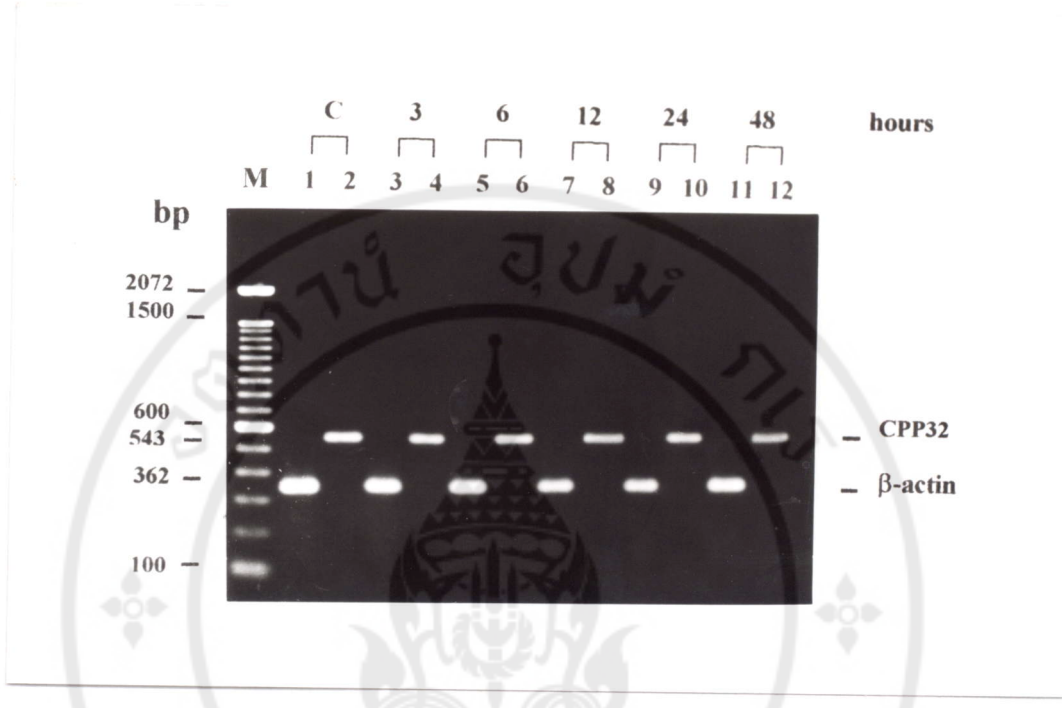

Figure 5.16 Expression of caspase-3 (CPP32) gene in LU-1 carcinoma cell line treated with VR-3848 from 3-48 hours. LU-1 cells were treated at indicated times with 20xGI_{50} of VR-3848 or vehicle (C) for 48 hours. Ethidium bromide-stained PCR amplification products after separating on 1% agarose gel electrophoresis are shown. β-actin gene expression was used as an internal standard. M is the 100 bp maker.

2.2 Activation of caspase-3 activity in human lung (LU-1) cancer cell line

To further characterize the relevance of caspase-3 activation in the VR-3848 and vinblastine-induced apoptosis, the effect of a caspase-3 specific inhibitor, Ac-DEVD-CHO, on apoptosis was performed. Cells were treated with VR-3848 and vinblastine using the same procedure as mentioned before in order to induce apoptosis. Following that, caspase-3 activity was measured in the absence and presence of 5 μM of Ac-DEVD-CHO and expressed as fold induction. The preliminary findings showed that at concentration $20xGI_{50}$ of 3848 and $2xGI_{50}$ of vinblastine stimulated a substantial induction of caspase 3 activity as a time-dependent manner (Table 5.2 and Figure 5.17). The substantial activation of caspase-3 activity was clearly observed between 12 and 48 hours of both treatments. However, VR-3848 stimulated an induction of caspase-3 activity in a greater extent, especially, at 24 and 48 hours of treatment. The caspase-3 activity in the untreated cells was low and remained constant throughout the experiment. In the presence of 5 μM of Ac-DEVD-CHO the activation of caspase-3 induced by VR-3848 and vinblastine was completely inhibited. Further, no significant effect was seen of the inhibitor itself on caspase-3 activity since the activity of caspase-3 did not significantly change after an addition of the inhibitor compared with the vehicle (data not shown). The findings suggest that caspase-3 should be involved in the VR-3848-induced apoptosis in LU-1 cells.

Table 5.2 Effects of caspase-3 inhibitor on the fold induction of caspase-3 in LU-1 cells. Cells were treated with $20xGI_{50}$ of VR-3848 and $2xGI_{50}$ of vinblastine (VBL) from 3 to 48 hours. Subsequently, cytosol protein was extracted and measured for caspase-3 activity in the presence and absence of a specific inhibitor. Results are means ± S.E.M. from three independent experiments.

Treatment	Fold induction of caspase-3 activity Incubation time (hours)				
	3	6	12	24	48
VR-3848, $20xGI_{50}$	1.65±0.15	1.83±0.19	11.21±1.04	34.45±0.99	43.30±4.99
VR-3848, $20xGI_{50}$ + Ac-DEVD-CHO	0.65±0.14	0.77±0.12*	0.64±0.12**	0.96±0.18**	1.55±0.33*
VBL, $2xGI_{50}$	1.59±0.17	2.19±0.18	7.11±0.58	11.62±0.67	14.39±1.67
VBL, $2xGI_{50}$ + Ac-DEVD-CHO	0.88±0.06	0.95±0.08*	0.96±0.06*	1.15±0.04**	1.22±0.05*

* $P<0.05$, as compared to 5 μM Ac-DEVD-CHO at the corresponding time.

** $P<0.01$, as compared to 5 μM Ac-DEVD-CHO at the corresponding time.

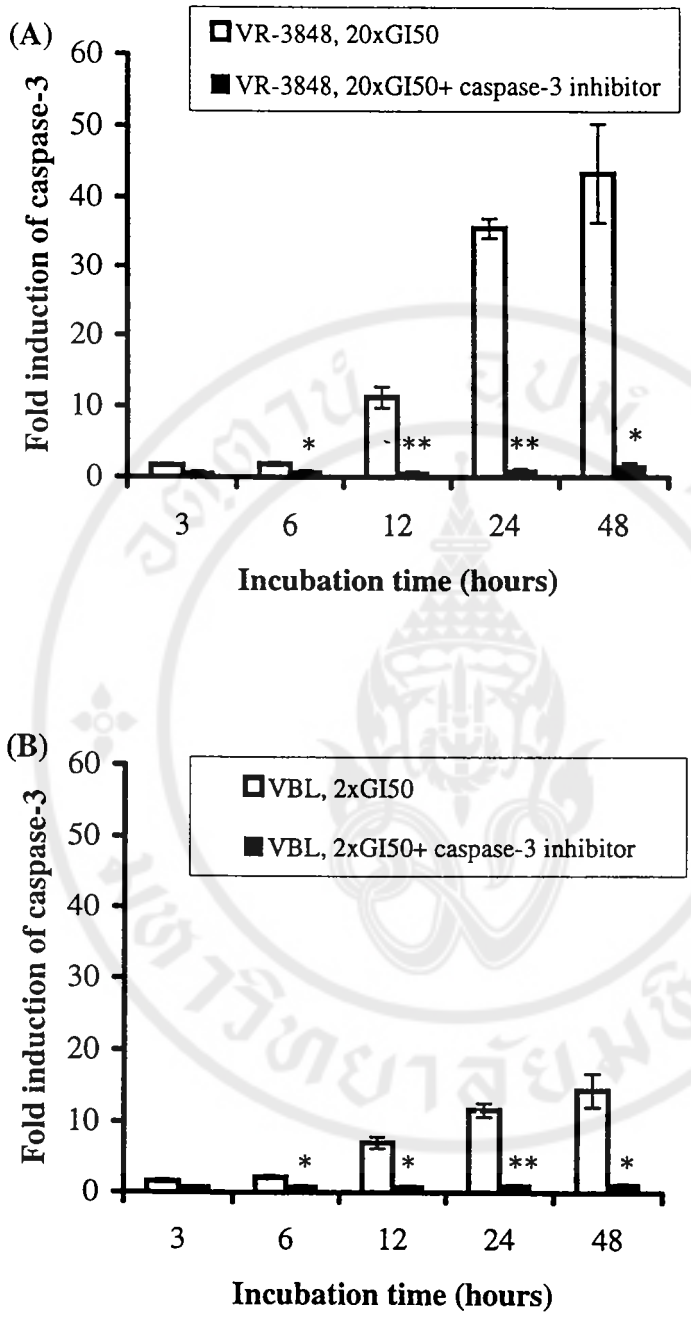

Figure 5.17 Effects of caspase-3 inhibitor on the fold activation of caspase-3 in apoptosis induced by VR-3848 (A) and vinblastine (B) in LU-1 cells. Cells were treated with $20xGI_{50}$ of VR-3848 (A) and $2xGI_{50}$ of vinblastine (B) for 3, 6, 12, 24, and 48 hours, respectively. Then, cytosol protein was extracted and measured for caspase-3 activity in the presence and absence of a specific inhibitor. Data are expressed as fold induction of caspase activity in each experiment. Each data value represents means± S.E.M. from three independent experiments.

CHAPTER VI

DISCUSSION

The mechanism of action of VR-3848, an unknown compound purified from *Euphobiaceae,* in inducing cell death was investigated in the present study using human lung (LU-1) cancer cell line as a model.

6.1 Growth inhibition assay

The responses of LU-1 cells at each step of vinblastine and VR-3848 exposure were quite similar, most cells initially died and floated. However, the cytotoxic effect of VR-3848 seems to be a delayed effect since those changes were clearly noticed after 24 hours of treatment. In contrast, dying cells were seen before 24 hours in vinblastine treatment. After repeating 3 experiments with vinblastine and VR-3848 for 48 hours found that the cells treated with VR-3848 is more cytotoxicity than vinblastine approximately 10 fold because the GI_{50} for VR-3848 and vinblastine are approximately 10 and 100 nM, respectively. The concentration of vinblastine that found to induce apoptosis in this study was 200 nM. This is similar to the dose that Tashiro *et al.,* (1998) (4) used in their experiment, 180 nM. Whereas they studied in small cell lung cancer cell, Ms-1 cells, the present work used LU-1 cell which is classified as non small cell lung cancer (personal communication).

6.2 Induction of apoptosis by VR-3848 in LU-1 cancer cell line

From the observation of floated cells after treatment of LU-1 cell with vinblastine and VR-3848 under phase contrast microscope prompted us to test the type of cell death caused by these agents. DAPI (4′, 6-Diamino-2-phenylindole), a DNA binding dye, staining is one method that is used to detect apoptosis in this work. The results of DAPI staining showed chromatin condensation, DNA fragmentation from large intact round shape to smaller structure of the so-called apoptotic bodies consistent with apoptotic changes. Therefore, morphological studies indicate that LU-1 cells developed apoptotic features after vinblastine and VR-3848 treatment. The findings obtained from DAPI staining show that when using the same concentration the effect of vinblastine in inducing apoptosis in LU-1 cells is quicker than that produced by VR-3848 treatment. For vinblastine treatment, condensed cells were found after 3 hours whereas fragmented DNA cells were firstly noticed at 12 hours and become a peak at 24 hours. For VR-3848 treatment, DNA condensation were seen at 12 hours and followed by DNA fragmentation at 24 hours. This indicates that disintegration of DNA is come after the condensation of DNA as many investigators have been reported previously (4, 95, 96). However, once the apoptosis has already initiated, the number of cell death caused by these two compounds showed the same degree of induction (Figure 5.10-5.12).

The result on the percentage of apoptosis induced by vinblastine 200 nM obtained in this report was about 34-36% between 24 and 48 hours. This is similar to the finding of Gibbson *et al.* (1999) (95). They reported that after exposing HEK 293 cells (human embryonic kidney 293 cells) to 1 μM of vinblastine for 24 hours, the peak

of apoptotic cells was approximately 40% when staining cells with propidium iodide. Therefore, DAPI staining which is used to detect the nuclei alterations during apoptosis in this study is appropriate.

Further, the result from DNA ladder formation which is one of a biochemical hallmarks of apoptosis confirmed that apoptosis is the cause of death in LU-1 cells after exposure to vinblastine and VR-3848. Furthermore, the apoptosis induced by VR-3848 is time- and -dose dependent. Similarly, vinblastine initiated apoptosis in a time-dependent manner.

6.3. Reverse Transcriptase-Polymerase Chain Reaction

The potential importance of caspases in LU-1 treated with VR-3848 was investigated in this study since it has not been studied before. The presence of the caspases gene in LU-1 cell was first examined here by reverse-transcription (RT) polymerase chain reaction (PCR), using primers designed from the coding sequences of the known genes of caspase homologs. The result showed that CPP32 gene is expressed in LU-1 cells. However, the expression of CPP32 in the control and VR-3848-treated LU-1 cells did not change within 48 hours. This is similar to the result of Droin *et al.* 1998 (97) who used RT-PCR to demonstrate the transcription for the nine pro-caspases in untreated K562 cells (the human leukemic cell line). Additionally, in the K562 cell line, the expression of the caspase-3 gene remained stable during the first 48 hours exposure to 100 μM etoposide. Therefore, RT-PCR analysis reveal that LU-1 cells express mRNA for caspase-3 and apoptosis induced by VR-3848 may not be regulated by transcription of caspase-3 gene.

6.4 Caspase-3 Activity

The role of caspase-3 contributes to the VR-3848-induced apoptosis was further investigated. Using 5 μM of the specific caspase-3 inhibitor, Ac-DEVD-CHO, the result indicates that DEVD dependent protease activity may be responsible for apoptotic characteristics in LU-1 cells induced by $20xGI_{50}$ of VR-3848 within 48 hours. This concentration of the inhibitor can completely inhibit the activation of caspase-3 induced by 200 nM of VR-3848 and vinblastine (Figure 5.17). It should be noted that the concentration of the tetrapeptide inhibitor used in this work is 5 μM which is less than those have been used previously (50 μM by Lie *et al.*, (1998) (91); and 100 μM by Simizu *et al.*, (1998) (96)].

Also it should be noted that at the same concentration of the test compounds was used. The fold induction of caspase-3 activity induced by VR-3848 is greater than that caused by vinblastine. This could be the fact that VR-3848 ($GI_{50} \simeq 10$ nM) is more potent than vinblastine ($GI_{50} \simeq 100$ nM). However, this finding did not go along well with the result of apoptotic study. Whereas the number of apoptotic cells reached the maximum at 24 hour after vinblastine treatment, the caspase-3-like enzyme activity was highest at 48 hours.

This suggests that other pathways may also be responsible in vinblastine-induced apoptotic for example, Bcl-2 phosphorylation which works upstream in the apoptotic pathway. This conclusion is based on the findings reported by Tashiro *et al.* (1998) (4) that vinblastine induced apoptosis in Ms-cells by inactivation of the antiapoptotic function of Bcl-2 by phosphorylation. This is followed by activation of caspase-3. In addition, Wang *et al.* (1998) (98) have shown that vinblastine activated

the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in apoptotic regulationof cancer cells. JNKs, also known as stress-activated protein kinase (SAPKs), are involved in a signal transduction pathway parallel to that of mitrogen-activated protein kinases. Furthermore, Simizu *et al.* (1998) (96) have examined whether generations of H_2O_2 is a critical event for the apoptotic pathway downstream of caspase-3(-like) protease activation by vinblastine, and scavenging of H_2O_2 caused cells to fail to undergo apoptosis. They found that the activation of caspase-3(-like) proteases by vinblastine casuses generation of H_2O_2 presumbly through the activation of NADPH oxidase, thereby inducing apoptosis. Therefore, H_2O_2 may function as a common mediator for apoptosis induced by vinblastine.

On the other hand, the activation of caspase-3 by VR-3848 seems to be a major mechanism in regulating of apoptosis since the levels of this enzyme precede the apoptotic event (Figure 5.13 and 5.17).

The morphological and biochemical changes in apoptotic cells observed in the present study should be due to caspase-3 after being activated by VR-3848 or vinblastine. This conclusion comes from the findings that caspase-3 has been demonstrated to be required for DNA fragmentation and the morphological alterations during apoptosis (75). This could be due to CAD (caspase activated deoxyribonuclease) which is shown to be the DNA endonuclease responsible for cleavage chromosomal DNA specifically during apoptosis (55, 99). Normally, the endonuclease function of CAD is suppressed by the inhibitor called ICAD which binds tightly to CAD. However, in the presence of caspase-3, ICAD will be cleaved and inactivated, the releasing active CAD then translocates into the nucleus where it

degrades the DNA (55, 99, 100). The results raise the possibility that ICAD degradation may be the caspase-3-dependent step in DNA fragmentation. Therefore, from caspase-3 study the result indicates that VR-3848 may regulate the caspase-3 level at the translation step. Consequently, it has been proposed that VR-3848 may induced apoptosis mediated by the activation of caspase-3. Detailed regulation in apoptotic pathway awaits for further investigation.

In the future, the involvement of caspase-3-like protease in the VR-3848 induced apoptosis should be reinvestigated. The cleavage of procaspase-3 to its active forms P17 and P12, and of its substrate (PARP) will be studied by western blotting and analysis with specific antibody. PARP [poly-(ADP-ribose) polymerase] is a 113 kDa protein that binds specifically at DNA strand breaks. PARP is also a substate for certain caspase activated during early stages of apoptosis. These protease cleave PARP to fragments of approximately 89 kDa and 24 kDa. Detection of the 89 kDa PARP fragment with Anti-PARP thus serves as an early marker of apoptosis.

Therefore, the data suggest that VR-3848 may induce cell death by apoptosis which is regulated by caspase-3 activation. VR-3848 may subsequently be developed to be a novel chemotherapeutic drug for cancer treatment in the future since the mutagenicity and teratogenicity effect could not be detected (personal communication).

CHAPTER VII

CONCLUSION

The present study intended to evaluate whether the inhibitory effect of VR-3848 on the growth of lung (LU-1) cancer cell line was due to apoptotic cell death and further investigate the possible role of caspase-3 (CPP32) on apoptosis induced by VR-3848. LU-1 cells were treated with three concentrations of VR-3848, GI_{50}, $10xGI_{50}$, and $20xGI_{50}$ for 3-48 hours, and the cells were detected for features of apoptosis by phase contrast microscope, DAPI staining, and agarose gel electrophoresis. The contribution of caspase-3 to the VR-3848-induced apoptosis was studied by RT-PCR and a fluorogenic assay. The findings were compared with those obtained from vinblastine ($2xGI_{50}$), a positive control, under the same condition.

It can be concluded from the results of the present work that:

1. VR-3848, an unknown compound purified from *Euphobiaceae*, is more potent than vinblastine about 10 fold as the GI_{50} concentrations for VR-3848 and vinblastine determined from the dose-response curves were approximately 10 and 100 nM, respectively.

2. LU-1 cells treated with vinblastine $2xGI_{50}$ for 3-48 hours demonstrated a significant of vinblastine-induced apoptosis ($p<0.0001$). The number of apoptotic cells, which were recognized as condensed cells and DNA fragmentation, was rapidly increased and reached the peak 36% within 24 hours. In addition, this dose of

vinblastine produced a DNA ladder formation, one of the biochemical hallmarks of apoptosis revealed in a agarose gel, at 48 hours after the peak of apoptosis.

3. Results from morphological and DNA analyses suggest that VR-3848 may exert the inhibitory effect on cancer cell growth by induction of apoptotic cell death which is in dose- and time-dependent manners.

4. LU-1 cells exposured to GI_{50} of VR-3848 caused a minimal effect on apoptosis. However, when increasing the concentration of VR-3848 up to $10xGI_{50}$, the percentage of apoptosis gradually increased from 3-12 hours, after that the number of apoptosis cell death rapidly increased and reached the peak 31% at 48 hours ($P<0.0001$), but this concentration did not display a DNA ladder formation.

5. Nevertheless, exposing LU-1 cells to $20xGI_{50}$ of VR-3848 for 3-48 hours demonstrated a significant VR-3848-induced apoptosis ($P<0.0001$). The pattern of changes in the number of apoptotic cells run parallely with that found in the $10xGI_{50}$ of VR-3848 treatment. The peak of apoptosis observed after 48 hours was about 41%. The formation of a DNA ladder was also revealed at 48 hours of treatment.

6. Studies the role of caspase-3 protease on the VR-3848-induced apoptosis using RT-PCR, the findings showed no difference in the expression of caspase-3 gene in cells treated with $20xGI_{50}$ of VR-3848 for 3-48 hours compared with that observed in the control cells. Therefore, apoptosis-induced by VR-3848 should not be regulated by caspase-3 at the mRNA level.

7. On the other hand, measurement of the CPP32-like activity using a fluorogenic assay showed that in the presence of 5 μM of the caspase-3 inhibitor, Ac-

DEVD-CHO, completely blocked the activity of CPP32-like protease enzyme in cells treated with the same concentration of VR-3848 that induced apoptosis.

8. Taken together, the findings from the present study demonstrate that the activation caspase-3 activity may involve in the VR-3848-induced apoptosis in human lung cancer cell line.

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APPENDIX I

SOLUTION FOR CELL CULTURE

Preparation 1 litre of MEM (Minimum essential medium) containing 5% bovine calf serum

	Weight (gm)	Volume (ml)
1. $NaHCO_3$	2.2	-
2. Powder MEM medium	9.5	-
3. Distilled water	-	930
4. Non-essential amino acids	-	10
5. Penicillin-streptomycin	-	10
6. Fungizone	-	1
7. Bovine calf serum (BCS)	-	50

Poured a pack of the powder medium and 2.2 gram $NaHCO_3$ in a flask. After an addition of distilled water, mixed well until they were completely dissolved. Then, filtered the medium through 0.22μ membrane filter. Subsequently, added 10 ml of Non-essential amino acids, 10 ml penicillin-streptomycin 1 ml fungizone, and supplemented with 50 ml of bovine calf serum. The growth medium was kept at 4°C until use. The pH of the medium should be around 7.2-7.4.

Preparation of Phosphate-Buffered Saline (PBS), Mg^{++} and Ca^{++} free

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

Added distilled water up to 1 litre.

The solution was sterilized by autoclaving at 121°C for 15 minutes at 15 pounds. The pH of PBS should be 7.4 and the solution was kept at room temperature.

APPENDIX II

TOTAL CELL COUNTING

A. PROCEDURE

The cell suspension, approximately 10 µl, was transferred to the hemocytometer chamber by carefully touching the edge of the coverslip with a Pasteur pipette and allowing the chamber to fill by capillary action without overfilling or underfilling it. Reloaded the pipette and filled the second chamber as described above. Then, the cells located in the four corners (1 mm^2 each) of the chamber were counted under a phase contrast microscope at the magnification of 10x objective (Figure II.A). To avoid counting the same cell twice, only the cells which lie on the top and left-hand lines of each square were counted, but not those on the bottom or right-hand lines (Figure II.B).

B. CALCULATION

The average of the two counts was calculated and the concentration of cell per ml was derived from the formula:

$$c = \frac{n}{v}$$

here, c = cell concentration (cells/ml),

n = number of cells counted, and

v = volume counted (ml).

Each square of the hemocytometer (with a coverslip in place) represents a total volume of 0.1 mm^3 or 10^{-4} ml since the depth of the chamber is 0.1 mm and assuming only the middle 1 mm^2 is used. Therefore:

the number of cells per ml = the average of cell counts per square x 10^4.

If the average cell counts per square is more than 60, the procedure should be repeated by adjusting the cell suspension to an appropriate concentration.

Thus, the number of cell per ml = the average counts per square x 10^4 x dilution factor

And the total cell counts was obtained as follows:

Total cells counts = cells per ml x the original volume of total cell suspension from which cell sample was removed.

Reference:

Freshney RI. Culture of animal cells: a manual of basic technique. 3rd ed. New York: Wisley-Liss; 1994: 269.

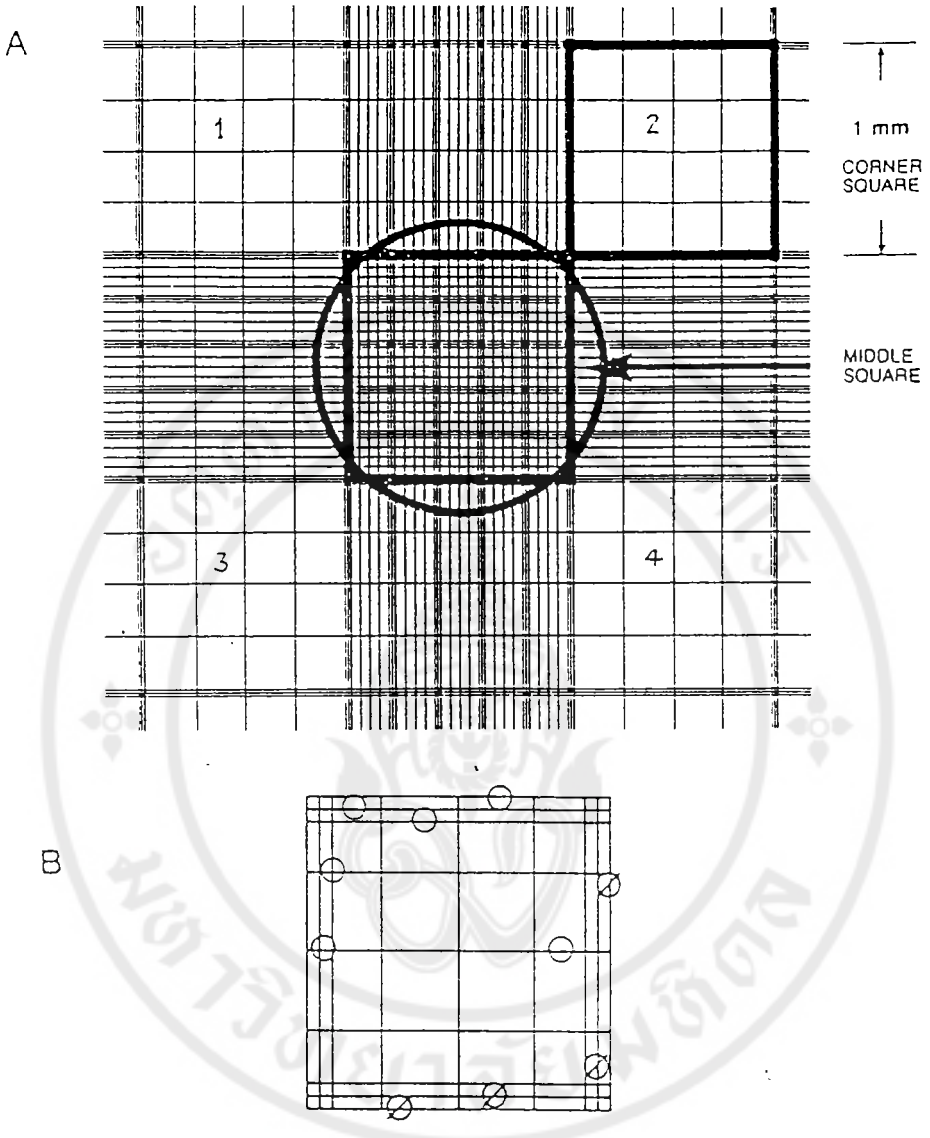

Figure II. A. Diagram showing the standard hemocytometer used in cell counting. While 9 chambers are contained in one chamber of the hemocytometer, only four squares in the corner are labeled here. B. The counted cells are presented as white circles whereas the uncounted cells are shown as cross circles. The number of averaged cell/ml of the cell suspension is obtained by multiplying the average cell count from 8 squares by 10^4 (101).

APPENDIX III

SULFORHODAMINE B ASSAY

PROCEDURE

1. Cell fixation

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

2. SRB assay

After that, the TCA-fixed cells were stained with 100 µl of 0.4% (w/v) SRB in 1% acetic acid for 30 minutes at room temperature, washed with 1% acetic acid for five times to remove excess SRB and air dried again. Finally, the stained cells were solubilized in 200 µl 10 mM unbuffered Tris base (pH 10) for 5 minutes on a gyratory shaker. The amount of bound SRB released was analyzed by measuring the absorbance at 515 nm using a microtitre plate reader (EL 312 microplate, Bio-Kinetic Reader).

Reference:

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

APPENDIX IV

CYTOTOXICITY ASSAY

1. Preparation of cell cultures

In brief, after growing up the cells, LU-1 cell suspension was prepared in growth medium and plated at a cell concentration of $0.65x10^4$ cells/190 µl/well from row A to row F of a 96-well plate (Appendix IV). Growth medium was added into row G and served as control blank (B) to route out the effect of serum proteins in growth medium. Cells were then incubated for 24 hours in a humidified atmosphere with 5% CO_2 at 37°C.

2. Treatment

Various concentrations of vinblastine and VR-3848 were prepared from 10 mM of stock solutions in DMSO by diluting with 0.5% (v/v) DMSO in distilled water. After 24 hours incubation, fresh medium was exchanged and 10 µl of working solution of the test compounds at each concentration was added into the 96-well plate (row B to row E) to give at indicated concentrations. Three wells were prepared for each concentration. In order to test the effects of DMSO on cell growth solvent control (SC) cells were prepared in the presence of 0.05% DMSO (the same concentration which applied with the treatment). Also the remaining wells in row A, cells were grown in the absence of test compounds and served as control cultures (C).

3. SRB assay

After a further 48 hours incubation period, the number of viable cell was quantitated by SRB staining as described previously in Appendix III. Dye uptake was quantified by measuring OD at 515 nm using an ELISA plate reader. Then, the GI_{50} concentrations of VR-3848 and vinblastine were determined from the dose-response curves, which were obtained from three independent experiments.

Table IV.1. Plating-out scheme for LU-1 cell line cytotoxicity test performed on 96-well plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C	C	C	C	C	C	C	C	C	C
B	V_1	V_1	V_1	V_5	V_5	V_5	T_1	T_1	T_1	T_5	T_5	T_5
C	V_2	V_2	V_2	V_6	V_6	V_6	T_2	T_2	T_2	T_6	T_6	T_6
D	V_3	V_3	V_3	V_7	V_7	V_7	T_3	T_3	T_3	T_7	T_7	T_7
E	V_4	V_4	V_4	V_8	V_8	V_8	T_4	T_4	T_4	T_8	T_8	T_8
F	SC	SC	SC	SC	SC	SC	SC	SC	SC	SC	SC	SC
G	B	B	B	B	B	B	B	B	B	B	B	B
H												

B: Control blank, i.e., no cells added to the well; C: Control cultures, i.e., cells were not exposed to test compound, but only to treatment medium; SC: Solvent control, i.e., cells were not exposed to test compound, but only to treatment medium containing 0.05% DMSO; T and V: Test culture 3 wells exposed to the same concentration of VR-3848 and vinblastine, respectively.

Reference:

Monks A, Scediero D, Skehan P, Shoemaker R, Paull K, and Vistica D, *et al.* Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 1991;83: 757-66.

APPENDIX V

ANALYSIS OF DNA DEGRADATION

1. DNA extraction from LU-1 cells

Briefly, approximately $2x10^6$ cell in exponential phase were collected from at a 25 cm^2 culture flask after washing with ice-cold PBS and detaching with trypsin. Following centrifugation of the cell suspension at 3,000 rpm 4°C for 15 minutes (Sorvall RT 6000D), the cell pellet was washed twice with ice-cold PBS. After that, the cells were lysed in 1.3 ml of lysis buffer containing 10 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.5% SDS, at 50°C for 3 hours in a shaking waterbath. The lysate was treated with 0.2 ml of proteinase K (0.1 mg/ml) at 50°C a further for 1 hour. Then, the lysate was added with an equal volume of a mixture of phenol : chloroform : isoamyl alcohol, 25 : 24 : 1), mixed thoroughly for 5 minutes by repeated inversion, and centrifuged at 12,000 rpm for 15 minutes. The upper aqueous phase was then carefully transferred into a fresh clean tube and repeated extraction with phenol : chloroform : isoamyl. The aqueous phase, which contains fragmented apoptotic DNA was collected and made to 3 M sodium acetate, and the nucleic acid was subsequently precipitated with two volumes of absolute ethanol, mixed by repeated inversion and kept at -80°C overnight. The fragmented DNA pellet obtained by centrifugation at 12,000 rpm for 15 minutes was washed in 70% ethanol, allowed to dry and dissolved in TE buffer containing 1 mM EDTA and 10 mM Tris-HCl, pH 8.0. The concentration of a DNA sample was quantitated by measuring the OD at 260 and 280

nm, respectively, using a spectrophotometer (Jasco Model 7850 UV/VIS Spectrophotometer) and calculated as follows:

$$\text{Total DNA concentration } (\mu g/\mu l) = \frac{OD_{260} \times \text{dilution factor} \times 50}{1{,}000}$$

In the present study, approximately 8-15 µg of DNA was obtained from $1x10^6$ of LU-1 cells. The purity of DNA sample is obtained from the ratio of OD260:OD280 reading number which it normally varies between 1.8-2.0 (90).

2. Agarose gel electrophoresis

Contaminated RNA was removed by digestion with RNase A (100 µg/ ml, 37°C for 1 hour). The DNA fragments were subsequently separated by electrophoresis in a 1.8% (w/v) horizontal agarose gel. The gel was prepared by dissolving 1.14 g of agarose in 63 ml 1xTBE, boiling and cooling to about 60°C before pouring into the electrophoresis chamber. 15 µg/lane of the DNA samples was loaded into the slots under TBE buffer and electrophoresed at 80 constant voltage. This took approximately 2 hours on an 8-cm "mini" gel.

3. DNA visualization

After electrophoresis, the gel was stained in 1 µg/ml of ethidium bromide for 15 minutes and destained in distilled water for 5 minutes and visualized on a UV transilluminator.

Reference:

Kaufman PB, William WU, Kim D, and Cseke LJ, editor. Molecular and cellular methods in biology and medicine. CRC Press, London, 1995; 1-26.

APPENDIX VI

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

1 RNA EXTRACTION FROM LU-1 CELLS

Total RNA was extracted from $1x10^6$ of LU-1 cells treated with VR-3848, $20xGI_{50}$ using TRIzol^R reagent according to the manufacturer's instruction (GIBCO, BRL, Grand Island). Briefly, cells were lysed in 1 ml of TRIzol^R reagent by incubating at room temperature for 15 minutes. Then, 0.2 ml of chloroform was added and mixed vigorously by hand for 15 seconds. Following centrifugation at 12,000 rpm for 15 minutes at 4°C, the colorless upper aqueous phase which contains mainly RNA was collected. The RNA was recovered by precipitating with 0.7 ml of isopropyl alcohol at room temperature for 10 minutes and centrifuged at 12,000 rpm for 10 minutes. The RNA pellet was collected, washed once with 1 ml of 75% ethanol, and centrifuged for 5 minutes at 9,700 rpm. The pellet was briefly dried and dissolved in RNase free water. The concentration of each RNA sample was performed by measuring OD at 260 and 280 nm using a spectrophotometer (Jasco Model 7850 UV/VIS Spectrophotometer). After measuring RNA sample(s) at 260 and 280, respectively, the ratio of OD260:OD280 reading number was calculated and used to indicate the purity of RNA sample. Normally it should vary from 1.8 to 2.0 (90). Then, the RNA concentration in a sample was calculated by the following:

$$\text{Concentration of RNA } (\mu g/\mu l) = \frac{OD_{260} \times \text{dilution factor} \times 40}{1{,}000}$$

Approximately 8-15 µg of RNA was obtained from $1x10^6$ of LU-1 cells.

2. cDNA SYNTHESIS

cDNA was synthesized by using 2 ng of total cellular RNAs as templates. Briefly, first, the reaction was performed at the temperature of 70°C for 10 minutes in the presence of 0.5 µg/µl of oligodT (12-18) and RNase free water. Then, the Superscript reverse transcriptase was added to 20 µl of cDNA mix containing 5x first strand buffer, 0.1 M DTT and 10 mM dNTP mix (GIBCO, BRL, Grand Island). For cDNA synthesis, the reaction was performed at 42°C for 50 minutes and the reverse transcriptase was subsequently inactivated at 70°C for 15 minutes. cDNA was stored at -80°C.

3. POLYMERASE CHAIN REACTION (PCR)

Next, the gene of interest and the internal control gene were amplified by PCR. Five µl of 0.02 µg/µl cDNA, and 0.5 mM of primer per reaction were added the manufacturer's instruction (Perkin-Elemer) PCR mix containing 0.2 mM dNTPs (Promega, USA) and followed by 0.5 U of Taq polymerase to make the total volume of 20 µl. The primer sequences were as follows: 5' ATG GAG AAC ACT GAA AAC TCA G 3' and 5' GTC ATC ATC AAC ACC ACT GTC T 3'. Each cycle of PCR included 15 seconds of denaturation at 94°C, 45 seconds of primer annealing at 57°C, and 45 seconds of extension at 72°C for a total of 30 cycles.

4. AGAROSE GEL ELECTROPHORESIS

Ten µl of PCR products was loaded and electrophoresed under TBE buffer on a 1% (w/v) horizontal agarose gel. The gel was prepared by dissolving 0.63 g of agarose in 63 ml 1xTBE, boiling and cooling to about 60°C before pouring into the electrophoresis chamber. The agarose gel was electrophoresed at 80 constant voltage for 1 hour and 40 minutes.

5. PCR PRODUCTS VISUALIZATION

The gel was stained in 1 µg/ml of ethidium bromide solution for 15 minutes and destained in distilled water for 5 minutes. The products were subsequently visualized as bands on a UV transilluminator and photographed.

Primers sequences used in this study

Name	human β-actin
Product size	362 bp
Primer	5′ ACT TCG AGC AAG AGA TGG CC 3′
	5′ GAG TAC TTG CGC TCA GGA GG 3′
PCR condition	94°C 5 min
	94°C 15 sec ⎫
	57°C 45 sec ⎬ 30 cycles
	72°C 45 sec ⎭
	72°C 10 min
Name	human CPP32

Product size 543 bp

Primer 5′ ATG GAG AAC ACT GAA AAC TCA G 3′

5′ GTC ATC ATC AAC ACC ACT GTC T 3′

PCR condition 94°C 5 min

94°C 15 sec
57°C 45 sec } 30 cycles
72°C 45 sec

72°C 10 min

References:

Kaufman PB, William WU, Kim D, and Cseke LJ, editors. Molecular and cellular methods in biology and medicine. CRC Press, London, 1995; 1-26.

Fernandes-Alnemri T, Litwack G, and Alnemri ES. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1β-converting enzyme. J Biol Chem 1994; 269 (49):30761-4.

APPENDIX VII

CASPASE-3 (CPP32) ASSAY

1. PROTEIN EXTRACTION

Briefly, approximately $10x10^6$ LU-1 cells in exponential phase were collected from a 10 cm diameter of petri dish after trypsinization with 0.05% trypsin. Cells were treated with VR-3848, $20xGI_{50}$, and vinblastine, $2xGI_{50}$, between 3 and 48 hours. Following incubation for 3, 6, 12, 24 and 48 hours, apoptotic cells were harvested using the same procedure as described in Experiment 4.3. The cell pellets were kept on ice, washed with ice-cold PBS and resuspended in 500 µl of hypotonic cell lysis buffer containing 100 mM Tris-HCl (pH 7.40), 2 mM EDTA, 20 mM EGTA and 10 µ M digitonin. The cells were lysed by subjecting them to four cycles of freezing and thawing according to the Promega's instruction. The cell lysates were centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was collected. Subsequently, the concentration of protein was measured by Bicinchoninic acid (BCA) assay based on the modified method of Smith *et al.* (1985) (93) using bovine serum albumin as a standard (Appendix VIII).

2. ASSAY FOR CASPASE-3 ACTIVITY IN CELL EXTRACTS

Total proteins (100 µg) from the control and treated cells were incubated at 37°C for 30 minutes in 1.6 ml of assay buffer [0.8 µM HEPES (pH 7.5), 0.8 µM sucrose, 8 mM DTT, and 0.08 nM CHAPS or (3-[(3-cholamidopropyl) dimethyl

ammonio]-1-propane-sulfonate). Following that, 10 μM of the substrate of CPP32, Ac-Asp-Glu-Val-Asp-MCA (Peptide Institute, Osaka, Japan) was added and incubated a further for 2 hours at 37°C. The release of MCA produced a yellow-green fluorescence that was mornitored by a spectrofluorometer (Shimadzu spectrofluorometer RF-500) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The results were expressed as fold induction of CPP32 activity, i.e., it was calculated from the ratio of MCA released from treated cells-blank to untreated cells-blank (91).

In addition, to further characterize the relevance of caspase-3 activation in the VR-3848 and vinblastine-induced apoptosis, in some experiments, the effect of a caspase-3-specific inhibitor, Ac-Asp-Glu-Val-Asp-CHO or Ac-DEVD-CHO, on apoptosis was examined. Caspase-3 activity was measured in the presence of 5 μM of the tetra peptides Ac-DEVD-CHO and the activity was compared with that obtained from the assays without the inhibitor.

References:

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, and Provenzano MD, *et al.* Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150 (1): 76-85.

Lei W, Yu R, Mandlekar S, and Keng AN. Induction of apoptosis and activation of interleukin 1β-converting enzyme/Ced-3 protease (caspase-3) and c-Jun NH_2-terminal kinase 1 by benzo(a) pyrene. Cancer Res 1998; 58(10):2102-6.

APPENDIX VIII

DETERMINATION OF PROTEIN CONCENTRATION USING BICINCHONINIC ACID

A. PRINCIPLE

Protein in the sample extracts was measured by the Bicinchoninic Acid (BCA) assay as previously described by Smith *et al.* (1985) (93). BCA is sodium salt which is a sensitive and stable water-soluble compound. The assay involves the binding between peptide bonds of the protein and the copper atoms (Cu^{1+}) present in the alkaline solution of BCA. This induces the formation of an intense purple complex, which is quite stable and its maximum absorbance can be measured against the blank at 562 nm. In addition, the color produced from this reaction increases in a proportional fashion over a broad range of increasing protein concentration. When compared to the standard Lowry method, the BCA assay shows more the advantages. It is more tolerance toward interfering substances, such as simple buffer salts, nonionic detergents, greater working reagent stability and increased sensitivity.

B. REAGENTS

Reagent A 1% BCA-Na_2, 2% $NaCO_3.H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH and 0.95% $NaHCO_3$ (pH 11.25)

Reagent B 4% (w/v) $CuSO_4.5H_2O$ solution

Reagent C Prepared by mixing 30 ml of Reagent A with 600 µl of Reagent

B (100 volume of Reagent A with 2 volume of Reagent B).

Standard protein 1 mg/ml Bovine Serum Albumin (BSA, Sigma Chemical Co., St. Louis, M.O., U.S.A.)

C. PROCEDURE

1. Prepared duplicate protein standards by pipetting 2-25 µg of standard BSA (1 mg/ml) into 5 ml glass tubes and adjusted the volume in the tubes to 100 µl with distilled water. Subsequently, added 2 ml of Reagent C into the tubes, mixed thoroughly by vortexing and incubated the standard protein mixture for 30 minutes at 37°C.
2. Prepared duplicate protein samples extracted from the cultured cells in the same manner as described for the standards except using 2-5 µg of protein.

Measured OD at 562 nm against a blank using 2 ml cuvettes in a spectrophotometer (Spectronic® Genesys™5 Spectrophotometer, Milton Roy Co., NY., USA).

Reference:

Smith PK, Krolm RI, Hermanson GT, Mallia AK, Gartner FH, and Provenzano MD, *et al.* Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150 (1): 76-85.

BIOGRAPHY

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