ผลของแอซิทิลเมโลโดรินอลและเปลาโนทอลต่อเซลล์มะเร็ง

นางสาวพรทิชา โลลุพิมาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF ACETYLMELODORINOL AND PLAUNOTOL ON CANCER CELL LINES

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พรทิชา โลลุพิมาน : ผลของแอซิทิลเมโลโดรินอลและเปลาโนทอลต่อเซลล์มะเร็ง (EFFECTS OF ACETYLMELODORINOL AND PLAUNOTOL ON CANCER CELL LINES) อ. ที่ปรึกษา: รศ. ดร. อมร เพชรสม, 62 หน้า. ISBN 974-53-1682-2.

งานวิจัยนี้มีจุดมุ่งหมายเพื่อศึกษาผลของสารแอซิทิลเมโลโดรินอลและเปลาโนทอลต่อเซลล์มะเร็งเพาะเลี้ยง 4 ชนิด (BT474, HepG2, KATOIII, และ SW620) และเซลล์ปกติ 2 ชนิด (CH-liver และ Hs27) เมื่อนำเซลล์เหล่านี้มาทดสอบกับสารที่ความเข้มข้นต่าง ๆ (ตั้งแต่ 0-10 μg/ml) แล้วบ่มเป็นเวลา 12, 24, 48, และ 72 ชั่วโมง วิเคราะห์เปอร์เซ็นต์ความมีชีวิตของเซลล์โดยวิธี MTT พบว่าสารทั้งสองชนิดมีฤทธิ์ยับยั้งการเจริญของเซลล์ที่ระยะเวลาบ่ม 48 ชั่วโมง ต่างจากกลุ่มควบคุม และระยะเวลาบ่ม 12-24 ชั่วโมง ที่ความน่าจะเป็นน้อยกว่า 0.01 แอซิทิลเมโลโดรินอลมีฤทธิ์ยับยั้งการเจริญขึ้นกับเวลาและความเข้มข้น โดยให้ค่า IC_{50} ดังนี้ BT474 1.43±0.39 μg/ml, HepG2 4.32±0.89 μg/ml, KATOIII 3.10±0.48 μg/ml, SW620 1.39±0.08 μg/ml, และ CH-liver 2.80±0.35 μg/ml แต่ไม่มีฤทธิ์ยับยั้งการเจริญต่อเซลล์ Hs27 ส่วนเปลาโนทอลมีฤทธิ์ยับยั้งการเจริญของเซลล์โดยขึ้นกับเวลาและความเข้มข้นของสารเช่นกัน โดยค่า IC_{50} คือ BT474 7.07±1.44 μg/ml, HepG2 6.11±1.15 μg/ml, KATOIII 10.36±2.19 μg/ml, และ SW620 6.90±0.68 μg/ml แต่ไม่มีฤทธิ์ยับยั้งการเจริญของเซลล์ปกติ นอกจากนี้ยังพบว่าฤทธิ์ยับยั้งการเจริญของสารแอซิทิลเมโลโดรินอลมีความเกี่ยวข้องกับการตายแบบ apoptosis ในเซลล์ SW620 จากการทดสอบด้วยการสังเกตการเปลี่ยนแปลงลักษณะรูปร่างของเซลล์ภายใต้กล้องจุลทรรศน์ และพบการแตกหักของดีเอ็นเอ จากนั้นยืนยันการตายแบบ apoptosis โดยบ่มเซลล์กับสารแอซิทิล-เมโลโดรินอล 5 μg/ml แล้วย้อมด้วยสีย้อม Annexin-V-FITC พบว่าจำนวนเซลล์ SW620 ที่ตายแบบ apoptosis เพิ่มขึ้นจาก 29.02±4.99% เป็น 56.38±3.73% เมื่อระยะเวลาในการบ่มเพิ่มจาก 12 เป็น 24 ชั่วโมงตามลำดับ ส่วนเซลล์ที่ตายแบบ necrosis ซึ่งย้อมด้วย propridium iodide นั้นมีจำนวนน้อยมากในช่วงเวลาดังกล่าว จากนั้นทดสอบกลไกการตายของเซลล์ในระดับโมเลกุล โดยศึกษาผลของสารแอซิทิลเมโลโดรินอลต่อการแสดงออกของ pro-apoptotic gene (*p53*) และ anti-apoptotic gene (*Bcl-2*) โดยวิธี RT-PCR พบว่ายีนทั้งสองมีการแสดงออกในเซลล์ SW620 ที่ไม่ได้บ่มกับสาร เมื่อมีการบ่มกับสารแอซิทิลเมโลโดรินอลจะไม่พบการเปลี่ยนแปลงระดับในการแสดงออกของยีน *p53* แต่ยีน *Bcl-2* มีระดับการแสดงออกลดลง ในช่วงเวลาบ่ม 4-10 ชั่วโมง จากการทดลองทั้งหมดสรุปได้ว่าสารแอซิทิลเมโลโดรินอลมีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งอย่างรุนแรงและชักนำให้เกิดการตายแบบ apoptosis ในเซลล์ SW620 และการลดลงของระดับการแสดงออกของยีน *Bcl-2* อาจเป็นกลไกสำคัญในการเกิดผลดังกล่าว ส่วนเปลาโนทอลมีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งในระดับปานกลางแต่ไม่มีผลต่อเซลล์ปกติ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา .....

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KEY WORD: AXETYLMELODORINOL / PLAUNOTOL / CYTOTOXIC / APOPTOSIS

PORNTICHA LOLUPHIMAN: EFFECTS OF ACETYLMELODORINOL AND PLAUNOTOL ON CANCER CELL LINES. THESIS ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph.D., 62 pp. ISBN 974-53-1682-2.

This study was aimed at determining effects of acetylmelodorinol and plaunotol on four human cancer cell lines (BT474, HepG2, KATOIII, and SW620) and two normal human cell lines (CH-liver and Hs27). Tested cells were cultured in the presence or absence of acetylmelodorinol and plaunotol at various concentrations (0–10 µg/ml) for 12, 24, 48, and 72 h and the percentages of cell viability were evaluated by MTT assay. The results indicated that both acetylmelodorinol and plaunotol have significant antiproliferative effect at 48 h of incubation, compared to control untreated cells and cells treated for 12-24 h ($p<0.01$). Acetylmelodorinol showed a dose and time-dependent inhibition of cell proliferation at 48 h of incubation with IC_{50} of 1.43±0.39 µg/ml against BT474, 4.32±0.89 µg/ml against HepG2, 3.10±0.48 µg/ml against KATOIII, 1.39±0.08 µg/ml against SW620, and 2.80±0.35 µg/ml against CH-liver. Acetylmelodorinol did not show antiproliferative effect against Hs27 cell. Plaunotol showed a dose and time-dependent inhibition of cell proliferation at 48 h of incubation with IC_{50} of 7.07±1.44 µg/ml against BT474, 6.11±1.15 µg/ml against HepG2, 10.36±2.19 µg/ml against KATOIII, and 6.90±0.68 µg/ml against SW620. However, plaunotol did not show antiproliferative effect against normal cells. In addition, antiproliferative effect of acetylmelodorinol was associated with apoptosis in SW620 cells as determined by morphological changes and oligonucleosomal DNA fragmentation. The confirmation of apoptosis assessed by Annexin-V-FITC staining showed that when SW620 cells were treated with 5 µg/ml of acetylmelodorinol, the number of apoptotic cells increased from 29.02±4.99 % to 56.38±3.73% at 12 and 24 h, respectively. Necrotic cells as determined by PI staining, was negligible during this incubation time. To investigate the molecular mechanism leading to apoptosis, we examined the expression of pro-apoptotic gene (*p53*), and anti-apoptotic gene (*Bcl-2*) by RT-PCR. Both genes were expressed in untreated SW620 cells. Acetylmelodorinol treatment did not change the level of expression of *p53* gene, but decreased the level of expression of *Bcl-2*, at 4-10 h incubation times. Taken together, these results suggested that acetylmelodorinol had strong antiproliferative, and apoptosis inducing effects in SW620 cells. The downregulation of *Bcl-2* may be the molecular mechanism underlying this effect. However, plaunotol showed moderate antiproliferative effect on human cancer cell lines but did not show any effect in normal cells and did not induce apoptosis.

Field of study Biotechnology Student's signature.....

Academic year 2004 Advisor's signature.....

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LIST OF ABBREVIATIONS

%	percentage
/	per
A	absorbance
bp	base pair
BT474	Human breast ductol carcinoma
$^{\circ}C$	degree celsius
CH-liver	human liver
cm	centimeter
CO_2	carbon dioxide
DEPC	Diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
FCS	fetal calf serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GLN	glutamine
g	gram
h	hour
H^+	hydrogen ion
HCl	hydrochloric acid
HepG2	Human liver hepatoblastoma
Hs27	Human liver
KATOIII	Human gastric carcinoma
Kb	kilobase
kg	kilogram
L	litre
M	molar

mg	Milligram
ml	Mililitre
mm	Millimeter
mM	Millimolar
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
MW	molecular weight
ng	nanogram
no.	number
OD	optical density
PBS	Phosphate buffer saline
PCR	polymarase chain reaction
pH	The negative logarithm or the concentration of hydrogen ions
RNA	Ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SW620	Human colon adenocarcinoma
Tris	tris(hydroxyl methyl) aminomethane
μg	Microgram
μl	Microliter
μM	Micromolar
/	per
%	percent
UV	ultraviolet light
V	voltage
vol	volume

CHAPTER I

BACKGROUND

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death of the host. Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These casual factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposures or mutations and detectable cancer. Currently cancer is treated by surgery, radiation, chemotherapy, hormones and immunotherapy. The American Cancer Society estimated that about 1,268,000 new cancer cases were diagnosed in the US in 2001. In the same year, 553,400 cancer patients were expected to die in the United States of America. These statistics translate to more than 1500 deaths per day. Cancer is the second leading cause of death in the US next to coronary heart disease. Breast cancer cases (31%) are the highest among adult females while prostate cancer (31%) is found to be the highest for adult males. Leukemia accounts for 31.5% of cancer cases among children between the ages of 0–14. The National Institutes of Health (NIH) estimates the overall costs for cancer, both direct and indirect, at 180.2 billion dollars in 2000.

At present, the cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most cancers. The development of new therapeutic approach to cancer remains one of the most challenging areas in cancer research. Many tropical plants have interesting biological activities with potential therapeutic applications. Flowers of *Melodorum fruticosum* Lour, family Annonaceae, have been used as a component of the Thai traditional medicine recipe' known as "Gaesorn Thung Gao" (เกสรทั้งเก้า) which are used as tonic stimulant and mild cardio tonic (Saralamp, 1992). The information in previous studies on the antiproliferation activity of Acetylmelodorinol, purified from petroleum ether extract of the flowers of *M. fruticosum*, on tumor cells has been based on MTT assay that measures cell viability. Acetylmelodorinol has high cytotoxic activity with an IC_{50} less than 3 μg/ml on tumor cell lines (Chaichantipyuth et al., 2001). Another substance used in this study is plaunotol, an acyclic diterpene alcohol extracted from a Thai medicinal plant

called *plau-noi (Croton Sublyratus* Kurz., family Euphobiaccae). Thai people have used plau-noi as traditional medicines for treatment of gastritis and gastric ulcer. Previously, it was reported that plaunotol have bactericidal effects against *Helicobacter pylori* and that cell death was associated with lysis of bacteria (Koga et al., 1996) and led to an increase in permeability and fluidities of membrane (Koga et al., 1998). In addition, plaunotol is called "a cytoprotective antiulcer agent" and has been reported to stimulate the release of endogenous secretin in humans (Shiratori et al., 1993).

From the traditional usages and previous data it was suggested that acetylmelodorinol and plaunotol are potential candidates as novel anticancer agents.

The purpose of this research

The purposes of this research are to study the effects of acetylmelodorinol and plaunotol on cell proliferation and apoptosis in human cancer cell lines and normal cells *in vitro*.

CHAPTER II

LITERATURES REVIEW

2.1 Cancer overview

Cancer is not just one disease but rather a group of diseases, all of which cause cells in the body to change and grow out of control. Cancer are classified either according to the kind of fluid or tissue from which they originate, or according to the location in the body where they first developed. In addition, some cancers are of mixed types. The following five broad categories indicate the tissue and blood classifications of cancer:

- **Carcinoma**

  A carcinoma is a cancer found in body tissue known as epithelial tissue that covers or lines surfaces of organs, glands, or body structures. Many carcinomas affect organs or glands that are involved with secretion, such as breasts that produce milk. Carcinomas account for 80 percent to 90 percent of all cancer cases.

- **Sarcoma**

  A sarcoma is a malignant tumor growing from connective tissues, such as cartilage, fat, muscle, tendons, and bones. The most common sarcoma, a tumor of the bone, usually occurs in young adults. Examples of sarcoma include osteosarcoma (bone) and chondrosarcoma (cartilage).

- **Lymphoma**

  Lymphoma refers to a cancer that originates in the nodes or glands of the lymphatic system, whose job is to produce white blood cells and clean body fluids, or in organs such as the brain and breast. Lymphomas are classified into two categories: Hodgkin's lymphoma and non-Hodgkin's lymphoma.

- **Leukemia**

  Malignant neoplasm of blood-forming tissues; characterized by abnormal proliferation of leukocytes; one of the four major types of cancer. White blood cells are needed to resist

infection. Red blood cells are needed to prevent anemia. Platelets keep the body from easily bruising and bleeding. Examples of leukemia include acute myelogenous leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, and chronic lymphocytic leukemia. The terms myelogenous and lymphocytic indicate the type of cells that are involved.

- **Myeloma**

  Myeloma is tumor of plasma cells growing in the bone marrow. Multiple myeloma (also known as myeloma or plasma cell myeloma) is a progressive hematologic (blood) disease. It is a cancer of the plasma cell, an important part of the immune system that produces immunoglobulins (antibodies) to help fight infection and disease. Multiple myeloma is characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of immunoglobulin.

Cancer is treated in several ways, depending on each person's medical condition and type of cancer. The traditional treatment of cancer patients involves a combination of surgery, radiotherapy and/or chemotherapy. Although surgery and radiation therapy are both key weapons in the fight against cancer, chemotherapy is also a vital approach and is the only approach possible for treating disseminated cancers. The ultimate goal of cancer chemotherapy is to kill cancer without killing the patient. This requires the development of selective drugs that can kill malignant tumor cells or render them benign without affecting normal cells (Boik, 1996: 1-14).

Different tumors have different aberrations in signaling and growth stimulation pathways that drive cancer growth. An understanding of these processes is a key to development of new anticancer agents and to identifying optimal treatment strategies and patient populations suitable for specific therapies.

Based on a mechanical approach, we can roughly classify the current anticancer chemotherapeutic approaches or agents as

a) Cytotoxic agents
b) Antibody targeting agents
c) Anti–hormonal agents
d) Signal transduction inhibition agents

e) Ras–inhibition agents

f) Cell cycle modulating agents

g) Apoptosis inducing agents

h) Angiogenesis inhibition agents

i) Anti–invasion agents

Cytotoxic agents are further subdivided as tubulin binders, DNA intercalators, antifolates, alkylating agents, and topoisomerase inhibitors; the most widely used anticancer drugs, such as taxol (paclitaxel or Taxol), doxorubicin, methotrexate, cisplatin and etoposide fall into each of these categories respectively (Boik, 1996).

2.2 Cytotoxic and viability assays

An intensive program to screen plant extracts and other natural materials for anticancer activity began in 1955 with funding from the United States National Cancer Institute (NCI) through the Cancer Chemotherapy National Service Center (CCNSC). Use of *in vitro* assay systems for screening of potential anticancer agents has been common practice almost since the beginnings of cancer chemotherapy. According to the NCI data, many plants extracted posse's cytotoxic activities. Definition of cytotoxicity refers to agents that are toxic to cells *in vitro.* Cytotoxic agents can be further divided into those with cytostatic effect (those that stop cell growth) and cytocidal effect (those that kill cells). The techniques of growing cells as a monolayer have been most frequently applied to the cytotoxicity testing of cancer cell lines. The National Cancer Institute now routinely measures the cytotoxicity of every compound under test against a panel of 60 human tumor cell lines, which are representative of major human tumor types. For each compound tested, the IC_{50} (concentration of drug needed to inhibit cell growth by 50%) is generated from the dose-response curves for each cell line. *In vitro* assays are highly useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells (Boik, 1996: 1-14).

2.2.1 Parameters which vary among different assays: (Freshney, 2000: 183-200)

2.2.1.1 Cell culture method

- **Seeding density**

Seeding density depends on cell size, growth rate, and assay duration. It must be determined individually for each cell type. In a 2-3 day assay, seeding densities are typically in the range of 5-25×10^3 cells per well in 96-well microtitre plates. Time zero values must remain within the linear range of the assay, typically 1.5-2.0 absorbance units.

- **Drug solubilization**

Stock solution of polar compounds are dissolved in water, buffer, or medium then diluted in complete growth medium to the final test concentration. Non-polar compounds are dissolved in a solvent such as dimethylsulfoxide (DMSO), methanol or ethanol (EtOH) and filter sterilized (0.22 μm pores). A 1:1 mixture of DMSO and EtOH is also a good solvent and it evaporates more slowly than EtOH and chemically sterilizes most test materials. DMSO is toxic to cells at concentrations above 0.1-1.0%. Ethanol is usually growth stimulatory in the 1-2% range. Different cells population exhibit different sensitivities to these organic solvents, and appropriate solvent controls should therefore always be included.

- **Drug incubation**

It is common procedure to incubate cells with drug solutions immediately after enzyme disaggregation of solid tissue, or harvesting of cell monolayer by trypsinization. There is evidence to suggest that susceptibility of cells to drug is altered by enzyme treatment and does not return to control levels until approximately 12 h after enzyme exposure. It may therefore be expedient to include a pre-incubation recovery period for freshly disaggregated cells to allow for this. Maintenance of pH at 7.4 is essential during the incubation period since alterations in pH will alter cell growth, and alkaline pH particularly will reduce cell viability.

2.2.1.2 Duration of drug exposure and drug concentration

Assay duration is determined by two factors: (i) the length of time cells need to respond to an experimental treatment; and (ii) the length of time that cells can grow before nutrient depletion sets in. Nutrient depletion typically develops within 3-4 days after plating unless

cultures are re-fed. Once it begins a progressive deterioration of cellular health and viability develops rapidly, and becomes a major artifact in data interpretation. Nutrient depletion can be calibrated by comparing the day-by-day growth kinetics of cultures that receive no feeding to cultures that are fed daily. The two curves begin to diverge when depletion sets in. This normally sets the upper limit to assay duration if cultures are not fed. If experiments must be continued for longer periods, then medium must be replaced. Above densities of near confluence, most types of cell require daily feeding. With cytotoxic assays, 36-48 h assay period following a 1-day recovery period is usually adequate to detect the effect of a drug while avoiding the need to re-feed in mid experiment.

2.2.1.3 Duration of recovery period after drug exposure

The inclusion of a recovery period following drug exposure may be important for three reasons:

(a) When metabolic inhibition is used as end-point, it allows the cells to recover from metabolic perturbations, which are unrelated to cell death.

(b) Sub lethal damage can be repaired and therefore not interfere with the assay result.

(c) Delayed cytotoxicity, perhaps not expressed until one to two cycles after drug treatment, can be measured.

Depending on the drug and the end-point, an absence of a recovery period can either underestimate the level of cell killing achieved. However, it is equally important that the recovery period is not too long, because cell killing can then be masked by overgrowth of a resistant population. In monolayer assays which monitor cell counts of precursor incorporation, the cells must remain in log phase of growth throughout the exposure and recovery period.

2.2.1.4 End-point used to quantitate drug effect

A variety of methods have been devised for measuring the viability or proliferation of cells *in vitro*. These can be subdivided into four groups:

1) **Reproductive assays** can be used to determine the number of cells in a culture that are capable of forming colonies *in vitro*. In these types of experiments, cells are plated at low densities and the number of colonies is scored after a growth period. These clonogenic

assays are the most reliable methods for assessing viable cell number. These methods very time-consuming and become impractical when many samples have to be analyzed.

2) **Permeability assays** involve staining damaged (leaky) cells with a dye and counting viable cells that exclude the dye. Counts can either be performed manually using a hemocytometer and stained with trypan blue. This method is quick, inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to determine the cell concentration (cell number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment. Or counts can be performed mechanically using for example a flow cytometer and propidium iodide. Alternatively, membrane integrity can be assayed by quantifying the release of substances from cells when membrane integrity is lost, *e. g.,* Lactate dehydrogenase (LDH) or ^{51}Cr

3) **Metabolic activity assays**: MTT reduction – a tetrazolium-based colorimetric assay for cell survival and proliferation

In 1983, a quantitative colorimetric assay for mammalian cell survival and cell proliferation was proposed by Mosmann. The assay is dependent on the reduction of yellow-colored tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form insoluble formazan which precipitates out of solution in the immediate vicinity of the reaction. The assay measure cell respiration and the amount of formazan product is proportional to the number of living cells present in culture and quantitated with an ELISA plate reader. The assay has been shown to be a simple, rapid alternative to counting cells by dye inclusion/exclusion, monitoring the release of ^{51}Cr from lysed cells, or incorporation of [3H]-thymidine into cellular DNA. The MTT assays have been used with a growing number of cell types including primary cultured cells as well as establish cell lines. This colorimetric microplate assay is cost effective because of the number of tests which can be performed at one time without the problem of radio-isotope and contaminated material disposal.

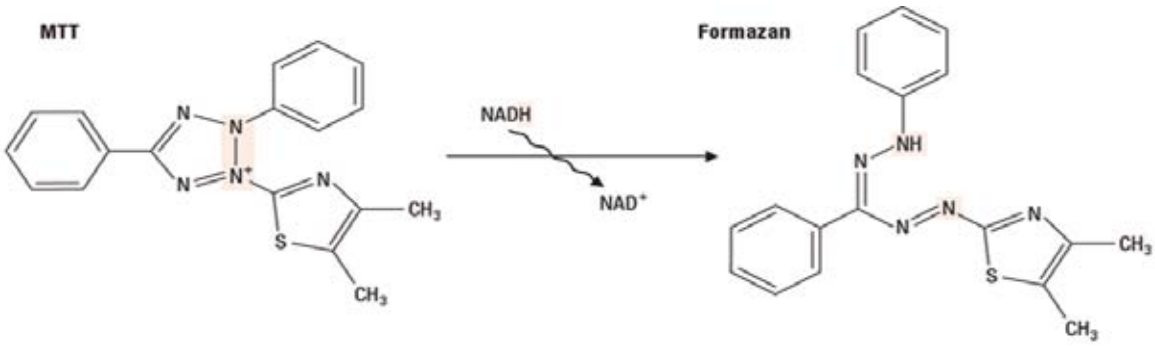

Figure 1: Molecular structure of MTT and its corresponding reaction products.

4) **Direct proliferation assays** use DNA synthesis as an indicator of cell growth. These assays are performed using either radioactive or nonradioactive nucleotide analogs. Their incorporation into DNA is then measured.

2.3 Modes of cell death (Studzinski, 1995: 143-191)

Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death.

2.3.1 Apoptosis

Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Due to rapid cell dehydration, cells which were originally round often become elongated or convoluted in shape and diminished in size. Chromatin condensation and the loss of distinct chromatin structure, which occurs in parallel with cell shrinkage, start at the nuclear periphery and followed by nuclear fragmentation. Distinct hyperchromicity and homogeneity characterize the stainability of DNA with fluorochromes such as 4,6-diamidino-2-phenylindole (DAPI), Hoechst 33342, propidiun iodide or (PI), in the fragmented nuclei. Nuclear fragments, together with the constituents of the cytoplasm (including intact organelles), are then packaged into so-called apoptotic bodies, which, enveloped in plasma membrane, detach from the dying cell and, *in vivo*, are phagocytosed by neighboring cells without an inflammatory response.

2.3.2 Necrosis

Necrosis ("accidental" cell death) is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. The early event of necrosis is swelling of cell mitochondria, followed by rupture of the plasma membrane and release of cell constituents, which include many proteolytic enzymes, necrosis, in contrast to apoptosis, triggers an inflammatory reaction in the tissue, often resulting in scar formation. DNA degradation is not as extensive as in the case of apoptosis, and the products of degradation are heterogeneous in size, and do not form any discrete bands during gel electrophoresis. Necrosis generally represents a cell's response to gross injury and is frequently induced by an overdose of cytotoxic agents. It has been observed, however, that certain cell types do respond even to pharmacological concentrations of some drugs or moderate doses of physical agents by necrosis rather than apoptosis though the reason for the difference in response is not entirely clear.

2.3.3 Differences between necrosis and apoptosis (Wyllie, 1999)

There are many observable morphological (Figure 2) and biochemical differences (Table 1) between necrosis and apoptosis. Necrosis occurs when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia), which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses. Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extra cellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extra cellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Zhao, 1997).

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy. Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation,

partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies), which contain ribosome, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Miyashita, 1995). Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyses. This terminal phase of *in vitro* cell death has been termed "secondary necrosis" (Figure 2).

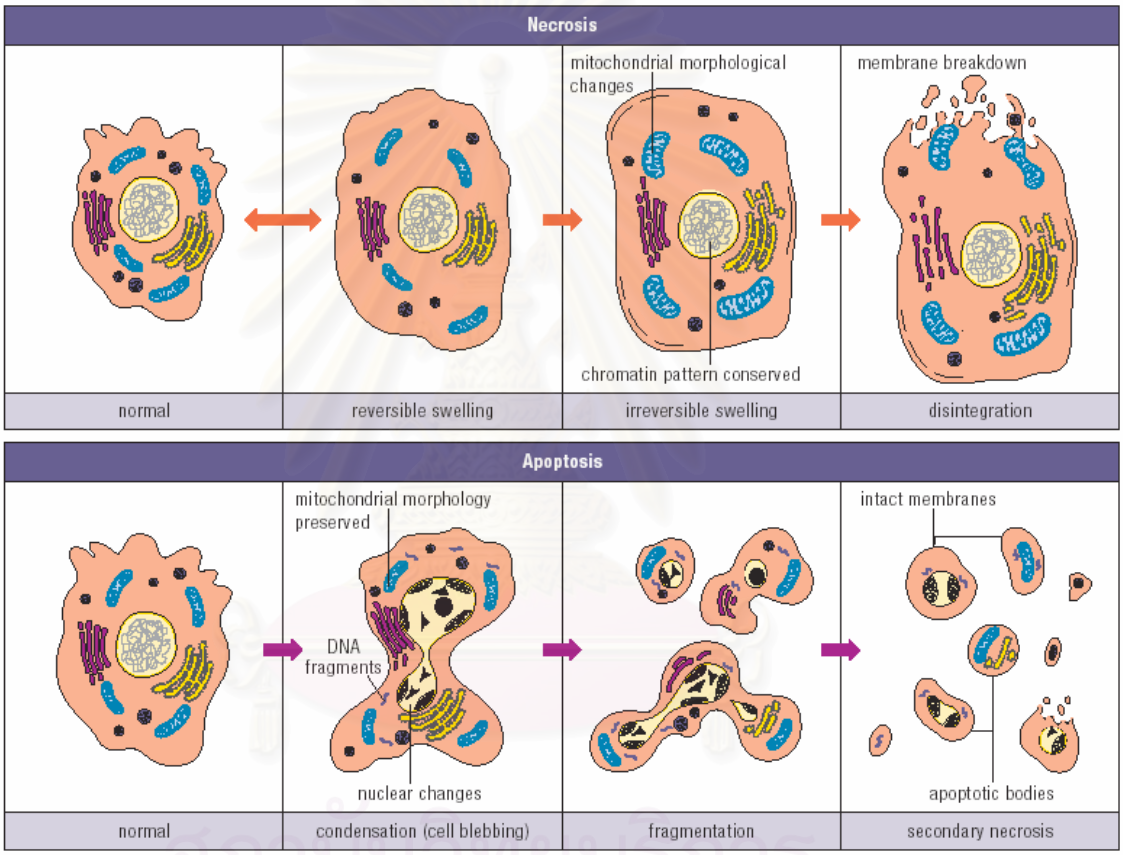

Figure 2: Illustration of the morphological features of necrosis and apoptosis (Wyllie, 1999)

Table 1: Different features and significance of necrosis and apoptosis

Necrosis	Apoptosis
Morphological features	
• Loss of membrane integrity • Begins swelling of cytoplasm and mitochondria • Ends with total cell lysis • No vesicle formation, complete lysis • Disintegration (swelling) of organelles	• Membrane blebing but no loss integrity • Aggregation of chromatin at the nuclear membrane • Begins with shrinking of cytoplasm and condensation of nucleus • Ends with fragmentation of cell into smaller bodies • Formation of membrane bound vesicles (apoptotic bodies) • Mitochondria become leaky due to pore formation involving proteins of the *Bcl-2* family.
Biochemical features	
• Loss of regulation of ion homeostasis • No energy requirement (passive process, also occurs at 4°C) • Random digestion of DNA (smear of DNA alter agarose gel electrophoresis) • Postlytic DNA fragmentation (=late event of death)	• Tightly regulated process involving activation and enzymatic steps • Energy (ATP)-dependent (Active process, does not occur at 4°C) • Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern alter agarose gel electrophoresis) • Prelytic DNA fragmentation • Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria • Activation of caspase cascade • Alterations in membrane asymmetry (i.e. translocation of phosphatidyl-serine from the cytoplasmic to the extracellular side of the membrane)
Physiological significance	
• Affects groups of contiguous cells • Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, Ischemica, metabolic poisons) • Phagocytosis by macrophages • Significance inflammatory response	• Affect individual cells • Induced by physiological stimuli (lack of growth factors, changes in hormonal environment) • Phagocytosis by adjacent cells or macrophages • No inflammatory response

2.3.4 Apoptotic Pathways

The apoptotic response is a complex biological process that requires the regulation of a variety of genes that interact with and respond to intracellular and extracellular stimuli.

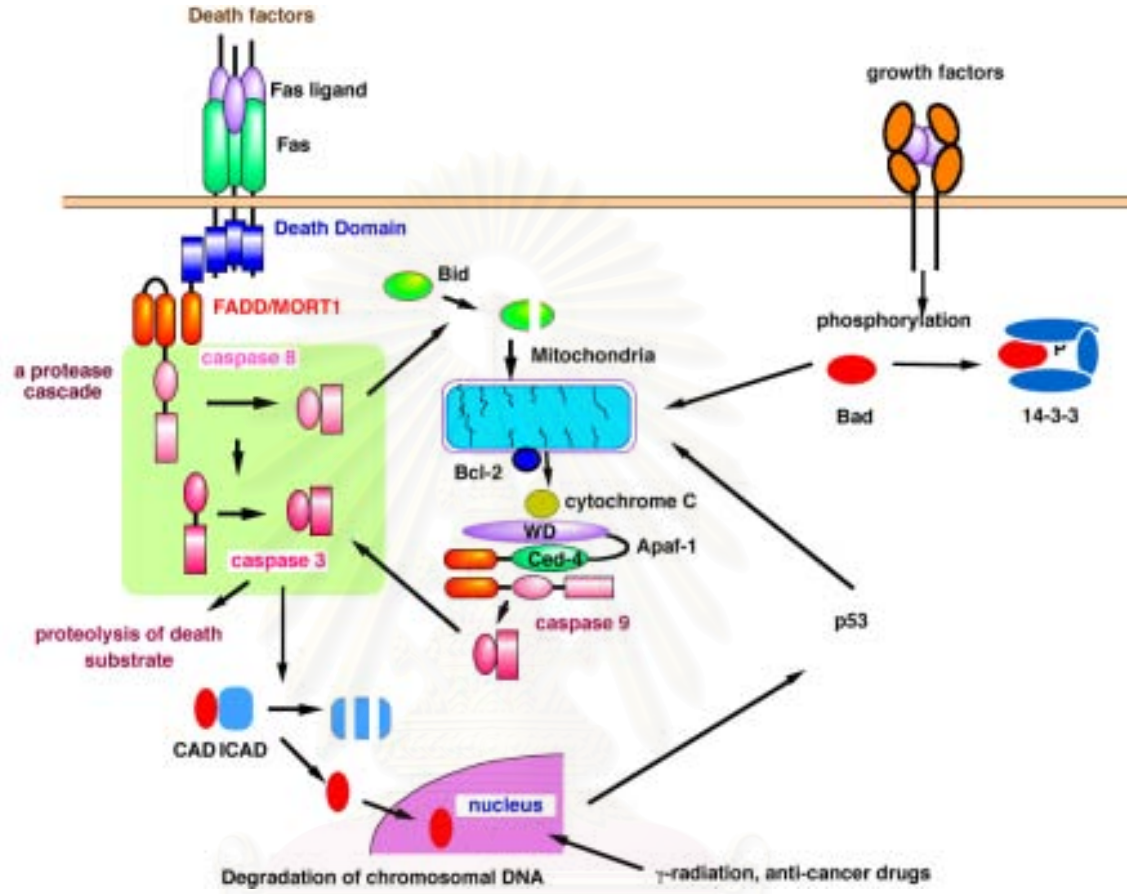

Figure 3: Apoptotic Pathways (Wyllie, 1999)

Apoptosis plays a central role during development and homeostasis in most organisms. The activation of apoptosis is associated with many diseases, such as cancer, acquired immunodeficiency syndrome (AIDS), and neurodegenerative disorders. The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times, but the activation of apoptosis is regulated by many different signals that originate from both the intracellular and the extracellular surrounding. Components in these signaling pathways are varied, and many agents have been characterized either as inducers or inhibitors of the apoptotic response. During the last few years, the cell death pathway has been extensively studied, and three protein families have been identified according to their functions in apoptosis. These

proteins are *Fas* and *Fas* ligands, *Bcl-2* (and analogs), and the ICE families (Shigekazu and Golstein, 1995).

Signal transduction for apoptosis

Inducers of apoptosis are organized into three groups (death factor, genotoxic anti-cancer drugs, and factor deprivation). Fas ligand, a representative of death factors, binds to Fas receptor, and causes its trimerization. The trimerized death domain in Fas cytoplasmic region recruits pro-caspase 8 through a FADD/MORT1 adaptor, and forms a DISC. The pro-caspase 8 is autoactivated at DISC, and becomes a mature active enzyme. Two routes have been identified to activate caspase 3 by caspase 8. In one route, caspase 8 directly processes pro-caspase 3 in the downstream, and caspase 3 cleaves various cellular proteins including ICAD. CAD is released from ICAD and degrades chromosomal DNA.

In another route, caspase 8 claves Bid, a pro-apoptotic member to *Bcl-2*, which translocate to mitochondria to release cytochrome C into the cytosol. *Bcl-2* or Bcl-xL, anti-apoptotic members of the *Bcl-2* family, inhibits the release of cytochrome C, the mechanism of which is not well understood. The cytochrome C then activates caspase 9 together with Apaf-1, and caspase 9 in turn activates caspase 3 (Wang, 2004). The genotoxic anti-cancer drugs such as etoposide and γ-radiation generate damage in chromosomal DNA. The signal seems to be transferred to mitochondria in a *p53*-dependent manner by as yet an identified mechanism. This releases cytochrome C from mitochondria and activated caspase 9 as scribed above (Shigekazu, 1997).

Cellular and Molecular Regulation

Apoptosis is mediated by a family of protease called caspases that are activated by processing from its inactive precursor (zymogen). Thirteen members of the human caspase family have been identified. Some of family members are involved in apoptosis, and this can be divided into two subgroups. The first group consists of caspase 8, caspase 9, and caspase 10, which contain a long prodomain at the N-terminus, and function as initiators of the cell death process (initiator caspases). The second group contains caspase 3, caspase 6, and caspase 7, which have a short prodomain and work as effectors, cleaving various death substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells. The other effectors molecule in apoptosis is apoptotic protease activating factor (Apaf-1), which, together with cytochrome C, recruits pro-caspase 9 to the mature enzyme (Shigekazu, 1997).

Caspase 3 activated downstream of the caspase cascade activates a specific DNase (CAD, caspase activated DNase). CAD is a complex with its inhibitor, ICAD (inhibitor of CAD), in proliferating cells. When caspase 3 is activated in apoptotic cells it claves ICAD to release CAD. CAD then causes DNA fragmentation in the nuclei (Enari et al., 1998).

Key elements of the apoptotic pathway (Wyllie, 1999):

Death receptors

Apoptosis have been found to induce via the stimulation of several different cell surface receptors in association with caspase activation. For example, the CD95 (APO-1, Fas) receptor ligand system is a critical mediator of several physiological and pathophysiological processes, including homeostasis of the peripheral lymphoid compartment and CTL mediated target cell killing. Upon cross-linking by ligand or agonist antibody, the Fas receptor initiates a signal transduction cascade which leads to caspase-dependent programmed cell death (Shigekazu and Golstein, 1995).

Membrane alterations

In the early stages of apoptosis, changes occur at the cell surface and plasma membrane. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell.

Protease cascade

Signals leading to the activation of a family of intracellular cysteine proteases, the caspases, and (Cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. Different members of caspases in mammalian cells have been identified. Among the best-characterized caspases is caspase-1 or ICE (**I**nterleukin-1-Converting Enzyme), which was originally identified as a cysteine protease responsible for the processing of interleukin.

Mitochondrial changes

Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial

membrane results in the redistribution of cytochrome C to the cytosol followed by subsequent depolarization of the inner mitochondrial membrane. Cytochrome C (Apaf-2) release further promotes caspase activation by binding to Apaf-1 and therefore activating caspase 9. AIF (apoptosis inducing factor), released in the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis.

DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca2+ and Mg2+-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments.

2.3.5 Methods for studying apoptosis (Wyllie, 1999)

Many anticancer drugs kill cells by apoptosis is there fore important in the evaluation of cytotoxicity. Apoptosis can be determined in a number of ways.

- Morphological criteria
- DNA laddering
- Alterations in membrane asymmetry. Detection of phosphatidyl serine in the outer membrane using annexin V conjugated to FITC or biotin. Phosphatidylserine translocates from the cytoplasmic to the extracellular side of the cell membrane when undergo apoptosis. This is an early marker of apoptotic cells.
- Activation of apoptotic caspases. This family of proteases sets off a cascade of events that disable a multitude of cell functions.
- Release of cytochrome C and AIF-1 into cytoplasm by mitochondria

Morphological changes: On the other hand, the bisbenzimidazole dye, Hoechst 33342 (and also acridine orange), penetrates the plasma membrane and stains DNA in cells; without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be

visualized by fluorescence microscopy. They are also visible in permeabilized apoptotic cells stained with other DNA binding dyes like DAPI.

Hoechst is a UV-excited blue bisbenzimidazole dyed which selectively intercalate into A-T rich regions of DNA, undergoing a fluorescence enhancement in the process. Unlike PI, the Hoechst dye is specific for DNA rather than for macromolecule or polynucleic acids generally. It provides a fluorescence signal that is linearly proportional to DNA content over a wide range of DNA values. Dive et al. have reported that during a short exposure to Hoechst 33342, apoptotic cells have stronger blue fluorescence compared to non-apoptotic cells. Co-staining of the cells with propidium iodide (PI) allows the discrimination of dead cells from apoptotic cells. One drawback of using any vital staining method for measuring apoptosis is the variability of active dye uptake in different cells and its possible change during certain treatments. Therefore, the ability of Hoechst 33342 to discriminate apoptotic cells from normal cells by increased uptake of dye has to be tested for each new cell system (Freshney, 2000).

Assays that measure DNA fragmentation: The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die.

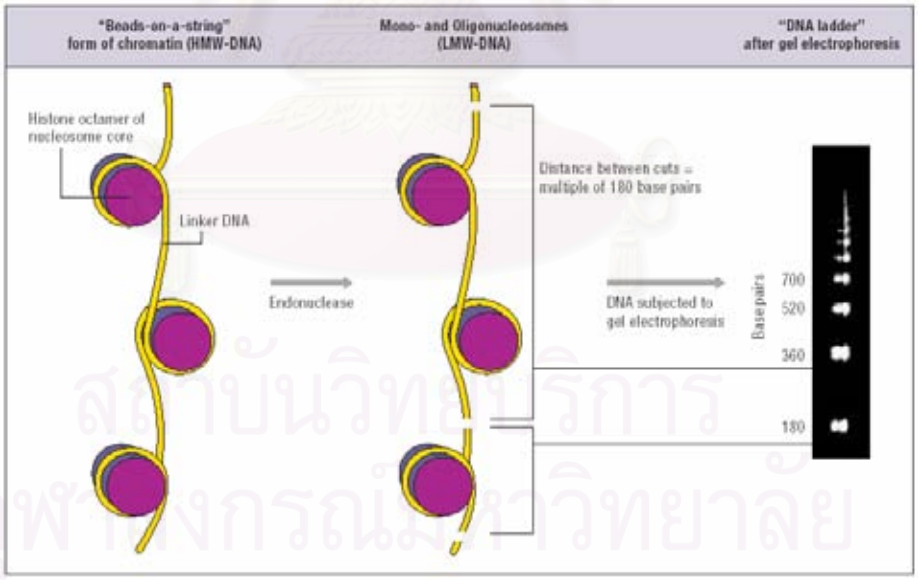

Figure 4: The biochemistry of DNA fragmentation and the appearance of the "DNA ladder" (Wyllie, 1999)

In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca2+ and Mg2+-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments (Figure 4). These DNA fragments reveal, upon agarose gel

electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit (Jarvis, 1994).

Assays that measure membrane alterations: In normal cells, the distribution of phospholipids is asymmetric, with the inner membrane containing anionic phospholipids (such as phosphatidylserine) and the outer membrane having mostly neutral phospholipids. In apoptotic cells however, the amount of phosphatidylserine (PS) on the outer surface of the membrane increases, exposing PS to the surrounding liquid. Two distinct modes of cell death, apoptosis and necrosis, can be distinguished on the basis of differences in morphological and biochemical characteristics. Under the electron microscope, cells undergoing apoptosis display cell shrinkage, apoptotic body formation, and chromatin condensation. Biochemically, the apoptotic process is charaterized by fragmentation of DNA into oligonucleosomal fragments. Furthermore, during the early stages of apoptosis, changes also occur at the cell surface membrane (Fadok et al., 1992). One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part to the outer layer of the plasma (Vermes et al., 1995), thus exposing PS at the external surface of apoptotic cells, where it can be specifically recognized by macrophage (Fadok et al., 1992).

Annexin V, a Ca^{2+} dependent phospholipid-binding protein, possesses high affinity for PS and can thus be used for detecting early apoptotic cells (Vermes et al. 1995, Homburg et al. 1995). Since annexin V can also detect necrotic cells as a result of the loss of membrane integrity, apoptotic cells have to be differentiated from these necrotic cells by the use of propidium iodide (PI). Indeed, PI selectively labels necrotic, but not apoptotic cells.

2.4 Acetylmelodorinol and plaunotol

2.4.1 Acetylmelodorinol

Acetylmelodorinol is isolated from petroleum ether extract of flowers of *Melodorum fruticosum* Lour., family Annonaceae. The information in previous studies on the antiproliferation activity of Acetylmelodorinol on tumor cells has been based on MTT assay that measures cell viability. Acetylmelodorinol has very high cytotoxic activity with an IC_{50} less than 3 μg/ml on five cell lines (Chaichantipyuth et al., 2001).

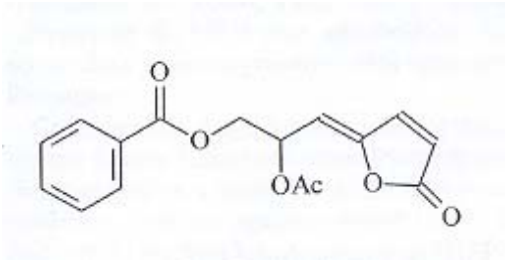

Figure 5: Structure of Acetylmelodorinol

2.4.2 Plaunotol

Plaunotol can be extracted from a Thai medicinal plant called *plau-noi* (*Croton Sublyratus* Kurz., family Euphobiaccae) and is often used as traditional medicines for the treatment of gastritis and gastric ulcer. In previously report plaunotol has been shown to posses bactericidal effects against *Helicobacter pylori* and that cell death was associated with lysis of bacteria (Koga et al., 1996), which may result from interaction of this compound with bacterial membrane (Koga et al., 1998) and led to an increase in permeability of membrane. Plaunotol has been called "a cytoprotective antiulcer agent" and has been reported to stimulate the release of endogenous secretin in humans (Shiratori et al., 1993).

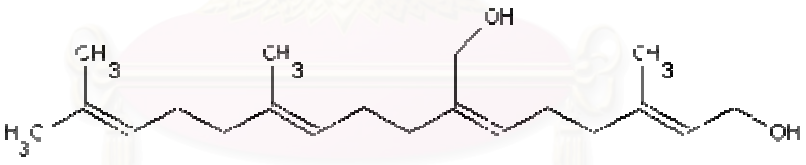

Figure6: Structure of plaunotol

CHAPTER III

MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemicals

100 base-pair DNA ladder	SibEnzyme
100 mM dATP, dCTP, dGTP, dTTP	SibEnzyme
Absolute ethanol	Mallinckrodt
Agarose gel	Sekem® LE agarose
Annexin-V-Fluos	Roche
Boric acid	Sigma
Chloroform	Merck
Diethyl pyrocarbonate sulfoxide (DEPC)	Sigma
Dimethyl Sulfoxide (DMSO)	Fluka
EDTA	Sigma
Ethidium bromide	Sigma
Fetal Bovine Serum	Biochrome AG
Glutaraldehyde	Sigma
Glycerol	Sigma
Glycine	Sigma
Hoechst 33342	Sigma
Hydrochloric acid	Merck
Isopropanol	LabScan Asia Co.
Methanol	Merck
MTT	Sigma
Phenol: chloroform: isoamyl alcohol (25:24:1 v/v)	Sigma
Potassium chloride	Merck
Potassium di-hydrogen phosphate	Carlo Erba
Ribonuclease A	Sigma
RPMI-1640 medium	Biochrome KG

Sodium acetate	Merck
Sodium carbonate	Merck
Sodium chloride	Fluka
Sodium citrate	Sigma
Sodium di-hydrogen phosphate	Carlo Erba
Sodium dodecyl sulfate (SDS)	Sigma
Sodium hydrogen carbonate	Merck
Sodium hydroxide	Merck
Tris (hydroxyl methyl)-aminomethane	Sigma
TRIZOL Reagent®	Gibco BRL
Trypan blue	The British Drug House, LTD.
Trypsin	Sigma

3.1.2 Eqiupments

-20°C Freezer	Sanyo
-80°C Freezer	Sanyo
Air pump	IWAKI
Autoclave: model RII	Udono
Automatic micropipettes P10, P20, P200, and P1000	Gilson Medical Electrical S.A.
Camera Pentax K1000	Asahi Opt. Co., LTD.
Centrifuge tube (50 ml)	Nunc
Fluorescence microscope	Nikon
Haemocytometer	Boeco
Inverted microscope	Olympus
Laminar flow Cabinet Model HS 124	ISSCO
Microcentrifuge tube	Sorenson
Microtiterplate 96 wells	Nunc
Microtiterplate reader / MCC/340	Titertek multiskan®
Pipette tips 10, 20, 200 and 1000 µl	Gilson Medical Electrical S.A.
Power supply	Bio-RAD Laboratories
Spectrophometer Spectronic Genesys5	Milton Roy
Syringe (10 ml)	Terumo

Tissue culture flask (25 cm^2)	Nunc
UV Transilluminator M-26	UVP
Vacuum Blotter 785	Bio-RAD Laboratories
Water bath SBS30	Stuart Scientific
Water jacket incubator	REVCO

3.2 Cell line and medium

Cell line was obtained from the American Type Culture Collection (Rockville, MD)

- BT474 (Human breast ductol carcinoma; ATCC no. HTB20)
- Hep-G2 (Human liver hepatoblastoma; ATCC no. HB8065)
- KATO III (Human gastric carcinoma; ATCC no. HTB 103)
- SW620 (Human colon adenocarcinoma; ATCC no. CCL 227)
- Hs27 (Human foreskin fibroblast; ATCC no. CRL1634)
- CH-liver (Human liver; ATCC no. CCL13)

Basal medium consisted of RPMI-1640 medium with 2 g/L sodium bicarbonate. Serum-supplemented medium was prepared by adding fetal bovine serum (FBS) to basal medium. Cells were routinely propagated in 5% (v/v) serum-supplemented medium at 37°C in an incubator with 5% CO_2.

3.3 Cell culture and treatments

Cell lines were maintained in RPMI 1640 medium supplemented with 5% (v/v) FBS, at 37 °C in 5% CO_2 incubator. Exponentially growing cells were detached from the cell culture surface by trypsinization with 0.25% of trypsin solution for 1-2 minutes at 37°C then single-cell suspension was produced. Viable cell number was assessed using a haemocytometer by counting the number of cells excluding trypan blue staining.

Acetylmelodorinol and plaunotol was dissolved in 100% DMSO and stored at -20 °C. Stock concentration (20 mg/ml) was diluted to various concentrations by medium. Final concentrations of DMSO did not exceed 0.1-1.0% and did not affect cell survival (Studzinski, 1995).

3.4 Cell proliferation assay

Exponentially growing cells were plated at density of 5×10^3 cells/well in 96-well plates and incubated at 37 °C to allow for cell attachment. After 24 h the cells were treated with various concentrations (0-10 µg/ml) of acetylmelodorinol and plaunotol. Doxorubicin hydrochloride was used as a positive control. The plates were incubated for selected exposure times of 12, 24, 48 and 72 h. Cell proliferation assay was performed by the MTT (3-(4,5– dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method (Carmichael et el., 1987 and Twentyman, 1987).

After each incubation period, MTT (5 mg/ml) was added at 10 µl/well and incubation was carried out for an additional 4 h at 37 °C in the dark. The solution was then discarded and 150 µl of DMSO and 25 µl of 0.1 M glycine buffer (pH 10.5) were added into each well to dissolve insoluble formazan crystal. Plates were then kept agitation for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative was analyzed on a microplate reader (Titertek multiskan® MCC/340) at a wavelength of 540 nm (Moongkarndi et al., 1991 and Studzinski, 1995). The percentage of cell viability was calculated according to the following formula.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells}\times 100}{\text{OD of control cells}}$$

The IC_{50} values were obtained by plotting the percentage of cell viability versus the concentrations.

Repeating experiments at selected time to confirm results were carried out and the IC_{50} were determined. All data were expressed as mean ±S.D. and analyzed by one-way of variance (ANOVA) using SPSS software (version 10.0 for Windows). Pearson correlation analysis was used between parameters $p < 0.01$ was considered statistically significant.

3.5 Analysis of chromosomal DNA fragmentation

3.5.1 DNA extraction

DNA was extracted from cancer cell lines using phenol/chloroform method. After cells were treated by acetylmelodorinol and plaunotol at 5 and 10 µg/ml for 48 h, cells (more than

1×10^6 cells) washed with PBS (Ca^{2+} and Mg^{2+} free) and resuspended in 40 µl of the TE buffer [10 mM Tris–Cl (pH 7.4), 1 mM EDTA]. A volume of 10 µl of RNaseA (10 mg/ml) was added to cell suspension and incubated at 37 °C for 2 h. DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1 v/v) by adding an equal volume and shaking vigorously for at least 10 seconds. The mixture was centrifuged at 12,000 rpm for 10 minutes and the upper aqueous phase was removed followed by the addition of one-tenth volumes of sodium acetate and two volumes of iced cold absolute ethanol, stored at -80°C for 40 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 5 min at room temperature and briefly washed twice with 70% ethanol. DNA pellet was air dried and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The DNA solution was incubated at 37°C for 1-2 h for complete redissolved and kept at 4°C until further needed.

3.5.2 Measuring the concentrations of extracted DNA using spectrophotometry

The concentrations of extracted DNA or RNA samples were estimated by measuring the optical density at 260 nm (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA, 40 µg/ml single stranded RNA and 33 µg/ml single stranded DNA (Sambrook et al., 2001). Therefore, the concentration of DNA/RNA samples were estimated in µg / ml by using the following equation,

[DNA] = OD_{260} × dilution factor × 50 (40 or 33 for RNA or single stranded DNA, respectively)

The purity of DNA samples can be evaluated from a ratio of OD_{260} / OD_{280}. The ratios of appropriately purified DNA and RNA were 1.8 and 2.0, respectively (Sambrook et al., 2001).

3.5.3 Agarose gel electrophoresis

An appropriate amount of agarose was weighted and mixed with an appropriate volume of 1x TBE buffer (89 mM Tric-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 60°C before poured into the gel mold. A comb was inserted. The gel was left to solidify. When needed, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of TBE buffer covering the gel for approximately 0.5 cm.

Added 3 µl of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) to each sample that contained 5 ng of extracted DNA, subsequently analyzed by 2% agarose gel at 100 volts for one h. DNA was visualized by staining with ethidium bromide.

3.6 Detection of phosphatidylserine in the outer membrane using annexin-V-Fluos staining

BT474, KATOIII, and SW620 cells (2×10^5 cells) were grown on cover slides and then treated with Acetylmelodorinol 5 µg/ml for 12 h and 24 h. After incubation, medium was removed and 100 µl of Annexin-V-FITC labeling solution (containing with 20 µl Annexin-V-FITC labeling reagent and 20 µl propidium iodide solutions in 1 ml HEPES buffer) was added. The cover slides was put on and incubated for 10-15 min at 15-25 °C. The cell lines were examined by fluorescence microscope.

3.7 Determination of morphological changes of cells

3.7.1 Observation of cells by phase contrast microscope

SW620 cells (2×10^5 cells) were cultured in 35 mm petridishes and treated with 5 µg/ml acetylmelodorinol for 48 h. After incubation, the medium was removed and cells were washed once with PBS. They were observed by phase contrast inverted microscope (Olympus, Germany) at 400X magnification.

3.7.2 Benzimidazole Ho33342 staining

SW620 cells (2×10^5 cells) were grown on culture slides and then treated with 5 µg/ml acetylmelodorinol and plaunotol for 24, 48 h. After incubation, the medium was removed and cells were washed with PBS (Ca^{2+} and Mg^{2+} free). Then cells were fixed with 1% glutaraldehyde for 2 h in the dark. Cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free) and then added 100 µl Hoechst33342 (1 µg/ml) was added to each slide and further incubated at 37 ˚C for 30 min in the dark. Living and apoptotic cells were visualized through blue filter of fluorescence microscope (Nikon, Germany) at 400X magnification (Ramonede and Tomas, 2002).

3.8 Reverse transcription polymerase chain reaction (RT-PCR)

Bcl-2 and *p53* gene products have been both linked to cell death by apoptosis. Thus, to study *Bcl-2* and *p53* gene expression patterns, was used competitive template reverse transcription-polymerase chain reaction (RT-PCR).

3.8.1 RNA extraction

After cells were culture in 3.5 cm diameter dish and treated with 5 µg/ml acetylmelodorinol for 0, 2, 4, 6, 8, and 10 h total RNA was extracted from treated cells using TRIZOL Reagent®. Discard culture medium and lysed cells directly in a culture dish by adding 1 ml of TRIZOL Reagent® to a 3.5 cm diameter dish, and passing cell lysate several time through a pipette. The mixture was left at room temperature for 5-10 minutes, before adding 0.2 ml of chloroform. The mixture was vortexed for 15 seconds and left at room temperature for 2-15 minutes and centrifuged at 12000 g for 15 minutes at 4°C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 minutes and centrifuged at 12000g for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12000g for 15 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in an -80°C freezer for long storage.

3.8.2 First stranded cDNA synthesis

The first stranded cDNA synthesized from 1 µg of total RNA extracted from SW620 cells, using ImProm-II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 µg of oligo dt and appropriated DEPC-treated H_2O in a final volume of 5 µl. The reaction mixture was mixed by pipetting and centrifuge briefly. The reaction tube was incubated at 70°C in a thermal cycler for 2 minutes. The tube cooled on ice for 2 minutes and briefly centrifuged. The first strand cDNA synthesis was synthesized by adding 2 µl of 5x First-Strand

Buffer (250 mM Tris-HCl, pH 8.5, 40 mM $MgCl_2$, 150 mM KCl and 5 mM Dithiothreitol), 1 µl of dNTP Mix (10 mM each of dNTPs), 1µl of sterile H_2O, and 1 µl of (20 units) AMV Reverse Transcriptase. The reaction was incubated at 42°C for 1.5 h in an air incubator. The tubes were placed on ice to terminate the first strand cDNA synthesis.

3.8.3 RT-PCR analysis

One microgram of the first strand cDNA was used as the template in a 25 µl PCR composing of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1 % Triton X-100, 2 mM $MgCl_2$, 100 mM of each dNTP, 0.5 µM of each primer and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, Finland). PCR was carried out using the conditions described in Table 2. Eight microliters of the amplification products are electrophoresed though 1.2-1.8% agarose gel depending on sizes of the amplification products and visualized under a UV translluminator after ethidium bromide staining (Sambrook et al., 2001).

Table 2 Primer sequences for PCR and amplification conditions for each target gene.

Primer	Sequence	Expected Size (bp)	Amplification conditions
Bcl-2	5'-GAGGATTGTGGCCTTCTTTG-3'	170	Denaturation at 94°C for 30 seconds, annealing at 55 °C for 45 seconds and synthesizing at 72 °C for 30 seconds for 35 cycles.
	5'- ACAGTTCCACAAAGGCATCC-3'		
P53	5'-CCCTCCTCAGCATCTTATCCG-3'	262	Denaturation at 94°C for 30 seconds, annealing at 57 °C for 45 seconds and synthesizing at 72 °C for 45 seconds for 35 cycles.
	5'-GGCACAAACACGCACCTCAAA-3'		
GAPDH	5'-ACCACAGTCCATGCCATC-3'	452	Changed according to different target genes.
	5'-TCCACCACCCTGTTGCTG-3'		

All initial denaturations were at 94 °C for 5 minutes. Finally, an additional extension step at 72 °C for 7 minutes was done.

3.8.4 Agarose gel electrophoresis

Appropriate volumes of PCR products were mixed with one-fourth volume of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) and loaded into the well. The 100 bp DNA ladder was used as the standard DNA marker. Quantitative image analysis of the PCR fragments was performed using the Gel Pro analyzer 3.1 program. The expression levels were calculated as the ratios of optical density of PCR products to those of *GAPDH* PCR products.

CHAPTER IV

RESULTS

1. Growth inhibition of cancer cell lines by Acetylmelodorinol and plaunotol

Acetylmelodorinol and plaunotol were tested for their *in vitro* cytotoxicity against BT474 (Human breast ductol carcinoma; ATCC no. HTB20), Hep-G2 (Human liver hepatoblastoma; ATCC no. HB8065), KATO III (Human gastric carcinoma; ATCC no. HTB 103), SW620 (Human colon adenocarcinoma; ATCC no. CCL 227), Hs27 (Human foreskin fibroblast; ATCC no. CRL1634), and CH-liver (Human liver; ATCC no. CCL13).

Cells were incubated for selected appropriate exposure times of 12, 24, 48, and 72 h. Cell viability was determined using MTT assay.

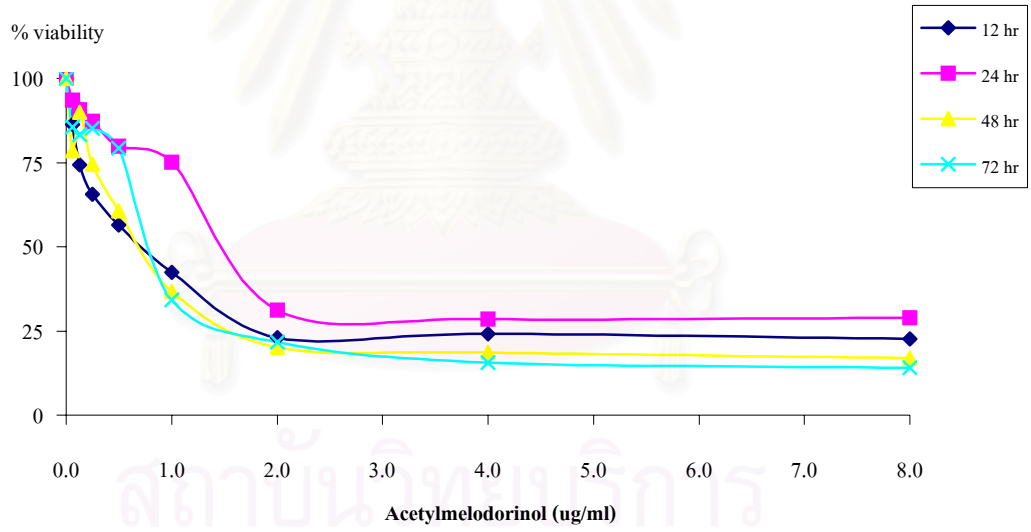

Figure 7 Effects of acetylmelodorinol on cell growth in BT474 cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-8 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

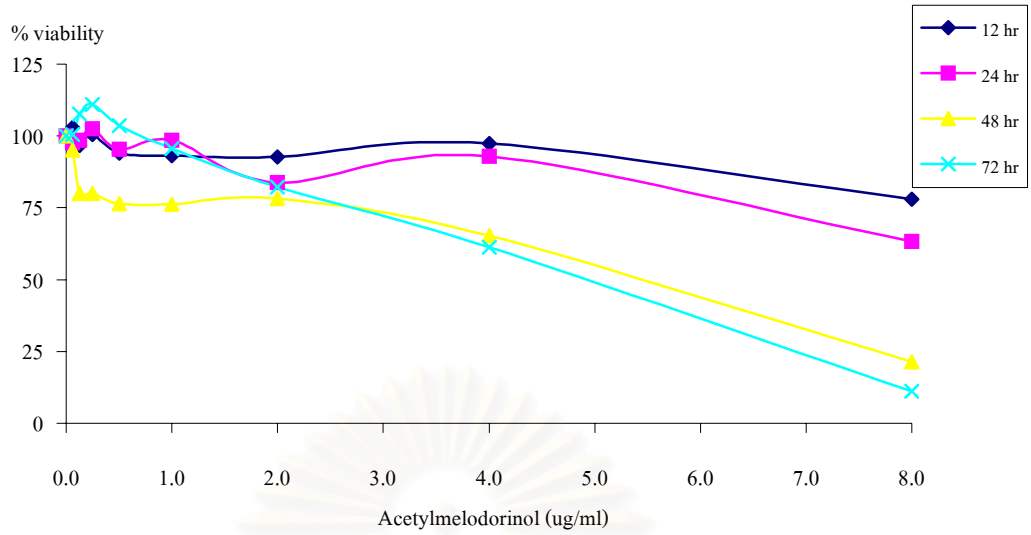

Figure 8 Effects of acetylmelodorinol on cell growth in Hep-G2 cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-8 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

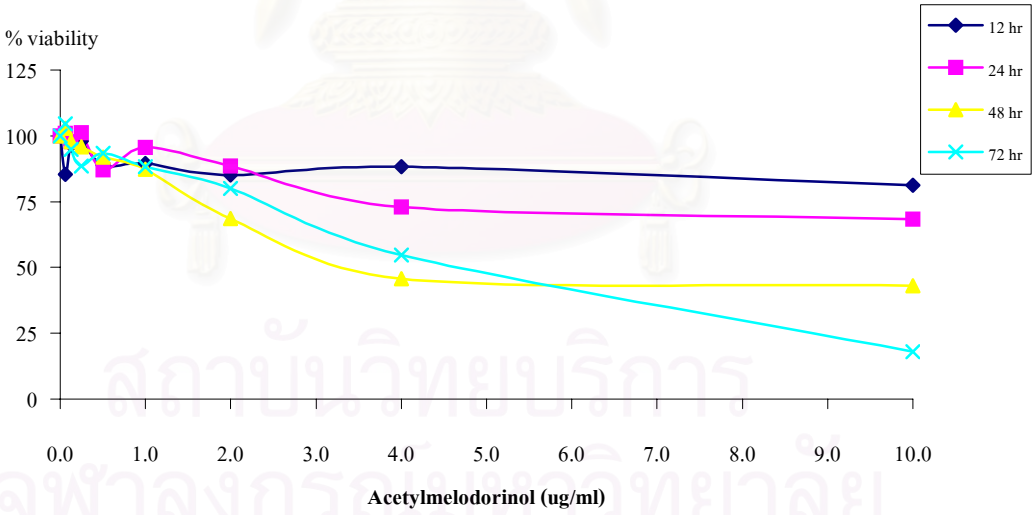

Figure 9 Effects of acetylmelodorinol on cell growth in KATOIII cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

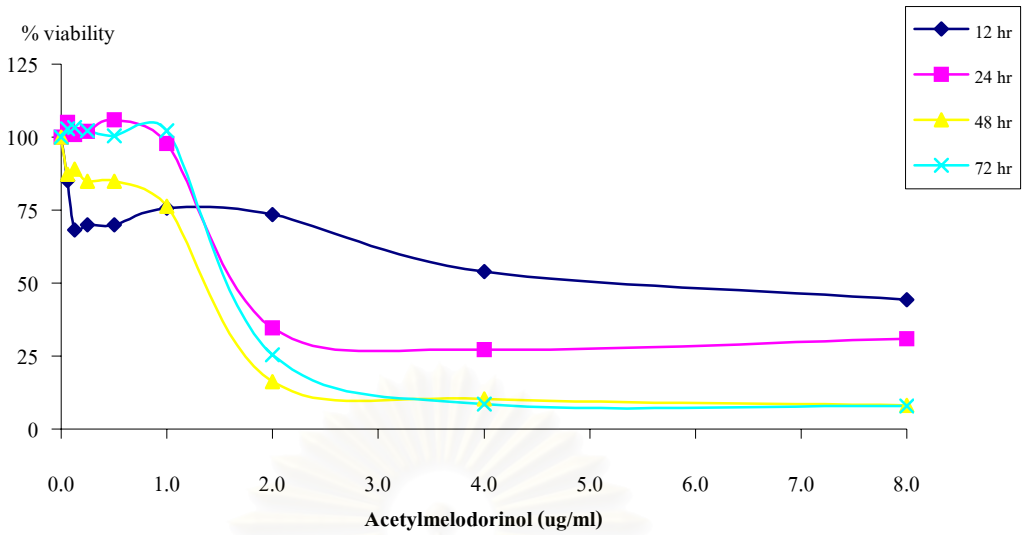

Figure 10 Effects of acetylmelodorinol on cell growth in SW620 cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-8 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

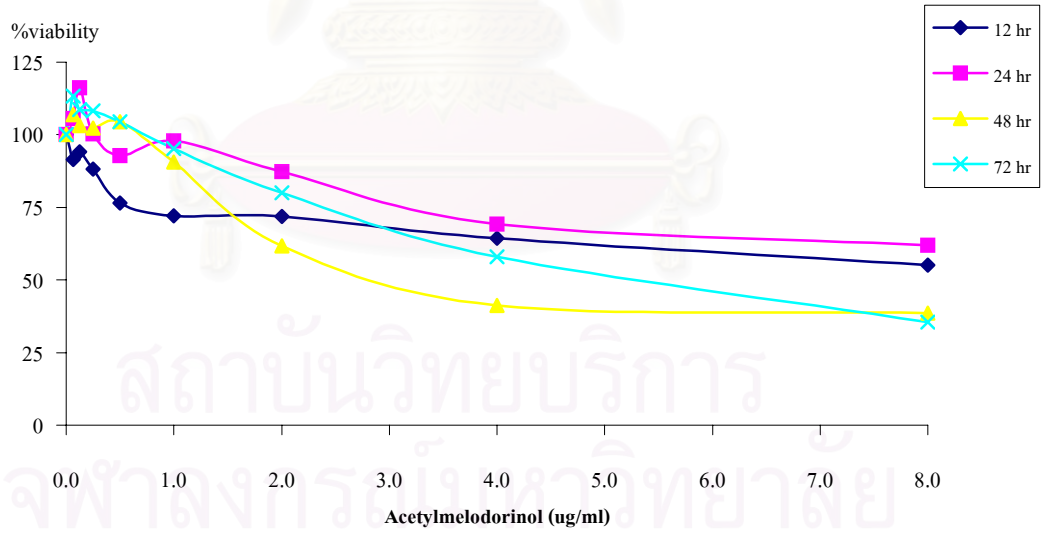

Figure 11 Effects of acetylmelodorinol on cell growth in CH-liver cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-8 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

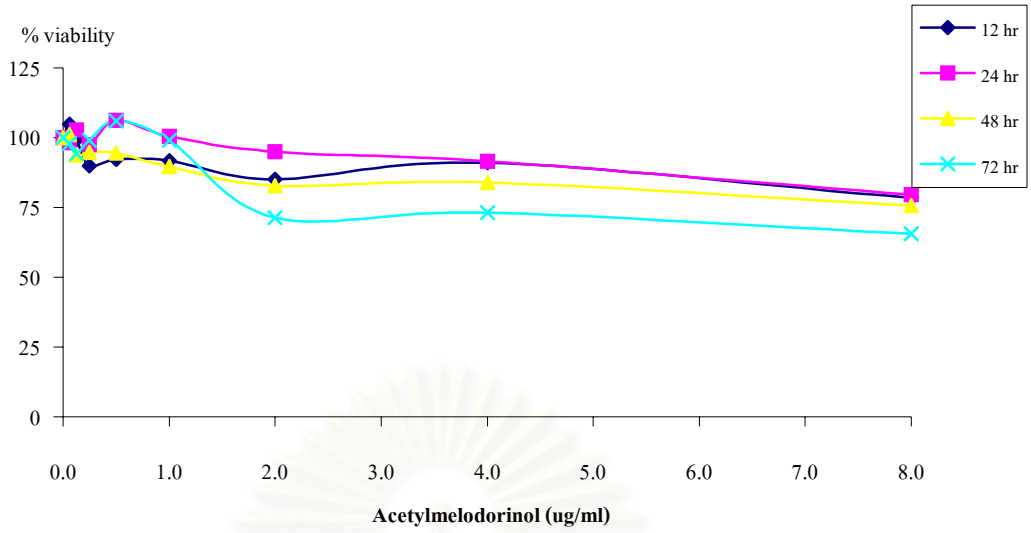

Figure 12 Effects of acetylmelodorinol on cell growth in Hs27 cells measured by MTT assay. The cells were incubated with acetylmelodorinol at various concentrations (0-8 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

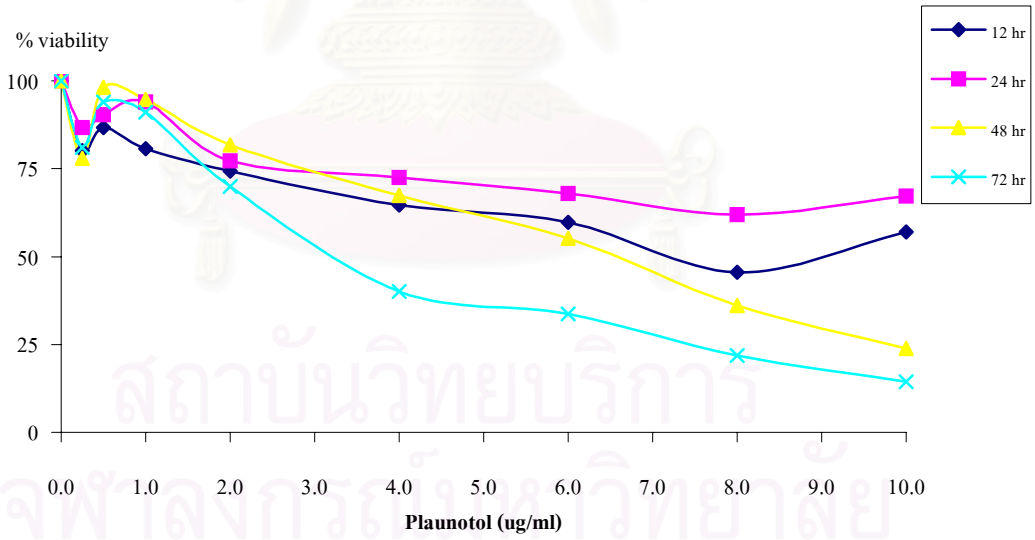

Figure 13 Effects of plaunotol on cell growth in BT474 cells measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

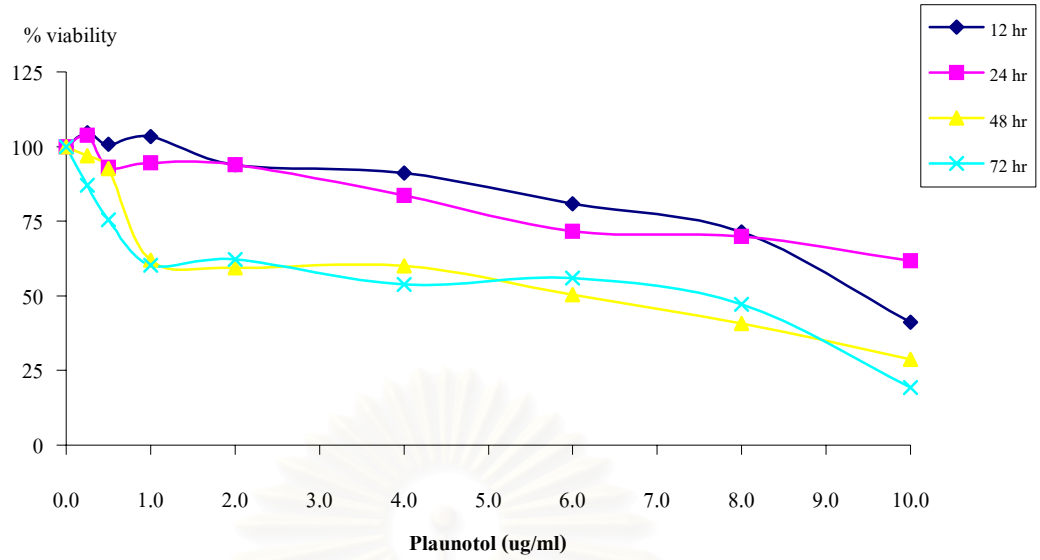

Figure 14 Effects of plaunotol on cell growth in Hep-G2 cells measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

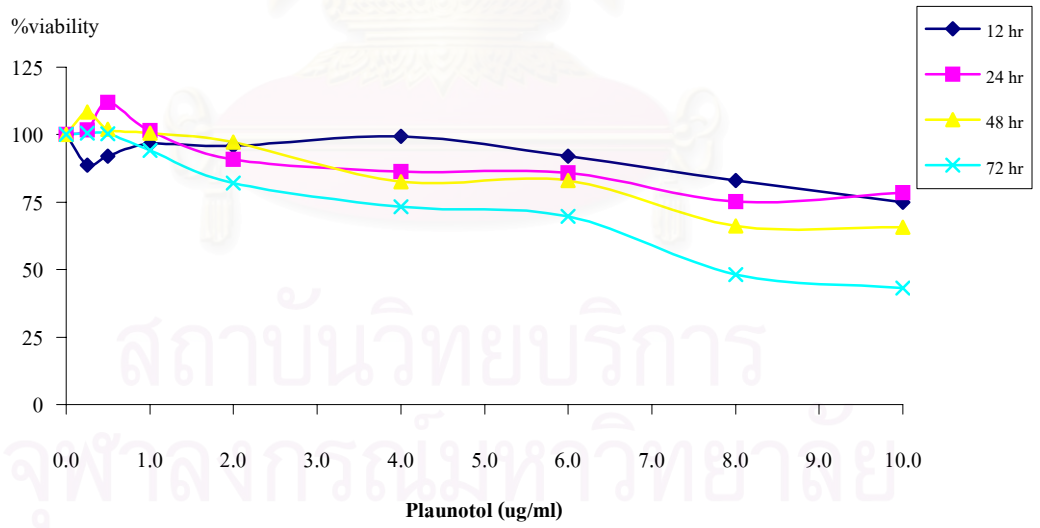

Figure 15 Effects of plaunotol on cell growth in KATOIII measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

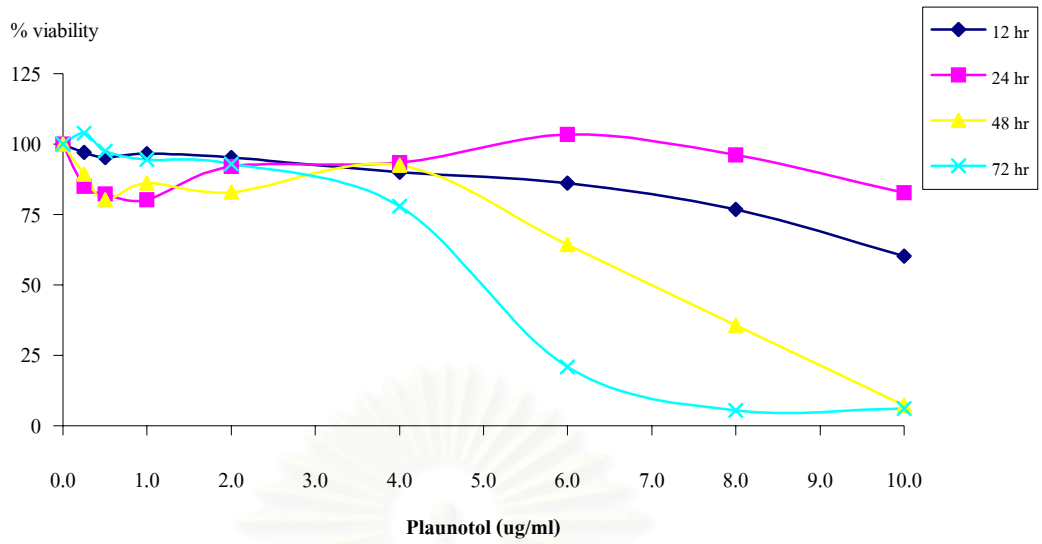

Figure 16 Effects of plaunotol on cell growth in SW620 measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

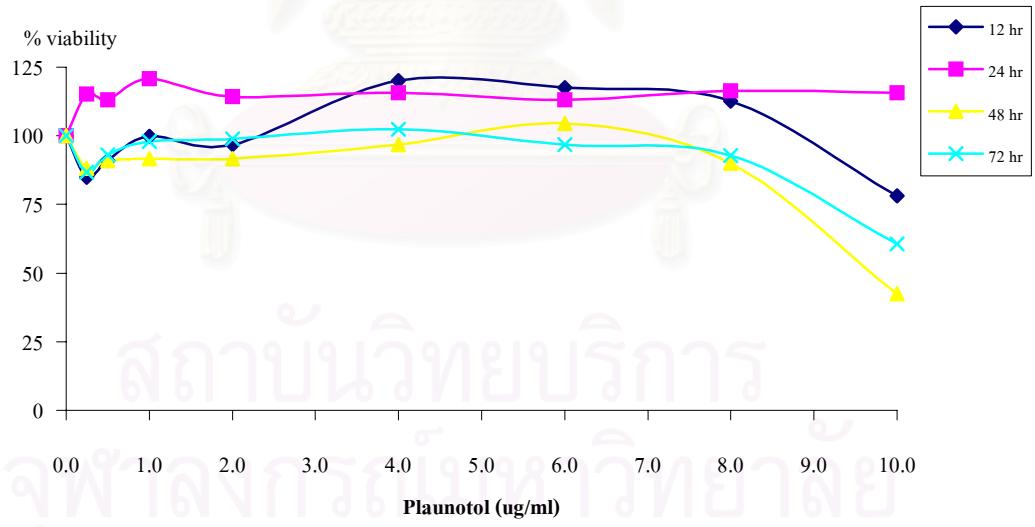

Figure 17 Effects of plaunotol on cell growth in CH-liver cells measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

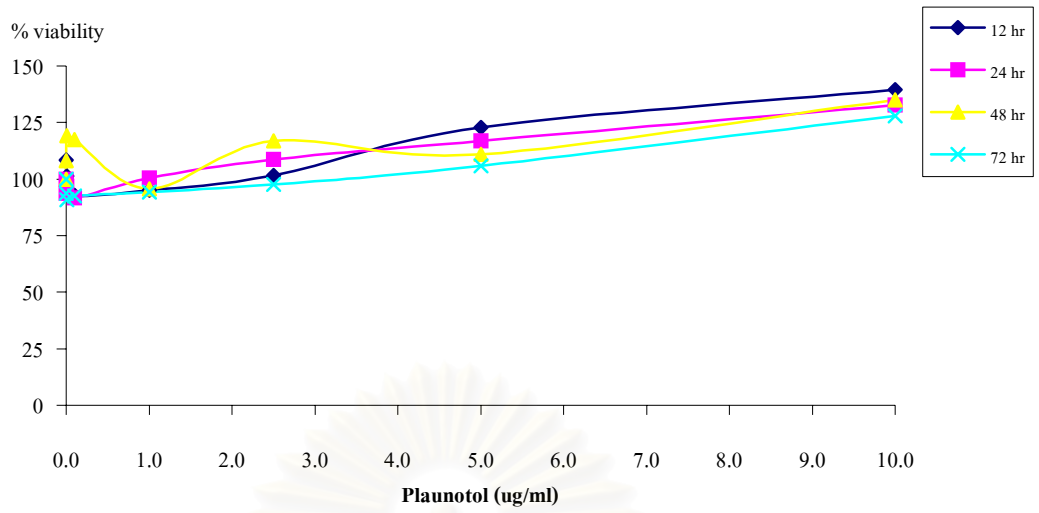

Figure 18 Effects of plaunotol on cell growth in Hs27 measured by MTT assay. The cells were incubated with plaunotol at various concentrations (0-10 μg/ml) and incubated for 12, 24, 48, and 72 h. The results represent the mean ± S.D. of triplicate determinations.

The results showed that acetylmelodorinol and plaunotol showed a dose and time-dependent inhibition of cell proliferation, and the stronger cytotoxicity was observed at 48 h incubation times than 12 and 24 h incubation times ($p<0.01$).

The confirmation of activity with incubation time of 48 h were repeated in three independent experiments and found that acetylmelodorinol showed stronger cytotoxicity than plaunotol in cancer cells line. However, plaunotol did not show antiproliferative effect in normal cells (CH-liver and Hs27) and acetylmelodorinol did not show antiproliferative effects only in Hs27 (fore skin fibroblast). Doxorubicin hydrochloride, anti cancer drug, was used as positive control. Their cytotoxicity data were tabulated in Table 3. The results represent the mean ± S.D. of triplicate determinations.

Table 3 Cytotoxicity data of acetylmelodorinol, plaunotol, and Doxorubicin (incubation times 48 h)

Compound	Cytotoxicity IC_{50} (μg/ml)					
	BT474	Hep-G2	KATO-III	SW620	CH-liver	Hs27
Acetylmelodorinol	1.43±0.39	4.32±0.89	3.10±0.48	1.39±0.08	2.80±0.35	>10
Plaunotol	7.07±1.44	6.11±1.15	10.36±2.19	6.90±0.68	8.14±1.22	>10
Doxorubicin*	>10	1.42±1.15	2.05±0.53	0.35±0.29	1.15±0.01	>10

Doxorubicin*: Doxorubicin hydrochloride was used as positive control.

2. Analysis of chromosomal DNA fragmentation

To examine apoptosis inducing potentials of acetylmelodorinol and plaunotol in cancer cell lines, we analyzed chromosomal DNA fragmentation by agarose gel electrophoresis. We found that in SW620 cells treated with 5 and 10 μg/ml of acetylmelodorinol, there was evidence of DNA fragmentation seen as the DNA ladder patterns. This result indicates that acetylmelodorinol induced cell death via an apoptotic pathway in SW620. In addition, KATOIII cells treated with acetylmelodorinol also exhibited the DNA ladder pattern, suggesting that acetylmelodorinol also induced apoptosis in this cell lines. However, acetylmelodorinol did not showed DNA ladder pattern when treated in other cancer cell lines and normal liver cells.

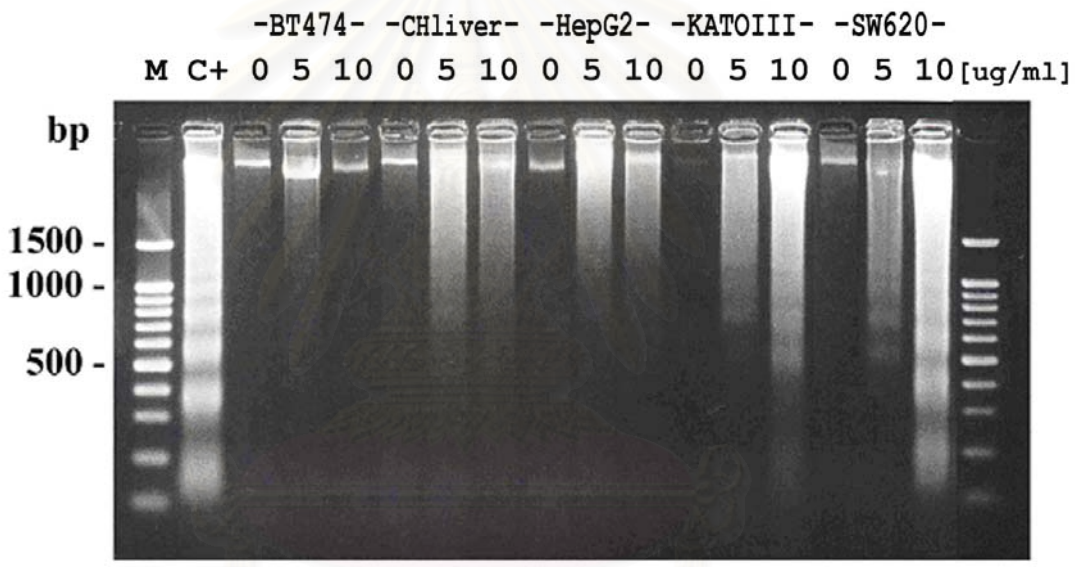

Figure 19 DNA fragmentation analysis of cancer cell lines treated with acetylmelodorinol for 48 h. Human cancer cell lines (BT474, HepG2, KATOIII, and SW620) and normal liver cells (CH-liver) treated with acetylmelodorinol for 48 h at 5 and 10 μg/ml. Lane M, 100 bp markers; lane C+, positive control (hybridoma cultured with serum deprivation).

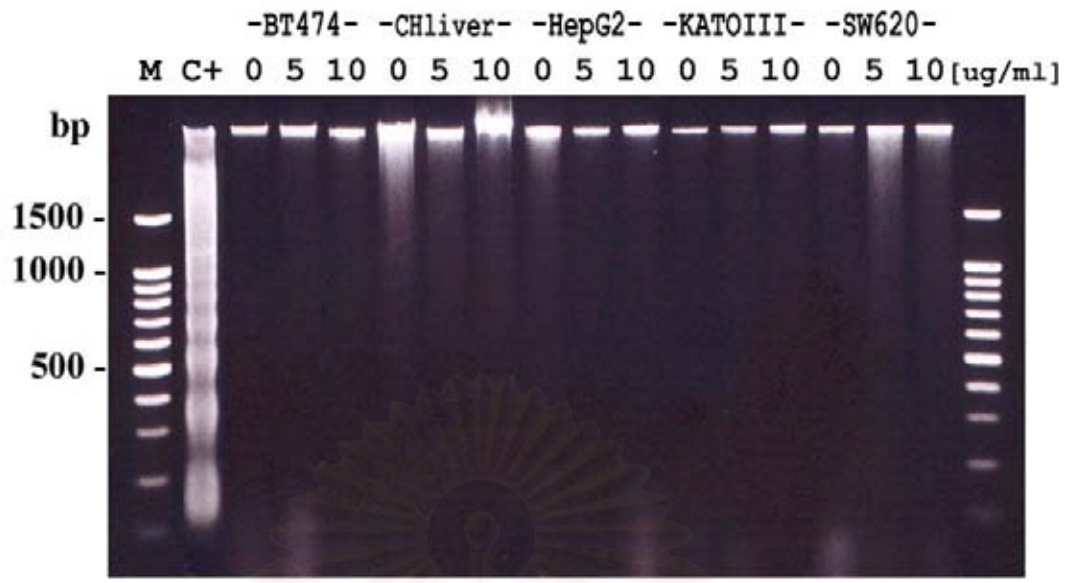

Figure 20 DNA fragmentation analysis of cancer cell lines treated with plaunotol for 48 h. Human cancer cell lines (BT474, HepG2, KATOIII, and SW620) and normal liver cells (CH-liver) treated with plaunotol for 48 h at 5 and 10 μg/ml. Lane M, 100 bp markers; lane C+, positive control (hybridoma cultured with serum deprivation).

No DNA fragmentation was observed in any cancer cell lines and normal liver cells, when treated with plaunotol (Figure 20). These results strongly suggest that plaunotol did not induced apoptosis in any cell lines studied.

3. Detection of phosphatidylserine in the outer membrane using Annexin-V-FITC staining

To confirm the induction of apoptosis by acetylmelodorinol in BT474, KATOIII, and SW620 cells, cells were treated with 5 μg/ml of acetylmelodorinol and analyzed by Annexin-V-FITC Apoptosis Detection Kit (Roche Molecular Biochemicals). Phosphatidylserine (PS) exposure on the surface of apoptotic cells was detected by fluorescence microscope after staining with Annexin-V-FITC. The cells were counter-stained with propidium iodide, analyzed and counted stained cells under fluorescence microscope in six field and means of average were plotted in histogram (Figure 21-23). The results are expressed as percentage of apoptotic Annexin-V-FITC positive cells and percentage of necrotic propidium iodide positive cells. Majority of acetylmelodorinol-treated BT474 cells became PI positive at 12 h after treatment, while a few numbers became Annexin-V positive. This result suggests that acetylmelodorinol induced BT474 cells to die by necrosis.

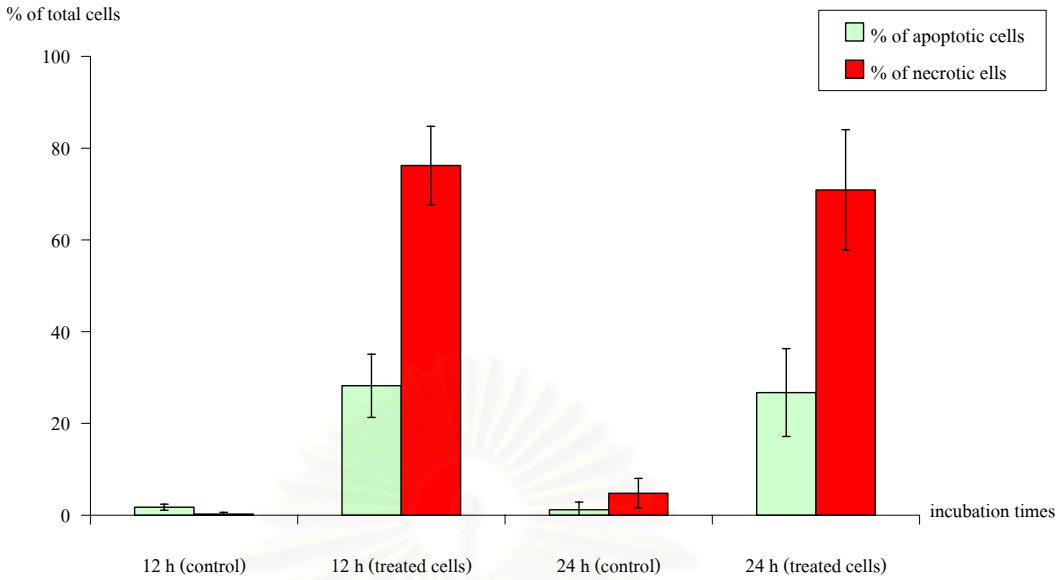

Figure 21 Characterization of cell death in BT474 cells treated with acetylmelodorinol 5 μg/ml for 12 h and 24 h. Cell death assessed by Annexin-V-FITC staining was expressed as percentage of apoptosis (green bar), necrotic cells assessed by PI staining (red bar). The results represent the means ± SD. of triplicate determination.

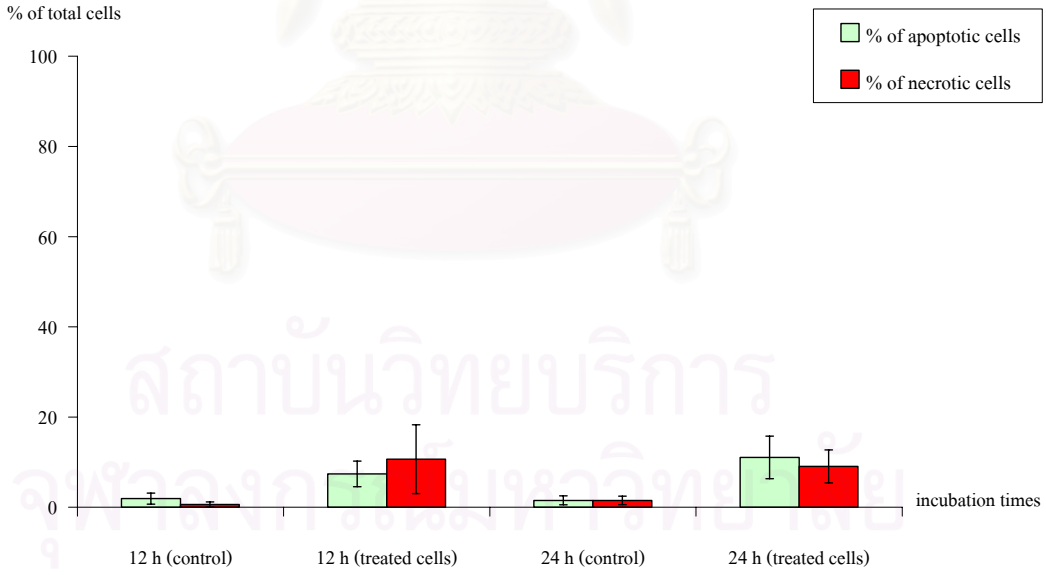

Figure 22 Characterization of cell death in KATOIII cells treated with acetylmelodorinol 5 μg/ml for 12 h and 24 h. Cell death assessed by Annexin-V-FITC staining was expressed as percentage of apoptosis (green bar), necrotic cells assessed by PI staining (red bar). The results represent the means ± SD. of triplicate determination.

For KATOIII cells treated with acetylmelodorinol (5 μg/ml) became PI/Annexin-V positive. This result does not agree with DNA fragmentation pattern. This may be due to the concentration that trigger DNA fragmentation is higher than that has been used in this experiment. When SW620 cell were treated with acetylmelodorinol, the number of apoptotic cells increased from 29.02% at 12 h to 56.38% at 24 h, however, while necrotic cells increased from 3.82% at 12 h to 20.18% at 24 h.

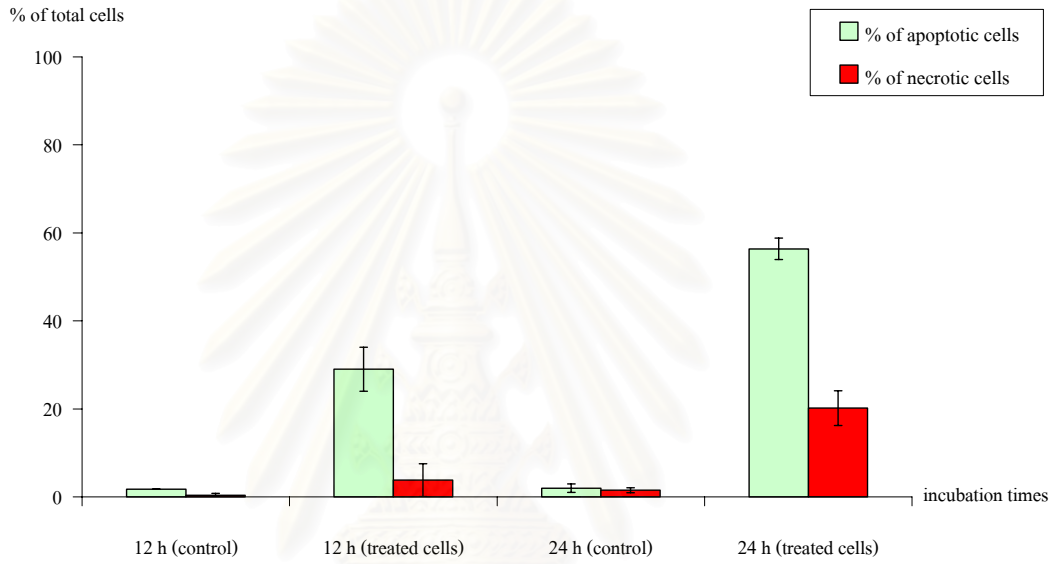

Figure 23 Characterization of cell death in SW620 cells treated with acetylmelodorinol 5 μg/ml for 12 h and 24 h. Cell death assessed by Annexin-V-FITC staining was expressed as percentage of apoptosis (green bar), necrotic cells assessed by PI staining (red bar). The results represent the means ± SD. of triplicate determination.

4. Observation of cell morphology by microscope

The results showed that only SW620 cells treated with acetylmelodorinol exhibited DNA ladder pattern and positive staining with annexin-V, indicating that acetylmelodorinol induced cell death by apoptotic pathway (Figure 19). Furthermore, these results were confirmed at microscopic level using phase contrast microscopy and fluorescence microscopy by Hoechst 33342 staining.

4.1 Phase contrast microscopy

Treatment of SW620 cells with 5 μg/ml acetylmelodorinol resulted in progressive morphological changes typical of apoptosis, including cells shrinkage, rounding, and detachment of cells from plate, as observed with phase contrast microscopy (Figure 24(B)).

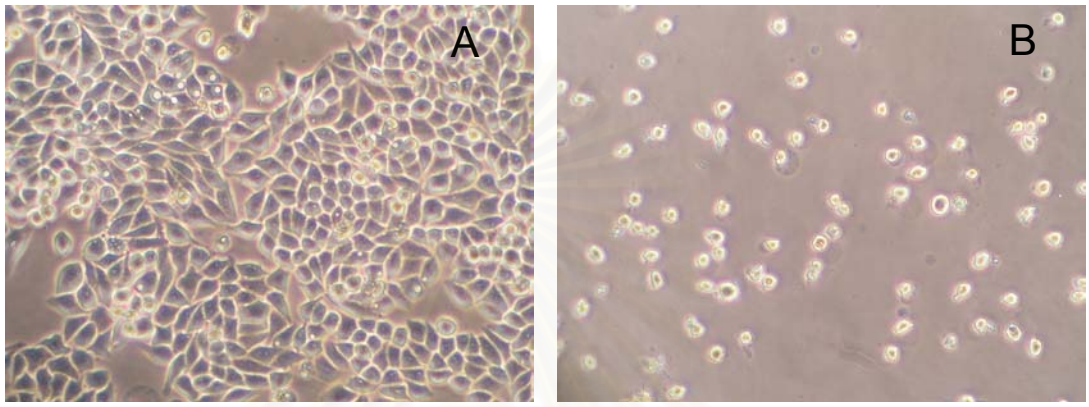

Figure 24 Morphological alterations of SW620 cells following expose to 5 μg/ml of acetylmelodorinol for 48 h. (A) Control untreated SW620 cells and (B) acetylmelodorinol-treated SW620 cells were observed by phase contrast inverted microscope (200X magnification).

4.2 Benzimidazole Hoechst 33342 staining

The nuclear morphological changes were observed by Hoechst 33342 staining. In control group, SW620 cells nuclei were round and stained homogeneously with Hoechst 33342 (Figure 25. In contrast to untreated cells, the apoptotic nuclei of SW620 cells gave stronger blue fluorescence and nuclear condensation and fragmented was evident typical of apoptotic bodies (Figure 26(C)). However, when SW620 cells were treated with plaunotol, they did not showed morphological changes indicative of apoptosis (Figure 27 (B), (C)).

Figure 25 Fluorescence microscopic analysis of SW620 nuclei stained with Hoechst 33342 (400X magnification). Control untreated SW 620 cells at 0 h (A), 24 h (B), and 48 h (C) incubation times.

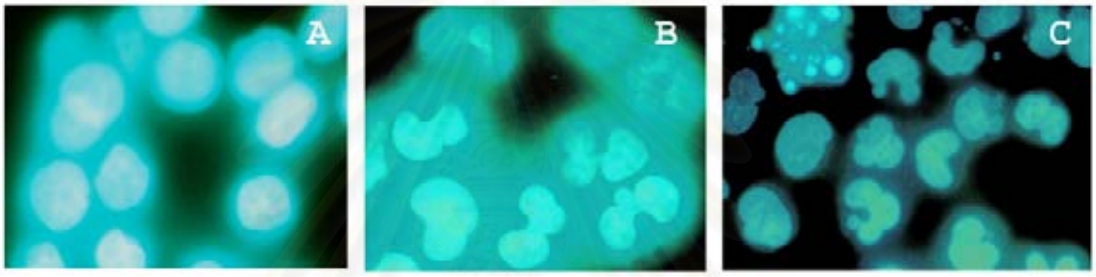

Figure 26 Fluorescence microscopic analysis of SW620 nuclei stained with Hoechst 33342 (400X magnification). (A) Control untreated cells; (B) SW620 cells treated with acetylmelodorinol 5 µg/ml for 24 h and (C) treated for 48 h.

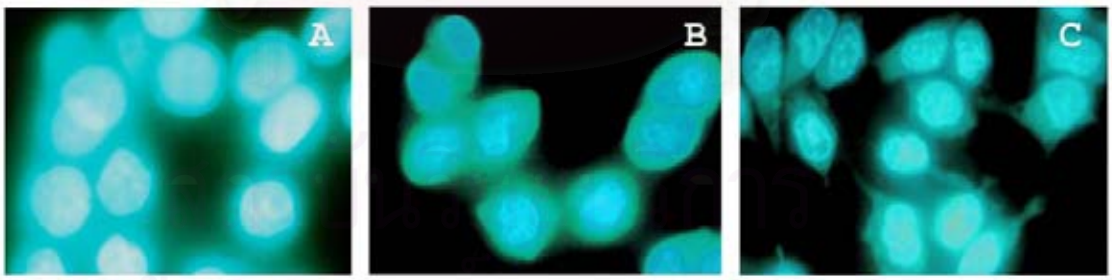

Figure 27 Fluorescence microscopic analysis of SW620 nuclei stained with Hoechst 33342 (400X magnification). (A) Control untreated cells; (B) SW620 cells treated with plaunotol 5 µg/ml for 24 h and (C) treated for 48 h.

5 Reverse transcription polymerase chain reaction (RT-PCR)

Levels of *Bcl-2* and *p53* mRNA were analyzed by RT-PCR and the housekeeping gene *GAPDH* were used as loading controls. Total extracted RNA was prepared from SW620 cells after treating with 10 μg/ml acetylmelodorinol for 0, 2, 4, 6, 8, and 10 h. Then analyzed by 1% agarose to determine quality of extracted RNA (Figure 28) showed intact RNA in lowest banded and 18S and 28S rRNA in predicted size 1.86 kb and 5.02 kb, respectively.

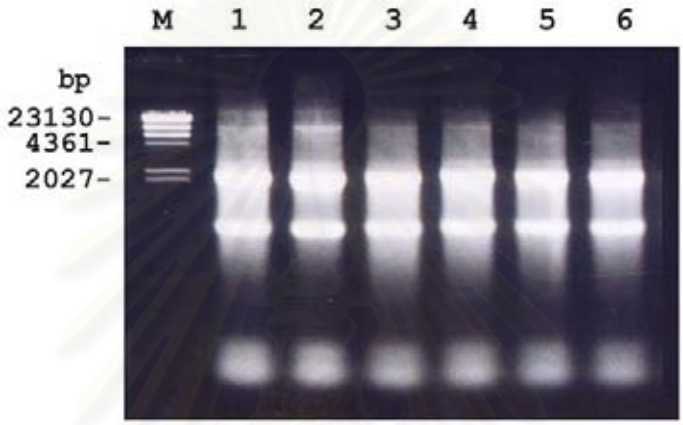

Figure 28 A 1.0% agarose gel electrophoresis showing the quality of total RNA extracted from SW620 cells treated with acetylmelodorinol (lanes 1-6) for 0, 2, 4, 6, 8, and 10 h respectively. Lane M = λ *Hind III* marker

The RT-PCR products from total RNA of acetylmelodorinol treated SW620 cells were analyzed by electrophoresis with ethidium bromide in 1.8% agarose gel. The results showed a single band of *Bcl-2* gene with a predicted size (170 bp) and *GAPDH* (452 bp) (Figure 29). The level of anti-apoptotic gene *Bcl-2* after treated with acetylmelodorinol was found to be down-regulation at 4-10 h of treatment.

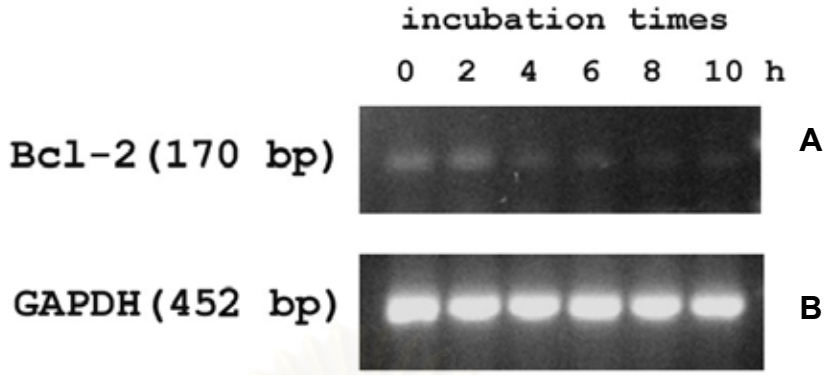

Figure 29 RT-PCR of the first strand cDNA synthesized from total RNA of SW620 treated with acetylmelodorinol for 0-10 h using primers *Bcl-2* (panel A). Positive amplification of *GAPDH* (panel B) was observed.

Quantitative image analysis of the PCR fragments was performed using the Gel Pro analyzer 3.1 program. The expression levels were calculated as the ratios of optical density of PCR products to those of *GAPDH* PCR products. The level of bcl-2 expression was significantly reduced by acetylmelodorinol treatment (Figure 30).

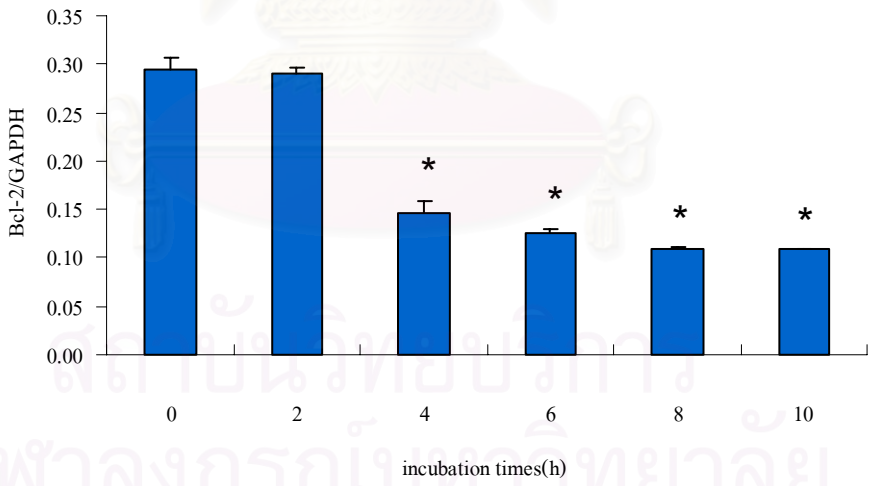

Figure 30 Comparison of the effect of acetylmelodorinol exposure for 0-10 h on *Bcl-2* mRNA levels in SW620 cells. Data are means ± SD. and level of significance from control (0 h) was determined by Student's *t*-test (*: $p < 0.01$).

The expression levels were significantly reduced after treated with acetylmelodorinol 10 μg/ml at 4, 6, 8, and 10 h of incubation, but no significant difference was found at 0 h (control) and 2 h of incubation ($p < 0.01$).

The RT-PCR products from total RNA of SW620 cells analyzed by electrophoresis with ethidium bromide in 1.8% agarose gel are shown a single band of *p53* gene with a predicted size (262 bp) and *GAPDH* (452 bp) (Figure 31).

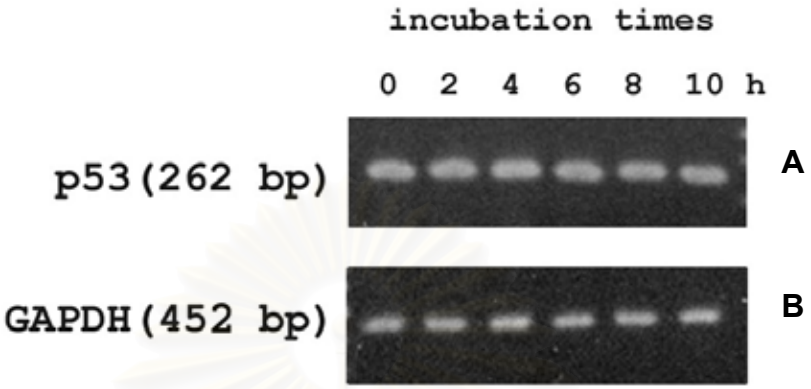

Figure 31 RT-PCR of the first strand cDNA synthesized from total RNA of SW620 treated with acetylmelodorinol for 0-10 h using primers *p53* (panel A). Positive amplification of *GAPDH* (panel B) was observed.

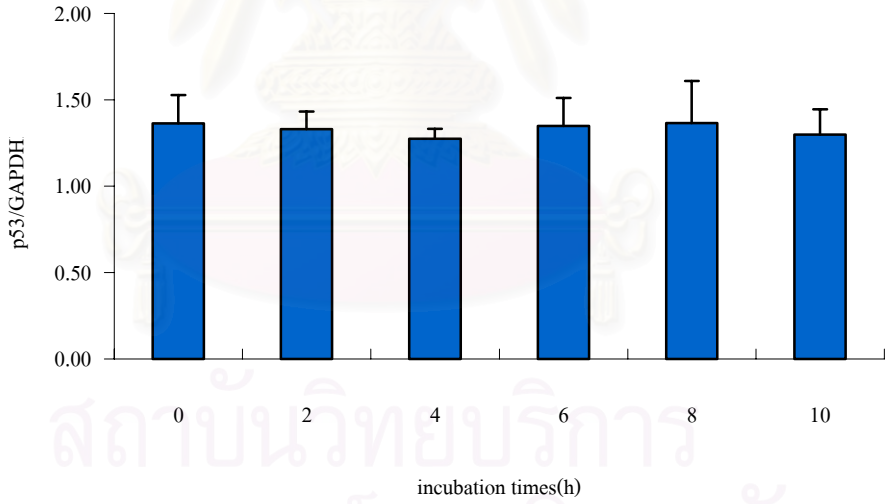

Figure 32 Comparison of the effect of acetylmelodorinol exposure for 0-10 h on *p53* mRNA levels in SW620 cells. Data are means ± SD. and level of significance from control (0 h) was determined by Student's *t*-test (*: $p < 0.01$).

When SW620 cells were treated with acetylmelodorinol, *p53* gene was expressed at high level but did not show significant difference as compared to the control group (0 h) ($p < 0.001$).

CHAPTER V

DISCUSSION

Previous studies have revealed that acetylmelodorinol possess cytotoxic effects and antitumor activity. Acetylmelodorinol was found to inhibit the proliferation of human cancer cell lines in dose-dependent manners with an IC_{50} less than 3 μg/ml (Chaichantipyuth et al., 2001). These results were consistent with our present findings that acetylmelodorinol inhibit the growth of cancer cell lines in a dose- and time-dependent manners. Our results demonstrated that at incubation times of 48 h, acetylmelodorinol and plaunotol showed statistically significant high antiproliperative effect, when compared to control untreated cells and incubation times of 12-24 h. Acetylmelodorinol showed stronger cytotoxic activity in cancer cell lines than plaunotol at 48 h of incubation. Plaunotol did not show antiproliferative effects in normal cells (CH-liver and Hs27), which was consistent with previous study reporting that plaunotol was a cytoprotective antiulcer agent (Koga et al., 1996).

To investigate whether apoptosis is involved in the cell death caused by acetylmelodorinol and plaunotol in cancer cell lines, morphological changes and DNA ladder pattern by agarose gel electrophoresis was investigated. The induction of apoptosis stimulates endonuclease that predicts double-strand DNA breaks into oligonucleosome length fragments, resulting in a typical ladder in DNA electrophoresis. Characteristics of apoptosis also include nuclear chromatin condensation, nuclear fragmentation, cytoplasmic vacuolation and condensation, extensive plasma membrane ruffling and the presence of apoptotic bodies (Raff, 1998 and Burlacu, 2003). Among cancer cell interested, only SW620 cells treated with acetylmelodorinol showed ladder pattern on agarose gel electrophoresis. These hallmark features of changes suggested that acetylmelodorinol caused apoptosis in SW620 colon cancer cell lines. When treating cells with plaunotol, ladder pattern was not observed, indicating that plaunotol did not induce cells death by apoptosis. Previously it was reported that plaunotol have bactericidal effects against *Helicobacter pylori* and that cell death was associated with lysis of bacteria (Koga et al., 1996) and led to an increase in permeability and fluidities of membrane (Koga et al., 1998). When treating with plaunotol, cells may die by cell lysis and did not affect the fragmentation of chromosomal DNA.

In addition, morphological changes of SW620 were observed by phase contrast microscope, which exhibited cytoplasmic membrane shrinkage, loss of contact with neighboring cells, membrane blebbing and apoptotic body formation. Moreover, morphological analysis of cells with Hoechst33342 staining displayed nuclear shrinkage, DNA condensation and fragmentation after treating cells with 5 μg/ml of acetylmelodorinol for 48 h.

In other situations, some features associated with apoptosis, such as non-specific internucleosomal DNA degradation, may accompany cell necrosis. Clearly, the use of a technique that based on the detection of such a feature will fail to identify the 'atypical' apoptotic or necrotic cell. Apoptotic cells exposed phosphatidyl-serine to the cell surface, which can be stained with fluorescence labeled Annexin-V (Vermes et al., 1995). To confirm the induction of apoptosis by acetylmelodorinol in BT474, KATOIII, and SW620 cells that show strong cytotoxic activity, cells were analyzed by Annexin-V-FITC staining and counter-stained with propridium iodide. The number of apoptotic cells (Annexin-V-FITC positive) and necrotic cells (propridium iodide positive) in control untreated cells at 12 h and 24 h incubation times were negligible. BT474 treated with acetylmelodorinol show 28.20% of apoptotic cells, 76.19% of necrotic cells and 26.72%of apoptotic cells and 70.90% of necrotic cells at 12 h and 24 h of incubation times, respectively. The results show that the majority cause of BT474 cells death with acetylmelodorinol was necrosis. When KATOIII cells were treated with acetylmelodorinol the cell death by apoptosis and necrosis were comparable. This result does not agree with DNA fragmentation pattern. This may be due to the concentration that trigger DNA fragmentation is higher than that has been used in this experiment. Unlike BT474 cells and KATOIII cell, when SW620 cells were treated with acetylmelodorinol, the number of apoptotic cells increased from 29.02% at 12 h to 56.38% at 24 h, while necrotic cells increased from 3.82% at 12 h to 20.18% at 24 h. This result confirmed that acetylmelodorinol induced cell death mainly via an apoptotic pathway in SW620.

Bcl-2 and *p53* are prototypic anti-apoptotic and pro-apoptotic gene, respectively. To study mRNA expression of *Bcl-2* and *p53* in the dying cells, we used competitive template reverse transcription-polymerase chain reaction (RT-PCR). The *Bcl-2* and *p53* gene has been implicated as a major player in apoptosis pathway (Dong, 2005). The regulation of *Bcl-2* at the transcriptional level seems to be a critical factor in the regulation of cell death (Burlacu, 2003) e. g. aspirin-induced apoptosis is associated with the down regulation of *Bcl-2* expression in HEC cells (Li, 2000). We compared the level of *Bcl-2* and *p53* gene after treated with

acetylmelodorinol and found that acetylmelodorinol did not affect the expression of *p53*, suggesting that regulation of apoptosis by acetylmelodorinol in SW620 cells did not depend on alteration in *p53* expression, but decreased the level of expression of *Bcl-2*.

p53 contributes to apoptosis induced by a variety of cellular stresses, including damage, oxidative stress and chemotherapeutics drugs (Steele et al., 1998). Because over 50% of all human tumors contain functionally mutant *p53* (Hollstein et al., 1996) and, *p53* mutations have been described in about 40% to 50% of colorectal carcinomas (Menzel, 2002). The role of p53 in apoptosis has led to concern that many tumors may escape p53-mediated cell death path way (Fisher, 1994). These results demonstrated that acetylmelodorinol can induce apoptosis in SW620 cells devoid of *p53*, mutation in *p53* gene may result in *p53*-independent apoptosis. Thus, acetylmelodorinol may be of utility in treating the ~50% of human cancers with mutant or absent *p53*.

These investigations suggested that acetylmelodorinol had strong antiproliferation, and induction of apoptosis in SW620 colon cancer cell lines. It indicates that acetylmelodorinol may be a potential candidate for anti-cancer drug development due to its apoptosis-dependent cytotoxic effects on SW620 colon caner cell lines. However, plaunotol show moderate antiproliferative effect on cancer cell line but show much less cytotoxicity against normal cells and not induced cell death by apoptosis.

CHAPTER VI

CONCLUSION

1. Acetylmelodorinol and plaunotol were tested for their *in vitro* cytotoxicity against four cancer cell lines (BT474, HepG2, KATOIII, and SW620) and two normal cells (CH-liver and Hs27) at various concentrations (0–10 μg/ml) for selected exposure times 12, 24, 48, and 72 h. The result indicated that at incubation times of 48 h, acetylmelodorinol and plaunotol had high antiproliperative effect, significant to control untreated cells and incubation times of 12-24 h (p<0.01).

2. The confirmation of activity at 48 h exposure time was found that acetylmelodorinol still show stronger activity for cancer cells line than plaunotol. However, plaunotol did not show antiproliferative effects in normal cells (CH-liver and Hs27).

3. Analysis of chromosomal DNA fragmentation indicated that only SW620 treated with acetylmelodorinol induced nuclear fragmentation, the formation of a DNA ladder pattern. This results indicated that acetylmelodorinol induced cell death was via an apoptotic pathway in SW620. However, no DNA fragmentation was observed in any tested cells with plaunotol therefore plaunotol did not induced any apoptotic death in any of cells tested.

4. The apoptotic and necrotic cell death was confirmed by Annexin-V-FITC staining, in control untreated cell the number of apoptotic and necrotic cells were negligible at 12 h and 24 h incubation times. When BT474 cells were treated with acetylmelodorinol, the number of cell death by necrosis was higher than that of apoptosis. On the contrary, when treated with acetylmelodorinol in KATOIII cells, the number of cells death by apoptosis and necrosis were comparable. When the incubation times was increased from 12 h to 24 h, the number of cell death by apoptosis by acetylmelodorinol treated was increase when treated SW620 cells with acetylmelodorinol increased the number of apoptotic cells when increased incubation times from 12 h to 24 h, respectively. The results show that acetylmelodorinol induced mainly cell death via an apoptotic pathway in SW620

5. Determination of morphological change by phase contrast microscopy by treated SW620 cells with acetylmelodorinol resulted in progressive morphological changes typical of apoptosis, including cells shrinkage, rounding, and detachment of cells from plate. Then, the nuclear morphological changes were observed by Hoechst 33342 staining. In contrast to untreated cells, the apoptotic nuclei of SW620 cells gave stronger blue fluorescence staining, condensed and fragmented showing the typical apoptotic bodies. However, when SW620 cells were treated with plaunotol, they did not showed morphological changes indicative of apoptosis. These results were consistent with the result from DNA fragmentation analysis.

6. Treating cells with acetylmelodorinol at various incubation times (0-10 h) did not affect the expression of *p53* genes, suggesting that regulation of apoptosis in SW620 cell may be not concerning to *p53* expression. The level of anti-apoptotic gene *Bcl-2* before and after treated with acetylmelodorinol were compared and it was found that acetylmelodorinol could down-regulated the expression of *Bcl-2*, suggesting that down-regulation of *Bcl-2* may be required for SW620 cell apoptosis induced by acetylmelodorinol.

Suggestions for the future work

- The mechanism that induced apoptosis by down-regulation of Bcl-2 by Western Blot should be investigated because the reduction in the Bcl-2 level is caused not only by the inhibition of Bcl-2 at the transcription level, but also by protein degradation.
- The other molecular mechanism leading to apoptosis by another gene e.g. Bax, Bak and Bid, which are in Bcl-2 family that indicated pro-apoptotic functions, should be investigated.
- The Caspase activity that played a pivotal role in the initiation and execution of apoptosis induced by various stimuli should be determined.
- The cells cycle in the presence of acetylmelodorinol should be investigated.

REFERENCES

Arden, N., and Betenbaugh, M.J. 2004. Life and death in mammalian cell culture: Strategies for apoptosis inhibition. TRENDS in Biotechnology 22(4): 174-180.

Boik, J. 1996. Cancer and Natural Medicine: A textbook of basic science and clinical research. Oregon Medical Press.

Burlacu, A. 2003. Regulation of apoptosis by *bcl-2* family proteins. Journal of Cellular and Molecular Medical 7(3): 249-257.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., and Mitchell, B. 1987. Evaluation of tetrazolium based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Research 47(4): 933-942.

Chaichantipyuth, C., Tiyaworanan, S., Mekaroonreung, S., Ngamrojnavanich, N., Roengsumran, S., Phuthong, S., Petsom, A., and Ishikawa, T. 2001. Oxidized heptenes from flowers of *Melodorum fruticosum*. Phytochemistry 58:1311-1315.

Chao, J.C., and Chu, C.C. 2004. Effects of *Ginkgo biloba* extract on cell proliferation and cytotoxicity in human hepatocellular carcinoma cells. World Journal of Gastroenterol 10(1): 37-41.

Chang, G.C., Hsub, S.L., Tsaia, L.R., Wub, W.J., Chend, C.Y., and Sheub. C.G. 2004. Extracellular signal-regulated kinase activation and *Bcl-2* down regulation mediate apoptosis after gemcitabine treatment partly via a *p53*-independent pathway. European Journal of Pharmacology 502: 169-183.

Davis, J.M., Navolanic, P.M., Weinstein-Oppenheimer, C.R., Steelman, S., Hu, W., Konopleva, M., Blagosklonny, M.V., and McCubrey, J.A. 2003. Raf-1 and *Bcl-2* induce distinct and common pathways that contribute to breast cancer drug resistance. Clinical Cancer Research 9:1161-1170.

Dong, M., Ma, G., Tu, W., Guo, K.J., Tian, Y.L., and Dong, Y.T. Clinicopathological significance of p53 and mdm2 protein expression in human pancreatic cancer. World Journal of Gastroenterol 11(14):2162-2165.

Enari, M., Hug, H., and Nagata, S. 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 375: 78-81.

Enari, M., Sakahira, H., Yogoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43-50.

Escribano, J. et al. 1999. The cytotoxic effect of a glycoconjugate extracted from corms of saffron plant (*Crocus sativa*) on human cell lines in culture. Planta Medica 66: 157-162.

Fadok, V. A., et al., 1992. Exposure of phosphatidyl serine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. Journal of Immunology 148:2207-2216.

Fikrat, I. A., Roberto, R. L., Alejandro, G. C., Felix, A. F., and Javier, E. A. 2001. Cytotoxic effect of three arsenic compounds in Hela human tumor and bacterial cells. Mutation Research 493:31-38.

Fisher, D.E. 1994. Apoptosis in cancer therapy: crossing the threshold. Cell. 78: 539-542.

Freshney, R.I. 2000. Culture of animal cell: A manual of basic technique. Wiley-Liss, Inc. New York.

Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R. and Harris, C.C. 1996. Somatic point mutations in the *p53* gene of human tumors and cell lines: updated compilation. Nucleic Acids Research. 24: 141-146.

Homburg, C.H.E., et al. 1995. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. Blood 85: 532.

Huang, D.S., Shen, K.Z., Wei, J.F., Liang, T.B., Zheng, S.S., and Xie, H.Y. 2005. Specific COX-2 inhibitor NS398 induces apoptosis in human liver cancer cell lines HepG2 through *Bcl-2*. World Journal of Gastroenterology 11(2): 204-207.

Itharat, A., Houghton, P.J., Eno-Amooquayec, E., Burke c, P.J., Sampsonb, J.H., and Raman, A. 2004. In vitro cytotoxic activity of Thai medicinal plants used traditionally to treated cancer. Journal of Ethnopharmacology 90(12): 2759-2763.

Jane, C.C., and Chia, C.C. 2004. Effects of *Ginkgo biloba* extract on cell proliferation and cytotoxicity in human hepatocellular carcinoma cells. World Journal of Gastroenterology 10(1):37-41.

Jarvis, W.A., Turner A.J., Povirk, L.F., Trayor, R.S., and Grant, S. 1994. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibition of protein kinase C. Cancer Research 54: 1707-1714.

Jiao, H., Ye, P., and Zhao, B. 2003. Protective effects of green tea polyphenols on human HepG2 cells against oxidative damage of fenofibrate. Free Radical Biology & Medicine 55(9): 1121-1128.

Jung, J. H., Chang, C. J., Smith, D. L., McLaughlin, J. L., Pummangura, S., Chaichantipyuth, C., and Patarapanich, C. 1991. Additional bioactive heptenes from *Melodorum fruticosum*. Journal of Natural Products 54 (2): 500-505.

Jung, J. H., Pummangura, S., Chaichantipyuth, C., patarapanich, C., Fanwick, P. E., Chang, C.-J., and McLaughlin, J. L. 1990a. New bioactive heptenes from *Melodorum friticosum* (Annonaceae). Tetrahedron 46 (15): 5043-5054.

Jung, J. H., Pummangura, S., Chaichantipyuth, C., patarapanich, C., and McLaughlin, J. L. 1990b. Bioactive constituents of *Melodorum fruticosum*. Phytochemistry 29(5): 1667-1670.

Kim, J.Y, Lee, K.W., Kim, S.H., Wee, J.J., Kim, Y.S., and Lee, H.J. 2002. Inhibitory effect of tumor cell proliferation and induction of G_2/M cell cycle arrest by panaxytriol. Planta Medica 68:119-122.

Koga, T., Kawada, H., Utsui, Y., Domon, H., Ishii, C., and Yasuda, H. 1996. Bactericidal effect of plaunotol, a cytoprotective antiulcer agent against Helicobacter pylori. Journal of antimicrobial Chemotherapy 38: 387-397.

Koga, T., Watanabe, H., Kawada, H., Takahashi, K., Utsui, Y., Doman, H., Ishii, C., Narita, T., and Yasuda, H. 1998. Interactions of plaunotol with bacterial membranes. Journal of Antimicrobial Chemotherapy 42:133-140.

Kogen, H., Tago, K., Arai, M., Minami, E., Masuda, K., and Akiyama, T. 1999. A highly stereoselective synthesis of plaunotol and its thiourea derivatives as potent antibacterial agents against *Helicobacter pylori*. Bioorganic Medical Chemistry Letters 9(10):1347-1350.

Lau, C.B.S., et al. 2004. Cytotoxic activities of *Coriolus cersicolor* (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis. Life Sciences 75:797-808.

Maliqueo, M.A., et al. 2004. Potential action of endrostenedione on the proliferation and apoptosis of stromal endometrial cells. Reproductive Biology and Endocrinology 2:81.

Menzel, T., Schauber, J., Kreth, F., Kudlich, T., Melcher, R., Gostner, A., Scheppach, W., and Luhrs, H. 2002. Butyrate and aspirin in combination have an enchanced effect on apoptosis in human colorectal cancer cells. European Journal of Cancer Preventation 11(2): 271-281.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65:55-63.

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., and Neungton, N. 2004. Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell lines. Journal of Ethanopharmacology 90:161-166.

Nakahara, T., et al. 2003. Acute and chronic effects of alcohol exposure on skeletal muscle c-myc, *p53*, and *bcl-2* mRNA expression. American Journal of Physiological Endocrinological Metabolism 285: E1273-E1281.

Palaga, T., Kataoka, T., and Nagai, K. 2004. Extracellular ATP inhibits apoptosis and maintains cell viability by inducing autocrine production of interleukin-4 in a myeloid progenitor cell line. International Immunopharmacology 4: 953-961.

Raff, M., 1998. Cell suicide for beginners. Nature 396(6707): 119-22.

Ramoneda, B.M., and Pe′rez-To′mas, R., 2002. Activation of protein kinase C for protection of cell against apoptosis induced by the immunosuppressor prodigiosin. Biochemical Pharmacology 63: 463-469.

Roengsumran, S., Petsom, A., Kuptiyanuwat, N., Vilaivan, T., Ngamrojnavanich, N., Chaichantipyuth, C., and Phuthong, S. 2001. Cytotoxic labdane diterpenoids from *Croton oblongifolius*. Phytochemistry 56:103-107.

Ruiz, C.R., Rubledo, G., Cano, E., Redondo, J.M., and Rivas, A.L. 2003. Characterization of *p53*-mediated up-regulation of CD95 gene expression upon genotoxic treatment in human breast cancer cells. The Journal of Biological Chemistry 27(34): 31667-31675.

Saralamp, P. ed. 1992. Medicinal plants in Siri Ruckhachati Garden. Bangkok: Amarin Printing Group.

Sambrook, Fritsch, Maniatis. 1989. Molecular cloning. Cold Spring Harbor Laboratory Press.

Shigekazu, N., Golstein, P., 1995. The Fas death factor. Science 267(5203): 1449-56.

Shigekazu, N., 1997. Apoptosis by death factor. Cell 88: 355-365.

Shiratori, K., Watanabe, S., and Takeuchi, T. 1993. Role of endogeneous prostaglandins insecretin- and plaunotol-induced inhibition of gastric acid secretion in the rat. American Journal of Gastroenterology 88(1): 84-89.

Steele, R.J., Thompson, A.M., Hall, P.A. and Lane, D.P. 1998. The p53 tumor suppressor gene. British Journal of Surgery. 85: 1460–1467.

Studzinski, G.P. 1995. Cell Growth and Apoptosis. IRL press, New York.

Syng-ai, C., Kumari, A.L., and Khar, A. 2004. Effect of curcumin on normal and tumor cells: Role of glutathione and *bcl-2*. Molecular Cancer Therapeutics 3(9): 1101-1108.

Tago, K., Miami, E., Masuda, K., Akiyama, T., and Kogen, H. 2001. Synthesis of plaunotol derivatives and their antibacterial activities against *Helicobacter pylori*. Bioorganic & Medicinal Chemistry 9:1781-1791.

Tiyawornan, S. 1998 Chemical Constituents of *Melodorum fruticosum* Flowers. Master's thesis, Department of Pharmacognosy, Graduated School, Chulalongkron University.

Tsujimoto, Y., 1998. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria. Genes to Cells 3: 696-707.

Tuchinda, P., Udchachon, J., Reutrakul, V., Santisuk, T., Taylor, W. C., Farnsworth, N.R., Pezzuto, J. M., and Kingkorn, A. D. 1991. Bioactive butanolides from *Melodorum fruticosum.* Phytochemistry 30(8): 2685-268.

Twentyman, P. R. and Luscombe, M. 1987. British Journal of Cancer 56:279-285.

Vermes, I., et al. 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin-V. Journal of Immunological Methods 184: 39.

Wang, X., et al. 2000. Cytotoxic effect of gossypol on colon carcinoma cells. Life Sciences 67: 2663-2671.

Wang, X. Z., et al. 2004. Effects of platelet-derived growth factor and interleukin-10 and *Fas/Fas*-ligand and *Bcl-2/Bax* mRNA expression in rat hepatic stellate cells *in vitro*. World Journal of Gastroenterology 10(18): 2706-2710.

Wu, Z., Lu, Y., Wang, B., Liu, C., and Wang, Z. 2003. Effects of bile acids on proliferation and ultrastructural alteration of pancreatic cancer cell lines. World Journal of Gastroenterol 9(12): 2759-2763.

Wyllie, A.H., 1999. Apoptosis and cell proliferation. Roche Molecular Biochemicals. Germany.

Yang, L.L., Lec, C.Y., and Yen, K.Y. 2000. Induction of apoptosis by hydrolysable tannin from *Eugenia jambos* L. on human leukemia cells. Cancer Letters 157:65-75.

Zhang, Y., Wu, L., Tashiro, S., Onodera, S., and Ikejima, T. 2004. Evodiamine induces tumor cell death through different pathways: Apoptosis and necrosis. Acta Pharmacologica Sinica 25(1): 83-89.

Zhao, Y., Cao, J., Ma, H., and Liu, J. 1997. Apoptosis induced by tea polyphenols in HL-60 cells. Cancer Letters 121: 163-167.

APPENDICES

APPENDIX A

REAGENT AND BUFFER PREPARATIONS

1. 0.1 M Phosphate buffer saline (Ca^{++}, Mg^{++} free)

- KH_2PO_4 0.21 g
- Na_2HPO_4 0.72 g
- NaCl 7.45 g
- KCl 0.21 g

Adjusted pH to 7.4 with 1 M HCl or 1 M NaOH and adjusted volume to 1 liter with distilled water.

2. 0.1 M Glycine buffer pH 10.0

- Glycine 7.5 g

Adjusted pH to 10.0 with 1 M HCl or 1 M NaOH and adjusted volume to 1 liter with distilled water.

3. 1 M Tris-HCl buffer, pH 8.0

- Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 7.4 with 1 M HCl or 1 M NaOH and adjusted volume to 1 liter with distilled water.

4. 1 M $MgCl_2$

Dissolve 203.3 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of H_2O. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

5. 3 M Sodium acetate (pH 5.2)

Dissolve 408.1 g of sodium acetate $\cdot 3H_2O$ in 800 ml of H_2O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

6. Ethidium bromide 10 mg/ml

Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4 $^{\circ}C$

7. TE buffer pH 7.4

- 10 mM Tris·Cl (pH 7.4)
- 1 mM EDTA (pH 8.0)

8. TE buffer pH 7.6

- 10 mM Tris·Cl (pH 7.6)
- 1 mM EDTA (pH 8.0)

9. TE buffer pH 8.0

- 10 mM Tris·Cl (pH 8.0)
- 1 mM EDTA (pH 8.0)

10. Tris-Borate (TBE)

Working solution

- 0.089 M Tris-borate
- 0.089 Boric acid
- 0.002 M EDTA

Concentrated stock solution (5x)

Per liter:

- Tris base 54 g
- Boric acid 27.5 g
- 0.5 M EDTA (pH 8.0) 20 ml

11. Equilibration of Phenol

Before use, phenol must be equilibrated to a pH of > 7.8 because the DNA partitions into the organic phase at acid pH. Wear gloves, full-face protection, and a lab coat when carrying out the procedure.

1) Store liquefied phenol at -20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1 %. This compound is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.

2) To the melted phenol, add an equal volume of buffer (usually 0.5 M Tris·Cl [pH 8.0] at room temperature). Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer, and when the phase is separated, aspirated as much as possible of the upper (aqueous) phase using glass pipette.

3) Add an equal volume of 0.1 M Tris·Cl (pH 8.0) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer and remove the upper phase as describes in step 2. Repeat until the pH of phenolic phase is > 7.8 (as measured with pH paper)

4) After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 M Tris·Cl (pH 8.0) containing 0.2% β-mercaptoethanol. The phenol solution may be stored in this form under 100 mM Tris·Cl (pH 8.0) in a light-tight bottle at 4 °C for periods of up to 1 month.

12. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform: isoamyl alcohol (24:1) is frequency used to remove proteins from preparation of nucleic acids. The chloroform denatures protein and facilitates the separation of the aqueous and organic phases,

and the isoamyl alcohol reduces foaming during extraction. Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol: isoamyl alcohol mixture may be stored under 100 m Tris·Cl (pH 8.0) in a light-tight bottle at 4 °C for period of up to 1 month.

13. Glycerol (70% v/v)

Dilute 7 volume of molecular biology grade glycerol in 3 volume of sterile pure H_2O. Sterilized the solution by passing it through a preprinted 0.22 μm filter. Store at 4 °C.

Biography

Miss Pornticha Loluphiman was born on September 11, 1979 in Nakornpathom. She graduated with the degree of Bachelor of Science from the Department of Biotechnology, faculty of Industrial Technology at Silpakorn University in 2000. In 2001, she has studied to Master degree of Science in Biotechnology at Chulalongkron University.