REGULATION OF APOPTOTIC EFFECTS OF ERYTHROCARPINE E, A NEW CYTOTOXIC LIMONOID EXTRACTED FROM *Chisocheton erythrocarpus*

NORLIZA BT SHAH JEHAN MUTTIAH

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR MALAYSIA
2010

REGULATION OF APOPTOTIC EFFECTS OF ERYTHROCARPINE E, A NEW CYTOTOXIC LIMONOID EXTRACTED FROM *Chisocheton erythrocarpus*

NORLIZA BT SHAH JEHAN MUTTIAH

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR MALAYSIA
2010

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisor, Associate Professor Dr. Noor Hasima Nagoor, for all the beneficial guidance offered, not excluding the invaluable advices, dedication, patience and confidence throughout the course of this study. I also would like to convey my sincere thank you to Professor Dr. Khalijah Awang and Mr. Chong for providing me with the compound and its chemistry information used in my study.

To Amma and the whole family, thank you very much for your love, concern, support and trust. I would like to express my special appreciation to my late father, Shah Jehan Muttiah because he is the person who triggered my interest to further my study to a Master level.

I also wish to thank my lab mates Mr.Lionel In, Ms. Norahayu Othman, Ms. Juliana Abu Bakar, Ms. Yap Seow Hui, Mr. Jeffry Yap Lim Hui, Mr. Phuah Neoh Hun and Mr. Tasyriq Che Omar. Not forgetting my friends, Ms. Sharon Leena Sarlus and Ms. Enisha Kalaimani, your kindness and our sweet memories will forever be cherished in my heart.

To Ranjitha Chandran and Kash, millions of thanks for your continous support and for sharing the laughters and sorrows. Without your encouragement I will never reach this level.

Last but not least, I would like to thank Dr. Eswary Thirthagiri and Dr. Ng Ching Ching who helped me with the completion of this thesis.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

^{1}H	Proton NMR
^{13}C	13-carbon NMR
AFP	α-fetoprotein
Annexin V-FITC	Annexin V Fluorescein conjugates
APAF- 1	Apoptotic protease-activating factor 1
Bcl-2	B-cell lymphoma 2
BEBM	Bronchial Epithelial Basal Medium
BH	Bcl-2 homology domains
Bmf	Bcl-2 modifying factor
BSA	Bovine Serum Albumin
CAD/CPAN	Caspases activated DNase
Calcein AM	Acetomethoxy derivate of calcein
Caski	Human Epidermoid Cervical carcinoma cells
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CEB4	Erythrocarpine E
Chk	Checkpoint kinase
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
Cyto C	Cytochrome c
dATP	Deoxyadenosine triphosphate
DD	Death domains

DED	Death effector domains
DISC	Death-inducing-signalling-complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	A caspase target
DR5	Transmembrane death domain containing receptor for TRAIL
DR5/KILLER	Tumor necrosis factor receptor superfamily
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
EthD-1	Ethidium homodimer - 1
FADD	Fas-Associated protein with Death Domain
Fas	Type II transmembrane protein
FasL	Fas ligand
G_0	GAP 0
G_0/G_1	G_0/G_1 phase transition
G_1	GAP 1
G_1/S	G_1/S phase transition
G_2	GAP 2
G_2/M	G_2/M phase transition
H	Hydrogen

HBV	Human hepatitis virus
HCC	Hepatocellular carcinoma
HCT116	Human colon cancer cells
HepG2	Human Hepatocyte Liver carcinoma cells
HPV	Human Papillomavirus
HPV16	Human Papillomavirus type 16
HPV18	Human Papillomavirus type 16
HRP	Horseradish peroxidase
HSC2	Human Oral squamous carcinoma cells
$HSC4^{COX2-}$	Human Oral squamous carcinoma cells
Hz	Hertz
IR	Infrared red
kDa	Kilo Dalton
M	Mitosis
MCF7	Human Breast adenocarcinoma cells
Mdm2	Murin double mouse 2
METase	Methioninase
ml	millilitre
mRNAs	Messenger ribonucleic acid
MS	Mass spectrum
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide

NCI	National Cancer Institute
NCR	National Cancer Registry
NER	Nuclear Extraction Reagent
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHBE	Normal human bronchial epithelial cells
NMR	Nuclear Magnetic Resonance
NK	Natural killer
Noxa	Pro-apoptotic member of the Bcl-2 protein family
p21	Cyclin-dependent kinase inhibitor 1A
p53	Phosphoprotein 53
PARP	Poly ADP ribose polymerase
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PIASg	Protein inhibitor of activated STAT
PMSF	Phenylmethanesulphonyl fluoride
pRB	Phospho retinoblastoma
PRIMA	p53 reactivation and induction of massive apoptosis
Pro-IL-18	Pro-inflammatory cytokines
Pro-IL-1ß	Pro-inflammatory cytokines
PGs	prostaglandins
PS	Phosphatidylserine

PUMA	p53 up-regulated modulator of apoptosis
RPM	Rotation per minute
RPMI 1640	Roswell Park Memorial Institute
S	Synthesis phase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser15	Serine 15
Ser26	Serine 26
SPIKE	Small protein with inherent killing effect
STAT	Signal Transducers and Activators of Transcription protein
Sub-G1	Sub GAP
T cells	T lymphocytes
tBID	Truncated Bid
TBP	TATA-binding proteins
TBST	Tris-buffered saline-0.05% (v/v) Tween20
T_C	Cytotoxic T cells
TNF	Tumor necrosis factor
TNF-R	Tumour necrosis factor receptor
TNF-α	Tumor necrosis factor-alpha
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein

TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

ABSTRACT

The aim of this study is to determine the cytotoxic and apoptotic effects of erythrocarpine E (CEB4), a new limonoid extracted from *Chisocheton erythrocarpus*. MTT assay, Live/Dead® Viability/Cytotoxicity assay, cell cycle analysis, annexin V analysis, PARP cleavage analysis, DNA fragmentation assay, sandwich ELISA assay and Western blot analysis were performed on five CEB4 treated cancer cell lines; $HSC4^{COX2-}$ and HSC2 (oral), CaSki (cervical), HepG2 (liver), MCF7 (breast) and NHBE (normal human bronchial epithelial cell line). CEB4 treated $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 cells demonstrated a cytotoxic effect and inhibited cell proliferation in a dose and time dependent manner. CEB4 had minimal effect on NHBE. Cell cycle analysis of treated cancer cells detected a hypodiploid sub-G1 peak in all the cancer cell lines expect in the HSC2 cells confirming CEB4 induced apoptosis. The shift in percentage in the G_0/G_1 phase in $HSC4^{COX2-}$, CaSki, and HepG2 cells suggested a possible G_0/G_1 arrest of the cancer cells upon treatment with CEB4 at 12 hours. Annexin V analysis by flow cytometry, PARP cleavage analysis, and DNA fragmentation results showed that CEB4 induces apoptosis in CEB treated cancer cell lines. The results of the ELISA analysis suggest that CEB4 induced apoptosis may be mediated by *p53*, a tumour suppressor gene. Western blot results demonstrated that CEB4 induce growth arrest and apoptosis in $HSC4^{COX2-}$ cells appeared to be mediated through regulation of p53 signalling pathway as there is an increase in expression of phosphorylated p53 and decrease in expression of the p53 inhibitor, Mdm2. CEB4 up-regulates pro-apoptotic gene, *Bax* and at the same time down-regulate the anti-apoptotic gene, *Bcl-2*. It also activates activator caspase 9 and executioner caspase 3, which are downstream molecules to induce apoptosis. These results demonstrated the cytotoxic and apoptotic ability of this compound and suggest its potential use as a cancer chemopreventive agent.

ABSTRAK

Kajian ini telah di jalankan untuk menyiasat sama ada erythrocarpine E (CEB4), ekstrak baru daripada *Chisocheton erythrocarpus*, mempunyai kesitotoksikan dan mekanisma kematian sel (apoptosis) ke atas lima jenis sel-sel kanser, mulut ($HSC4^{COX2-}$ dan HSC2), servik (CaSki), hati (HepG2), payu dara (MCF7) dan sel normal dari epitelia bronkus manusia (NHBE). Kesan ketoksikan dan mekanisma kematian sel ditentukan melalui cherakin MTT, asei Live/Death, analisa kitaran sel, analisa annexin V, analisa PARP, asei DNA fragmentasi, assei sandwich ELISA dan asei Western blot. CEB4 mempunyai kesan sitotoksik keatas $HSC4^{COX2-}$, HSC2, CaSki, HepG2 dan MCF7 serta menghalang proliferasi sel bergantung pada dose dan masa. CEB4 memberi kesan minima pada epitelia bronkus manusia normal, NHBE. Analisis kitaran sel sel kanser kecuali sel HSC2 menunjukkan puncak sub-G1 di mana mengesahkan apoptosis oleh CEB4 keatas sel sel kanser ini. Pengurangan sel sel kanser di fasa G_0/G_1 pada $HSC4^{COX2-}$, CaSki, dan HepG2 selepas rawatan dengan CEB4 selama 12 jam mencadangkan kemungkinan CEB4 menghalang proliferasi sel di fasa G_0/G_1. Analisa annexin V, analisa PARP dan asei DNA fragmentasi menunjukkan CEB4 menginduksi apoptosis keatas semua sel. Analisa ELISA p53 menunjukkan CEB4 mungkin menyebabkan kematian sel-sel kanser yang dimediasi oleh p53 suatu gene □tumour suppressor'. Analisa Western menunjukkan kematian keatas sel kanser mulut, HSC4 $^{COX2-}$ telah dimediasi melalui regulasi laluan isyarat p53 kerana terdapat penambahan dalam ekspresi posforilasi p53 dan penurunan dalam ekspresi penghalang p53, Mdm2. CEB4 meningkatkan regulasi gen pro-apoptotik *Bax,* dan pada masa yang sama menurunkan regulasi gen anti-apototik, *Bcl-2*. Ia juga mengaktifkan kaspas pengaktifan 9 dan kaspas eksekusi 3, iaitu molekul protein yang terlibat di akhir mekanisma kematian sel. Keputusan ini membuktikan yang sitotoksik dan kemampuan apoptotik sebatian ini menyarankan potensinya digunakan sebagai agen rawatan kanser.

CHAPTER 1.0: INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer

According to the National Cancer Institute (NCI), cancer is a term used for a group of diseases in which cells in a part of the body start to grow out of control form a mass of tissue. Transformed cells are able to invade other tissues. Cancer cells often travel to other parts of the body, where they begin to grow and form new tumors that replace normal tissue. This process is called metastasis. There are more than 100 different types of cancer and the name of most cancers comes from the name of the organ or type of cell that the cancer started to arise from or also known as the primary site.

The main categories of cancer include:

- **Carcinoma** - cancer that begins in the skin or in tissues that line or cover internal organs.
- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to produced and enter the blood.
- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system.
- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord.

There are several views which explain the mechanisms of how cancers arise and two interrelated yet contrasting views will be discussed here. One view gives the proposition for the formation of cancers due to the rare simultaneous acquisition of two interdependent conditions on the same cell at the same time. These conditions are deregulated cell proliferation and suppressed apoptosis. Having either one condition will not be sufficient to form cancer because deregulated proliferation alone induces cell death. On the other hand, suppressed apoptosis without deregulated proliferation would not expand uncontrollably. Therefore, the combination of these two conditions forms a sort of a basic foundation for all neoplasms to arise (Evan and Littlewood, 1998).

The second view states that most types of human cancer have been suggested to have acquired capabilities or traits through a multistep process. The process alters the genome of cancer cells at multiple sites with changes such as point mutations to even changes in chromosome complement and each alteration would confer one or another type of growth advantage which is similar to Darwinian evolution (Hanahan and Weinberg, 2000).

Essentially, there are six acquired capabilities from tumorigenesis that give a cancer cell its genotype and they are: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory (antigrowth) signals, 3) escape from programmed cell death (apoptosis), 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (Hanahan and Weinberg, 2000).Acquisition of each of these six capabilities during tumorigenesis represents the breakdown of an intrinsic anticancer defence mechanism in cells and tissues. The particular sequence where these capabilities are acquired varies widely whether among same or different tumour

types. The mutations that occur in oncogenes or tumour suppressor genes are also variable where it can either be early in some tumorigenesis pathways or late in others (Hanahan and Weinberg, 2000).

However, both views state the importance of acquiring certain conditions or capabilities before a normal somatic cell has the selective advantage to transform into a cancerous cell and overcome the multiple anticancer defences (Hanahan and Weinberg, 2000).This implies that the probability of getting cancer is very low and yet we see cases of it happening very often. The reason for such a 'paradox' might be due to the "wear away" of mechanisms that limit somatic cell proliferation through prolonged and random accumulation of multiple mutations (Evan and Littlewood, 1998).

1.2 Cancer statistics

Cancer can affect everybody and is considered the highest killer after cardiovascular diseases (Chuah *et al.*, 2006). Kamangar *et al.,* 2006 reported a worldwide estimation of 11 million cancer incidences, 7 million cancer deaths and about 25 million individuals were living with cancer. Cancer of lung, stomach, colon and rectum, liver, and oesophagus are seen as being of the highest incidence, while cancer of prostate, female breast, uterine and cervix are common sex-specific cancer.

A total of 21,464 cancer cases comprising 9,400 males and 12,064 females were diagnosed among Malaysians in Peninsular Malaysia according to the Second Report of the National Cancer Registry (NCR) in 2003.Cancer can occur at all ages. The median age at diagnosis for cancer in Malaysia for males and females was 59 year old and 53

year old, respectively in 2003. Leukemia, brain cancer, lymphoma, connective tissue cancer and kidney cancer are the five top incidences of cancers in children of 0-14 years old (Lim and Halimah, 2004).

In the group of young adults of 15-49 years old, the common cancers were nasopharynx, leukemia, lymphoma, lung, colon and rectum in men, and cancers of the breast, cervix, ovary, uterus, thyroid gland and leukemia in women. In the group of people 50 years old and above, cancers of the lung, colon, rectum, nasopharynx, prostate and stomach were common among men, while cancers of the breast, cervix, colon, uterus, lung and rectum occurred frequently in women (Lim and Halimah, 2004).

1.2.1 Oral cancer

According to the World Health Organization (WHO), 267,000 cases of oral cancers were newly diagnosed worldwide, around 40% (108,843 cases) occurred in India, Pakistan, Bangladesh, and Sri Lanka. South Asia has almost twice incidence and mortality rates of oral cancer than global rates (Ahluwalia, 2005). In Peninsular Malaysia, the NCR reported that for the year 2002, oral cancer was ranked 21st and 16th most common cancer among males and females respectively. However, oral cancer incidence has increased to 19th for males, and 16th for females in 2003 (Lim and Halimah, 2004).

A nationwide prevalence study on oral mucosal lesions were carried out in 1993/4 and reported that there was a variation seen in the occurrence of oral pre-malignancy among the ethnic groups. The Indians, followed by the indigenous people of Sabah and Sarawak were identified as groups with higher risk for oral cancer and pre-cancer. It has been observed that both of these ethnic groups chewed betel quid.

1.2.2 Cervical cancer

Squamous cell carcinoma and adenocarcinoma are the two main types of cervical cancer where 80 to 90% of them are of the squamous cell type while the adenocarcinoma type makes up the remaining 10 to 20%. There is also a less common type of cervical cancer where it has features of both mixed squamous cell carcinomas and adenocarcinomas (Syrjanen, 1989).

The single most important cause of cervical cancer would be due to infection from a virus called Human Papillomavirus (HPV), and about 95% of cervical cancers have been found to be HPV positive (Syrjanen, 1989). Studies found that the two oncogenes that are capable of inducing epithelial cell immortalization in culture alone, and increase cellular transformation with other oncogenes are the E6 and E7. Both genes are found in high-risk HPV strains such as HPV16 and HPV18 (Pecoraro *et al.,* 1989). The E6 and E7 oncogenes work to promote cell transformation by binding to two important tumour suppressor genes p53 and pRB respectively and disrupting their normal cellular function (Scheffner *et al.*, 1990).

The estimated number of new cases and deaths due to cervical cancer in the US according to the NCI registry in 2008 was 11,070 and 3,870 respectively while in the UK there are 2,800 new cases each year. In Malaysia, cervical cancer is the second most frequent cancer being diagnosed in females at 12.9% ((Lim and Halimah, 2004).

1.2.3 Breast cancer

Breast cancer is the second most common cancer and the most common cancer among women worldwide, with an estimated 1,152,161 new cases and 411,093 deaths per year (Kamangar *et al.*, 2006). Hormones, exposures, family history, alcohol consumption, early menarche, late menopause, low parity and postmenopausal obesity are established risk factors for breast cancer (Kolonel *et al.*, 2004).

Lim and Halimah, 2004 reported that breast cancer was the most commonly diagnosed type of cancer among females in Peninsular Malaysia, with prevalence of 3,738 female breast cancer cases with 31.0% new cases in the year 2003. Breast cancer was the most common cancer in all ethnic groups and all age groups in females starting from the age of 15 years.

1.2.4 Liver cancer

Primary liver cancers are uncommon among people in the Western countries but are more common in Africans and Asians, and it should not be confused with secondary liver cancer which is a metastasis of a cancer from another part of the body. Most primary liver cancer starts in liver cells called hepatocytes and this type of cancer is called hepatocellular carcinoma or malignant carcinoma while children may develop childhood hepatocellular carcinoma.

The secondary sites that liver cancer usually spread to would be the lymph nodes, lungs and bones. In the US, the estimated number of new cases and deaths from liver and intrahepatic bile duct cancer are 21,370 and 18,410 respectively in the NCI registry, 2008 while in the UK, 3,120 people were diagnosed with liver cancer in 2005 accounting for only 1% of all cancers diagnosed. In Malaysia, liver cancer accounts for 4% of all cancers in males and in females 1.3% of all cancers (Lim and Halimah, 2004).

1.3 Cancer cell lines

1.3.1 Oral HSC4^{COX2-} and HSC2 cancer cell lines

The cell lines used to represent oral cancers in this study are HSC4^{COX2-} and HSC2. There was no additional information on the two cell lines in the literature due to lack of characterization work. However, the difference between the two cell lines is that HSC2 has constitutively high COX-2 gene activity while HSC4^{COX2-} only expressed normal levels (Akita *et al.,* 2006). Cyclooxygenase-2 (COX-2) is an inducible enzyme that regulates prostaglandin synthesis and is overexpressed at sites of inflammation and in several epithelial cancers (figure1.1). COX-2 is involved in the regulation of apoptosis, angiogenesis, and tumour cell invasiveness, which appear to contribute to its effects on tumorigenesis.

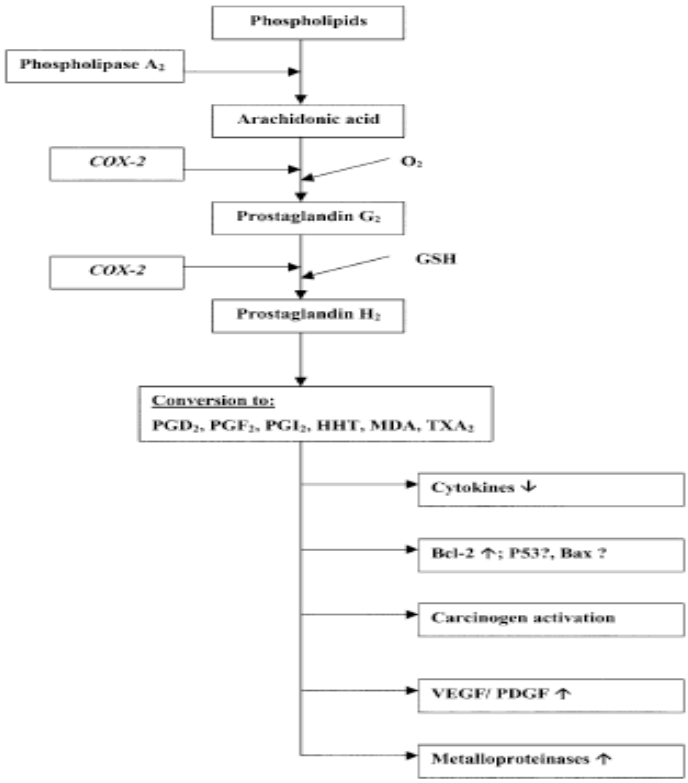


Figure 1.1: Biochemical pathways catalyzed by COX-2. (Adapted from Cyclooxygenase-2: a novel target for cancer chemotherapy. Dempke *et al.,* 2001)

1.3.2 Cervical CaSki cancer cell line

CaSki cancer cell lines have epithelial-like morphology. It was extracted from a 40 year old Caucasian female with an epidermoid carcinoma of the cervix metastatic to the small bowel mesentery. Almost all of the cervical cancers are caused by the infection of double-stranded DNA viruses, human papillomaviruses (HPV) on epithelial cells of the skin and the anogenital or oropharyngeal mucosa. The most common type of human papillomaviruses (HPVs) are HPV types 16, 18, 31, and 33 which encode two oncoproteins, E6 - early protein 6 and E7 - early protein 7 (Yee *et al*., 1985).

Both oncoproteins play major roles in contributing to the carcinogenic process and are capable of altering proliferation and apoptosis, as well as inducing immortalization of primary human keratinocytes. These proteins are capable of interacting and facilitating the proteolytic degradation of cellular proteins that regulates the cell cycle clock and apoptosis such as p53 and pRb, respectively, which are potential mechanisms by which these viral proteins induce tumours (Wu *et al*., 2006).

1.3.3 Breast MCF7 cancer cell line

The cancer cell line used to represent breast cancer in this study was MCF7 which was derived from a pleural effusion discovered after the removal of nodules in the chest wall of a 69 year-old Caucasian woman in 1970 (Dickson *et al*., 1986).

Some of the characteristics of the cell line would be the expression of E-cadherin (Hiraguri *et al*., 1998), epidermal growth factor receptor (Biscardi *et al*., 1998), estrogen receptor (Hall *et al*., 1990), and progesterone receptor (Sutherland *et al*., 1988). In addition, the cell line is tested negative for expression of basic fibroblast growth factor (Li and Shipley, 1998) and vimentin (Sommers *et al*., 1989).

1.3.4 Liver HepG2 cancer cell line

HepG2 is a human liver carcinoma cell line that was derived from the liver biopsy of a 15 year old Caucasian male with a well differentiated hepatocellular carcinoma (Aden *et al.*, 1979). HepG2 is an aneuploid cell line with a modal number of 55 chromosomes which range from 50-60 chromosomes. HBV surface antigens have not been detected in HepG2 (Kim and Kim, 2004).

Maruyama *et al.,* 2007 reported that the expression levels of albumin and α-fetoprotein (AFP) mRNAs remain unchanged, while the levels of expression of cytochrome P450 (CYP) genes such as CYP3A4, CYP3A5, and CYP3A7 mRNAs decreased gradually in HepG2. The mRNA expression of major CYP isoforms including CYP3As such as CYP1A2, CYP2A6, CYP2B6, CYP2C (2C9 and 2C19), CYP2D6, and CYP2E1 can be detected in HepG2 cells.

1.4 Cancer and apoptosis

Two major mechanisms of cell death are apoptosis and necrosis. In apoptosis, or programmed cell death, the cells undergo death to control cell proliferation in response to DNA damage (Ghobrial *et al.,* 2005).

The term apoptosis is based on the morphological characteristics of the dying cells, which include cellular shrinkage, membrane blebbing and eventually fragmentation into membrane bound apoptotic bodies (Figure 1.2). During apoptosis, the cell membrane loses its asymmetry, and phosphatidylserine (PS) becomes exposed on the cell surface. This PS exposure act as signal for macrophages, which can mediate the effective clearance of apoptotic cell. This type of cell death does not trigger inflammation (de Bruin *et al*., 2008).

In contrast, necrosis is generally known as a "non-specific" form of cell death, characterised by breaking of the plasma membrane which results in an unfavourable localized inflammatory response and damage to neighbouring cells and tissues (Kroemer *et al.*, 1998).

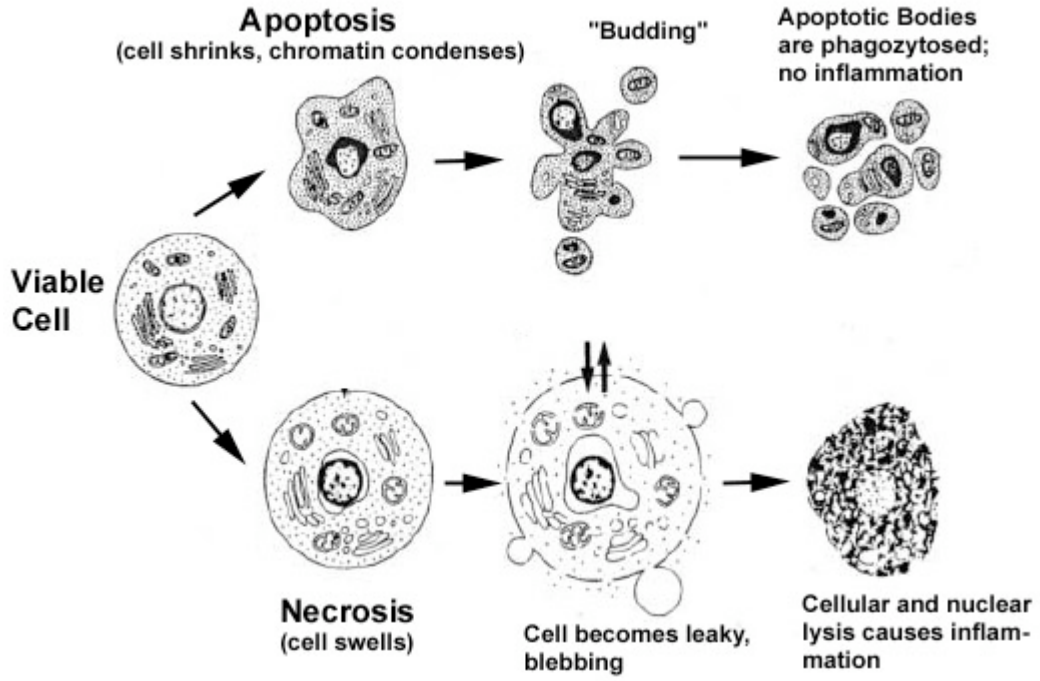


Figure1.2: Hallmarks of the apoptotic and necrotic cell death process. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytised without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation. (Adapted from Apo Review: Introduction to Apoptosis. Gewies, 2003)

1.4.1 Apoptosis signaling

Apoptosis is a highly regulated cell death program which requires the interaction of complex machinery controlled by complex pathways including extrinsic, intrinsic, p53, ubiquitin/proteosome and nuclear factor kappa B pathways and also by Bcl-2 family members. Among the several pathways identified, extrinsic or cytoplasmic and intrinsic or mitochondrial pathways are among two major pathways that are involved in apoptosis (Ghobrial *et al*., 2005).

1.4.2 The extrinsic and intrinsic pathway

The extrinsic pathway involves engagement of particular 'death' receptors that belong to the tumour necrosis factor receptor (TNF-R) family and, through the formation of the death-inducing-signalling-complex (DISC) (Ashkenazi and Dixit., 1998), leads to a cascade of activation of caspases, which in turn induces apoptosis (figure 1.2). The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization and release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Cytochrome c, apoptotic protease-activating factor 1 (Apaf- 1) and procaspase 9 then form a complex termed the apoptosome, in which caspase 9 is activated and promotes activation of caspases 3, 6 and 7. Activation of these caspases triggers activation of downstream apoptotic proteins and induce apoptosis (Nicholson and Thornberry, 2003).

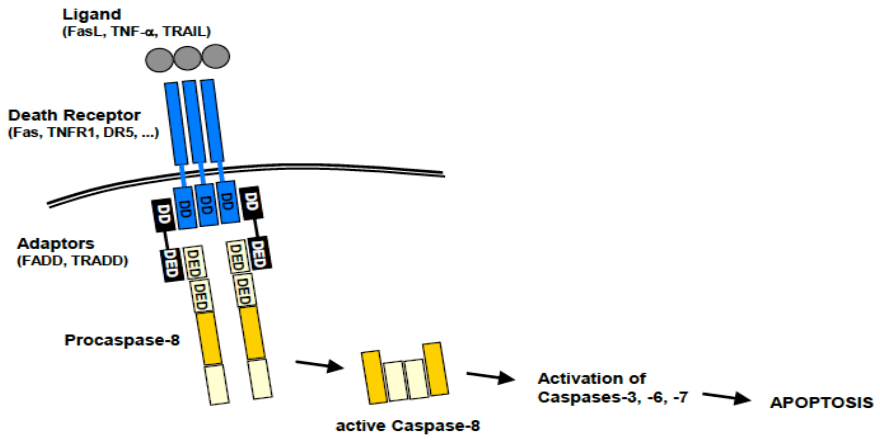


Figure 1.3: Receptor-mediated caspase activation at the DISC. Upon ligation by its cognate ligand, the trimeric death receptor recruits adaptor molecules via its cytoplasmic death domains (DD). Besides possessing DDs, the adaptors additionally contain death effector domains (DED) which recruit procaspase 8 to the receptor complex which now is called the death-inducing signalling complex (DISC).The initiator caspase 8 cleaves and thereby activates effector caspases for the execution of apoptosis. (Adapted from Apo Review: Introduction to Apoptosis. Gewies, 2003).

Death receptors of the extrinsic pathway belong to the tumour necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5. The TNFR family consist of cysteine rich extracellular sub-domains which recognize specific ligands, resulting in the trimerization and activation of particular death receptor which contains a conserved sequence known as the death domain (DD).

Adaptor molecules such as FADD or TRADD are recruited to the DDs of the activated death receptor which forms the death inducing signalling complex (DISC) (Figure 1.3). The adaptor FADD also contains death effector domains (DED) which through homotypic DED-DED interaction seize procaspase 8 to the DISC. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death (Gewies, 2003).

p53 can activate the extrinsic apoptotic pathway through the induction of genes encoding by transmembrane proteins such as Fas and DR5. Fas is activated by binding of its ligand, FasL, which is expressed predominantly by T cells (Muzio, 1998). p53 induces *Fas* mRNA expression by binding to elements found in the promoter and first intron of the *Fas* gene (Muller *et al.,* 1998). This induction occurs in response to γ-irradiation, and is strictly tissue specific, demonstrated in the spleen, thymus, kidney and lung, but not in heart and liver (Bouvard *et al.,* 2000).

In addition to stimulating *Fas* transcription, overexpressed p53 may enhance levels of Fas at the cell surface by promoting trafficking of the Fas receptor from the Golgi (Bennett *et al.,* 1998). This may allow p53 to rapidly sensitize cells to Fas induced apoptosis.

The second member of the TNF-R receptor family that is induced by p53 is *DR5/KILLER*, the death-domain-containing receptor for TNF-related apoptosis-inducing ligand (TRAIL). *DR5* is induced by p53 in response to DNA damage and in turn promotes cell death through caspase 8 (Wu *et al.,* 1997). *DR5* induction is cell type specific. Whole body γ-irradiation induces *DR5* expression in the spleen, small intestine and thymus (Burns *et al.,* 2001), which is reliable with DR5 participating in the p53-mediated response to DNA damage in these tissues.

In some cases the signal from the activated receptor does not generate strong signal for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways also known as the intrinsic apoptotic pathway (Figure 1.4). The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins, which direct the release of cytochrome c from the mitochondria.

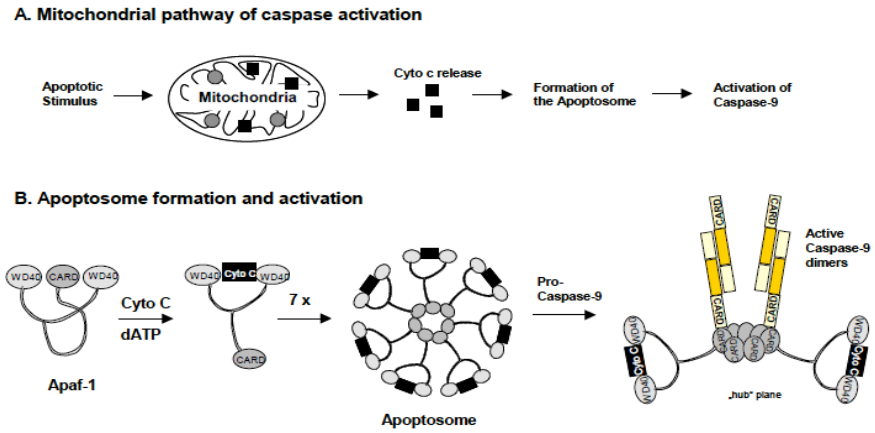


Figure 1.4: Mitochondria-mediated caspase activation at the apoptosome. A. Apoptotic stimuli trigger the release of apoptogenic factors from the mitochondrial intermembrane space to the cytosol, such as cytochrome c which induces the formation of the apoptosome and the activation of procaspase-9. B. By the action of cytochrome c (Cyto C) and dATP the Apaf-1 protein adopts a conformation that allows the formation of the apoptosome. Procaspase-9molecules can bind to the inner "hub" region of the apoptosome and are activated by dimer formation. Active caspase-9 dimers further mediate activation of effector caspases. (Adapted from Apo Review: Introduction to Apoptosis. Gewies, 2003)

The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase 8 and in its condensed form (tBID) translocates to the mitochondria where work together with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial pro-apoptotic factors into the cytosol.

Cytosolic cytochrome c binds to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7 fold symmetry that triggers the activation of the initiator procaspases 9.

The dimerization of procaspases 9 molecules at the Apaf-1 scaffold is responsible for caspase 9 activation. Activated caspase 9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase 3, 7, and 6, resulting in cell death. p53 promotes cytochrome c release through the induction of target genes encoding BH3-only proteins. Importantly, p53 also induces Apaf-1 expression through a response element within the Apaf-1 promoter (Figure 1.5).

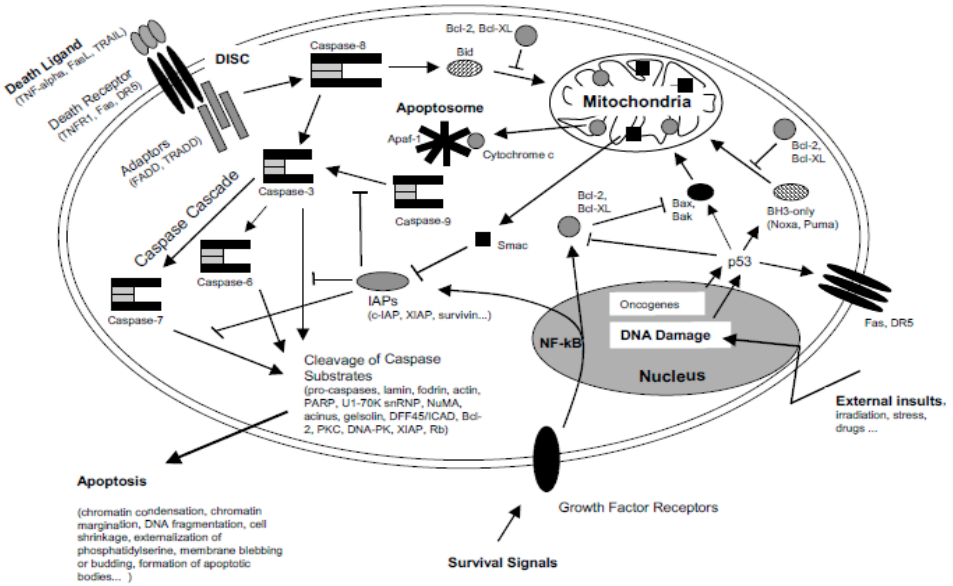


Figure 1.5: Schematic representation of some major apoptotic signalling pathways. Apoptosis can be induced in response to various signals from inside and outside the cell, e.g. by ligation of so called death receptors or by cellular stress triggered by oncogenes, irradiation or drugs. Organelles such as mitochondria, nucleus, ER and lysosomes are important in apoptotic signalling pathways, and hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors. (Adapted from Apo Review: Introduction to Apoptosis. Gewies, 2003).

1.5 Bcl-2 family members

Bcl-2 protein family are categorized into two major groups; anti-apoptotic and pro-apoptotic based on their functional properties. Anti-apoptotic protein members such as Bcl-2 and Bcl-X_L act as repressor of apoptosis, which suppresses the release of cytochrome c from the mitochondrion whereas pro-apoptotic proteins such as Bax and Bad act as promoter to induce apoptosis. Besides that, the Bcl-2 family proteins also can be divided according to their BH3 domain organization.

The BH3 domain, which is present in all members and is essential for heterodimerization among members, is the minimum domain required for the pro-apoptotic function. Anti-apoptotic proteins such as Bcl-X_L, Bcl-2, Bcl-XL, Bcl-w, possess the domains BH1, BH2, BH3, and BH4. The pro-apoptotic group of Bcl-2 members can be divided into two subgroups: the Bax-subfamily consists of Bax, Bak, and Bad possess the domains BH1, BH2, and BH3, whereas the BH3-only proteins such as Bid, Bim, Bik, Bad, Bmf, NOXA, PUMA and Spike have only the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action (Haupt *et al*., 2003).

In mutagenesis studies, inactive Bcl-2 which undergoes mutation in the BH1 domain which retains the BH3 domain, was unable to heterodimerize with the full-length Bax, and hence cannot inhibit apoptosis.

However, the Bcl-2 can bind to the C-terminally condensed Bax with complete set of BH3 domain to induce apoptosis. It can be suggested that there are some conformation constraints in the full-length Bax that does not allow the binding of Bcl-2. It is demonstrated that the BH3 domain in Bax is responsible for the binding between Bax and Bcl-2.

1.5.1 Regulation of Bcl-2 family members

It is essential to maintain a proper balance and regulation between the anti-apoptotic proteins and pro-apoptotic proteins, as improper regulation of expression of the apoptotic genes may affect the viability of normal cells, hence leading to the development of cancerous cells. The Bcl-2 family genes, including Bax, NOXA, PUMA and the most recently identified, Bid are p53 targets. Bax is a cytosolic monomer in viable cells but during apoptosis changes its conformation, integrates into the outer mitochondrial membrane and oligomerizes. Bax was the first member of this group shown to be induced by p53, but p53-responsive elements have only recently been clearly identified in the *Bax* gene (Thornborrow *et al.,* 2002). In response to stress activation, Bax forms a homodimer and releases cytochrome c from the mitochondria (Skulachev, 1998), which results in caspase 9 activation (Cory and Adams, 2002).

In contrast, anti-apoptotic Bcl-2 members seize pro-apoptotic Bcl-2 members by binding to their BH3 domains and prevent Bax or Bak activation or oligomerization and thus inhibit mitochondrial pro-apoptotic events where over expression of Bcl-2 or Bcl-X_L potently inhibits apoptosis (Figure 1.6).

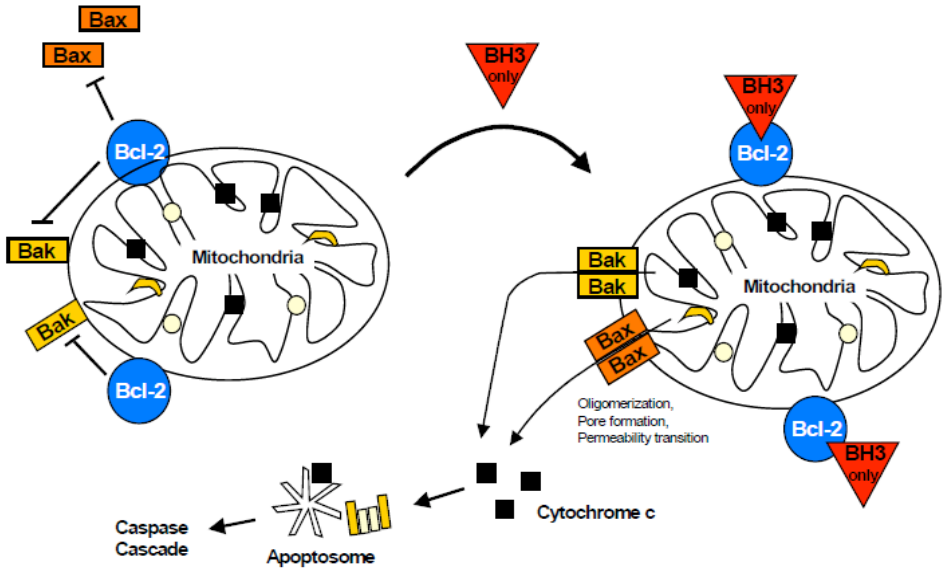


Figure 1.6: Regulation of apoptosis by the Bcl-2 family. In a viable cell, the pro-apoptotic Bcl-2 family members Bax, Bak, and BH3-only proteins are antagonized by anti-apoptotic members such as Bcl-2. In response to an apoptotic stimulus, BH3-only members are activated by transcriptional up-regulation (Bax, NOXA, and PUMA), sub-cellular relocalization (Bim, Bmf), dephosphorylation (Bad), or proteolysis (Bid). Activated BH3-only proteins prevent anti-apoptotic Bcl-2 members from inhibiting pro-apoptotic members. In consequence, pro-apoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade. (Adapted from Apo Review: Introduction to Apoptosis. Gewies, 2003).

Role of Bax in apoptosis induction has recently been offered in the context of PUMA. The PUMA gene is also directly induced by p53 in response to DNA damage, through p53-responsive elements within the first intron of PUMA. In humans, PUMA encodes two BH3-domain-containing proteins, PUMA-a and PUMA-b (Nakano and Vousden, 2001). A vital balance between PUMA and p21 has been identified to determine the onset of arrest, or death, in response to exogenous p53 expression. If p21 is disrupted, the cells die through apoptosis and if PUMA is disrupted, apoptosis is prevented. Bax is absolutely required for PUMA-mediated apoptosis. PUMA expression promotes mitochondrial translocation and mulitmerisation of Bax, and ends in apoptosis induction (Yu *et al.,* 2003).

Thus, although p53 can bind to the Bax promoter, the affinity is weak in contrast to p21 and PUMA binding. Bax thus participates in the death response as an indirect target of p53 through PUMA (Yu *et al.,* 2003). Another p53 target gene, Noxa, contains a single p53-responsive element in its promoter and is induced in response to X-ray irradiation. Thus, in response to DNA damage, p53 activates the intrinsic mitochondrial apoptotic pathway by inducing the expression of at least three Bcl-2 pro-apoptotic family members, shifting the balance towards pro-apoptotic effects.

1.6 p53 signalling pathways

Apoptosis is known as a major obstacle that must be overcome by tumour cells to allow them to survive and proliferate in such stressful conditions. Tumours obtain resistance to apoptosis through several strategies, frequently observed, for example the loss-of-function mutations of the phosphoprotein 53 (p53) tumour suppressor proteins. The tumour suppressor p53 is a transcription factor that has a critical role in preventing cancer. Since p53 can induce apoptosis by activating transcription of pro-apoptotic Bcl-2 proteins in the circumstance of DNA damage, non-functional p53 can directly be linked to a failure to induce apoptosis after cellular stress (de Bruin *et al.,* 2008)

Depending upon the stimulus and the state of the cell, activation of p53 can lead to either the halting of cellular proliferation and DNA repair, or induction of apoptosis. While the primary stimulus for activating p53 is DNA damage, other cellular stresses including metabolite deprivation, physical damage, heat shock, and loss of oxygen can also lead to p53 activation (Blatta and Glicka, 2001).

Among the factors that activate p53 in the absence of DNA damage are expression of oncoproteins, non-genotoxic drugs, ribonucleotide pool depletion and hypoxia (Oleinik, 2005).

In a normal state, the cell has low levels of p53 because of the metabolic instability of inactivated p53. Phosphorylation takes place in order to activate p53 after DNA damage or cellular stress. A large number of kinases phosphorylate p53, including casein kinases, extracellular-signal-related kinases, JNK, protein kinase C, and Raf1 kinase. However, the response to DNA damage is most likely mediated by DNA-PK (a caspase target), the product of the ataxia-telangiectasia gene (ATM), and the checkpoint kinase Chk2. The phosphorylation state of p53 is also controlled by the action of protein phosphatases 1 and 2A, in that inhibition of these enzymes by okadaic acid leads to phosphorylation of p53 (Blatta and Glicka, 2001).

Once phosphorylated, p53 then acts as a transcription factor to enhance and suppress the transcription of several genes involved in apoptosis. First, p53 increases the transcription of several genes that control the redox state of the cell. The synthesis of these genes leads to the production of reactive oxygen species that subsequently cause mitochondrial apoptosis.

p53 also up-regulates the transcription of Bax and suppress Bcl-2 transcription, altering the Bcl-2: Bax ratio which promotes apoptosis via a mitochondrial pathway . The Bcl-2 family of proteins direct mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic.

It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via modification of mitochondrial membrane permeability (Elmore, 2007). Besides p53 up-regulates the transcription of the Fas receptor which likely leads the cell to apoptosis induced by exposure to FasL.

Mutations in p53 have been shown to eliminate its functions in promoting apoptosis, cell-cycle arrest and DNA repair, thus leading to cancer development and progression. Activation of p53 results in the transactivation of many target genes that regulate these biological processes. Thus, loss of DNA-binding function of p53 results in amelioration of p53 dependent transcription, and hence, target genes required for the efficient execution of the biological processes are not activated. The significance of the DNA-binding property of p53 in regulating many of its biological functions is highlighted by the large percentage (~90%) of mutations found in DNA-binding domain (DBD) of p53 in human cancers (Vikhanskaya *et al.,* 2006).

p53 is also able to promote apoptosis through transcription-independent apoptotic mechanisms. Under certain conditions, p53 induces apoptosis in the absence of transcription or protein synthesis. Furthermore, transcriptionally inactive mutants of p53 can induce apoptosis in certain cell types and PIASg (protein inhibitor of activated STAT), which blocks binding of p53 to DNA, does not inhibit p53-mediated apoptosis.

In general, the transcription independent apoptotic activities of p53 have been demonstrated in transformed cells rather than in normal cells (e.g. lymphocytes or fibroblasts). Apparently, these activities of p53 require cooperation with other apoptotic factors for instance E2F-1 (a transcription factor in the retinoblastoma protein pathway) (Haupt *et al.,* 2003).

1.7 Caspases

Caspases, or cysteine-aspartic proteases, are a family of cysteine proteases, which play essential roles in apoptosis (programmed cell death), necrosis and inflammation. Cell death classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity. In the cell, caspases are synthesized as inactive zymogens procaspases, which at their N-terminus carry a prodomain, followed by a large and a small subunit which are sometimes separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit.

Eleven caspases have so far been identified in humans. There are two types of apoptotic caspases, initiator caspases and executioner caspases. The pro-apoptotic caspases can be divided into the group of initiator caspases including procaspases 2, 8, 9 and 10, and group of executioner caspases including procaspases 3, 6, and 7 (Earnshaw *et al.,* 1999).

1.7.1 Caspase activation

Caspases catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide - QACRG, and the caspases specifically cleave their substrates after Asp residues. So far, 7 different caspases have been identified in Drosophila, and 14 different members of the caspase-family have been described in mammals, with caspases 11 and 12 only identified in the mouse (Denault, 2002).

Caspases 1, 4, 5, 11 and 12 appear to be mainly involved in the proteolytic maturation of pro-inflammatory cytokines such as pro-IL-1ß and pro-IL-18 and their contribution to the execution of apoptosis remains questionable (Denault, 2002). Caspases 9 and 2 respond to changes in mitochondrial potential, whereas caspases 8 and 10 sense activation of death receptors. These initiator caspases cleave the pro-enzyme forms of the effector caspases 3, 6 and 7, allowing digestion of essential targets that affect cell viability (MacLachlan and El-Deiry, 2002).

Caspases 2, 3, 6, 7, 8, 9 and 10 have been recognized to play important roles in the apoptotic signalling machinery (Earnshaw et al., 1999). p53 can activate the caspase cascade by both transcriptions of the dependent and independent mechanisms. In response to g-irradiation, p53 can activate caspase 8 (Ding *et al.,* 1998).

p53 also stimulates caspase 6 through a more conventional mechanism. In response to DNA damage, p53 directly induces caspase 6 expression through a response element within the third intron of the gene (MacLachlan and El-Deiry, 2002). Caspase 6 cleaves the nuclear envelope protein lamin A and several transcription factors (Galande *et al.,* 2001). Caspase 6 plays an important role in p53 induced neuronal cell death and is the major protein involved in the cleavage of the amyloid precursor protein (LeBlanc *et al.,* 1999).

1.8 Treatments of cancer

Treatment for cancer can be localised, where the cancer cells in the tumour and the surrounding area is being directly targeted by chemotherapy and immunotherapy or systemic, where the treatment drugs will travel through the bloodstream and reach cancer cells all over the body such as hormone therapy.

Surgery or drug treatments are the primary treatment for many solid tumours. Benign growth or early detected cancer can be cured by surgery by removing all cancerous cells and in combination with radiation to completely kill any remaining cancer cells directly by damaging them with high energy beams. Radiation can be either external or internal. In the external form, the radiation comes from a machine that aims the rays at the tumour. In internal radiation, radioactive material is sealed in needles, seeds, or wires and placed directly in or near the tumour.

Chemotherapy is a word used for a wide array of drugs used to kill cancer cells. Chemotherapy drugs work by damaging the dividing cancer cells and preventing their further replication without killing normal host cells and tissues. Primary chemotherapy or neoadjuvant chemotherapy is a chemotherapy used before surgery. The purpose of this chemotherapy is to reduce the size of the tumour. The more common use of chemotherapy is adjuvant therapy. In this treatment, chemotherapy is given after surgery to destroy any remaining cancer cells and prevent cancer from persisting. Chemotherapy is also used in conjunction with radiation therapy.

In hormonal treatments, the drugs are designed to prevent cancer cell growth by preventing the cells from receiving signals essential for their continued growth and division. Thus the growth of the tumour slows and survival may be extended for several months or years.

Antibodies are also used to target cancer cells. Specific inhibitors are quite new in the treatment of cancer. The antibodies work by several different mechanisms, either depriving the cancer cells of necessary signals or causing the direct death of the cells. As a result of their specificity, antibodies are considered as a type specific inhibitor. They target specific proteins that primarily promote growth of cancer cells. Inhibition of these processes prevents cancer cell growth and division.

High intensity focused ultrasound (HIFU) is also found to be the current treatment for cancer. It is a highly precise medical procedure using high-intensity focused ultrasound to heat and destroy tumour tissues rapidly.

The use of vaccines is also important in cancer treatment. Cancer vaccines are to stimulate the body's defences against cancer. Vaccines usually contain proteins or antigens found on or produced by cancer cells. Administration of these proteins aims to increase the immune response of the body against the cancer cells, especially in enhancing the cytotoxic T (T_C) and natural killer (NK) cells responses.

1.8.1 p53-mediated cancer therapy

Stimulation of disabled p53 pathways has been suggested as a potential mode for cancer therapy. Potential approaches include introducing wild-type p53 genetically, allowing abnormal p53 molecules to perform wild type functions, or intervening to activate directly targets in the p53 apoptotic pathways. Gene therapy based on the introduction of wild-type p53 (Wen *et al.,* 2003) and elimination of mutant p53-expressing cells is undergoing clinical trials. Restoration of wild-type conformation to structurally distorted p53 DNA-binding mutants has been demonstrated by using peptide constructs and small molecular weight synthetic molecules. Synthetic peptides derived from the C-terminus of p53 can induce p53-dependent apoptosis in tumour cells and restore the specific DNA-binding and transcription functions to mutant p53 in vitro (Selivanova *et al.,* 1999).

In vivo activity of these peptides has been associated with an increase in the levels of the Fas receptor on the cell surface, through a p53-dependent mechanism. (Kim *et al.,* 1999). Other small synthetic peptides derived from a p53- binding protein have been introduced to restore sequence specific DNA-binding activity to mutant p53 (Friedler *et al.,* 2002).

p53 reactivation and induction of massive apoptosis (PRIMA) is a small molecular weight molecule that provokes apoptosis in a transcription-dependent manner through conformational manipulation of p53 mutants to restore sequence-specific DNA binding (Bykov *et al.,* 2002). CP-31398 is another small synthetic molecule with the capacity to restore wild-type p53 function by inducing apoptosis or growth arrest induction. CP-31398 has been suggested to trigger apoptosis through the intrinsic pathway by increasing DR5 cell surface exposure and reduction in p53-ubiquitination with this molecule (Wang *et al.,* 2003).

1.8.2 ELISA and p53 tumour suppressor gene

An early event in apoptosis is DNA fragmentation and release of nucleosomes into the cytoplasm (Salgame *et al.,* 1997). An enzyme-linked immunosorbent assay (ELISA) for apoptosis is based upon the detection of cytoplasmic nucleosomes. The nucleosome is the basic unit of chromatin and results from the ordered association of histones and DNA. The double antibody sandwich ELISA is based upon the specific recognition of nucleosomes by a pair of monoclonal antibodies (mAb).

Nucleosomes in cytoplasmic lysates are captured onto ELISA plates coated with a first mAb reacting with an exposed epitope of histone H2B. The bound nucleosomes are then detected by a second mAb specific for the complex formed by the H2A–H2B dimer and DNA. ELISA could detect as low a number of apoptotic cells as possible. ELISA is easy to perform, extremely sensitive, and particularly suited to detect apoptosis in a large number of samples (Salgame *et al.,* 1997).

ELISA has been widely employed to measure p53 levels, for example in determining the amount of p53 in tumour tissues and in the nervous system, as well as for detecting p53 mutants. ELISA analysis has also been used to detect and quantitate auto-antibodies against mutant p53 in cancer patients. ELISA also used to identify p53/DNA complex formation due to role of p53 and DNA binding properties. The results indicate that detection of p53/DNA binding properties opens new possibilities for analyzing the function of p53 in a wide variety of circumstances (Jagelska *et al*., 2002).

1.8.3 Current efforts in improvement of cancer treatments

Although many treatments are currently used in treating cancer, some of them still have several limitations. In chemotherapy and radiation therapy, the selected target cells that are in the process of proliferation; both malignant and non malignant cells are subject to cytotoxic or immunotherapeutic effects.

Researchers have been able to create new targeted therapies for cancer treatment to minimize the harmful effects of therapy on healthy cells through new understandings and theories of how cancer cells survive, and metastasize cancer therapy may be more effective if the proteins associated with the specific pathways such as cell membrane receptors, signalling pathways, enzymatic activity, and regulatory cell growth controls for cancer transformation and progression are interrupted or blocked within the cell's macro-environment.

The benefits of more selective agents include a higher therapeutic index and a lower toxicity profile than conventional therapy. In chemotherapy, their toxicity is not limited to cancer cells only, but the normal cells are also harmed. Cancer cells are difficult to target because they are so similar to normal cells. Both proliferate using the same cellular machinery, and destroying that machinery in one destroys it in the other. Rapidly dividing cells are particularly affected because their high proliferation rate makes them dependent on frequent DNA synthesis. Thus chemotherapy has been highly successful on fast-growing cancers such as testicular cancer and some forms of leukemia.

Chemotherapy is not considered remedial although it can induce complete responses because treated patients will generally develop recurrent disease requiring additional therapy which can cause worsening immune dysfunction. A modified form of chemotherapy, isolated limb perfusion, is being studied as an alternative to traditional chemotherapy when the melanoma occurs on an arm or leg. Isolated limb perfusion involves temporarily stopping the flow of blood to the affected limb with a tourniquet and administering a high dose of chemotherapeutic medication to the affected area. It is believed that high doses can more effectively destroy the cancerous cells. This has been beneficial for some patients.

Vascular endothelial growth factor (VEGF) has emerged as a key target for the treatment of cancer. As the ligand to the VEGF receptor, it plays a central role in promoting tumour angiogenesis. Over-expression of VEGF leads to poor outcomes in patients with breast cancer and other tumours.

Preclinical studies have shown that the humanized monoclonal antibody to VEGF, bevacizumab can reduce tumour angiogenesis and inhibit the growth of solid tumours, either alone or in combination with chemotherapy.

1.9 Natural compounds and cancer

Natural compounds are being investigated extensively worldwide as potential anti-cancer agents. 48 of 65 the drugs (74%) that were approved for cancer treatment have been extracted from natural sources, and created by structural modification, or by the synthesis of new compounds, designed following a natural compound as model (Gordaliza, 2007). Most of these natural anticancer agents available today are derived from plants, animals, marine organisms and microorganisms. Some currently used examples of plant-derived compounds are vincristine, irinotecan, etoposide and paclitaxel, while bleomycin and doxorubicin are microbial-derived compounds and citarabine, derived from a marine source (da Rocha *et al*., 2001).

Limonoids are unique highly oxygenated triterpenoid compounds long recognized as significant biologically active natural compounds. Citrus limonoids appear in large amounts in citrus juice and citrus tissues as water soluble limonoid glucosides or in seeds as water insoluble limonoid aglycones (Ozaki *et al.,* 1995). Over 300 limonoids have been isolated to date. Past work has established a wide range of biological activities for these compounds including insect antifeedant and growth inhibiting characteristics.

Although best known for their insecticidal properties a variety of medicinal effects in animals and humans has been illustrated including some anti-carcinogenic effects on in-vitro human tumour cell lines and test animals.

Other limonoid properties include antifungal, bactericidal, and antiviral effects. Extracts of many citrus species are used traditionally in the herbal medicines of the Far East. The fruits and bark of the Chinaberry (*Melia azedirach*) have been used as a treatment for a variety of ailments in small doses, as it can be toxic to humans. Some of the most exciting applications of limonoids and compounds derived from them are their use in the treatments of specific cancers. Limonin, nomilin, 12- hydroxyamdorastatin, and isofraxinellone are limonoids or their derivatives that have been shown successful in treatments with in vitro bioassays on human tumour cell lines (Bagge, 2008).

Several citrus limonoids have recently been subjected to anti-cancer screen procedures utilizing laboratory animals and human breast cancer cells in culture. In mice, it was found that five limonoid aglycones (limonin, nomilin, obacunone, isoobacunoic acid) induced significant amounts of glutathione-S-transferase (GST) in the liver and intestinal mucosa (Lam and Hasegawa *et al.,* 1994). GST is a major detoxifying enzyme system which catalyzes the conjugation of glutathione with many potentially carcinogenic compounds which are highly electrophilic in nature.

Most recently, several limonoid aglycones and a mixture of limonoid glucosides were administered in vitro to estrogen dependent and estrogen independent human breast tumour cell lines (Guthrie *et al.,* 2000).

The results showed that the limonoids were equally potent as tamoxifen for inhibiting the proliferation of estrogen dependent breast cancer cells, and more effective than tamoxifen for activity against estrogen-independent cancer cells. Limonin and obacunone fed to rats have also been shown to increase GST and quinine reductase activities in liver and colon mucosa and was correlated with the prevention of colon carcinogenesis in rats (Tanaka *et al.,* 1999).

The insecticidal properties of many limonoids, especially C-seco, and many more widely distributed intact apo-euphol skeleton, 14,15- epoxide limonoids, are driving many recent studies in the extraction and isolation of these compounds from new sources due to continued exposition of limonoid compounds against organisms other than insects. Indications of the antifungal, bacteriacidal, protisticidal and antiviral characteristics suggest a broader role for these compounds. With over 300 similar compounds, the bioassay of known limonoids for their anticancer and anti-mutagenic activities will promote much more investigation for cancer treatment.

1.9.1 Erythrocarpine E (CEB4) - a novel natural compound from *Chisocheton erythrocarpus*

Erythrocarpine E (CEB4) is a new cytotoxic limonoid which is dichloromethane extracted from the bark of *Chisocheton erythrocarpus* Hiern from Meliaceae family. This newly extracted limonoid have been shown to have cytotoxic activity against P-388 murine leukemia cells at IC_{50} of 18μg/ml. It is a A, B, D-seco heptacyclic limonoid having mexicanolide type skeleton with cinnamoyl group as side chains at C-3 (Awang *et al*., 2007). CEB4 with a molecular formula of $C_{36}H_{38}O_9$ with the presence of functionalities such as a b-furyl ring, a methoxy group, a conjugated d-lactone ring, and

a cinnamoyl group. The Me-29 was suggested to be oxidized and cyclized with C-1 to form an oxygen-bridge in the ring A. It is different to the typical 4, 29, 1-carbon-bridge sequence found in the phragmalin skeleton which was biogenetically and structurally closely related to mexicanolide skeleton (Awang *et al*., 2007). The structure of CEB 4 is shown below:

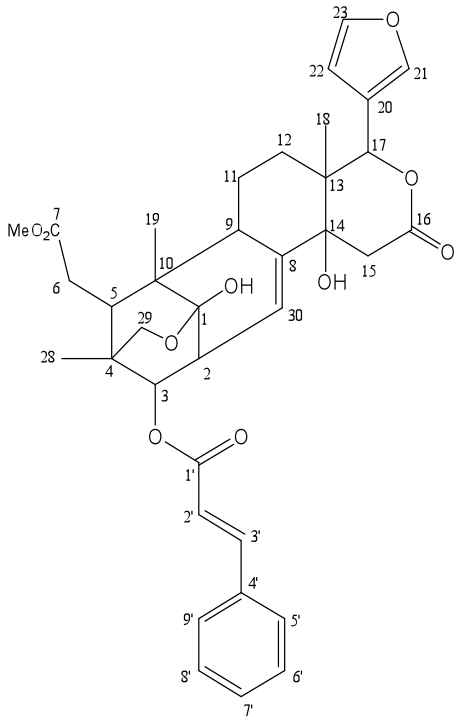


Figure 1.7: Structure of erythrocarpine E (CEB4)

1.9.2 Objectives of the study:

Erythrocarpine E (CEB4), extracted from *Chisocheton erythrocarpus* Hiern is being investigated as a potential natural compound to induce apoptosis in tumour cell lines. Therefore, this study was carried out with the aim:

1. To investigate effects of CEB4 on cell viability using MTT assay and fluorescence microscopy.

2. To investigate effects of CEB4 on cell cycle progression and apoptosis using flow cytometry.

3. To assess whether CEB4 treatment of cancer elicits an apoptotic response, using Western blot analysis of PARP cleavage and DNA fragmentation analysis.

4. To determine if p53 and its associated regulatory proteins function to elicit the apoptotic response observed following CEB4 treatment of cancer cells.

CHAPTER 2.0: MATERIALS AND METHODS

2.1 Materials and methods of CEB4 compound

2.1.1 Plant material:

Chisocheton erythrocarpus Hiern was collected from Hutan Simpan Terenas, Kedah, Malaysia. Voucher specimens (KL 4863) were deposited in the Herbarium of Chemistry Department, University of Malaya.

2.1.2 Botanical Aspect of *Chisocheton erythrocarpus* Hiern

This plant is commonly known as Rongga in Malaysia. It is a big tree that can grow up to 80 feet tall with the branchlets velvety tomentose. Its leaves are 12 to 15 inches long with 4-6 pairs of coriaceous elliptic-oblong or broad ovate leaflets. The flowers are about 0.35 inches long with its petals elliptic silky outside and stamen-tube shorter silky below. The seeds are ex-arillate to about 1 inch and are orange red in colour.

2.1.3 Extraction and isolation

Dried ground barks (1.3kg) were extracted successively with hexane, dichloromethane, and methanol. The dichloromethane extract (4.7g) was repeatedly subjected to silica gel column chromatography using solvent mixture of hexane: ethyl acetate (7:3) and final purification was done by PTLC using the same solvent system.

Erythrocarpine E
$[\alpha]_D$: -128° (*c* 0.8, $CHCl_3$)

IR (KBr): 3413, 2928, 1700, 1201 cm^{-1}
UV/Vis λmax (MeOH): 297 nm
^1H NMR (400 MHz, $CDCl_3$): 2.95 (1H, dd, J=7.1, 10.1 Hz, H-2), 4.91 (1H, d, J=10.1 Hz, H-3), 2.92 (1H, m, H-5), 2.31 (2H, m, H-6), 2.34 (1H, dd, J=3.2, 10.2 Hz, H-9), 1.54 (1H, m, H-11), 1.68 (1H, m, H-11), 1.17 (1H, m, H-12), 1.89 (1H, m, H-12), 2.76 (2H, dd, J=18.1, 18.1 Hz, H-15), 5.46 (1H, s, H-17), 0.88 (3H, s, Me-18), 1.02 (3H, s, Me-19), 7.64 (1H, s, H-21), 6.29 (1H, br s, H-22), 7.30 (1H, d, J=1.5 Hz, H-23), 0.62 (3H, s, Me-28), 3.46 (1H, d, J=9.3 Hz, H-29), 3.88 (1H, d, J=9.3 Hz, H-29), 5.53 (1H, dd, J=1.7, 7.1 Hz, H-30), 3.65 (3H, s, 7-Ome), 6.25 (1H, d, J=15.7 Hz, H-2'), 7.58 (1H, d, J=15.7 Hz, H-3'), 7.19 (1H, m, H-5'), 7.18 (1H, m, H-6'), 7.19 (1H, m, H-7'), 7.18 (1H, m, H-8'), 7.19 (1H, m, H-9').

^{13}C NMR (90 MHz, $CDCl_3$): 97.3 (C-1), 44.8 (C-2), 74.9 (C-3), 36.2 (C-4), 34.9 (C-5), 31.7 (C-6), 174.0 (C-7), 140.5 (C-8), 43.6 (C-9), 41.4 (C-10), 19.0 (C-11), 28.2 (C-12), 41.2 (C-13), 78.9 (C-14), 39.4 (C-15), 168.8 (C-16), 76.9 (C-17), 14.8 (C-18), 14.7 (C-19), 120.3 (C-20), 146.2 (C-21), 109.8 (C-22), 142.6 (C-23), 15.5 (C-28), 67.9 (C-29), 122.1 (C-30), 52.1 (7-Ome), 166.4 (C-1'), 117.0 (C-2'), 146.2 (C-3'), 134.4 (C-4'), 128.3 (C-5'), 128.5 (C-6'), 129.9 (C-7'), 128.5 (C-8'), 128.3 (C-9').
HRMS-FAB: m/z $[M + H^+]$ = 633.3703

2.2 Cell lines and culture conditions

Human epidermoid cervical carcinoma cells (CaSki) and human hepatocyte liver carcinoma (HepG2) cells were obtained from Medical Faculty of University of Malaya. Human breast adenocarcinoma cells (MCF7), human oral squamous carcinoma cells (HSC2 and $HSC4^{COX2-}$) were obtained from Cancer Research Initiative Foundation, Subang Jaya Medical Center (CARIF, SJMC). Normal human bronchial epithelial cells (NHBE) were obtained from Cancer Research Initiative Foundation, Subang Jaya Medical Center (CARIF, SJMC).

CaSki, HepG2, HSC2 and $HSC4^{COX2-}$ cells lines are culture flask attached cells and were grown in Dulbeco's Modified Eagle Medium (DMEM) (Lonza, USA) culture

media while the MCF7 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640). Heat activated 10% (v/v) fetal bovine serum (Lonza,USA) that contains a rich variety of protein was added to the culture media to maintain growth of the cells. The NHBE were cultured with Bronchial Epithelial Basal Medium (BEBM) with 10% FBS and 1% penicillin/ streptomycin.

Cancer cells were plated on a 25.0 cm^2 culture plate (Nunc, Denmark) and incubated in a carbon dioxide (CO_2) incubator (Memmert, Gemany) with high relative humidity (95%) , stable temperature (37ºC), controlled CO_2 levels (5%) and controlled pH (7.2-7.4). Culture media had buffer system that contained appropriate amount of bicarbonate where 5% atmosphere CO_2 levels helped maintain culture media in a proper pH (7.0 to 7.6). These essential variables create an ambient environment for mammalian cells to thrive. Culture media were also supplemented with 100 units/mL penicillin or streptomycin (Lonza, USA) to prevent growth of bacteria and do not have an adverse effect on the viability of cells.

2.2.1 Subculturing and maintenance of adherent cell lines

The culture medium of the cell lines grown in 25.0 cm^2 filter cap culture flask (Nunc, Denmark) was removed and discarded using an aspirator. The cell monolayer was then rinsed with sterile autoclaved 1X Phosphate Buffer Saline (PBS) (Lonza, USA) to wash off any remaining culture medium. The solution in the culture flask was removed and discarded using an aspirator. Next, 3.0 ml of dissociating agent 0.25% trypsin (Lonza, USA) was added to the culture flask. The culture flask was then incubated at 37°C in 5% CO_2 incubator (described in 2.2) for approximately 10 minutes

to allow the cells in the culture flask to detach. The progress of cell detachment was checked every 5 minutes using an inverted microscope (Labscope, China).

Then 3.0 ml of the appropriate growth medium was added to the culture flask. Any remaining cells were washed from the bottom of the culture flask and a quick check was done under the inverted microscope to look for single cells in the suspension. If they were not mostly single cells, more vigorous pipetting was done on the suspension. All the suspension from the culture flask was then transferred to a labeled 15.0 ml Falcon tube and the tube was centrifuged for 10 minutes at 1500 RPM using Centrifuge 5702 (Eppendorf, USA). The supernatant in the tube was then discarded and the pellet was resuspended in 4.0 ml to 6.0 ml of the appropriate growth medium. The cell suspension was then collected for counting or passaging into prepared culture flasks.

2.2.3 Cell count

The cell suspension was gently mixed and a 20.0 µl of the suspension was aliqouted into a 1.5 ml microcentrifuge tube and 20.0 µl of trypan blue (Sigma Aldrich, USA) stain was then added to this aliquot and mixed well. The tube was allowed to stand for about 3 minutes. After 3 minutes, 10.0 µl of the mixture was then removed and loaded on a clean hemacytometer (Germany) to be counted. The counting was done under an inverted microscope. Dead cells will be seen as blue while viable cells will be seen as bright spheres.

The counting was done and the actual concentration of the cell suspension (number of cells/ml) was calculated according to the formula:

$$\frac{\text{(Total number of cells counted)(Dilution factor)}}{\text{(Proportion of chamber counted)(Volume of chamber)}} \times 1000$$

From the concentration, the volume of suspension required for cell plating was calculated. Approximately 1.0 x $10^4$of cell suspension was then transferred to a new 25cm^2 culture flask with 3.0 ml of new growth medium being added and the culture flask was then stored for routine maintenance of cell lines in 37°C and 5% CO_2 .

2.3 Cytotoxicity and viability assay

2.3.1 MTT assay

A cytotoxicity assay of CEB4 isolated by Chemistry Department of University Malaya was done on normal cell line and five cancer cell lines. Cells at a volume of 100.0µl/well were plated on a 96-well plate (Nunc, Denmark). The maximum density for each well was about 1.0 x 10^4 cells with culture medium and cell based controls. Cells were incubated for a period of 24 hours to allow for reattachment to the wells. All of the experiments were plated in triplicates and the results of the assays were presented as means ± SD.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was prepared by adding 50.0 mg of MTT (Merck, Germany) to 10.0 ml of 1X PBS in a 15 ml tube. The solution was stored at 2 to 8°C in the dark until further use.

The solution of the compound CEB4 was prepared by adding dimethyl sulfoxide (DMSO) (Merck, Germany). The concentration of stock solution of CEB4 is 10.0mM and the concentration of working solution of CEB4 is 1.0mM. After 24 hours the cells were treated with the CEB4 at different concentrations of 0.0, 5.0, 10.0, 20.0, 30.0, and 40.0 μM (working solution) for 6, 12, 18 and 24 hours for each concentration of CEB4. 10.0 μl of MTT solution was added to each well at the end of each incubation time point and was mixed by gently tapping the plates and incubated for approximately 2 hours at 37°C.

After the incubation hours, the media was removed and 200.0 μl of DMSO was added to each well and the plates were left to incubate overnight to allow the formazan crystals to dissolve. Once the crystals were dissolved, the absorbance values of the wells were measured using Sunrise microplate reader (Tecan, Switzerland) at 570 nm wavelength.

2.3.2 Live and dead assay

The objective of this assay is to observe cell-mediated cytotoxicity. The cytotoxicity result would give a visual confirmation of the MTT assay results. We used a LIVE/DEAD® Viability/Cytotoxicity Kit which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium bromide homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells. In brief, cells (1.0×10^5 per well) were incubated in 12-well plates, treated with CEB4 for 12 hours using IC_{50} values of each cell lines and then stained with the assay reagents for 30 minutes at ambient temperature. Cell viability was observed under a fluorescence microscope.

2.4 Flow cytometry analysis

2.4.1 Cell cycle analysis

Cell cycle analysis was performed using PI based staining methods. CEB4 treated and untreated cells were fixed in ice-cold 70% (v/v) ethanol and kept at -30°C. Staining of nuclear DNA content was conducted by adding PI (50.0g/ml) and RNase A (10.0mg/ml), followed by incubation at 37°C for 30 minutes in the dark. Fluorescence from a population of 1.0 x 10^4 cells was detected using a flow cytometer (BD FACSCalibur^{TM}, USA) at 488 nm wavelength.

2.4.2 Annexin V assay

The objective of this analysis is to determine whether the cytotoxic effect in the MTT assay is apoptotic. Detection of apoptosis was conducted using the Annexin V-FITC/PI apoptosis detection kit. Briefly, both CEB4 treated and untreated cells were harvested by trypsinization, washed in PBS and stained with annexin-V FITC conjugate and PI according to the manufacturer's protocol (Calbiochem ,Merck). Cells (1.0 x 10^4) were then analyzed by flow cytometry (BD FACSCalibur^{TM}, USA) using BD CellQuest acquisition and analysis software.

2.5 DNA fragmentation assay

Total DNA was extracted from both untreated cells and cells treated with CEB4 for 12hours and 24 hours using the Suicide Track TM DNA Ladder Isolation Kit according to the manufacturer's protocol. Isolated DNA was analysed on a 1.0% agarose gel electrophoresis and stained with ethidium bromide. Fragmentation of DNA was observed under UV illumination and visualized using a gel documentation system (Alpha Inotech, USA)

2.6 Sandwich ELISA assay

The cells were both grown in a two 20.0cm^2 culture plate separately (Nunc, Denmark) in CO_2 incubator until reached 80 % confluency. The confluency of cells on culture plate was verified using an inverted microscope (Labscope, China). Culture plates were then washed with autoclaved sterile 1 X PBS (Lonza, USA). New culture DMEM medium was added to the cell plates. Two of the cell culture plates were treated with CEB4 according to IC_{50} value (Table 2.2) and incubated in CO_2 incubator (Memmert, Germany) for 6 and 12 hours separately. Another cell culture plate was not treated with any CEB4 and act as a control. Cell lysates were harvested according to the manufacturer's protocol (Cell Signalling Technology, USA). The relative percentage of phosphorylated p53 at each time point was calculated according to the formula:

$$\frac{\text{absorbance of phosphorylated 53}}{\text{absorbance of total p53}} \text{ X } 100\ \%$$

2.7 PARP cleavage and expression of p53 and other p53 signalling related apoptotic proteins by Western analysis

Cancer cells were grown in a 10.0 cm^2 dish, until cell density reached 80% confluency. The CEB4 treated cells were then washed once with ice-cold PBS and NE-PER® Nuclear and Cytoplasmic Extraction Kit (Pierce, USA) was used to extract nuclear and cytoplasmic proteins separately instead of whole cell lysates. The protein concentration was determined by the Bio- Rad DC protein assay (Bio-Rad Laboratories, USA). Equal amounts of protein (20.0 mg/lane) were subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane.

The membranes were subsequently incubated with the corresponding primary antibodies, as indicated: Equal amount of cytoplasmic and nuclear extracts of each cell lines was probed against 9 antibodies: β-actin, PARP, p53, phospho-p53, Bax, Bcl-2, MDM2, caspase 3, Caspase 9 antibodies (Cell Signalling Technology) were probed with for 1 hour at room temperature.

After extensive washing in TBST buffer, respective secondary antibody, anti-rabbit IgG antibodies linked to horseradish peroxidase (Zymed Laboratories Inc, CA) was added with the blocking buffer for another 1 hour. Antibody bound proteins were detected by the ECL western blotting analysis system (Amersham Pharmacia Biotech UK Limited).

CHAPTER 3.0: RESULTS

3.1 MTT viability assay

The objective of these MTT assays was to assess cytotoxicity of CEB4 on the five human cancer cell lines and normal cell line and to determine the inhibition concentration (IC_{50}) of CEB4 on these human cancer cell lines. MTT assays were performed in time and dose dependent manner on oral ($HSC4^{COX2-}$ and HSC2), cervical (CaSki), breast (MCF7), liver (HepG2) and normal human bronchial epithelial (NHBE) cell lines, which acted as positive control throughout this study.

It was also of interest to investigate on which of the five different cancer cell lines (available in the laboratory) that CEB4 has the strongest cytotoxic effect. Results (figure 3.1 a-b) indicated that CEB4 induced cytotoxicity on the treated cancer cells in a dose and time dependent manner. CEB4 also shows minimal cytotoxicity effect on NHBE.

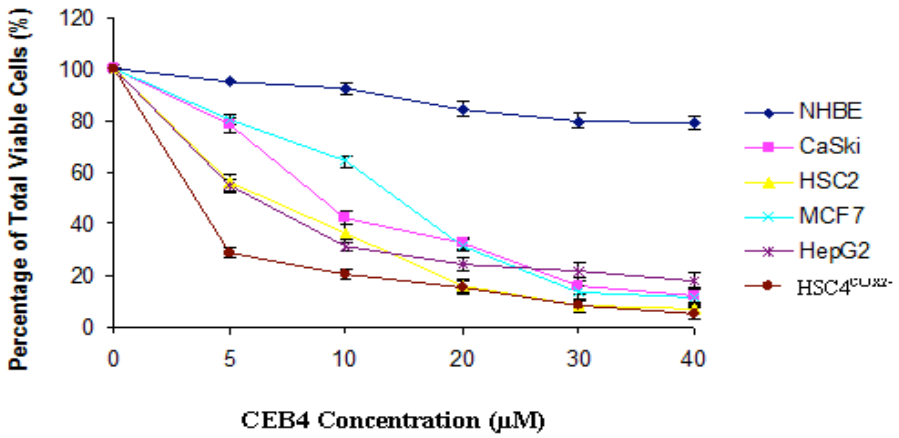


Figure 3.1(a): Dose-dependent MTT assay graph. Comparison of total relative cell viability (%) between various cancer cell lines and normal cell line (NHBE) after treatment with CEB4 at 24 hours with different concentrations (0 to 40.0µM). Results were expressed as total percentage of viable cells. Each value is the mean ±SD of three determinations.

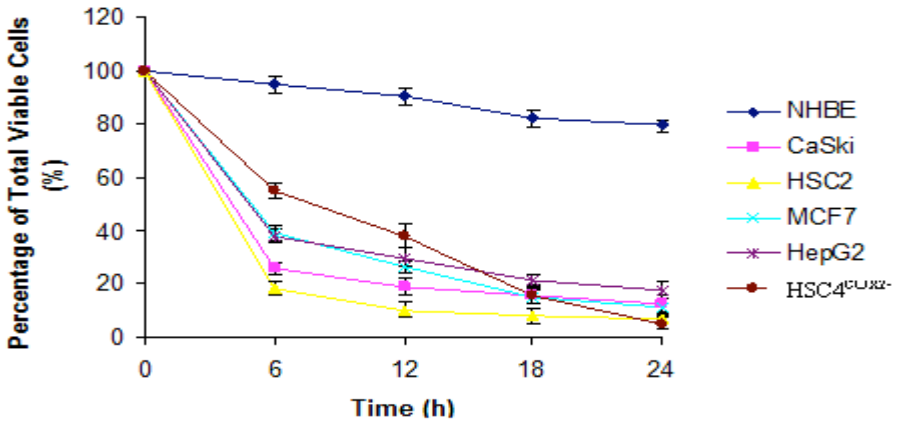


Figure 3.1(b): Time-dependent MTT assay graph. Comparison of total cell viability (%) between various tumour cell lines and normal cell line (NHBE) after treatment with 40.0μM of CEB4 at 0, 6, 12 and 24 hours. Results were expressed as total percentage of viable cells. Each value is the mean ±SD of three determinations.

Figure 3.1(a) shows percentage of total viable cancer cells of HSC4^{COX2-} HSC2, CaSki, HepG2 and MCF7 and normal cell line, NHBE, against CEB4 concentration at 5.0, 10.0, 20.0, 30.0, 40.0 μM at 24 hours incubation. Percentage of viable cancer cells decrease after 24 hours treatment. The sensitivity of the cancer cell line can be observed in ascending order: HepG2, MCF7, CaSki, HSC2 and highest levels of cytotoxicity were observed in oral squamous carcinoma cells, HSC4^{COX2-}. The result shows that CEB4 has cytotoxic effect on all five human cancer cells lines, affecting the percentage of total viable cells in a dose dependent manner.

Figure 3.1(b) shows percentage of total viable CEB4 treated cancer cells of $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 decreasing at a concentration of 40μM at 6, 12, 18 hours and at 24 hours incubation almost 100% killing was achieved. The highest degree of killing was observed in $HSC4^{COX2-}$. The results demonstrated that the cytotoxic effects of CEB4 on all five human cancer cell lines were also time dependent. Viability of all cancer cells reduced and reaches only 50% at 12 hours as incubation concentration at 50% (IC_{50}) were taken at 12 hours.

Table 3.1: The IC_{50} value for each cancer cell line

Cell lines	IC_{50} (μM)
$HSC4^{(COX2-)}$	18
HSC2	20
Caski	25
HepG2	30
MCF7	26

Therefore further experiments were conducted using IC_{50} value as shown in table 3.1 to treat all the cancer cell lines respectively. Minimal cytotoxic effect of CEB4 was observed on NHBE cells where approximately 80.0% cell viability after 24 hours of incubation at 40.0μM. This reinforces that cytotoxic effect of CEB4 was only on cancer cells. Viability of cells treated with DMSO without CEB4 insignificantly affected (<1.0 %) (data not shown), indicating that cytotoxicity was induced by CEB4 and not the solvent used to dissolve the compound. Both time and dose dependent

assays supported the need to further investigate the apoptotic effects of CEB4 and its potential as an anti-cancer drug for the treatment of cancer.

3.2 Live and dead assay

The objective of this assay was to observe cell-mediated cytotoxicity. The cytotoxicity result would give a visual confirmation of the MTT assay results. Live and dead assay was performed by using LIVE/DEAD® Viability/Cytotoxicity Assay Kit to observe viability and cytotoxicity of untreated and CEB4 treated of cancer cells and NHBE respectively. This kit provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability - intracellular esterase activity and plasma membrane integrity.

The figure 3.2 shows the untreated cells and treated cells after treatment with CEB4 at 12 hours using dyes that recognized parameters of cell viability – intracellular esterase activity and plasma membrane integrity. Calcein AM (green dye) stains live cells while ethidium homodimer (red dye) stains dead cells.

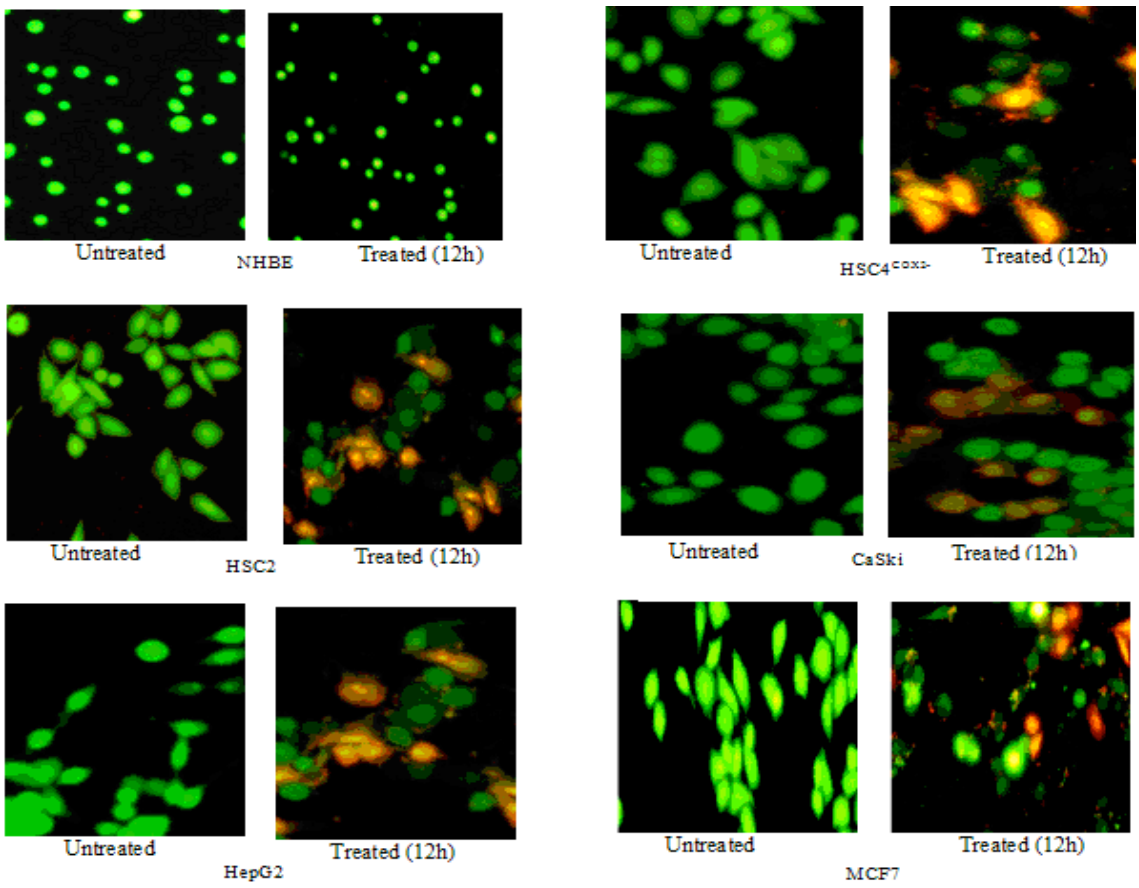


Figure 3.2: Detection of cytotoxicity by using The LIVE/DEAD® Viability/Cytotoxicity Assay Kit on NHBE (Magnification X 300), $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 (Magnification X 500).

The five CEB4 treated cancer cell lines were observed under fluorescence microscope and cell death was observed in all the cell lines. The control experiment with the normal cells, NHBE, showed no killing after treatment with CEB4. Therefore the result of this assay gives a visual confirmation of the MTT assay.

3.3 Measurement of cell cycle by flow cytometry analysis

CEB4 treated and untreated cancer cells and NHBE cells were stained separately with propidium iodide and the DNA contents were analyzed by flow cytometry to compare cell cycle progression before and after CEB4 treatment. All the five cancer cell lines; $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 were treated with CEB4 at their

respective IC_{50} value at 12 hours incubation. Only $HSC4^{COX2-}$, CaSki, HepG2 and MCF7 except in HSC2 cells (figure 3.3 f) showed cell cycle progression as demonstrated in the flow cytometry analysis (figures 3.3 b-e), respectively. There were no changes observed in the normal cell line NHBE (figure 3.3 a).

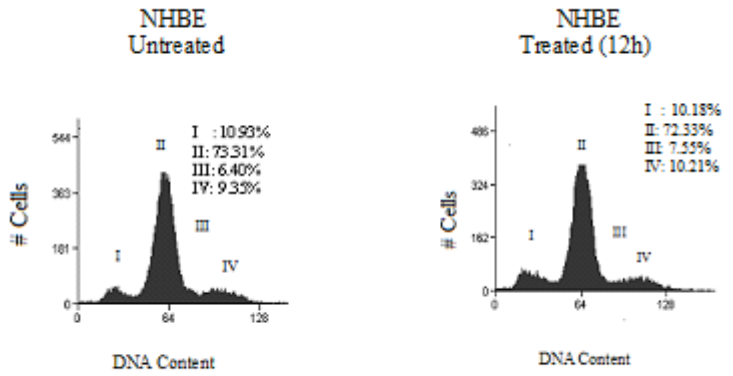
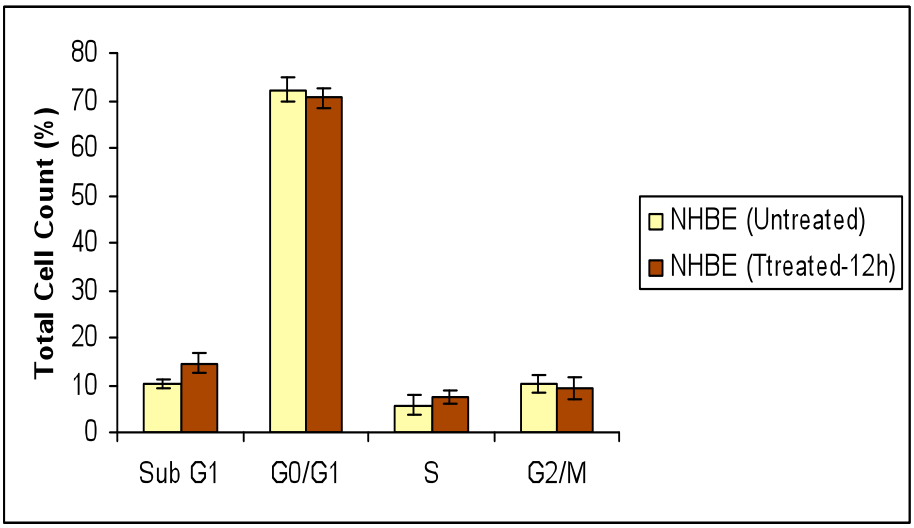


Figure 3.3(a): Cell cycle distribution of NHBE using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

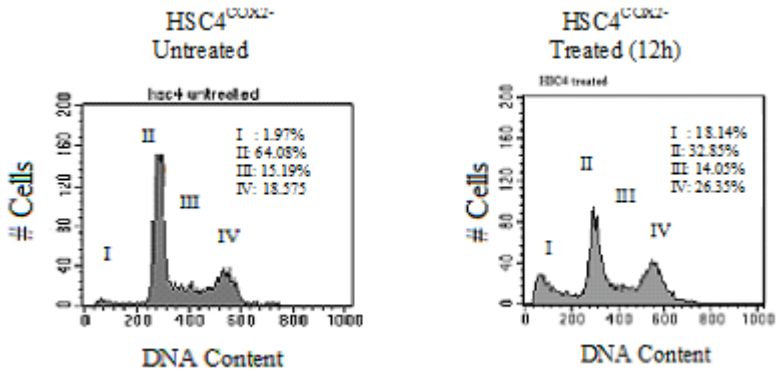
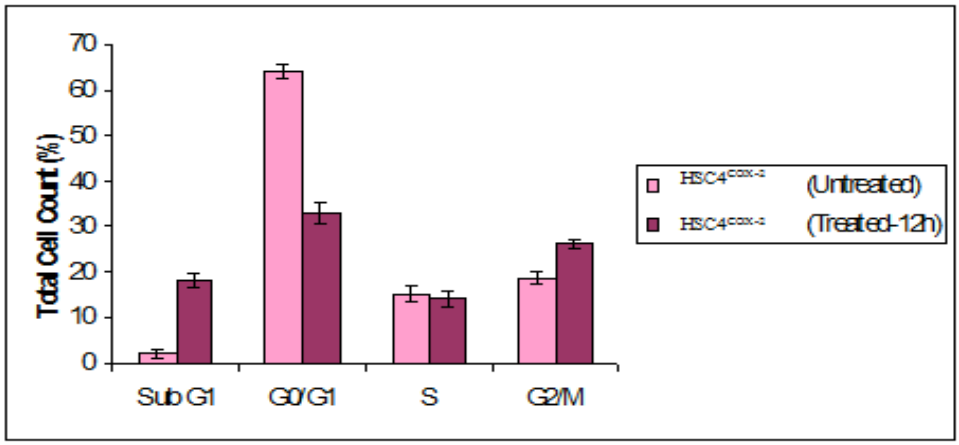


Figure 3.3(b): Cell cycle distribution of $HSC4^{COX2-}$ using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

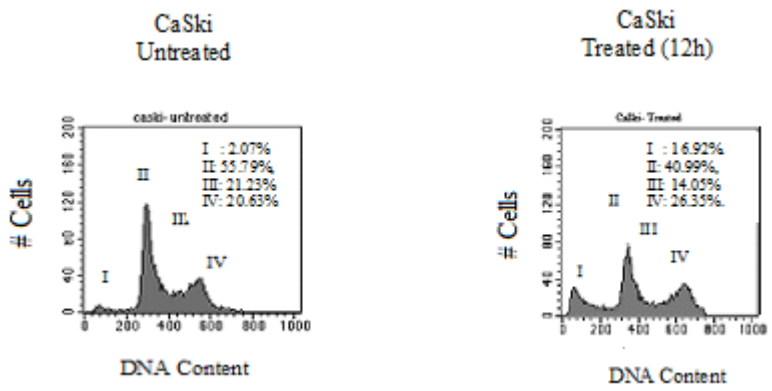
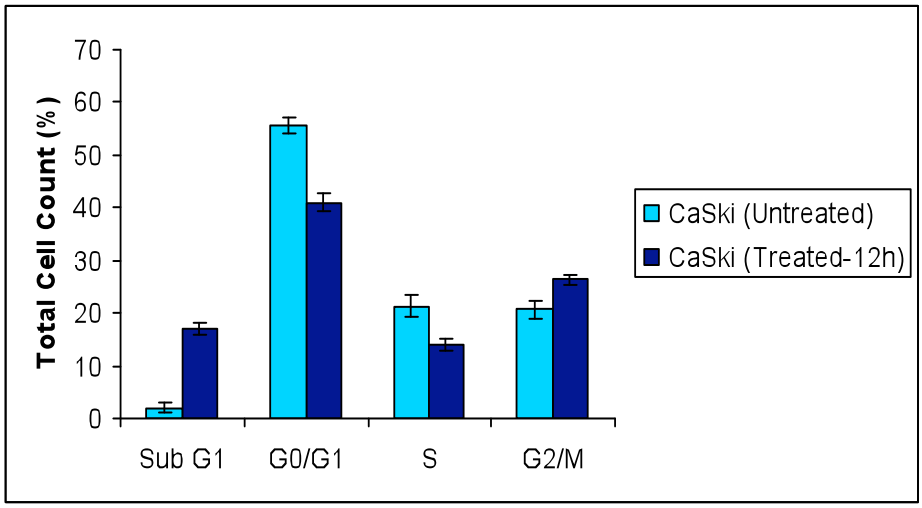


Figure 3.3(c): Cell cycle distribution of CaSki using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

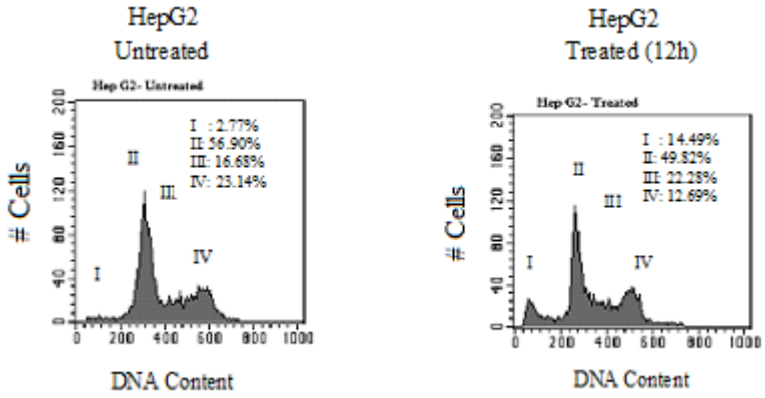
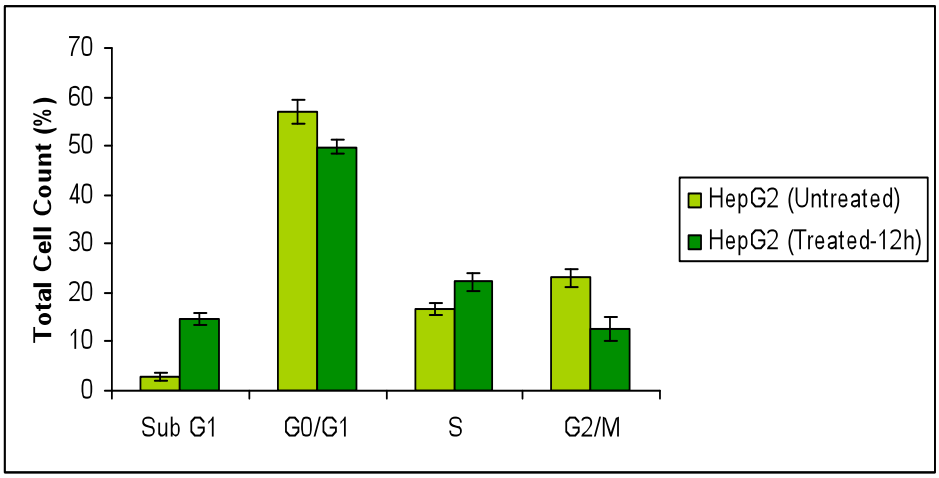


Figure 3.3(d): Cell cycle distribution of HepG2 using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

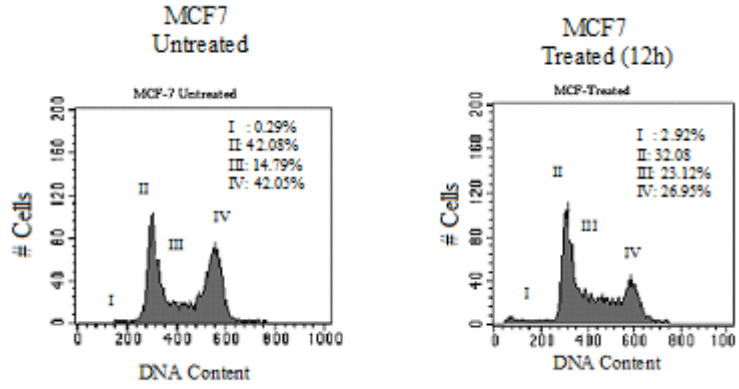
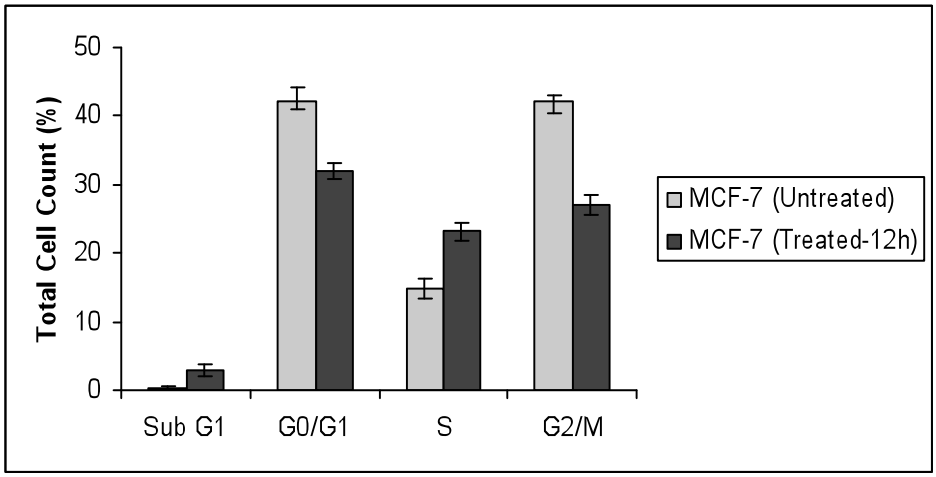


Figure 3.3(e): Cell cycle distribution of MCF7 using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

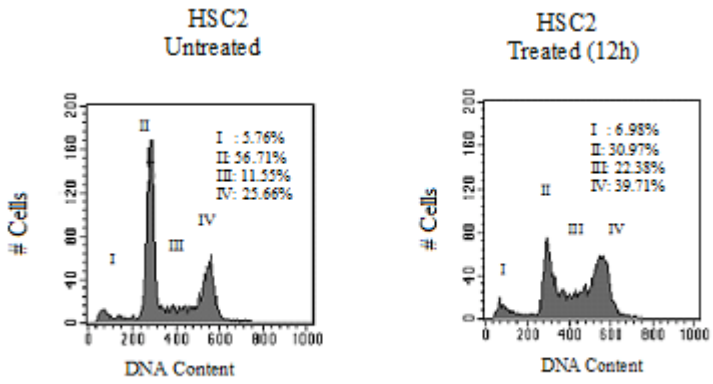
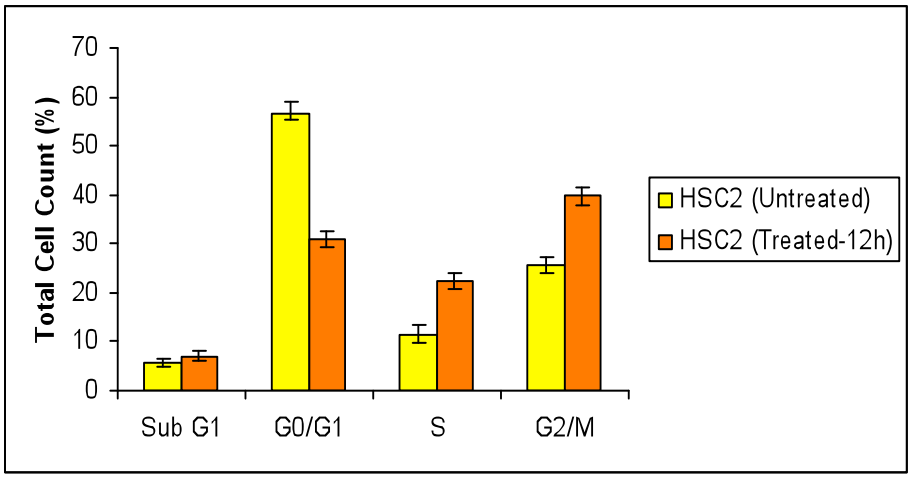


Figure 3.3(f): Cell cycle distribution of HSC2 using flow cytometry after staining with propidium iodide (PI) before and after CEB4 treatment for 12 hours. I: Sub-G_1; II: G_0/G_1; III: S; IV: G_2/M. All experiments are a representative of a 10000 cell population and mean values of three independent replicates are plotted (n=3). The percentage of cells in all cell cycle phases is indicated.



Bar graph representing mean values from independent triplicate experiments.

In analysing the percentage of cells in all the cell cycle phases; sub-G_1, G_0/G_1, S and G_2/M, the difference between the treated and untreated cell lines was used in predicting the cell cycle arrest induced by CEB4 on the five tumour cell lines.

Percentage population of HSC4^{COX2-} cells at sub-G_1 before treatment was 1.97% and increased to 18.14%, and at G_0/G_1 the population cell cycle decreased from 64.08% to 32.85% after treatment.

Percentage population of CaSki cells at sub-G_1 before treatment was 2.07% and increased to 16.92%, and at G_0/G_1 the population cell cycle decreases from 55.79% to 40.99% after treatment.

Percentage population of HepG2 cells at sub-G_1 before treatment was 2.77% and increased to 14.49%, and at G_0/G_1 the population cell cycle decreased from 56.90% to 49.82% after treatment.

In the analyses on the HSC4^{COX2-}, CaSki and HepG2 cells the reduction of G_0/G_1 phase suggested that CEB4 inhibits cell cycle progression effects at the G_0/G_1 phase.

In MCF7 cells, even though there was a decrease in the G_0/G_1 phase, but only a slight increase in the sub-G_1 phase was observed with an increase from 0.29% - 2.92%.

In HSC2 cells even though there was a decrease in the G_0/G_1 phase, there was no change in the sub-G_1 phase.

Therefore the increase in the sub-G_1 peak in all the cancer cell lines; HSC4^{COX2-}, CaSki, HepG2 and MCF-7 cells except HSC2 suggested ongoing apoptosis which was not observed in the NHBE normal cells.

3.4 Annexin V assay by flow cytometry analysis

The Annexin V assay provides a simple and effective method to detect apoptosis at a very early stage. This assay takes advantage of the fact that phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, and that the annexin V protein has a strong, specific affinity for PS. CEB4 treated cancer cells and NHBE cells at their respective IC_{50} values for 12 hours incubation was examined by flow cytometry.

As shown in figures 3.4 b-f, treatment of the tumour cell lines, at their IC_{50} concentrations of CEB4 at 12 hours induced apoptotic cell death in all cell lines. There was no changes in NHBE cells (figure 3.4 a). After treatment of all five human tumour cell lines with CEB 4 for 12 hours, there was a shift in the viable cells population to early and late stage of apoptosis (II and III) followed by secondary necrosis (IV).

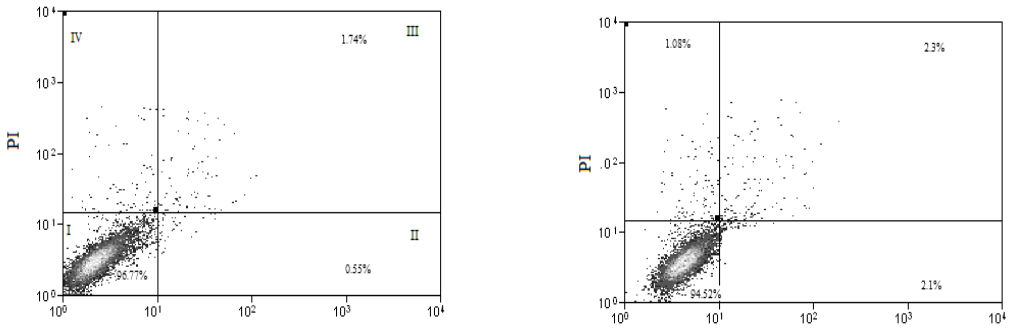
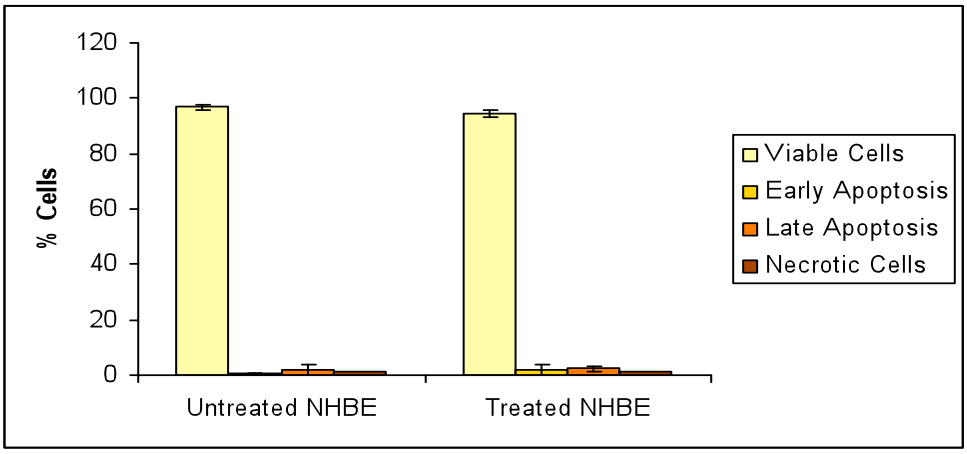


Figure 3.4 (a): Detection of apoptosis using annexin V-FITC and PI dual staining on NHBE. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).



Bar graph representing mean values from independent triplicate experiments.

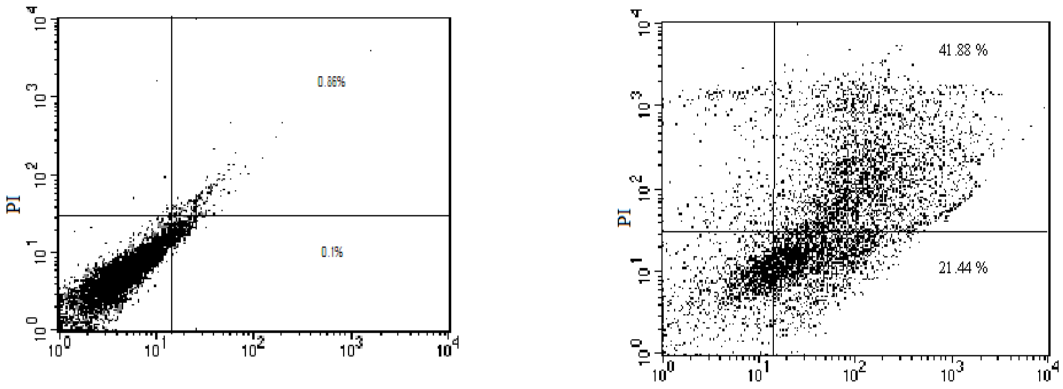
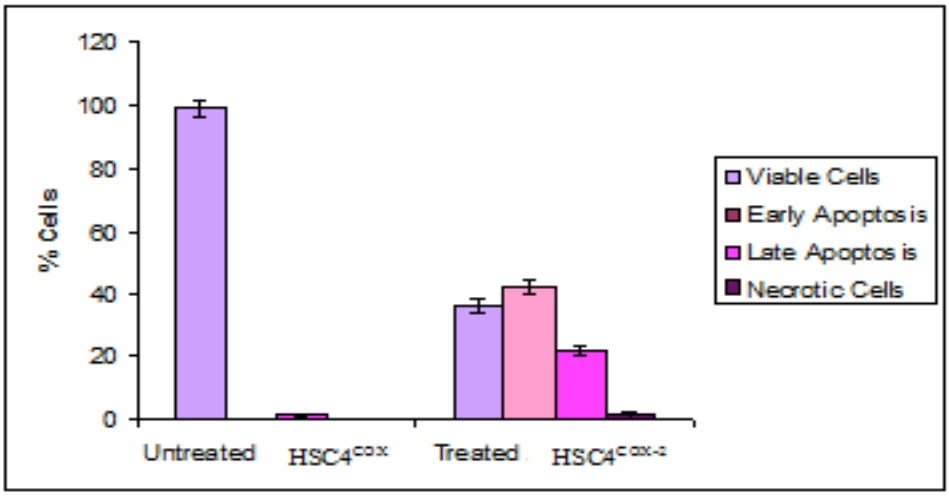
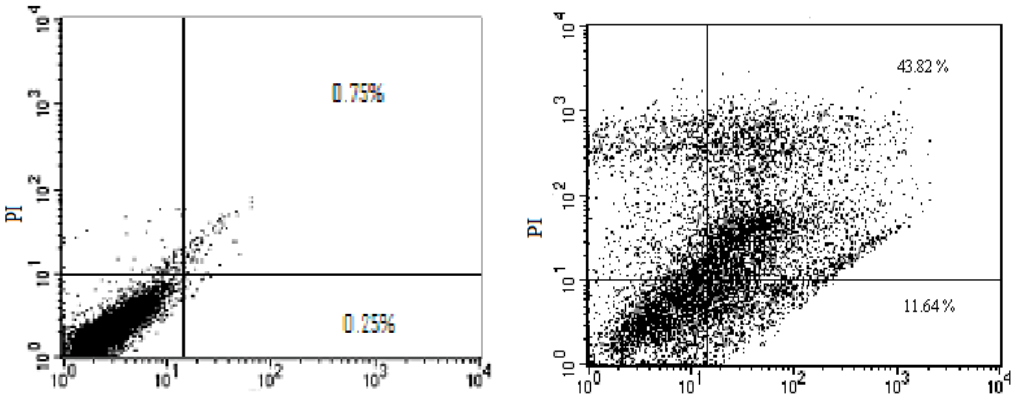


Figure 3.4 (b): Detection of apoptosis using annexin V-FITC and PI dual staining on $HSC4^{COX2}$. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).

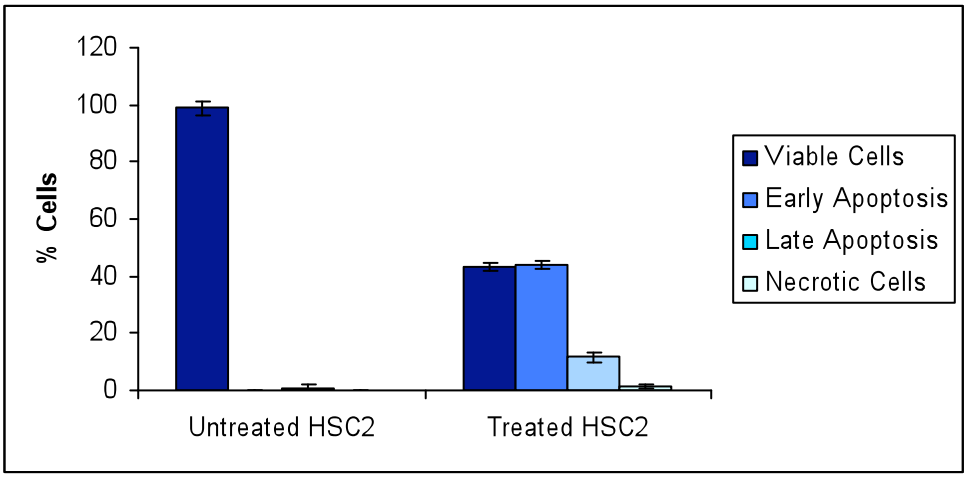


Bar graph representing mean values from independent triplicate experiments.



Annexin V-FITC Annexin V-FITC

Figure 3.4 (c): Detection of apoptosis using annexin V-FITC and PI dual staining on HSC2. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).



Bar graph representing mean values from independent triplicate experiments.

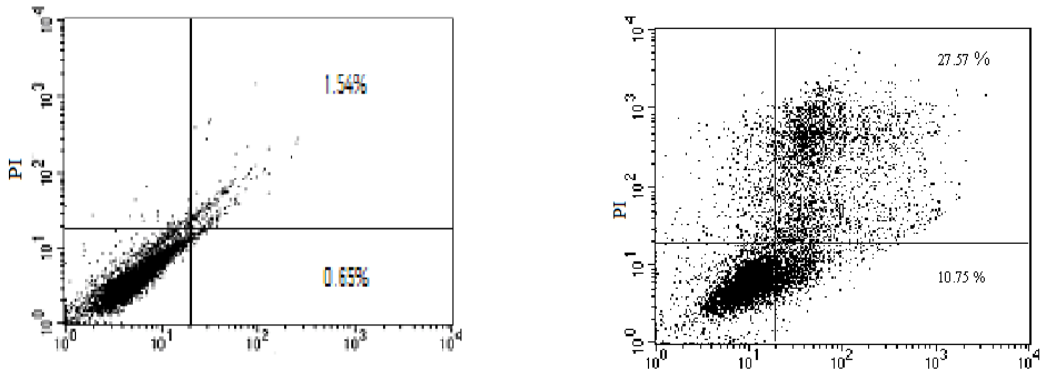
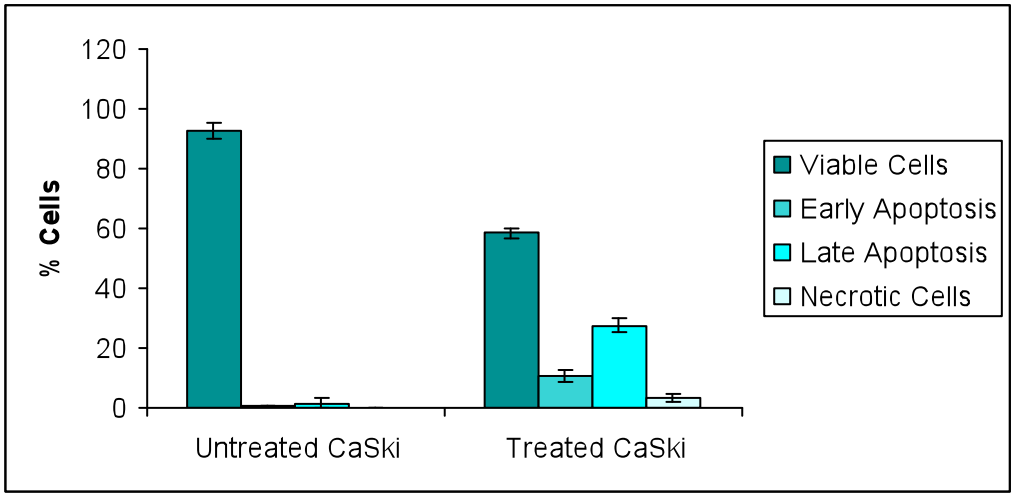


Figure 3.4 (d): Detection of apoptosis using annexin V-FITC and PI dual staining on CaSki. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).



Bar graph representing mean values from independent triplicate experiments.

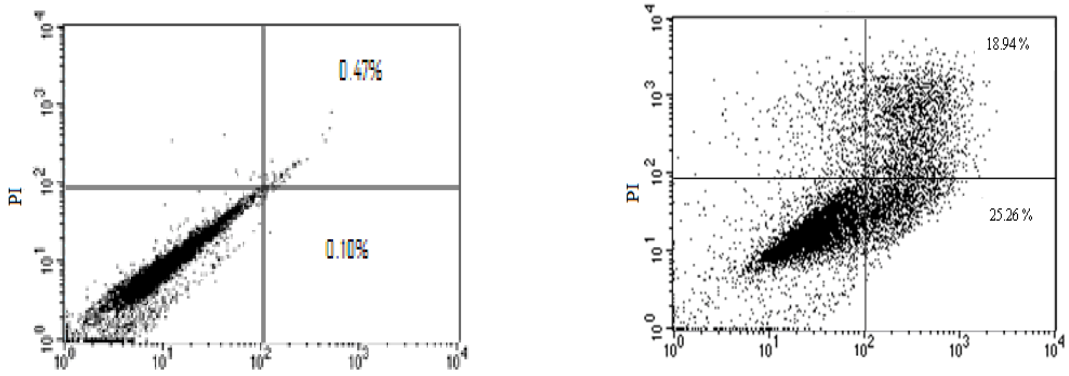
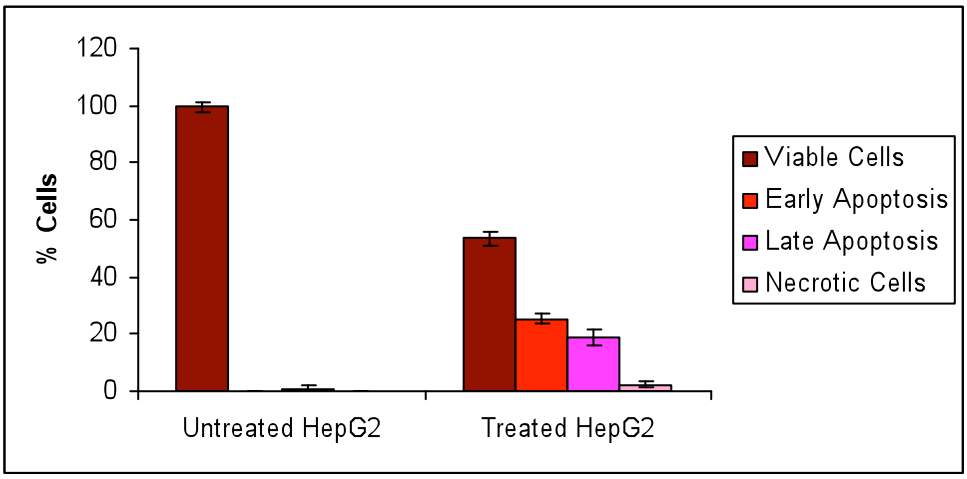


Figure 3.4 (e): Detection of apoptosis using annexin V-FITC and PI dual staining on HepG2. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).



Bar graph representing mean values from independent triplicate experiments.

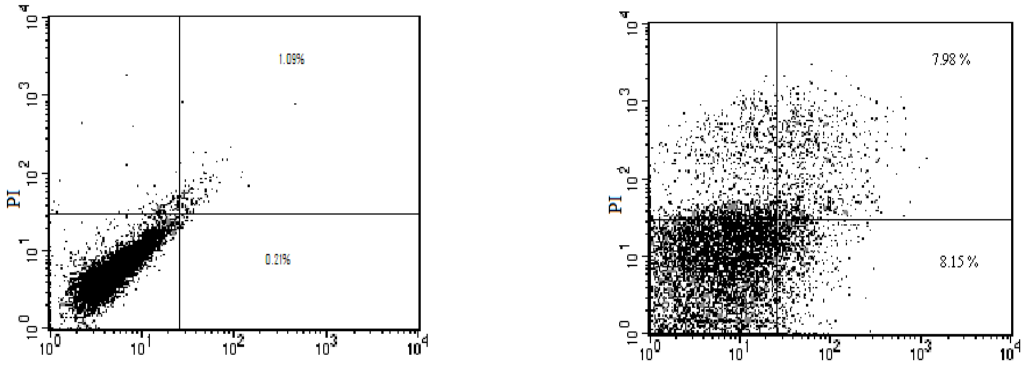
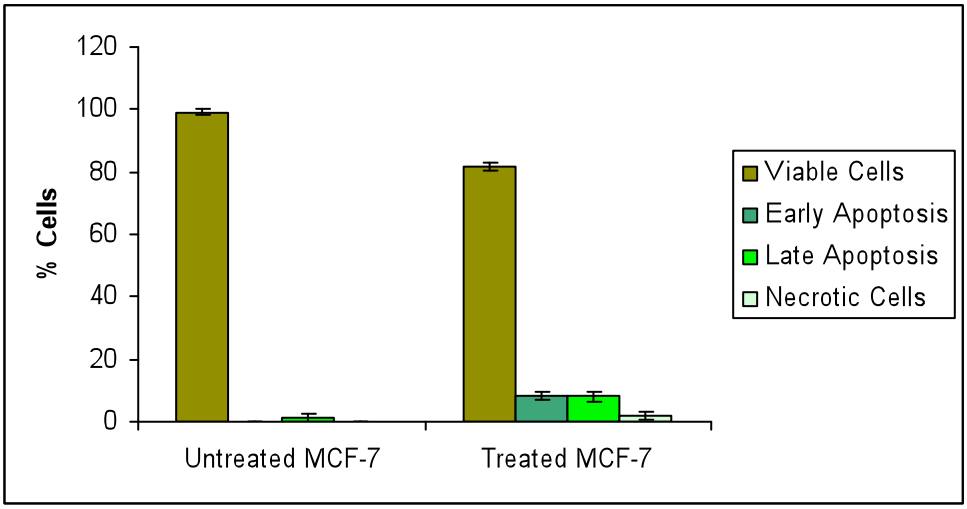


Figure 3.4 (f): Detection of apoptosis using annexin V-FITC and PI dual staining on MCF7. Untreated cells (left panel) before and treated cells (right panel) after CEB4 treatment for 12 hours. Quadrants were designed as follows – I: non-stained cells indicating live cells; II: annexin V stained cells indicating early apoptosis; III: annexin V and PI stained cells indicating late apoptosis; and IV: PI stained cells indicating 2'-necrosis. All dot plots are a representation of an equal cell population (n=10,000).



Bar graph representing mean values from independent triplicate experiments.

In analysing the shift in the viable cells populations (quadrant 1) to early (quadrant 2) and late stage of apoptosis (quadrant III) followed by secondary necrosis (quadrant IV), the highest percentage of early and late apoptotic cells were observed in $HSC4^{COX2-}$ cells with 63.32% followed by HSC2 with 55.46%. The three other cancer cell lines; CaSki, HepG2 and MCF7 showed lower levels of cell death with <50.00% of the cancer cell population being apoptotic.

3.5 PARP analysis by Western blots

To further confirm apoptosis in the five human tumour cell lines, we studied the involvement of caspases in CEB4 induced apoptosis by investigating the expression and degradation of a nuclear protein, poly (ADP-ribose) polymerase (PARP). Proteolytic cleavage of PARP from a 116 kDa polypeptide to an 85 kDa large fragment is a typical marker for the onset of apoptosis. In figure 3.5 degradation of PARP was observed in a time dependent manner in all five of the CEB4 treated human tumour cell lines. These results support that CEB4 induces caspase activation and apoptosis in all five human tumour cell lines.

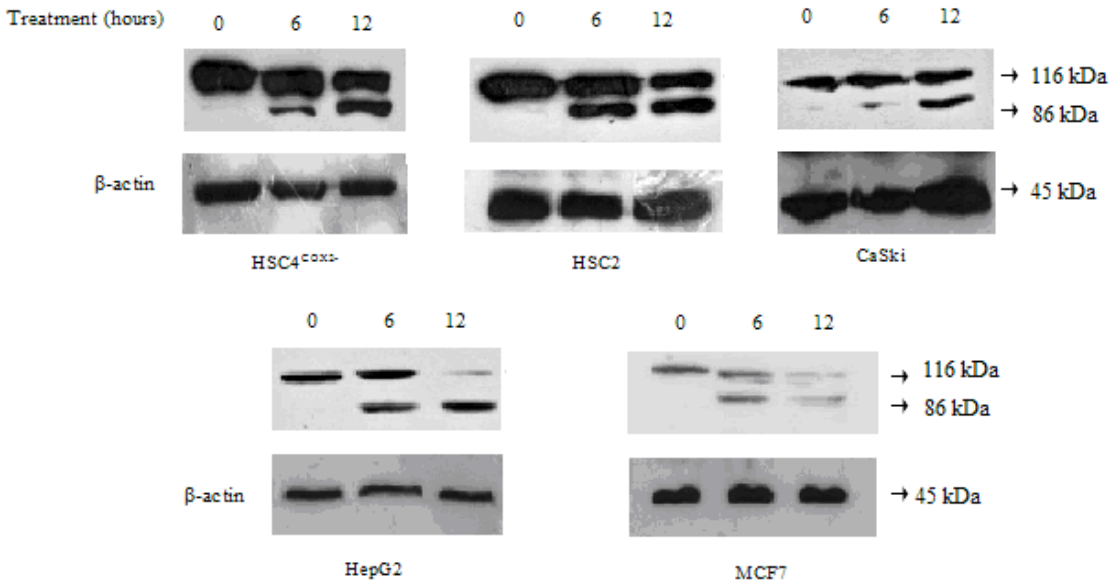


Figure 3.5: Induction of PARP degradation by CEB4 on HSC4^{COX2-}, HSC2, CaSki, HepG2 and MCF7.Cancer cell lines were cultured in media and incubated with CEB4 at different incubation time. Equal amounts of cellular proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and PARP degradation from its native form (116kDa) to the cleaved form (89kDa) was detected by Western blot analysis.

The PARP cleavage is prominent in the two oral cancer cell lines, HSC4^{COX2-} and HSC2 compare to in the other three cell lines CaSki, HepG2 and MCF7. Therefore the Western analysis of PARP cleavage confirms that CEB4 induces apoptosis in these cancer cells.

3.6 DNA fragmentation assay

This assay was performed to detect DNA cleavage in cancer cell lines to confirm results of flow cytometry and PARP analysis on HSC4^{COX2-} and HSC2 cells. The apoptotic program is manifested in a diverse manner, and besides PARP cleavage, DNA undergoes cleavage from a high to low molecular weight DNA that run rapidly upon gel electrophoresis, forming a "DNA ladder".

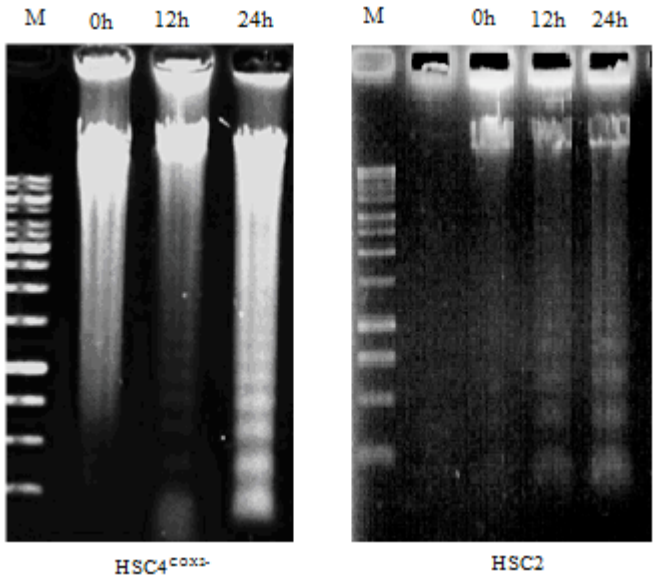


Figure 3.6: Confirmation of apoptosis mediated cell death through observation of DNA laddering using DNA fragmentation assay on HSC4^{COX2-} and HSC2. M: 100bp DNA size marker.

As seen in the gel figure 3.6, DNA from untreated HSC4^{COX2-} cells (0 hour) presented no fragmentation. The untreated DNA (0 hour) was the negative control in this study. A slight DNA degradation was observed due to possible errors occurring during the cell harvesting and DNA extraction procedures. DNA from 12 and 24 hours of CEB4 treated HSC4^{COX2-} cells showed DNA laddering on 0.1% agarose gel.

DNA from untreated HSC2 cells (0 hour) presented no fragmentation. DNA from 12 and 24 hours of CEB4 treated HSC2 cells also showed DNA laddering on 0.1% agarose gel.

Therefore, the compound CEB4 was confirmed to induce apoptotic mediated cell death as observed through the activation endonucleases-mediated nucleosome excision leading to the observation of DNA laddering of about 180-200 base pairs. Figure 3.6 (a-b) demonstrated partial and complete fragmentation of $HSC4^{COX2-}$ and HSC2 genomic DNA after 12 and 24 hours of CEB4 treatment respectively, which represent one of the major hallmark of apoptosis.

3.7 Sandwich ELISA assay

As CEB4 was shown to induce apoptosis in flow cytometry, Western analysis of PARP cleavage and DNA fragmentation, therefore the next pertinent question would be – how does it mediate its apoptotic effects?

In the development of cancer, the tumour suppressor gene, p53 which controls apoptosis is frequently mutated and as a result the cancer cells would be protected from undergoing apoptosis.

As activation (phosphorylation) of p53 leads to apoptosis, restoration of its function is an attractive therapeutic strategy to suppress tumour cell growth. To determine whether p53 plays an important role in the induction of apoptosis in the CEB4 mediated anti-proliferative effect on the five tumour cells, the level of this protein was assayed by Sandwich ELISA technique.

Results from the ELISA assay shows that treatment of tumour cells with CEB4 stimulates phosphorylation of p53 at Ser15, as detected by PathScan® Phospho-p53 (Ser15) Sandwich ELISA kit, without affecting the level of total p53 protein, as detected by PathScan® Total p53 Sandwich ELISA kit.

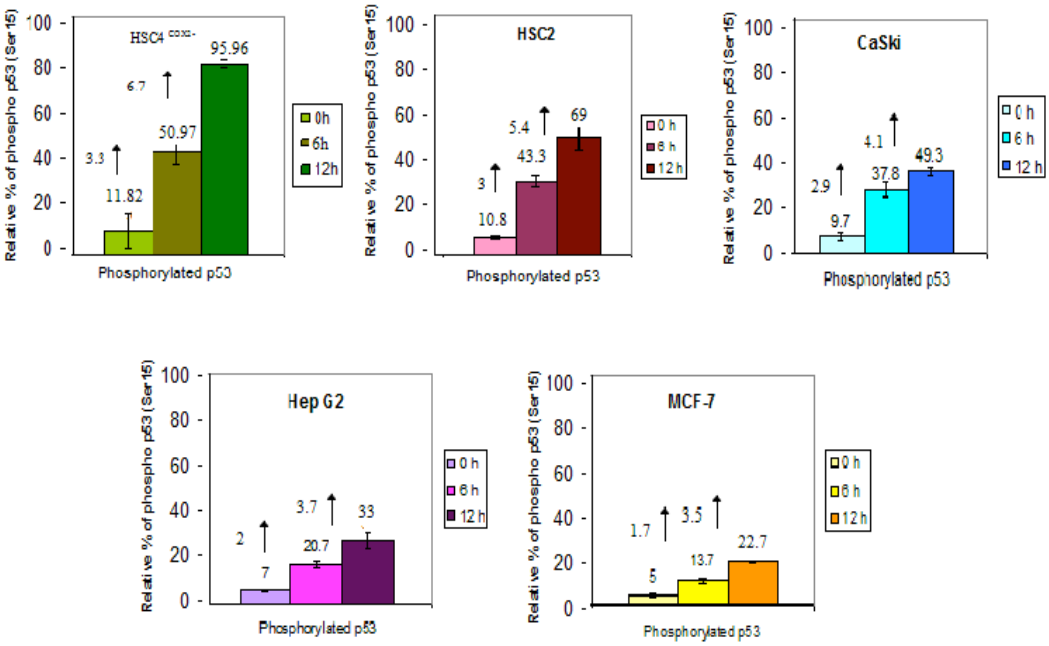


Figure 3.7: Relative percentage of phosphorylated p53 (Ser15) in HSC4^{COX2-}, HSC2, CaSki, HepG2 and MCF7 by using PathScan® Phospho-p53 (Ser15) Sandwich ELISA kit. Each value is the mean ±SD of three determinants.

As observed in the graph figure 3.5, a 3.3 fold increase which is 39.2% of phosphorylated p53 was observed in 6 hours CEB4 treated HSC4^{COX2-} cells and a 6.7 fold increase which is 84.14 % was observed in 12 hours compared to the untreated HSC4^{COX2-} cells.

A 3.0 fold increase, which is 32.5% of phosphorylated p53, was observed in 6 hours CEB4 treated HSC2 cells and a 5.4 of fold increase, which is 58.5%**,** were observed in 12 hours compared to the untreated HSC2 cells.

With the CaSki cells the level of phosphorylated p53 was not high in comparison to the two oral tumour cells where only a 2.9 fold increase, which is 28.1% of phosphorylated p53, was observed in 6 hours CEB4 treated CaSki cells and a 4.1 fold increase, which is 39.6%**,** was observed in 12 hours compared to the untreated CaSki cells. In HepG2 and MCF7 cells, the increment of phosphorylated p53 was less than 35% compared to untreated HepG2 and MCF7 cells.

In this ELISA analysis, the highest increment of phosphorylated p53 is in $HSC4^{COX2-}$ cells, followed by HSC2, CaSki, HepG2 and MCF7 which is in accordance with the apoptotic analysis carried out in this study.

3.8 Expression of p53 and other p53 signalling related apoptotic proteins by Western blot analysis

The results of the p53 ELISA analysis suggest that CEB4 anti-cancer regulation may be mediated by this important tumour suppressor gene. The aims of the Western analysis on CEB4 treated $HSC4^{COX2-}$ cells were to analyze expression of p53 related apoptotic proteins and to understand their regulation modulated by this compound.

The expression of p53 and other p53 signalling related apoptotic proteins such as total p53, phosphorylated p53, Mdm2, Bcl-2, Bax, Caspase 3 and 9 were labeled with respective monoclonal antibodies and analyzed by western blots.

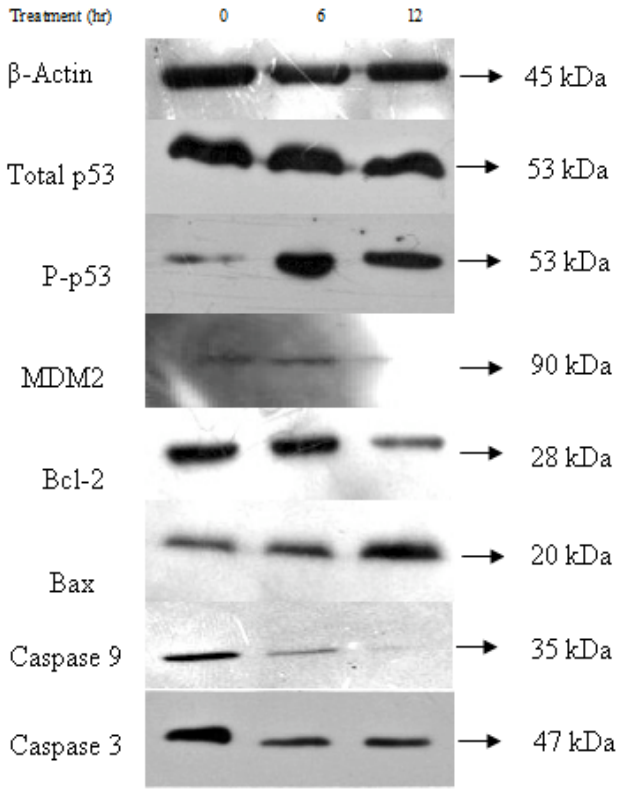


Figure 3.8: Modulation of p53 and p53 signalling related apoptotic protein expression by CEB4 in HSC4^{COX2-}.

p53 and its apoptotic related proteins levels were measured by semi- quantitative Western blot analysis after normalizing with β-actin.

Results observed in Figure 3.8 showed that upon CEB4 treatment, expression of p53 increased from 6 hours to 12 hours, expression of the inhibitor of p53, Mdm2, decreased after 6 hours and further decline after 12 hours. The level of total p53 was high in HSC4^{COX2-} cells and remained almost unaltered after CEB4 treatment.

On the other hand, the level of the pro-apoptotic Bax increased significantly after 6 hours and attained a peak at 12 hours after CEB4 treatment while the expression level of anti-apoptotic Bcl-2 decreased simultaneously with the change in expression of Bax. Interestingly, the increase in expression of phosphorylated p53 was observed together with the increase of Bax expression in $HSC4^{COX2-}$ cells as a result of CEB4 treatment.

In investigating further downstream caspases, the expression of the activator caspase 9 and the executioner caspase 3 decreased as they were cleaved but, only one of the cleaved fragments was observed in the Western blot analysis.

CHAPTER 4.0: DISCUSSION

Plants have a long history of use in the treatment of cancer. Plant-derived compounds have been an important source of several clinically useful anti-cancer agents. These include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (taxol®) (Cragg *et al.,* 2005). Malaysia has vast potential in production of such drugs due to its mostly untapped biodiversity and its traditional use of plants in treatment of various diseases.

Currently, the desired cytotoxic effect of most chemotherapeutic drugs is to induce apoptosis in cancer cells. Apoptotic mediated cell death is favored because it does not involve the sudden release of pro-inflammatory mediators which would lead to induction of inflammation, as compared to necrosis with its sudden release of pro-inflammatory mediators and induction of inflammation (Vakkila and Lotze *et al.,* 2004). There is also much accumulated knowledge about apoptosis and the mechanisms that these drugs induce the apoptotic pathway in various cancer types.

In this study, erythrocarpine E (CEB4) a new cytotoxic limonoid was isolated from the dichloromethane extract of the bark from *Chisocheton erythrocarpus* Hiern which was collected from Hutan Simpan Terenas, Kedah, and Malaysia. This plant is commonly known as Rongga in Malaysia. It is a big tree that can grow up to 80 feet tall with the branchlets velvety tomentose. Its leaves are 12 to 15 inches long with 4-6 pairs of coriaceous elliptic-oblong or broad ovate leaflets. The flowers are about 0.35 inches long with its petals elliptic silky outside and stamen-tube shorter silky below. The seeds are ex-arillate to about 1 inch and are orange red in colour.

CEB4 was repeatedly purified over silica gel column chromatography using solvent mixture (hexane: ethyl acetate) with increasing polarity identified as erythrocarpine E or CEB4 through spectroscopic methods; UV, IR, NMR, and MS.

MTT assays were performed to assess cytotoxicity of CEB4 on the various cancer cell lines; oral ($HSC4^{COX2-}$ and HSC2), cervical (CaSki), liver (HepG2), breast (MCF7) and normal human bronchial epithelial (NHBE) cell line and the inhibition concentrations (IC_{50}) of CEB4 were determined on these cancer cell lines. CEB4 treatment was performed in time and dosage dependent manner on the five cancer cell lines. The NHBE cell line acts as positive control throughout this study. CEB4 had cytotoxic effects on all five human cancer cells lines with the percentage of total viable cells observed in time and dose dependant manner. IC_{50} values were assessed at 12 hours because viability of all cancer cells reduced and reaches only 50% at 12 hours. The IC_{50} values were summarized in table 3.1 and all further experiments were conducted using these IC_{50} values as shown in table 3.1 to treat all the cancer cell lines respectively.

Minimal cytotoxic effect of CEB4 was observed on NHBE cells where approximately 80.0% cell viability after 24 hours of incubation at 40.0μM. This reinforces that the cytotoxic effect of CEB4 was only on cancer cell lines. Viability of cells treated with DMSO without CEB4 insignificantly affected (<1.0 %) (data not shown), indicating that cytotoxicity was induced by CEB4 and not the solvent used to dissolve the compound. Both time and dose dependant assays supported the need to further investigate the apoptotic effects of CEB4 and its potential as an anti-cancer drug for the treatment of cancer.

LIVE/DEAD® Viability/Cytotoxicity Assay Kit was used to further confirm the cytotoxic effect of CEB4 on the normal and five cancer cell lines. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Therefore the determination of cell viability depends on these physical and biochemical properties of cells.

These cytotoxicity results provided a visual confirmation of the MTT assay results and cell death were seen in all cell lines. The control experiment with the normal cells, NHBE, showed no killing after treatment with CEB4.

Evidence from these two results showed that indeed this drug induces cytotoxic effects on all the five cell lines and the effect was cell type dependent, in which some cell lines were more sensitive to the compound as compared to others. Both time and dose dependent assays supported the need to further investigate the apoptotic effects of CEB4 and its potential as an anti-tumour drug for the treatment of cancer. Its minimal cytotoxic effect on the normal NHBE cells was also desirable as it implies its safety in being a potential chemotherapeutic agent.

Apoptosis is a major mode and desirable cell death in response to cytotoxic drug treatment. Induction of apoptosis and inhibition of tumor cell proliferation have been used as markers for evaluation of phytochemical anti-cancer activities. Many chemotherapy agents causes disturbance in the cell cycle progression or induce apoptosis in cancer cells. The cell cycle is simply the program for cell growth and cell proliferation (Tolis *et al*., 1999). Thus, cell cycle modulation could serve as an effective method in investigating the regulation of cell proliferation.

CEB4 treatment on the five cancer cells indicates suppression of viability of cancer cell could be partially explained by the bioactive effects of this compound on cell-cycle control and apoptosis induction. All the five cancer cell lines; $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 were treated with CEB4 at their respective IC_{50} values at 12 hours incubation but only CEB4 treated $HSC4^{COX2-}$, CaSki, HepG2 and MCF7 showed cell cycle progression as demonstrated in the flow cytometry analysis. There were no changes observed in HSC2 cells. CEB4 also did not show any cytotoxic effect on NHBE cell line.

In analysing the percentage of cells in all the cell cycle phases; sub-G_1, G_0/G_1, S and G_2/M, the difference between the treated and untreated cell lines was used in predicting the cell cycle arrest induced by CEB4 on the five tumour cell lines. In the present study, treatment with the purified natural compound CEB4 on $HSC4^{COX2-}$, CaSki and HepG2 cells results in reduction of G_0/G_1 phase, which suggest that CEB4 inhibits cell cycle progression at the G_0/G_1 phase. Detection of hypodiploid (apoptotic) sub-G_1 peak population of cells in the cancer cell lines; $HSC4^{COX2-}$, CaSki, HepG2 and MCF-7 cells except HSC2 confirms CEB4 role as an inducer of apoptosis.

As it stands results from cell cycle analysis is insufficient to conclude that CEB4 has any effect on cell cycle progression. It is possible that the 12 hours time point is insufficient for the cell cycle progression to complete in order to assess effect of CEB4 on cell cycle. This could be reason there were no changes observed in the HSC2 cells.

Three types of assays were conducted to confirm apoptotic effect of CEB4 in the five human cancer cells; annexin V, PARP cleavage and DNA fragmentation assays. Extensive studies have been conducted to gain knowledge about cell proliferation in comparison to the occurrence, duration and frequency of apoptosis in tumours. The changes from early stages to the end-stage apoptotic bodies that undergo rapid phagocyte takes only a few hours without the unwanted inflammatory reaction. Changes on the surface of apoptotic cells, such as the expression of thrombospondin binding sites, loss of sialic acid residues and exposure of phosphatidylserine (PS) have been difficult to recognize. PS is a negatively charged phospholipid that is predominantly present in membrane leaflets facing the cytosol. Surface exposure of PS has been reported for activated platelets and senescent erythrocytes and recently it was shown in cells undergoing apoptosis.

Annexin V has the biological property of binding to phospholipids in a Ca^{2+} dependent way. Annexin V binds preferentially to phospholipid species such as PS. During apoptosis PS is translocated to the external surface of the cell membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane itself remains intact. On the other hand, necrosis is accompanied by loss of cell membrane integrity and leakage of cellular constituents into the environment. Therefore the measurement of annexin V binding, executed simultaneously with a dye exclusion test,

would provide a perfect assay to detect apoptotic cells and to discriminate between apoptosis and necrosis (Vermes *et al.,* 1995).

It was seen in earlier studies by Shounan *et al.,* 1998, that during apoptosis, the plasma membrane undergoes changes that allow phagocytes to recognize and engulf these cells before they rupture. The apoptotic stimulus externalization of PS occurs earlier than the nuclear changes.

Thus, the FITC conjugated annexin V binding assay of PS detects early phases of apoptosis before the loss of cell membrane integrity. This allows the measurement and scoring of individual apoptotic cells by fluorescence activated cell sorting FACS and fluorescence microscopy and makes an ideal test for the measurement of cell-mediated cytotoxicity.

Further, when annexin V apoptosis assay is combined by double staining with propidium iodide (PI) staining, it can analyze morphology changes, detection of phospholipid asymmetry and plasma membrane integrity. When membrane integrity is lost, as seen in the later stage of cell death resulting from either the apoptotic or the necrotic processes, the PI staining becomes positive. Therefore when results are only annexin V-positive, it only contains apoptotic cells whereas cells stained with both Annexin V-FITC and PI is referred to as late apoptotic (Nigam *et al*., 2008).

The effectiveness of CEB4 on the five human cancer cell lines and normal human bronchial epithelial cell line (NHBE) for apoptosis induction was examined by flow cytometry determination of annexin V-positive cells at 12 hours after treatment with this drug. An increase in cellular staining with FITC conjugated annexin V served as an early marker for apoptosis. The cells were simultaneously stained with PI to

investigate loss of cell membrane integrity. The double staining procedure distinguishes early stage apoptotic cells (annexin V-positive) from late stage apoptotic cells (annexin V-positive, PI-positive).

Upon exposure of all five human cancer cell lines to CEB4 treatment for 12 hours, the population of cells indicated a shift from viable cells to early stage and late stage apoptosis followed by secondary necrosis. In analysing the flow cytometry results, the annexin V-positive apoptotic cells after 12 hours of treatment with CEB4, the early apoptotic cells were higher in the two oral cell lines, $HSC4^{COX2-}$ and HSC2 in contrast to the liver cells (HepG2), cervical cells (CaSki), and breast cells (MCF7) cells. In this preliminary study of flow cytometry, it certainly suggests CEB4 to be most effective on the two oral cell lines $HSC4^{COX2-}$ and HSC2.

In both $HSC4^{COX2-}$ and HSC2, nearly equal amount of early apoptotic cells, approximately 40.0% were observed upon treatment with CEB4, with a slightly higher percentage, 21.44% of late apoptotic cells for $HSC4^{COX2-}$ compared to the 11.64% for HSC2.

In contrast, CaSki and HepG2 showed lower apoptotic effects after treatment with CEB4 with amounts less than 30% in the second quadrant, with an even lesser amount (approximately 16%) in MCF7 cells.

The flow cytometry analysis confirmed that cytotoxicity in the MTT assay was apoptotic, and that CEB4 induced apoptosis in $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7 cells.

Apoptosis is normally characterized by morphological and biochemical changes. Morphological changes include membrane blebbing, cellular shrinkage and chromatin condensation. Biochemical changes involve an activation of cascades of proteases such as caspases and endonucleases, cleavage of poly-AD ribose polymerase (PARP) and fragmentation of genomic DNA (Jessie *et al.*, 1999).

PARP is a DNA binding protein that recognizes DNA strand breaks and is implicated in DNA repair and in the apoptotic response of cells. PARP functions by adding poly (ADP-ribose), in an ATP and NAD dependent manner, to various proteins in response to DNA damage and has been shown to directly interact with DNA polymerase.

Caspase 3 is known to be involved in a cascade of cleavage events that disable key homeostatic and repair enzymes including PARP, which serves as a biological substrate for caspases. Caspases are activated during apoptosis and in turn cleaves PARP and correlates well with chromatin condensation and precedes the ability to detect actual DNA fragmentation. Proteolytic cleavage of PARP from a 116 kDa polypeptide to an 85 kDa fragment is a typical marker for the onset of apoptosis (Pillai *et al.,* 2004).

There are many studies carried out to identify the events that occur during early stages of apoptosis and to establish a reliable hallmark of apoptosis. One of it by Bursztajn *et al.,* 2000, showed that an increase in PARP protein and the cleavage of PARP are early signs of apoptosis that occur before any morphological and other measurable parameters of apoptosis can be detected. This increase in caspase 3 activity

precedes PARP cleavage. Changes in PARP protein and the appearance of PARP cleavage product was observed 1 hour after exposure to staurosporine.

They showed for the first time that caspase 3 activity and PARP cleavage is readily detectable at a much earlier time point than any other morphological and biochemical indices of apoptosis or cytotoxicity tested. Therefore, caspase 3 activity and PARP cleavage can serve as an early hallmark of apoptosis.

To further confirm apoptosis in the five human cancer cell lines, we studied the CEB4 treated cancer cells by investigating the expression and degradation of this nuclear protein, PARP from a 116 kDa polypeptide to an 85 kDa fragment. In this study, degradation of PARP was observed in a time dependent manner in the cancer cell lines treated with CEB4. The results support that CEB4 induces apoptosis in all five of the human cancer cell lines and maybe through caspase activation. However the intensity of band in Western blotting of PARP protein was high in the two oral cell lines, $HSC4^{COX2-}$ and HSC2. The protein band was seen at a lower intensity in the other three cell lines CaSki, HepG2 and MCF7. Therefore the Western analysis of PARP confirms the flow cytometric analysis.

The apoptotic program is manifested in a diverse manner, and besides PARP cleavage, DNA undergoes cleavage from a high to low molecular weight DNA that run rapidly upon gel electrophoresis, forming a “DNA ladder”. Cleavage of PARP prevents its enzymatic function in DNA repairing. This is followed by activation of a calcium/magnesium-dependent endonucleases, and results in inter-nucleosomal DNA fragmentation.

Cleavage of DNA fragmentation factor is also associated with inter-nucleosomal DNA fragmentation. During the inter-nucleosomal DNA fragmentation, nuclear DNA was sequentially degraded to about 300 kb, 50 kb, and then about 200 bp fragments (Nigam *et al.,* 2008).

DNA fragmentation is a late event in apoptosis that is frequently used to confirm apoptotic death, where the fragmented DNA is released into the cytoplasm as a DNA-histone complex (Jessie *et al.*, 1999).

The cancer cell lines with the prominent results for flow cytometry and PARP analyses, $HSC4^{COX2-}$ and HSC2, were further investigated using the DNA fragmentation assay. As seen in the gel, DNA from untreated $HSC4^{COX2-}$ and HSC2 cells (0 hour) presented no fragmentation. The untreated DNA was the negative control in this study.

A slight DNA degradation was observed which may be due to degradation during the cell harvesting and DNA extraction procedures. Alternatively, the smearing in the agarose gel picture was possibly caused by random DNA fragmentation, which occurs in necrosis. Theoretically, DNA fragmentation process in late stage of apoptosis would be very similar to random DNA fragmentation in necrosis. This random and non-specific DNA fragmentation, together with asynchrony of apoptotic entry was proposed as a possible reason for the occurring of mixed smearing and laddering of DNA fragments together in gels (Collins *et al.*, 1997). This may explain the CEB4-induced early apoptosis as clear laddering and smearing for late apoptosis or necrosis.

Therefore CEB4 was confirmed to induce apoptotic mediated cell death. The occurring of DNA ladder for treated HSC4^{COX2-} and HSC2 cell can be explained by the DNA degradation in an inter-nucleosomal pattern. Indeed, the DNA cleavage during apoptosis occurred at sites between nucleosome, which are protein-containing structures that occur in chromatin at approximately 200 bp intervals (Collins *et al.*, 1997).

HSC4^{COX2-} and HSC2 demonstrated partial and complete fragmentation of genomic DNA after 12 and 24 hours of CEB4 treatment respectively, which represent one of the major hallmark of apoptosis.

Apoptosis is a highly synchronized cell death program which requires the communication of complex pathways including extrinsic, intrinsic, p53, and nuclear factor kappa B pathways, and also by Bcl-2 family members. The tumour suppressor p53 is a protein that has a critical role in preventing cancer where common target for genetic alterations in human cancer, with point mutations or deletions in the p53 gene present in more than 50% of cases. The status of the p53 gene is considered to be an important determinant of tumour sensitivity to anticancer therapies. Phosphorylated p53 induces either cell cycle arrest or programmed cell death through transcriptional activation of inhibitor of cell cycle or pro-apoptotic proteins (Viktorsson *et al*., 2005).

As CEB4 was shown to induce apoptosis in flow cytometry, Western analysis of PARP cleavage and DNA fragmentation, Sandwich ELISA technique was used to determine whether tumour suppressor gene p53 played an important role in the induction of apoptosis which is commonly mutated in cancer in the CEB4 treated five cancer cell lines

Results from the ELISA assay shows that treatment of tumour cells with CEB4 stimulates phosphorylation of p53 at Ser15, detected by PathScan® Phospho-p53 (Ser15) Sandwich ELISA kit without affecting the level of total p53 protein, detected by PathScan® Total p53 Sandwich ELISA kit.

Sandwich ELISA technique was performed on the five tumour cell lines, $HSC4^{COX2-}$, HSC2, CaSki, HepG2, and MCF7 treated with CEB4 at post 12 hours treatment. In this ELISA analysis, the highest increment of phosphorylated p53 is in $HSC4^{COX2-}$ cells, followed by HSC2, CaSki, HepG2 and MCF7, which are in accordance with the apoptotic analysis carried out in this study.

It would be interesting to investigate if CEB4 induces phosphorylation sites at other serine residue than at only Ser15, such as serine residues 20, 37, or 40, by using sandwich ELISA or Western blotting techniques.

In addition, CEB4 might also induce phosphorylation event on other molecules that could trigger cell to undergo apoptosis. Hence it is possible that modification on other proteins such as Mdm2 may contribute to the stability of p53 since there is evidence that the phosphorylation mechanism itself maybe insufficient to activate p53 protein (Maya *et al*., 2001).

One possible mechanism for CEB4 to phosphorylate Mdm2 is via ATM kinase. Rami *et al.,* 1999, showed that ATM phosphorylates the carboxyl terminal end of Mdm2 by blocking the Mdm2 from targeting p53 for degradation. Therefore CEB4 might play a role in inducing phosphorylation of Mdm2 by ATM and eventually increase stability of p53 proteins to trigger apoptosis on human cancer cell lines. In verify this assumption; further investigations needs to be carried out to examine phosphorylation of Mdm2 by ATM-dependent manner on CEB4 treated human cancer cell lines.

In addition, CEB4 might probably induce the ubiquitination of Mdm2 in treated human cancer cell lines. Degradation of Mdm2 will promote the activation of tumour suppressor protein, p53, to trigger the cell to undergo apoptosis by transcriptionally regulating pro-apoptotic genes. A natural product, parthenolide, an anti-inflammatory agent, was shown to induce ubiquitination of Mdm2 in treated cells and subsequent activation of p53 (Gopal *et al.,* 2009).

Besides phosphorylation, other post-translational modification such as acetylation could promote p53 stability. Li *et al.,* (2002) showed that acetylation of p53 at C-terminal domain had abrogated the Mdm2 mediated ubiquitylation and degradation of p53. In addition acetylation of p53 proteins by acetylases p300 could enhance the sequence-specific DNA binding activity (Liu *et al*., 2000). Accordingly this might then suggest that acetylation may be a physiologic response to activate p53 as a tumor suppressor protein and to activate anti-apoptotic proteins. In contrast, suppression of p53 acetylation would render its transactivation properties.

Thus different modification such as acetylation of p53 proteins and consequently leading to p53 dependent apoptosis could be a possible explanation that no elevation in the level of phosphorylated p53 (Ser15) was observed in HepG2 and MCF7 treated tumour cell lines as in both cell lines cell death was seen. In order to verify this possibility, further investigation can be done by using acetyltransferase assay to evaluate the HepG2, MCF7, CaSki and HSC2 cells after treatment with CEB4.

The mechanism and order of events of anti-proliferation, induction of apoptosis and p53 expression by CEB4 towards human tumour cells deserves further investigation. Therefore in this study, the mechanism of action of CEB4 on the apoptotic effects; the expression of total p53 in the cancer cell and phosphorylated p53 at Ser 15 and several apoptotic related proteins (e.g. Mdm2), anti apoptotic protein (e.g. Bcl-2), pro-apoptotic protein (e.g. Bax), the activator caspase 9 and the executioner caspase 3 were quantified by Western blot analysis on oral squamous cell line, $HSC4^{COX2-}$ which showed highest cytotoxic and apoptotic effect upon treatment with CEB4. However the mechanism of apoptosis induction is still unknown. Signaling pathway of activating p53 protein is complex as until today, a complete pathway describing the triggering of p53 activation is still unknown. Therefore the exact mechanisms of how CEB4 modulating p53 activation remains to be elucidated.

p53 and its apoptotic related proteins levels were measured by semi- quantitative Western blot analysis after normalizing with β-actin. Results shows that upon CEB4 treatment, expression of p53 increases from 6 to 12hours, expression of the inhibitor of p53, Mdm2, decreases after 6 hours and further decline after 12 hours. It is an important point that CEB4 can reduce Mdm2 for it to be a possible anti-cancer agent.

Mdm2 has been considered a major p53 regulator. Mdm2 is known to function as an oncoprotein and its mode of action is to antagonize p53 and thereby prevent entrance of a cell into cell cycle arrest or prevent entrance into the apoptotic suicide program with a final outcome that favours an increase in cell number. The Mdm2 protein was shown to bind and inhibit p53. Mdm2 can inhibit p53 in two major ways, first, by interacting with p53 and block the basal transcription machinery and transcriptional co-activators such as p300.

Second, through ubiquitin-mediated proteasomal degradation regulate the p53 stability. Inhibiting Mdm2 activity in tumours that express wild-type p53 has been considered an attractive anticancer strategy for many years. Analysis of the Mdm2-p53 interface by crystallography indicated that only 3 N-terminal amino acids in p53 are essential for Mdm2-p53 interactions (Toledo *et al.,* 2007). Amplification of the Mdm2 gene is now reported in more than 10% out of 8000 human cancers from various sites.

This study shows activation of p53 signaling related apoptotic proteins and up-regulation and down-regulation of p53 signaling proteins. β-actin and total p53 protein were used as internal control. Treated $HSC4^{COX2-}$ with CEB4 showed the phosphorylated p53 at Ser15 were up-regulated while the Mdm2 was down regulated, where the results show that CEB4 may induce apoptosis via p53 signaling pathway which is phosphorylated at Ser15. Direct experimental evidence for the actual phosphorylation of p53 by CEB4 at Ser15 is unknown. However, phosphorylation of p53 by CEB4 at Ser15 might lead to reduced interaction of p53 with its negative regulator, oncoprotein Mdm2.

On the other hand, the level of the pro-apoptotic Bax increases significantly after 6 hours and attained a peak at 12 hours after CEB4 treatment, while the expression level of anti-apoptotic Bcl-2 decreases simultaneously with the change in expression of Bax. Interestingly, the increase in expression of p53 was observed together with the increase of Bax expression in $HSC4^{COX2-}$ cells as a result of CEB4 treatment. Since it is known that p53 can transactivate Bax expression, it is perhaps a possibility that the CEB4 induces $HSC4^{COX2-}$ apoptosis via the p53-dependent pathway in which Bax is a downstream molecule.

Bcl-2 family proteins are one of the regulators of apoptosis and they represent a critical checkpoint within most apoptotic pathways by acting upstream at irreversible damage in cells. At least 15 bcl-2 family members have been identified so far in mammalian cells. They function either as pro-apoptotic (Bax, Bak, and Bad) or anti-apoptotic (Bcl-2, Bcl-X_L) regulators. These proteins form heterodimers of anti- and pro-apoptotic members thereby influencing one another's function. The ratio of anti-apoptotic and pro-apoptotic proteins determines how cells respond to apoptotic or survival signals. Over expression of Bcl-2 and Bcl-X_L enhances cell survival by suppressing apoptosis and over expression of Bax accelerates cell death upon growth factor withdrawal **(**Hsu *et al*., 1997).

Since apoptosis is a complex pathway that can be regulated by many factors, only two apoptotic genes were targeted in this study. Western analysis results show up-regulation in Bax expression and down-regulation in Bcl-2 expression in the CEB4 treated $HSC4^{COX2-}$. It has been proposed that the balanced ratio between Bcl-2 and Bax

regulates the apoptotic pathway by dimerizing with each other or themselves (Matsumoto *et al.,* 2004).

An interesting explanation for the role of Bax in apoptosis induction has recently been offered in the context of PUMA. The PUMA gene is also directly induced by p53 in response to DNA damage. In humans, PUMA encodes two BH3-domain-containing proteins, PUMA-a and PUMA-b (Yu *et al.,* 2001). A vital balance between PUMA and p21 has been identified to determine the onset of arrest, or in response to exogenous p53 expression and also hypoxia in human colorectal cancer cells. Growth arrest through activation of p21 is the normal response to p53 expression in these cells. If p21 is disrupted the cells die through apoptosis and if PUMA is disrupted, apoptosis is prevented. Therefore Bax is absolutely required for PUMA mediated apoptosis. PUMA expression promotes mitochondrial translocation and multimerization of Bax, end in apoptosis induction (Yu et al., 2003).

Thus, although p53 can bind to the Bax promoter, the affinity is weak in contrast to p21 and PUMA binding (Kaeser and Iggo, 2002). Bax thus participates in the death response as an indirect target of p53 through PUMA (Yu *et al.,* 2003). In response to DNA damage, p53 activates the intrinsic mitochondrial apoptotic pathway by inducing the expression of at least three Bcl-2 pro-apoptotic family members, shifting the balance towards pro-apoptotic effects (Oda *et al.,* 2000).

Therefore, the exact target of CEB4 on the apoptotic gene remains to be elucidated. Hence, further study on other bcl-2 family genes is needed to be carried out to figure out the activity and effect of CEB4 in targeting the apoptotic gene to modulate apoptosis.

The mechanism of action of CEB4 in apoptotic cells includes not only cellular and molecular biological features, such as cell shrinkage and DNA fragmentation, but also activation of p53 signaling molecules and several apoptotic related proteins were observed which leads to activation of caspase cascade, which is a well known molecular mechanism for the induction of apoptosis.

One of the major signaling pathways involved in apoptotic cell death includes the intracellular caspases, a family of structurally related cysteine proteases. Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular proteins, which are characteristically proteolyzed during apoptosis. In response to stress activation, Bax forms a homodimer and releases cytochrome c from the mitochondria (Skulachev *et al.,* 1998), which results in the activator caspase 9 activation. For example, caspase 2, 3, 6, 7 and 9 can cleave poly (ADP ribose) polymerase (PARP).

In obtaining further insight into the mechanism of CEB4 action, we studied the expression of activator caspase 9 and executioner caspase 3 – the two major players in apoptotic cell death. In the investigation further downstream caspases, the expression of the activator caspase 9 and the executioner caspase 3 decreased as they are cleaved, results of this experiment suggest the possibility that the mechanism of CEB4 induced apoptosis in cancer cells involve the activation of both these caspases .

However the smaller cleaved fragments were not detected in this analysis. The caspase 3 antibody detects endogenous levels of full length caspase 3 (35 kDa) and the large fragment of caspase 3 resulting from cleavage (17 kDa), while the caspase 9 antibody detects endogenous levels of full length caspase 9 (47 kDa) and large

fragments of caspase 9 (37 kDa and 35 kDa). Caspases have been shown to be activated during apoptosis in many cell systems and play critical roles in both the initiation and the execution of apoptosis.

The activation of activator caspase 9 upon CEB4 treatment may activate other caspases leading to an expanding cascade of proteolytic activity within the cell and to activate executioner caspase 3. This results in the digestion of structural proteins in the cytoplasm, chromosomal DNA degradation and phagocytosis of the cell. Cell death is said to follow a classical apoptotic mode if execution of cell death is dependent on caspase activation. This perhaps is the cause of apoptosis to the tumour cells as seen in the apoptotic analysis.

According to Calviello *et al.,* 2003, treatment of HL-60 cells with Tocopheryl quinine (TQ) resulted in activation of upstream caspase 9 and of the downstream caspase 3. The early activation of caspase 9 suggests that the TQ pro-apoptotic effect may be elicited mainly via the mitochondrial pathway. It is known that cytochrome *c* released from mitochondria into the cytosol binds to the apoptotic protease activating factor (Apaf) complex and triggers the activation of procaspase 9 to the active caspase 9. The close association of the release of cytochrome *c* from mitochondria with the concurrent increase in caspase 9 provides evidence that TQ induces apoptosis in HL-60 cells through the mitochondrial pathway. TQ also induce apoptosis by the activation of the downstream caspase 3, which has been shown to play an important role in apoptosis. The study shows that caspase 3 activation was preceded by the activation of caspase 9, the apical caspase of the intrinsic mitochondrial pathway of apoptosis.

Another study shows that paclitaxel, a Taxane derivative, triggers apoptosis not through caspase 10, but via caspase 9 activation by apoptosome. In their study they used different cellular models with deficiencies in key regulators of apoptosis to elucidate the mechanism of paclitaxel-induced cell death.

Caspase 3 activity has been described to be essential for drug-induced apoptosis. Recent results suggest that in addition to its downstream executor function, caspase-3 is also involved in the processing of upstream caspase 8 and 9. Blanc *et al.,* 2000, have published data to suggest that caspase 3 is not only involved in executing the apoptosis but is also essential for the processing of upstream procaspase 9 in cisplatin-induced apoptosis.

According to Enari *et al.,* 1998, caspase 3 seems to play a central role in chemotherapy-induced apoptosis. It is specifically required for DNA fragmentation leading to the typical apoptotic pattern of DNA laddering. In some cell lines, caspase 3 plays a direct role in proteolytic cleavage of cellular proteins responsible for progression to apoptosis. Lu *et al.,* 2009, has shown that caspase 3 is involved in apoptosis and the activation of caspase 3 is considered to be the final step in many apoptosis pathways.

The activation of caspase 3 induces PARP cleavage, chromosomal DNA break and finally the occurrence of apoptosis. These results show for the first time that Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, isolated from the traditional Chinese herb *Artemisia annua*, is recommended as the first-line anti-malarial drug with low toxicity can inhibit proliferation and induce apoptosis via caspase 3 dependent mitochondrial death pathway in ASTC-a-1 cells.

All these studies reinforce that association of activator caspase 9 and the executioner caspase 3 may lead to apoptosis. The CEB4 treated HSC4 $^{COX2-}$ shows activation of caspase 9 and 3 which are shown to be activated during apoptosis in many cell systems and play critical roles in both the initiation and the execution of apoptosis. So CEB4 may induce apoptosis via the mitochondrial pathway. Hence, further study on apoptosis via mitochondrial pathway is needed to be carried out to figure out the activity and effect of CEB4 in targeting the apoptotic gene to modulate apoptosis.

Western blot results demonstrated that CEB4 causes growth arrest and apoptosis in human oral cancer cell line, HSC4 $^{COX2-}$, and the apoptotic ability of CEB4 appeared to be mediated by the regulation of p53, phosphorylated p53, Mdm2, Bcl-2, Bax, activator caspase 9 and the executioner caspase 3.

Therefore since CEB4 shows activation of p53 tumour suppressor protein, modification of CEB4's structure to increase its cytotoxic ability can be carried out to improve activation of p53. It would be interesting to understand the exact mechanism on how CEB4 stimulate phosphorylation of p53 with an aim to enhance CEB4's potential as an anticancer agent.

The apoptotic pathway is one of the most sophisticated pathways discovered in the cell to date. Its activity is tightly regulated and monitored by the cell. Recent advances and understanding of the apoptotic pathway have led to better and more innovative treatments against cancer and other diseases. However, the detailed mechanism of the apoptotic pathway is still waiting to be elucidated.

In summary, CEB4 was found to have cytotoxic and apoptotic effect on $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7. These results show that CEB4 may inhibit dose and time dependent cell proliferation and may induce apoptosis via p53 signaling pathway. CEB4 up-regulates pro-apoptotic genes and at the same time down-regulate the anti-apoptotic genes. It also activates activator caspase 9 and executioner caspase 3, which are downstream molecules to induce apoptosis. Furthermore, our data provides evidence of potential implications for the rational application of CEB4 as a novel potential anticancer drug against human cancer although much work is needed before CEB4 can be fully utilized in clinical treatments.

CHAPTER 5.0: CONCLUSION

The apoptotic pathway is one of the most sophisticated pathways discovered in the cell to date. Its activity is tightly regulated and monitored by the cell. Recent advances and understanding of the apoptotic pathway have led to better and more innovative treatments against cancer and other diseases. However, the detailed mechanism of the apoptotic pathway is still waiting to be elucidated.

Natural compounds are being investigated extensively worldwide as potential anti-cancer agents. Most of the natural anti-cancer agents available today are derived from plants, animals, marine organisms or microorganisms. In this study, erythrocarpine E (CEB4), extracted from *Chisocheton erythrocarpus* Hiern is being investigated as a potential natural compound to induce apoptosis in cancer cell lines.

CEB4 were treated on five cancer cell lines; oral ($HSC4^{COX2-}$ and HSC2), cervical (CaSki), liver (HepG2), breast (MCF7) and normal human bronchial epithelial (NHBE) cell line. The NHBE cell line acts as positive control throughout this study. MTT assay, Live/Dead® Viability/Cytotoxicity assay, cell cycle analysis, annexin V analysis, PARP cleavage analysis, DNA fragmentation assay, Sandwich ELISA assay and Western analysis were performed on these CEB4 treated tumour cell lines.

In summary, the MTT assay analysis confirmed that CEB4 has cytotoxic effect on $HSC4^{COX2-}$, HSC2, CaSki, HepG2 and MCF7, and has minimal effect on normal cell line, NHBE. These results show that CEB4 treatment indicates suppression of viability of cancer cells in a dose and time dependent manner. The LIVE/DEAD®

Viability/Cytotoxicity Assay Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. The IC_{50} values were used to treat the five tumour cell lines and cell death was seen in all cell lines. The control experiment with the normal cells, NHBE, showed no killing after treatment with CEB4. Therefore the result of this assay gives a visual confirmation of the MTT assay.

Cell cycle analysis suggested that cell cycle arrest in CEB4 treated cancer cells at G_0/G_1 for $HSC4^{COX2-}$, CaSki and HepG2. In the analysis on MCF7 and HSC2 cells, CEB4 does not have any significant arrest effect at either the G_0/G_1 or G_2/M phases. Detection of hypodiploid (apoptotic) sub-G_1 peak population of cells in the cancer cell lines; $HSC4^{COX2-}$, CaSki, HepG2 and MCF-7 cells except HSC2 confirms CEB4 role as an inducer of apoptosis.

Annexin V analysis, PARP cleavage analysis and DNA fragmentation assay were performed to determine whether the cytotoxic effect in the MTT assay is apoptotic. In annexin V analysis CEB4 treated cancer cells at their IC_{50} of CEB4 for 12 hours, induced apoptotic cell death in all cancer cell lines. There were no changes in the normal cell line NHBE. The flow cytometry analysis confirms that cytotoxicity in the MTT assays caused apoptotic mediated cell death. PARP cleavage analysis further confirmed apoptosis where Western blots results show PARP cleavage in all five CEB4 treated cancer cell lines.

Due to limitation of CEB4 compound and the cancer cell lines with the prominent results for flow cytometry and PARP analysis, $HSC4^{COX2-}$ and HSC2, were further investigated using the DNA fragmentation assay. The compound CEB4 was confirmed to induce apoptotic mediated cell death as observed through the activation endonucleases-mediated nucleosome excision leading to the observation of DNA laddering of about 180-200 base pairs. $HSC4^{COX2-}$ and HSC2 cells demonstrated fragmentation of genomic DNA after 12 and 24 hours of CEB4 treatment respectively, which represent one of the major hallmarks of apoptosis.

Results from the ELISA assay shows that treatment of tumour cells with CEB4 stimulates phosphorylation of p53 at Ser15, detected by PathScan® Phospho-p53 (Ser15) Sandwich ELISA kit without affecting the level of total p53 protein, detected by PathScan® Total p53 Sandwich ELISA kit. The highest increment of phosphorylated p53 is in $HSC4^{COX2-}$ cells, followed by HSC2, CaSki, HepG2 and MCF7 which are in accordance with the apoptotic analysis carried out in this study.

The result of the p53 ELISA analysis suggest that CEB4 anti-cancer regulation may be mediated by this important tumor suppressor gene. The aim of the Western analysis on CEB4 treated $HSC4^{COX2-}$ cells were to analyze expression of p53 related apoptotic proteins and to understand their regulation modulated by this compound. The expression of p53 and other p53 signaling related apoptotic proteins such as total p53, phosphorylated p53, Mdm2, Bcl-2, Bax, activator caspase 9 and executioner caspase 3 were labeled with respective monoclonal antibodies were analyzed.

Western blot results demonstrated that CEB4 causes growth arrest and apoptosis in HSC4^{COX2-}, and the apoptotic effect of CEB4 appeared to be mediated by the regulation of p53, phosphorylated p53, Mdm2, Bcl-2, Bax, activator caspase 9 and executioner caspase 3. Induction of apoptosis by CEB4 may involve p53 signaling pathway because there is an increased expression of p53 and decreased in expression of the p53 inhibitor, Mdm2. CEB4 up-regulates pro-apoptotic genes, Bax, and at the same time down-regulate the anti-apoptotic genes, Bcl-2. It also activates activator caspase 9 and executioner caspase 3, which are downstream molecules to induce apoptosis.

These results demonstrate the cytotoxic and apoptotic ability of erythrocarpine E or CEB4. It warrants further investigation because of its potential use as a cancer chemopreventive agent.

REFERENCES

1. Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., Knowles, B. B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature*, **282: 615-16.**

2. Akita, Y., Kozaki, K., Takeo, T., Ohmura, M., Nakagawa, A., Yanagishita, T., Kazaoka, Y., Nozaki, T., Yokoo K., Shinohara, M., Watanabe, D., Kawai, T., Yamada, S., Tamada, Y., Ohura, K., Matsumoto, Y. (2006). Enhancement of the photodynamic effects on human oral squamous cell carcinoma cell lines by treatment with calcipotriol. *Journal of Dermatological Science Supplement,* **2 57-64**.

3. Ashkenazi, A. and Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science*, **281: 1305-1308.**

4. Awang, K., Lim C.L., Mohamad, K., Morita, H., Hirasawa, Y., Takeya, K., Thoison, O., A Hadi, A.H. (2007). Erythrocarpines A-E, new cytotoxic limonoids from Chisocheton erythrocarpus. *Biorganic & Medicinal Chemistry*, **15: 5997 -6002.**

5. Bagge, D. (2008). Limonoids: Pesticide to anticancer applications from secondary metabolites of the Rutaceae and Meliaceae. *Colorado State University.*

6. Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R.,Weissberg, P. (1998). Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science,* **282, 290-293.**

7. Biscardi, J. S., Belsches, A. P. and Parsons, S. J. (1998). Characterization of human epidermal growth factor receptor and c-Src interactions in human breast tumor cells. *Molecular Carcinogenesis*, **21: 261-72.**

8. Blanc, C., Deveraux, Q.L., Krajewski, S., Janicke, R.U., Porter, A.G., Reed, J.C., Jaggi, R., Marti, A. (2000). Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. *Cancer Research,* **60: 4386-4390**.

9. Blatta, N. B. and Glicka G. D. (2001). Signaling Pathways and Effector Mechanisms Pre-Programmed Cell Death. *Bioorganic & Medicinal Chemistry* **9: 1371–1384.**

10. Bouvard, V., Zaitchouk, T., Vacher, M., Duthu, A., Canivet, M., Choisy-Rossi, C., Nieruchalski, M., May, E. (2000). Tissue and cell-specific expression of the p53-target genes: *bax*, *fas*, *mdm2* and *waf1/p21*, before and following ionising irradiation in mice. *Oncogene.* **19, 649-660.**

11. Burns, T. F., Bernhard, E. J. and El-Deiry, W. S. (2001). Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. *Oncogene,* **20, 4601-4612.**

12. Bursztajn, S., Feng, J.J., Berman, S.A., Nanda, A.2000). Poly (ADP-ribose) polymerase induction is an early signal of apoptosis in human neuroblastoma. *Molecular Brain Research*, **76: 363–376.**

13. Bykov, V. J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K. G., Selivanova, G. (2002). Restoration of the tumor suppressor functions to mutant p53 by a lowmolecular- weight compound. *Nature of Medicine*, **8: 282-288**.

14. Calviello, G., Nicuolo, F. D., Piccioni, E., Marcocci, M.E.,Serini, S., Maggiano, N., Jones, K. H., Cornwell, D.G., Palozza, P.(2003) γ-Tocopheryl quinone induces apoptosis in cancer cells via caspase-9 activation and cytochrome c release. *Carcinogenesis*, **24, 3, 427-433**.

15. Chuah, C. H., Mok, J. S. L., Liew, S.L., Ong, H. C., Yong, H. S., Goh, S. H. (Eds.). (2006).101 Plants to Fight Cancer. *An SBC Publication*.

16. Collins, J.A., Schandl, C.A., Young. K.K., Vesely, J., Willingham, M.C., (1997) Major DNA fragmentation is a late event in apoptosis. *The Journal of Histochemistry & Cytochemistry,* **45: 923-934.**

17. Cory, S. and Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Review of Cancer*, **2:647-656**.

18. Cragg, G.M., Newman, D.J., (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmcology*, **100: 72-79.**

19. da Rocha, A. B., Lopes, R. M. & Schwartsmann, G. (2001).Natural products in anticancer therapy. *Current Opinion in Pharmacology*, **1: 364-369.**

20. de Bruin, E. C. and Medema, J. P. (2008). Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treatment Reviews*, **doi:10.1016/j.ctrv.2008.07.001.**

21. Denault, J.B and Salvesen, GS (2002). "Caspases: keys in the ignition of cell death." *Chemistry Review*, **102(12): 4489-500.**

22. Dempke, W., Rie, C., Grothey, A., Schmoll, H.J. (2001).Cyclooxygenase-2: a novel target for cancer chemotherapy. *Cancer Research Clinical Oncology*, **127: 411-417.**

23. Dickson, R. B., Bates, S. E., Mc Manaway, M. E. and Lippman, M. E. (1986). Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Research*, **46: 1707-13.**

24. Ding, H. F., McGill, G., Rowan, S., Schmaltz, C., Shimamura, A.,Fisher, D. E. (1998). Oncogene-dependent regulation of caspase activation by p53 protein in a cell-free system. *Journal Biology& Chemistry*, **273:28378-28383.**

25. Earnshaw, W.C., Martins, L.M, and Kaufmann, S.H.(1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual Review Biochemistry*, **68: 383-424.**

26. Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicology & Pathology*, **35(4): 495–516.**

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

28. Evan, G., and Littlewood, T. (1998). A matter of life and cell death. *Scienc,* **281: 1317-1322**.

29. Friedler, A., Hansson, L. O., Veprintsev, D. B., Freund, S. M., Rippin, T. M., Nikolova, P. V., Proctor, M. R., Rudiger, S. ,Fersht, A. R. (2002). A peptide that binds and stabilizes p53 core domain: chaperone strategy for rescue of oncogenic mutants. *Proc. Natl. Acad. Sci. USA*, **99: 937-942.**

30. Galande, S., Dickinson, L. A., Mian, I. S., Sikorska, M.,Kohwi- Shigematsu, T. (2001). SATB1 cleavage by caspase 6 disrupts PDZ domainmediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Molecular and Cellular Biology*, **21: 5591-5604**.

31. Gewies, A.(2003). Introduction to Apoptosis.*ApoReview*, **1-26**.

32. Ghobrial, I. M., Witzig, T. E. and Adjei, A. A. (2005). Targeting Apoptosis Pathways in Cancer Therapy. *Journal of Clinical Cancer*, **55: 178-194**.

33. Gopal, Y. N. V., Chanchorn, E., and Dyke, M. W. V. (2009). Parthenolide promotes the ubiquitination of MDM2 and activates p53 cellular function. *Molecular Cancer Therapy*, **8: 552.**

34. Gordaliza, M. (2007).Natural products as lead to anticancer drugs. *Clinical & Translational Oncology*, **9: 767-776**.

35. Guthrie, N., Hasegawa, S., Manners, G. and Vandenberg,T. (2000). Inhibition of human breast cancer cells by citrus limonoids. In: Citrus Limonoids: *Functional Chemicals in Agriculture and Food. ACS Symposium*, **758: 164-174**.

36. Hall, R. E., Lee, C. S., Alexander, I. E., Shine, J., Clarke, C. L., Sutherland, R. L. (1990). Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels. *International Journal of Cancer*, ***46*: 1081-87.**

37. Hanahan, D. & Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell,* **100: 57-70.**

38. Haupt, S., Berger, M., Goldberg, Z., Haupt, Y. (2003).Apoptosis – the p53 network. *Journal of Cell Science*, **116: 4077-4085**.

39. Hiraguri, S., Godfrey, T., Nakamura, H., Graff, J., Collins, C., Shayesteh, L., Doggett, N., Johnson, K., Wheelock, M., Herman, J., Baylin, S., Pinkel, D., Gray, J. (1998). Mechanisms of inactivation of E-cadherin in breast cancer cell lines. *Cancer Research*, **58: 1972-77.**

40. Hsu Y.T., Wolter,K.G., Youle, R.J., (1997).Cytosol-to-membrane redistribution of Bax and Bcl-X_L during apoptosis. *Proc Natl Acad Sci U S A*, **94 (8): 3668–3672.**

41. Jagelska´, E., Bra´zda, V., Pospisilova´, S., Vojtesek, B.,Palecek, E. (2002). New ELISA technique for analysis of p53 protein/DNA binding properties. *Journal of Immunological Methods*, **267: 227– 235**.

42. Jessie, L., Au S., Wientjes, M. G. (1999). Kinetics of hallmark biochemical changes in Paclitaxel-induced apoptosis. *AAPS Pharmsci*, **1: 1-8.**

43. Kamangar, F., Dores, G. M., & Anderson, W. F(2006). Pattern of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *Journal of Clinical Oncology*, **24, 2137-2150.**

44. Kaeser, M. D. and Iggo, R. D**.** (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. *Proc. Natl. Acad. Sci. USA,* **99, 95-100**.

45. Kolonel, L. N., Altshuler, D. & Henderson, B. E. (2004). The multiethnic cohort study: exploring genes, lifestyle and cancer risk. *Nature Review of Cancer*, **4: 519-527.**

46. Kim, A. L., Raffo, A. J., Brandt-Rauf, P. W., Pincus, M. R., Monaco, R., Abarzua, P.,Fine, R. L. (1999). Conformational and molecular basis for induction of apoptosis by a p53 C-terminal peptide in human cancer cells. *Journal of Biological Chemistry*, **274: 34924-34931.**

47. Kim, J. R. & Kim, C. H. (2004). Association of a high activity of matrix metalloproteinase-9 to low levels of tissue inhibitors of metalloproteinase-1 and -3 in human hepatitis B-viral hepatoma cells. *International Journal of Biochemistry & Cell Biology*, **36(11): 2293-2306.**

48. Kroemer, G., Dallaporta, B. & Resche-Rigon, M.(1998).The mitochondrial death/life regulator in apoptosis and necrosis. *Annual Review of Physiology,* **60, 619-642.**

49. Lam, K.T., and Hasegawa, S. (1989). Inhibition of benzo[a]pyrene-induced forestomach neoplasia in mice by citrus limonoids. *Nutrition and Cancer,* **12: 43-47**.

50. LeBlanc, A., Liu, H., Goodyer, C., Bergeron, C., Hammond, J. (1999).Caspase-6 role in apoptosis of human neurons, amyloidogenesis, and Alzheimer's disease. *Journal of Biological Chemistry*, 274**: 23426-23436**.

51. Lim, G. C. C. & Halimah, Y. (Eds). Second Report of the National Cancer Registry. Cancer Incidence in Malaysia 2003. *National Cancer Registry*, Kuala Lumpur 2004.

52. Li, S. and Shipley, G. D. (1991). Expression of multiple species of basic fibroblast growth factor mRNA and protein in normal and tumor-derived mammary epithelial cells in culture. *Cell Growth Differentiation*, **2: 195-202**.

53. Li, M.Y., Luo, J.Y. Brook, C.L. & Gu, W. (2002).Acetylation of p53 Inhibits Its Ubiquitination by Mdm2. *Journal of Biological Chemistry*, **277(52): 50607-50611**.

54. Liu, Y., Colosimo, A. L., Yang, X. J. & Liao, D.Q. (2000).Adenovirus E1B 55-Kilodalton Oncoproteins Inhibits p53 Acetylation by PCAF. *Molecular and Cellular Biology*, **20(15): 5540-5553.**

55. Lu.Y.Y., Chen, T.C., Qu. J.L., Pan W. L., Sun.Lei., Wei X. B., (2009). Dihydroartemisinin (DHA) induces caspase-3-dependent apoptosis in human lung adenocarcinoma ASTC-a-1 cells. *Journal of Biomedical Science*, **16:16doi:10.1186/1423-0127-16-16.**

56. MacLachlan, T. K. and El-Deiry, W. S. (2002). Apoptotic threshold is lowered by p53 transactivation of caspase-6. Proc. Natl. Acad. Sci. USA. **99: 9492-9497.**

57. Maruyama, M., Matsunaga, T., Harada, E. & Ohmori, S.(2007). Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biological & Pharmaceutical Bulletin,* **30, 2091-2097.**

58. Matsumoto, H., Wada, T., Fukunaga, K., Yoshihiro, S., Matsuyama, H., Naito, Katsusuke. (2004).Bax to Bcl-2 Ratio and Ki-67 Index are Useful Predictors of Neoadjuvant Chemoradiation Therapy in Bladder Cancer. *Japanese Journal of Clinical Oncology*, **34:124-130**.

59. Maya, R. et al. (2001).ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes & Development*, **15: 1067–1077**.

60. Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber,M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M. et al. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *Journal of Experimental Medicine.* **188, 2033-2045.**

61. Muzio, M. (1998). Signaling by proteolysis: death receptors induce apoptosis. *International Journal of Clinical Laboratory Research,* **28, 141-147.**

62. Nakano, K. and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Molecular Cell.* **7, 683-694**.

63. Nicholson, D. W. and Thornberry, N. A. (2003). Life and death decisions. *Science,* **299: 214-215.**

64. Nigam, M., Ranjan, R., Srivastava, S., Sharma, R.,Balapure, A. K.,(2008) Centchroman induces G0/G1 arrest and Caspase-dependent Apoptosis involving Mitochondrial Membrane Depolarization in MCF-7 and MDA MB-231 Human Breast Cancer *Cells, Life Sciences*, **82:577–590.**

65. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T.,Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, **288: 1053-1058.**

66. Oleinik, N. V., Krupenko, N. I., Priest, D. G.,Krupenko, S. A. (2005). Cancer cells activate p53 in response to 10-formyltetrahydrofolate dehydrogenase expression. *Journal of Biochemistry,* **391: 503–511.**

67. Ozaki, Y., Ayano, S., Inaba, N., Miyake, M., Berhow, M.A.,Hasegawa, S. (1995). Limonoid glucosides in fruit, juice and processing by-products of Satsuma Mandarin (Citrus unshiu Marcov.). *Journal of Food Science*, **60: 186-189.**

68. Pillai, G. R., Srivastava, A. S., Hassanein, T.I., Chauhan, D.P., Carrier, E. (2004).Induction of apoptosis in human lung cancer cells by curcumin). *Cancer Letters*, **208:163–170**.

69. Rami, K., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y. & Shkedy, D. Rapid ATM dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage(1999). *Proc. Natl. Acad. Sci. USA*, **96(26), 14973–14977.**

70. Salgame, P., Varadhachary, A. S., Primiano, L. L., Fincke, J. E., Muller, S. ,Monestier, M. (1997) An ELISA for detection of apoptosis. Nucleic Acids Research, **25: 3**.

71. Selivanova, G., Ryabchenko, L., Jansson, E., Iotsova, V.,Wiman, K. G. (1999). Reactivation of mutant p53 through interaction of a C-terminal peptide with the core domain. *Molecular and Cellular Biology*, **19: 3395-3402.**

72. Scheffner, M., Werness, B.A., Huibregtse, J. M., Levine, A. J., Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types16 and 18 promotes the degradation of p53. *Cell,* **63: 1129-36**.

73. Sommers, C. L., Walker-Jones, D., Heckford, S. E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S. and Gelmann, E. P. (1989). Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. *Cancer Research*, ***49*: 4258-63.**

74. Shounan, Y., Feng, X., O'Connell, P. J.(1998). Apoptosis detection by annexin V binding: a novel method for the quantitation of cell-mediated cytotoxicity. *Journal of Immunological Methods*, **217: 61–70.**

75. Skulachev, V. P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Letters,* **423: 275-280.**

76. Sutherland, R. L., Hall, R. E., Pang, G. Y., Musgrove, E. A.,Clarke, C. L. (1988). Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. *Cancer Research***, 48: 5084-91**.

77. Syrjanen, K. J. (1989). Epidemiology of human papillomavirus (HPV) infections and their associations with genital squamous cell cancer. *Review article. APMIS*, ***97:957-70.***

78. Tanaka, T., Kohno, H., Kawaqbata, K., Honjo, S., Miyake, M. and Wada, K. (2000). Citrus limonoids obacunone and limonin inhibit the development of a precursor lesion, aberrant crypt foci for colon cancer in rats. In: Citrus Limonoids: Functional Chemicals in Agriculture and Foods. *ACS Symposium*, **758: 145-163.**

79. Thornborrow, E. C., Patel, S., Mastropietro, A. E., Schwartzfarb, E. M. and Manfredi, J. J. (2002). A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine bax genes. *Oncogene*, **21: 990-999**.

80. Toledo, F., and Wahl, G. M. (2007). MDM2 and MDM4: p53 regulators as targets in anticancer therapy. *International Journal of Biochemistry & Cell Biology*, **39 (7-8): 1476–1482**.

81. Tolis C, Peters G.J, Ferreira C.G, Pinedo H.M, Giaccone G.(1999) Cell Cycle Disturbances and Apoptosis Induced by Topotecan and Gemcitabine on Human Lung. Cancer Cell Lines. *European Journal of Cancer*, **Vol. 35, No. 5, pp. 796±807.**

82. Vakkila, J., and Lotze, M.T. (2004). Inflammation and necrosis promote tumour growth. *Nature Review Immunology*, **4: 641-48.**

83. Vermes, Is&m., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., (1995). A novel assay for apoptosis Flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labelled expression on Annexin V. *Journal of Immunological Methods*, **184:39-51.**

84. Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M.,Sabapathy, K. (2007). Cancer-derived p53 mutants suppress p53-target gene expression—potential mechanism for gain of function of mutant p53. *Nucleic Acids Research,* **35: 6.**

85. Viktorsson, K., Lewensohn, R., and Zhivotovsky, B. (2005).Apoptotic pathways and therapy resistance in human malignancies. *Advance Cancer Research*, **94: 143–196.**

86. Wang, W., Takimoto, R., Rastinejad, F., El-Deiry, W. S. (2003). Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. *Molecular and Cellular Biology*, **23: 2171-2181.**

87. Wen, S. F., Mahavni, V., Quijano, E., Shinoda, J., Grace, M., Musco-Hobkinson, M. L., Yang, T. Y., Chen, Y., Runnenbaum, I., Horowitz, J. et al. (2003). Assessment of p53 gene transfer and biological activities in a clinical study of adenovirus-p53 gene therapy for recurrent ovarian cancer. *Cancer Gene Therapy*, **10: 224-238**.

88. Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R. et al. (1997). KILLER/DR5 is a NA damage-inducible p53-regulated death receptor gene. *Nature Genetic,* **17, 141-143.**

89. Wu, S. F., Wang, S. X., Wang, W., Xi, Ling. & Tian, X. Reversal of the malignant phenotype of the cervical cancer CaSki cells through Adeno-associated virus-mediated delivery of HPV16 E7 antisense RNA. *Clinical Cancer Research*. **12, 2032-2037 (2006).**

90. Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R. Howley, P. M. (1985). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Journal of Pathology*, **119: 361-366.**

91. Yu, J., Zhang, L., Hwang, P., Kinzler, K. W.,Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular Cell*, **7: 673-682.**

92. Yu, J., Wang, Z., Kinzler, K. W., Vogelstein, B., Zhang, L. (2003). PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc. Natl. Acad. Sc, USA,* **100: 1931-1936**.