STUDIES OF THE ANTI-CANCER POTENTIAL OF FLAVONOIDS IN HUMAN NASOPHARYNGEAL CARCINOMA CELLS

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SUMMARY

Epidemiological studies have demonstrated that consumption of food rich in fruits and vegetables results in low incidence of cancers. Although it is not clear which components in fruits and vegetables are responsible for this preventive anti-cancer property, evidence point towards the presence of fibres, vitamins, minerals, polyphenols, terpences, alkaloids and phenolics in fruits and vegetables as the contributing factors.

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavour and colour to fruits and vegetables. When consumed in our daily life, flavonoids are able to provide beneficial effects like anti-oxidative, anti-viral, anti-tumour and anti-inflammatory activities.

The molecular mechanism underlying the anti-tumour activity of flavonoids has been extensively studied. However their effects on nasopharyngeal carcinoma (NPC) cells are relatively less studied. Therefore, in this study, we systematically investigated the anti-tumour property of two common flavonoids namely luteolin and quercetin on two NPC cell lines, CNE2 and HK1 including (i) the effects of quercetin on cell growth inhibition and apoptosis and (ii) the effects of luteolin on cell cycle arrest and (iii) the sensitisation effect of luteolin and quercetin on apoptosis induced by cancer chemotherapeutics.

We first identified the mechanism underlying quercetin-mediated cell cycle arrest in NPC cells. Quercetin was able to inhibit the transcription factor E2F-1 by keeping pRb in the hypophosphorylated form. E2F-1 is a transcription factor controlling the expression of cyclin E, the cyclin requires for S phase

progression. In addition, quercetin was able to induce apoptosis in CNE2 and HK1 by up-regulating the expression of Bad and Bax.

Next we investigated the molecular mechanisms underlying the cell cycle arrest induced by luteolin in CNE2 and HK1 cells and our study demonstrated the following: (i) Luteolin inhibited cell cycle progression at G1 phase and prevented entry into S phase in a dose- and time-dependent manner; (ii) Luteolin treatment led to down-regulation of cyclin D1 via enhanced protein phosphorylation and proteasomal degradation, leading to reduced CDK4/6 activity and suppression of retinoblastoma protein (Rb) phosphorylation, and subsequently inhibition of the transcription factor E2F-1. (iii) Lastly, luteolin was capable of suppressing Akt phosphorylation and activation, resulting in de-phosphorylation and activation of glycogen synthase kinase-3beta (GSK-3β). Activated GSK-3β then targeted cyclin D1, causing phosphorylation of cyclin D1 at Thr^{286} and subsequent proteasomal degradation. Since Akt is often over-activated in many human cancers including NPC, it is thus believed that data from this study support the potential application of luteolin as a chemotherapeutic or chemopreventive agent in human cancer.

In the third part of this study, we examined the sensitisation effect of quercetin and luteolin, both used at sub-cytotoxic concentrations on apoptosis induced by vincristine, a commonly used cancer therapeutic agents, in both CNE2 and HK1 cells. Data from this part of our study thus provide experimental evidence for potential application of combination therapy using these two flavonoids.

In conclusion, the present study provides evidence to support the potential application of flavonoids like luteolin and quercetin as chemopreventive or chemotherapeutic agents.

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LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin D
AIF	Apoptosis-inducing factor
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex/cyclosome
Apaf-1	Apoptotic-activating factor-1
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia telangiectasia and Rad3 related
ATP	Adenosine triphosphate
ATR	Ataxia and rad3 related
Bcl-2	B-cell lymphoma-2
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
BrdU	Bromodeoxyuridine
BRUCE	BIR repeat-containing ubiquitin-conjugating enzyme
CAD	Caspase-activator deoxyribonuclease
CAK	Cdk-activating kinase
CARD	Caspase recruitment domain
CDH1	CDC20 homologue 1
Cdks	Cyclin-dependent kinases
CHX	Cycloheximide
CKIs	Cyclin-dependent kinase inhibitors
c-FLIP	Cellular Fas-associated DD-like interleukin (IL)-1-converting enzyme inhibitory protein
CREB	Cyclic-AMP response element-binding protein

COX-2	Cyclooxygenase-2
CP110	Centrosomal protein of 110 kDa
DD	Death domain
DED	Death effector domain
DHFR	Dihydrofolate reductase
DIABLO	Direct IAP protein-binding protein of low pI
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulphoxide
DRs	Death receptors
DTX	Docetaxel
EBNA	EBV-determined nuclear antigens
EBV	Epstein-Barr virus
EDAR	Ectodysplasin A receptor
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-2-gallate
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Emil	Early mitotic inhibitor
Endo G	Endonuclease G
ERK	Extracellular signal regulated kinase
FADD	Fas-associating protein with death domain
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FKHR	Forkhead transcription factor

5-FU	5-Fluorouracil
GLI	Glioma-associated oncogene
GPCRs	G protein-coupled receptors
GSK-3β	Glycogen synthase kinase-3β
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible transcription factor
HLA	Human leucocyte antigen
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of caspase-activator deoxyribonuclease
IGFR	Insulin-like growth factor receptor
IKK	Inhibitor of NF-κB kinase
IMS	Mitochondrial inter-membrane space
IP	Immunoprecipitation
JNK	Jun N-terminal kinase
LiCl	Lithium chloride
LMP	Latent membrane proteins
LPH	Lactose phorizin hydrolase
Lu	Luteolin
MADD	Mitogen-activated kinase-activating death domain
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MMP	Metalloproteases
MOMP	Mitochondrial outer membrane permeabilisation

mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa B
NPA	Nuclear protein mapped at the AT locus
NPC	Nasopharyngeal carcinoma
NPM/B23	Nucleophosmin
OMM	Outer mitochondrial membrane
ORC	Origin recognition complex
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PDK1/2	3-phosphoinositide-dependent protein kinase 1 / 2
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4, 5-bisphosphate
PIP_3	Phosphatidylinositol-3, 4, 5-triphosphate
PKB	Protein kinase B
Plk	Polo-related kinase
PMSF	Phenylmethanesulfonylfluoride
pRb	Retinoblastoma
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PTP	Mitochondrial permeability transition pore
PTX	Paclitaxel

Qu	Quercetin
PVDF	Polyvinylidene difluoride
RAIDD	RIP-associated ICH-1 homologous protein with a death domain
Rheb protein	Ras homology enriched in brain protein
RIP	Receptor interacting protein
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
S6K	S6 Kinase
SCF	Skp, Cullin, F-box containing complex
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondrial activator of caspases
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TGF-β	Transforming growth factor-β
TK	Thymidine kinase
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNF-receptor associated death domain
TRAIL1	TNF-related apoptosis-inducing ligand 1
TRIS	Tris(hydroxymethyl)aminomethane
TSC2	Tuberous sclerosis protein 2
VCR	Vincristine
VGEF	Vascular endothelial growth factor

XIAP	X-linked inhibitor of apoptosis

LIST OF PUBLICATIONS

Ong CS, Zhou J, Ong CN, Shen HM (2010). Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3β-cyclin D1 pathway. **Cancer Letters** 298; 167-75

Ong CS, Tran E, Nguyen TTT et al (2004). Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. **Oncology Reports** 11; 727-33

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Ong CS, Zhou J, Ong CN, Shen HM. Involvement of the Akt-GSK-3β-cyclin D1 pathway in luteolin-induced G1/S arrest in human nasopharyngeal carcinoma. **Conference on Recent Development of Chinese Herbal Medicine.** January 25 – 26, 2010, Nanyang Technological University, Singapore.

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Literature review

Chapter 1

LITERATURE REVIEW

1.1 Cancer

1.1.1 Introduction

Cancer has one of the highest mortality rates worldwide despite great effort by research and industry in this field. It causes up to 7 million deaths worldwide based on a 2007 global study and is also the second leading global killer in the world, accounting for 12.5% of all deaths (Garcia et al., 2007). Although there are significant advances in cancer treatment over the past decades, current therapeutics have not changed and the decrease in mortality relies mostly on early detection and prevention rather than the consequence of effective therapeutics (Etzioni et al., 2003; Jemal et al., 2010).

An important aspect of cancer control and management resides in the epidemiology of the disease. Epidemiological studies have linked certain types of cancer among certain groups of people (Haenszel and Kurihara, 1968; Kolonel et al., 2004; Ziegler et al., 1993) and populations that consume food rich in fruits and vegetables have a lower incident rate of cancer development (Block et al., 1992; Reddy et al., 2003; Willett, 2000). Fruits and vegetables contain high fibre content, vitamins, minerals as well as components like polyphenols, terpenes, alkaloids and phenolics. The last group of components are the phytochemicals and flavonoids and these agents have been found to suppress inflammatory processes that can lead to transformation, hyperproliferation and the initiation of tumourigenesis.

Tumourigenesis is a multi-step process that can be triggered by many factors amongst them carcinogens including environmental antigens,

inflammatory agents and tumour promoters (Mathers et al., 2010). These carcinogens are known to activate intracellular pathways linked to cell division and growth; angiogenesis and anti-apoptosis. Dietary agents like phytochemicals and flavonoids are known to act on some of the intracellular pathways which not only prevent but can also be used as therapy of cancers (Aggarwal and Shishodia, 2006).

1.1.2 Cancer initiation and progression

Over the last decades, many key genes responsible for tumourigenesis have been identified. In addition, mutations to these genes have also been mapped and the pathway through which they act characterised. Cancer initiation and progression is regarded as a multi-step process involving progressive genetic alterations that leads to the transformation of normal cells into highly malignant precursors (Bertram, 2000).

Genetic alterations resulting in tumourigenesis are seen in three types of genes; oncogenes, tumour-suppressor genes and stability genes (Ponder, 2001; Stratton et al., 2009; Volgelstein and Kinzler, 2004). Unlike certain diseases like muscular dystrophy whose manifestation is due to a mutation to one gene, cancer development is caused by defects in several genes. Mammalian cells however have ways to safeguard themselves against the potentially lethal effects of cancer gene mutations; only when several genes are defective does an invasive cancer develop (Balmain et al., 2003; Bell, 2010). In this sense, one would think of mutated cancer genes that contribute to, rather than causing cancer.

Genomic instability and natural selection have been linked to the development of pre-malignant cells. In order for this group of cells to to reach the

biological endpoints characterised by malignant growth, self-sufficiency in growth signals, resistance to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis must occur (Hanahan and Weinberg, 2000; Sieber et al., 2003). With mutation and genomic instability working hand-in-hand, spontaneous and environmental DNA damage occur. These play important roles in the initiation and progression of neoplasms. On the other hand, cells do exhibit biological responses that will protect them from the consequences of mutations, most critically those that bring about cell cycle arrest and/or cell death. The cell cycle arrest checkpoints provide time for DNA repair before cell cycle progression is resumed, or if the damage is too extensive, apoptosis will be activated (Friedberg et al., 2004).

Mutations that lead to defective DNA sensing mechanism can also compromise the cell's DNA damage response. This can result in malignant transformation as observed in disorders like ataxia telangiectasia (AT), Li-Fraumeni syndrome, Nijmegen breakage syndrome and Fanconi anaemia (Motoyama and Naka, 2004). These include genes that encode for protein kinases like ATM (Ataxia-telangiectasia-mutated) and ATR (Ataxia telangiectasia and Rad3 related) and their downstream effector kinases like Chk1 and Chk2; and transcription factor p53 that can convey the damage signal to the various pathways that implement appropriate biological activities like DNA repair, cell cycle arrest and apoptosis (Shiloh, 2003).

Although the majority of cancers are triggered by mutational events, it is still not fully understood how cancer cells acquire so many mutations and chromosomal abnormalities that are observed in most cancers (Loeb et al., 2008).

There is evidence that genetic instability in cancers exists at two levels. The first form of instability is observed at the nucleotide level in a small subset of cancers which results in base substitutions or deletions or insertions of a few nucleotides. The second form of instability which is observed in most cancers is at the chromosomal level that results in losses and gains of whole chromosomes or part of (Lengauer et al., 1998). Chromosomal instability in some cancers leads to aneuploidy and a loss of heterozygosity which is associated with the inactivation of tumour suppressor genes (Michor et al., 2005).

Thus cancer cells can be viewed as cells that possess "mutator phenotype" to makes them more susceptible to small mutations which affect their growth regulatory genes (Bignold, 2004; Loeb, 1991). A second possibility in cancer initiation is that cancer cells start out more prone to genomic instability compared to normal cells. Mutations in these cells occur at a normal rate, but due to certain epigenetic events, they divide at a higher frequency rate compared to normal cells, thus leading to an accumulation of genetic mutations within this group of cells (Tysnes and Bjerkvig, 2007).

1.1.3 Alterations in cancer genomes and signal transduction

Mutations to proto-oncogenes lead to the constitutive expression of these genes in cells which are not seen in the wild-type genes. Oncogene mutation and activation can result from chromosomal translocations, gene amplification or from subtle intragenic mutations affecting crucial resides that regulate the activity of the gene product (Nambiar et al., 2008).

Mutations to tumour-suppressor genes work in the opposite way to that seen in oncogenes, namely a reduction in gene products or activities is observed.

Such inactivation arise from missense mutations at sites that are essential for tumour-suppressor activity, mutations that lead to the formation of truncated protein and also from deletions or insertions or epigenetic silencing of these genes (Negrini et al., 2010).

Oncogene and tumour-suppressor gene mutations result in similar activities; neoplasms in which cells are stimulated to undergo cell division and at the same time inhibiting cell death or cell cycle arrest. This increase in cell number is caused by activating genes that drive the cell cycle and inhibiting normal apoptotic processes or by facilitating the provision of nutrients to cells through enhanced angiogenesis.

The third group of genes termed stability genes or caretakers also promotes tumourigenesis when altered. However they promote tumourigenesis in a different manner compared to oncogenes and tumour-suppressor genes (Maynard et al., 2009; Rassool et al., 2007; Wimmer and Etzler, 2008) . Stability genes include those involved in DNA repair that are called into action to perform mismatch repair, nucleotide-excision repair and base-excision repair.

Mutation to these three groups of genes can occur in the germline or to a single somatic cell. The former will result in a genetic disposition to cancer and in the latter to sporadic tumours (Volgelstein and Kinzler, 2004). As a result of intensive cancer research over the past decade, it is established that cancer-gene mutation affects critical pathways which results in tumourigenesis. For instance, several cancer genes directly control the retinoblastoma (Rb) pathway that controls cell division. These include the genes that encode for proteins that are involved in the transition from a resting stage (G0 or G1) to a replicating stage (S) of the cell cycle like cyclin dependent kinase 4 (cdk4), cyclin D1, pRb and p16

(Classon and Harlow, 2002; Ortega et al., 2002; Sherr, 2000). In this instance, the genes encoding Rb and p16 are tumour suppressor genes inactivated by mutation and cdk4 and cyclin D1 are oncogenes activated by mutation. A second well documented pathway affected by alteration to the tumour suppressor genes and oncogenes is the one that is controlled by the TP73 protein. p53 is a transcription factor that inhibits cell growth and stimulates cell death when induced by cellular stress (Oren, 2003; Prives and Hall, 1999; Vogelstein et al., 2000). Disruption of this pathway can be brought about by a mutation to the p53 gene that inactivates its ability to bind specifically to its cognate recognition sequence, amplification of the *MDM2* gene and infection with DNA tumour viruses whose products bind to p53 and inactivate it (Volgelstein and Kinzler, 2004).

In addition to the Rb and p53 pathways, there are other pathways that have a role in many tumour types including those that involve adenomatous polyposis coli (APC) (Kwong and Dove, 2009; Wasch et al., 2010), glioma-associated oncogene (GLI) (Liao et al., 2009; Lo et al., 2009), hypoxia-inducible transcription factor-1 (HIF-1) (Dales et al., 2010; Kimbro and Simons, 2006) , phosphoinositide 3-kinase (PI3K) (Carnero, 2010; Courtney et al., 2010), SMADs (Nagaraj and Datta, 2010; Yang and Yang, 2010) and receptor tyrosine kinases (RTKs) (Rosell et al., 2010; Saif, 2010).

1.2 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a head and neck cancer of epithelial origin. Although it occurs sporadically in the western hemisphere, it is endemic in South China and Southeast Asia with an incidence rate of between 15 and 50 per 100 000 in man (Ho, 1978). There is an intermediate incidence among the

Alaskan Eskimos (Nutting et al., 1993), Arabs of North Africa (Parkin et al., 1997) and parts of the Middle East (Steinitz et al., 1989). Chinese emigrants exhibit a high incidence of this disease but the rate among ethnic Chinese born in North America is lower than their counterparts in China (Buell, 1974). These studies imply that both environmental and genetic factors play important roles in the development of NPC. One of the environmental factors is a diet consisting of preserved food, particularly at an early age (Armstrong et al., 1998; Yu and Henderson, 1987; Yu et al., 1988; Yuan et al., 2000). These findings have been further verified when rats fed with preserved food like salted fish developed nasal cavity carcinoma in a dose-dependent manner (Zheng et al., 1994). A change in lifestyle due to rapid economic development which leads to a decrease in intake of preserved food has resulted in a statistically significant decrease in incidence rate of NPC in Singapore and Hong Kong (Luo et al., 2007).

Certain human leucocyte antigen (HLA) subtypes have been associated with NPC indicating a strong genetic factor in the development of NPC (Goldsmith et al., 2002; Tse et al., 2009; Yu et al., 2009).

NPC is classified based on histology into three types (Shanmugaratnam and Sobin, 1991). Type 1 NPC is a keratinising squamous carcinoma which is characterised by the presence of well-differentiated cells that produce keratin. Type 2 is a non-keratinising squamous carcinoma with cells of varying degree of differentiation but does not produce keratin. Type 3 is also a form of non-keratinising squamous carcinoma but is less differentiated, with highly variable cell types. Types 2 and 3 NPC are Epstein-Barr virus (EBV) associated and have better prognoses compared to Type 1. However, recent data indicate that most NPC tumours, regardless of their histologic subtype, have comorbid EBV

infections, demonstrating a close association between EBV infection and NPC (Burgos, 2005; Raab-Traub, 2002). The presence of EBV latent genes encoding for the latent membrane proteins (LMP1, LMP2A and LMP2B) and EBV-determined nuclear antigens (EBNA1 and EBNA2) are prevalently expressed in NPC (Tsao et al., 2002). Moreover, LMP1, an oncogene that brings about cell immortalisation is present in 80 – 90% of NPC tumours (Lin et al., 2001).

The carboxyl-terminal region of LMP1 has been demonstrated to up-regulate pathways that promote cellular proliferation like the PI3K/Akt, NF-κB (nuclear factor-kappa B), MAP (mitogen-activated protein)) kinase, ERK (extracellular signal regulated kinase), p38 and JNK (Jun N-terminal kinase) and JAK/STAT (signal transducer and activator of transcription) (Shi et al., 2006). Activation of transcription factors downstream of these pathways including NF-κB and β-catenin leads to uncontrolled cell proliferation via c-Myc (Luo et al., 1997), cyclin D1 and cyclin E (Chou et al., 2008; Hwang et al., 2002; Tao et al., 2005) expressions; and inhibition of tumour suppressor proteins, p16, p27 and p53 (Chen et al., 2004; Chou et al., 2008; Hwang et al., 2003; Makitie et al., 2003) (Figure 1.1). LMP1-positive cells have greater mobility, leading to higher metastatic potential (Ozyar et al., 2004) and faster disease progression (Liu et al., 2003).

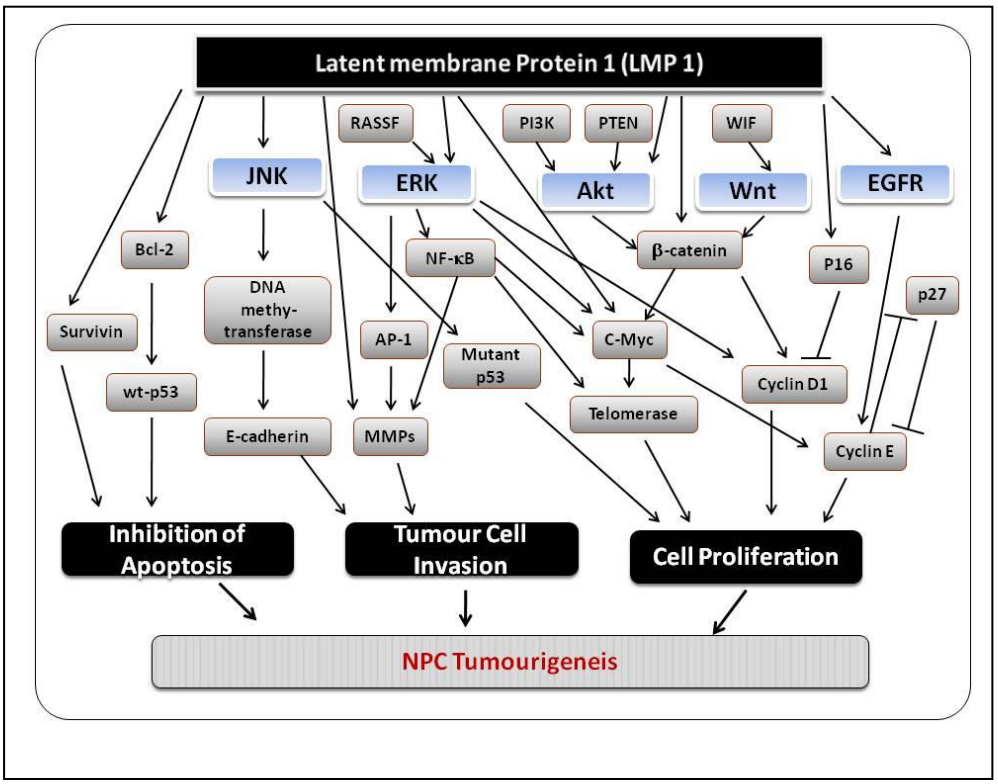


Fig 1.1: Overview of the molecular mechanisms involved in NPC development (adapted from (Chou et al., 2008))

PI3K is involved in a wide variety of cellular pathway including the regulation of cell proliferation via Akt. Over-activation of PI3K has been implicated in numerous cancers including NPC. In NPC, this over-activation occurs by various mechanisms (Morrison et al., 2004). LMP1 can also activate Akt directly (Morrison and Raab-Traub, 2005). A third possible mechanism is by down-regulating the expression of phosphatase and tensin homology (PTEN) (Pedrero et al., 2005), an inhibitor of PI3K. Akt is critical in cell growth and survival as it activates the mechanism for cell proliferation and inhibits apoptosis and is a key protein in tumourigenesis (Song et al., 2005a).

Like in all cancers, development of NPC involves amongst the various processes, the deregulation of the cell cycle. The LMP1 plays a critical role in the abnormal deregulation of key proteins in cell cycle regulation. Proteins that

enhanced cell cycle progression like c-Myc, cyclin D1, ERK, epidermal growth factor receptor (EGFR) and mutant p53 are up-regulated (Hwang et al., 2002; Luo et al., 1997; Yang et al., 2001b). At the same time, inhibitors of cell cycle like p16 and p27 are down-regulated (Hwang et al., 2002; Hwang et al., 2003; Makitie et al., 2003).

Cyclin D1 is responsible for cell progression through G1 (reviewed in Section 1.3 of this chapter). Over-expression of cyclin D1 allows cells with damaged DNA to transverse the G1/S checkpoint without cell cycle arrest, thereby increasing the risk of tumourigenesis (Robles et al., 1996; Zhou and Elledge, 2000). In NPC, cyclin D1 is over-expressed (Xie et al., 2000) and this is due to constitutive expression of active Ras and Raf proteins, low level of p16, the cyclin-dependent kinase (cdk) inhibitor (CKI) of cdk4/6-cyclin D (Kerkhoff and Rapp, 1998; Song et al., 2005b). Moreover, LMP1 induces over-expression of EGFR that can directly activate cyclin D1 transcription (Tao et al., 2005).

As cdk2-cyclin E controls cell cycle at S phase, deregulation of cyclin E expression leads to rapid progression of the cell through this phase and consequent increase in chromosomal instability (Spruck et al., 1999). An increase in cyclin E activity had been reported in a number of head and neck tumours, including NPC and laryngeal and oral cancers (Ioachim et al., 2004; Tao et al., 2005). This increase in cyclin E expression in NPC is due to LMP1-induced nuclear location of EGFR, which binds to the promoter of cyclin E and subsequent increase in its expression (Tao et al., 2005).

NPC is responsive to radiotherapy for which there is a high local control rate after radical radiotherapy (RT) (Fang et al., 2007a; Lu and Yao, 2008). However, concurrent radiotherapy and chemotherapy (chemoradiotherapy)

demonstrates a statistically significant reduction in failure and cancer-specific deaths compared with radiotherapy alone (Lee et al., 2010a). Chemotherapeutics used in chemoradiotherapy include 5-fluorouracil (5-FU) (Azli et al., 1992), vincristine (VCR) (Kwong et al., 2004), docetaxel (DTX) (Ngeow et al., 2010) and paclitaxel (PTX) (Chan et al., 2004).

While radiotherapy and chemoradiotherapy are the conventional treatment for NPC, there are now novel potential treatments that specifically target the molecular aberrations of NPC that lead to cell inhibition and apoptosis. As cyclin D1 is up-regulated in NPC, cyclin D1 offers a possible target protein. Cyclooxygenase (COX-2) is over-expressed in NPC and inhibitors of this protein are able to inhibit the growth of NPC cell lines in a dose-dependent manner by reducing the level of cyclin D1 in these cells (Chan et al., 2005). In addition, other novel potential agents for NPC control and management includes the flavonoids (reviewed in section 1.6 of this thesis)**.**

1.3 Cell cycle

The cell cycle consists of two major phases based on morphological features observed in cells; the M phase and the interphase. However, based on biochemical features, it comprises the S phase and the M phase with two gap phases namely G1 and G2 between the S and M phases (Fig 1.2). The gap or G phases allow cells to ready themselves before entry into the S and M phases. Cell division in eucaryotes is governed by three key proteins; the cyclin-dependent kinases (CDKs) and their specific cyclins; and the cyclin-dependent kinase inhibitors (CKIs).

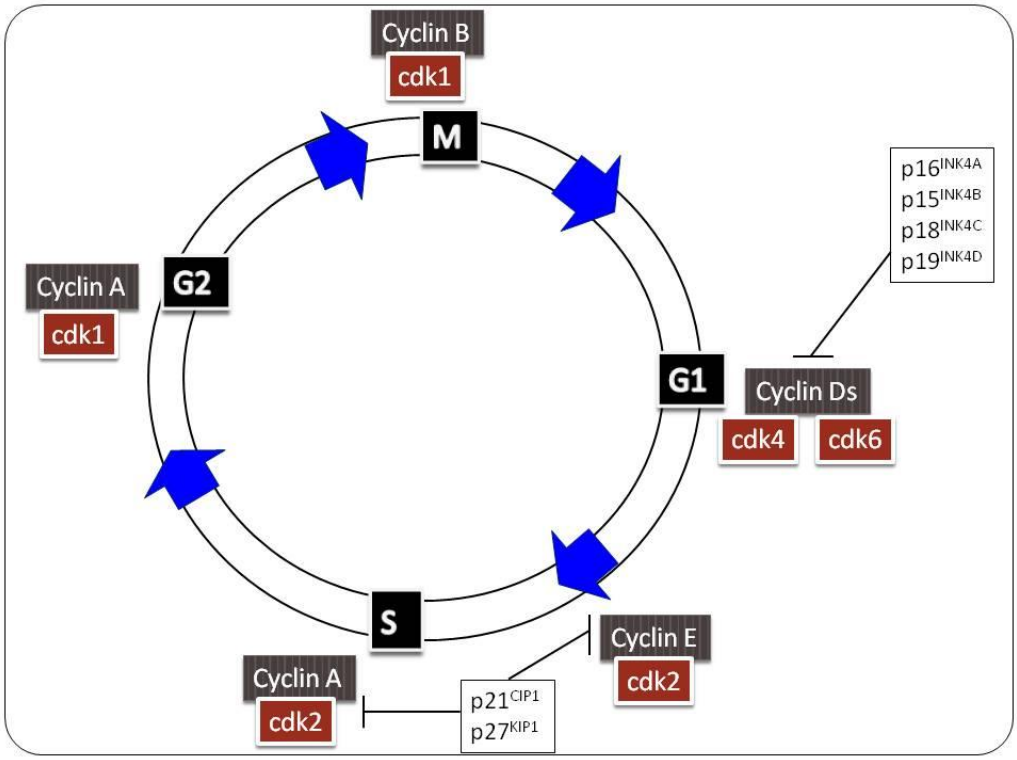


Fig 1.2: The cell cycle and the respective control mechanisms (adapted from (Malumbres and Barbacid, 2009))

1.3.1 Cdks and their corresponding cyclins as the key regulators of the cell cycle

Active cdk is made up of a protein kinase subunit whose catalytic subunit activity requires the presence of a regulatory cyclin subunit. Cyclins are expressed and degraded at specific time during the cell cycle and by this process, regulating the kinase activity in a systematic and controlled manner. Human cells possess 13 different loci encoding cdks and 25 loci for cyclins (Malumbres and Barbacid, 2005). However, only a certain subset of cdk-cyclin complexes is directly involved in cell cycle progression. These include the three interphase cdks (cdk2, cdk4 and cdk6), a mitotic cdk (cdk1) and 10 cyclins belonging to the A, B, D and E type cyclins. In addition, cell cycle progression requires the presence of the cdk7-cyclin H which is also referred to as cdk-activating kinase

(CAK) since this complex phosphorylates and activates the various cdk-cyclin complexes (Kaldis et al., 1998).

The pattern of cyclin expression varies with a cell's progression through the cell cycle and this pattern of specific cyclin expression is an indication of the phase of the cell cycle (Grana and Reddy, 1995; Johnson and Walker, 1999) (Fig 1.2). In a mammalian cell, cdk4 and cdk6 associated with cyclin Ds will drive the cell's progression through the G1 phase (Matsushime et al., 1992; Meyerson and Harlow, 1994). Cyclin E associates with cdk2 at the G1/S transition to drive the cell into the S phase (Koff et al., 1992). S phase and G2 phase progression are driven by the cdk2-cyclin A complex and the cdk1-cyclin A complex respectively (Pagano et al., 1992). Finally, progression of cells through mitosis is dependent on cdk1-cyclin B (Nigg, 2001).

During the late S and G2 phases of the cell cycle, cells prepare for mitosis by up-regulating the level of cyclins A and B. Both cyclins A and B are able to bind to cdk1 separately (Stark and Taylor, 2006). As the level of cyclin B increases, it forms a complex with cdk1 where the complex will remain in the cytoplasm. When cells are ready for mitosis, this complex of cdk1-cyclin B will translocate to the nucleus where it will bring about mitosis and cytokinesis (Takizawa and Morgan, 2000). Entry into mitosis is determined by the presence and activity of cdk1-cyclin B, which is regulated by its phosphorylation status, brought about by activating phosphorylation at Thr^{161}; and inhibitory phosphorylation at Thr^{14} and Thr^{15} (Fig 1.3). Phosphorylation at Thr^{161} and Thr^{14} and Thr^{15} are mediated by cdk-activating kinase (CAK) (Pines, 1995), Myt1 (Liu et al., 1997) and Wee1(Parker and Piwnica-Worms, 1992) respectively. At the onset of mitosis, both Thr^{14} and Thr^{15} residues are dephosphorylated by cdc25, a

phosphatase enzyme (Draetta and Eckstein, 1997). Complete Cdc25 activation requires phosphorylation at several sites within the cdc25 amino terminal domain and it is catalysed by two kinases; the polo-related kinase (Plk) (Lobjois et al., 2009) and cdk1-cyclin B (Hoffmann et al., 1993). The ability of cdk1-cyclin B to phosphorylate and activate cdc25 serves as a positive feedback loop.

Cdk1-cyclin B activity is also controlled by its sub-cellular location in the cell. During interphase, cdk1-cyclin B is found entirely in the cytoplasm (Pines and Hunter, 1991, 1994). In the late prophase, most cdk1-cyclin B complex will be translocated from the cytoplasm to the nucleus (Hagting et al., 1999; Takizawa and Morgan, 2000) (Fig 1.3). Cyclin B is continuously translocated into and out of the nucleus with help of an export receptor, Crm1 (Yang et al., 1998). During interphase, the rate of export exceeds the rate of import, leading to an accumulation of cdk1-cyclin B in the cytoplasm.

Cdc25, like cdk-cyclin B, is also localised in the cytoplasm during interphase and will re-localise to the nucleus during prophase. Localisation of cdc25 in the cytoplasm is controlled in part by the rate of import/export between the cytoplasm and nucleus. However during interphase, cdc25 is sequestered in the cytoplasm by a phosphoserine-binding protein, 14-3-3 (Peng et al., 1998; Peng et al., 1997) (Fig 1.3). To interact with 14-3-3, cdc25 must be phosphorylated at the Ser^{216} residue (in human). However, little is known about the identity of the kinases and phosphatases that act on Ser^{216}. There are strong indications that Chk1 and Chk2 are possible candidates as both enzymes are able to phosphorylate cdc25 at ser^{216} *in vitro*. Moreover, in the presence of DNA damage, Chk1 and Chk2 are able to mediate cell cycle arrest at G2 (Furnari et al., 1999; Peng et al., 1997).

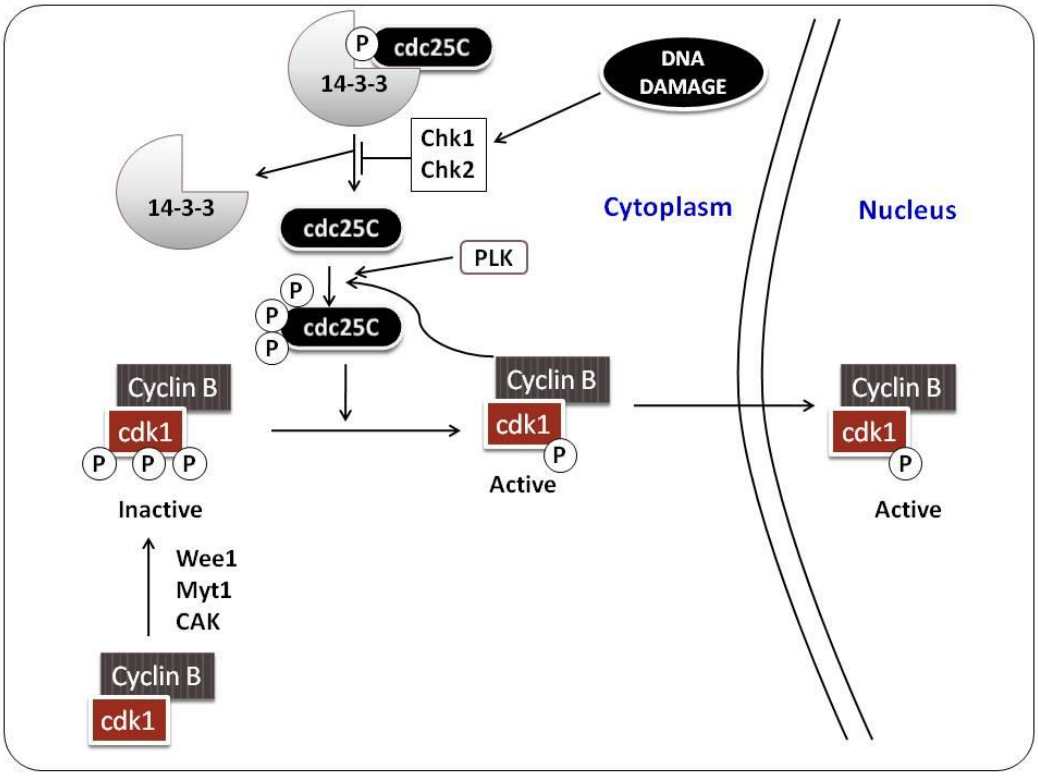


Fig 1.3: Molecular mechanisms controlling the activation of cdk1-cyclin B and cdc25c at the onset of mitosis (adapted from (Takizawa and Morgan, 2000))

1.3.2 Substrates of cdks

Although numerous cdk substrates have been identified, the detailed molecular mechanism on how cdk-mediated phosphorylation has only been well characterised for some of these substrates. Activated cdks are serine/threonine kinases whose activities are proline-directed, i.e cdks require a proline adjacent to the phosphorylated serine or threonine residue at the carboxyl-terminal (Songyang et al., 1994; Songyang et al., 1996; Srinivasan et al., 1995). In addition, near the serine and threonine phosphorylation sites, the recognition motif also possesses a positively charged lysine or arginine three positions downstream of the phosphorylated site (Songyang et al., 1994; Songyang et al., 1996; Srinivasan et al., 1995).

Different cdks may share common substrates but act on different phosphorylation sites within the substrate and thus regulating different aspects of this substrate function. A good illustration is the phosphorylation of pRb by cdk4-cyclin D1 and cdk2-cyclin E (Harbour et al., 1999). The mechanism underlying this selectivity is unclear, but may be linked to the cyclin subunits binding to distinct region of pRb.

Another mechanism to control cdk substrate specificity involves differential sub-cellular localisation of the cdks and their cyclins. Newly synthesised cyclins E and A will localise and complex with their respective cdks in the nucleus and thus act on substrates in the nucleus (Ohtsubo et al., 1995; Pines and Hunter, 1991). In the case of cyclin B1, it is translocated between the cytoplasm and nucleus during the cell cycle where synthesis of both cyclins B1 and B2 is initiated during the interphase and localised in the cytoplasm. During the prophase, cyclin B1 migrates from the cytoplasm to the nucleus but cyclin B2 remains in the cytoplasm (Draviam et al., 2001; Pines and Hunter, 1994).

1.3.2.1 Cdk substrates at the G1-S phase

The major cdk4/6-cyclin D1 substrate is pRb (Ezhevsky et al., 2001; Lundberg and Weinberg, 1998) (Fig 1.4). pRb, a tumour suppressor prevents cell entry into the G1/S cell progression by inhibiting the transcription factor E2F (Attwooll et al., 2004); and this inhibitory effect can be lifted by cdk4/6-cyclin D1-mediated phosphorylation (Adams, 2001). Initial phosphorylation of pRb by cdk4/6-cyclin D complexes leads to partial activation of E2F, which allows for the transcription of the *cyclin E* gene by the E2F transcription factor (Geng et al., 1996). The newly synthesised cyclin E interacts and activates cdk2 which will

further phosphorylate pRb, resulting in the complete activation of E2F. The active E2F will subsequently up-regulate the expression of numerous genes for cell cycle progression and these include *CDC6* (Hateboer et al., 1998); *DHFR* (dihydrofolate reductase) (Blake and Azizkhan, 1989; Noe et al., 1997); *TK* (thymidine kinase) (Dou et al., 1994); *DNA polymerase* α (Izumi et al., 2000); and *cyclin E* (Geng et al., 1996).

Cdk4/6-cyclin D and cdk2-cyclin E inactivate pRb through sequential phosphorylation at different sites, resulting in the progressive loss of pRb-mediated E2F inhibitory function. The initial phosphorylation by cdk4/6-cyclin D occurs at the amino acid position 788 and 795 of pRb, which destabilises its interaction with E2F (Rubin et al., 2005) and subsequent dissociation from histone deacetylases (HDACs) (Ferreira et al., 2001) (Fig 1.4). Subsequent phosphorylation of pRb during late G1 phase by cdk2-cyclin E leads to complete dissociation of E2F from the pRb-E2F complex (Harbour et al., 1999) (Fig 1.4).

Cdk2-cyclin E is also involved in the phosphorylation and activation of NPA (nuclear protein mapped at the AT locus), an important regulator in histone expression and synthesis (Zhao et al., 1998).

Centrosomes play a central role in sister chromatid segregation during mitosis. Following cytokinesis, each daughter cell inherits one centrosome. Therefore before mitosis, it is necessary to duplicate the centrosome. Cdk2-cyclin E initiates centrosome duplication by phosphorylating the centrosomal proteins NPM/B23 (nucleophosmin) and CP110 (centrosomal protein of 110 kDa) which allows the dissociation of NPM/B23 from the centrosome and subsequent duplication (Okuda et al., 2000; Tokuyama et al., 2001).

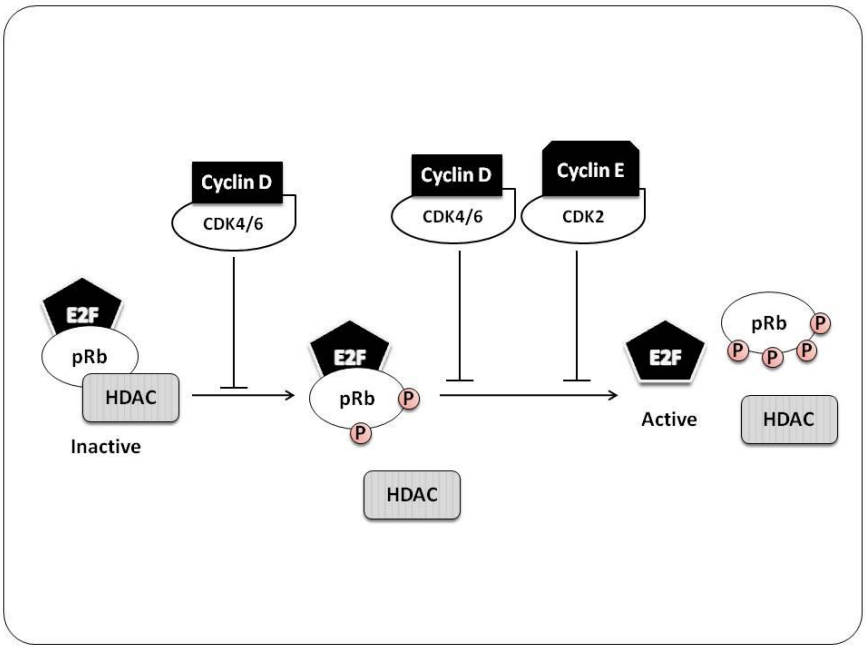


Fig 1.4: Inhibition of pRb activity by cdk4/6-cyclin D and cdk2-cyclin E phosphorylation (adapted from (Schwartz and Shah, 2005))

1.3.2.2 Cdk substrates at the S phase

As cells enter the S phase, DNA replication is initiated at numerous origins simultaneously. Each DNA replication origin consists of initiator proteins collectively termed ORC (origin recognition complex) which will interact with replicator elements within the DNA (Hamlin et al., 1994). The ORC serves as a base for protein-protein interactions to bring about DNA replication. In order to prevent polyploidy in cells, DNA is not allowed to replicate twice in the S phase and this is regulated by the cdks. Phosphorylation status of ORC changes throughout the cell cycle, with the ORC being hypophosphorylated during the early G1 and increasingly being phosphorylated as cells progress from the G1 to S phase (Li et al., 2004). Several proteins which regulate the ORC are also phosphorylated by the cdks. For instance, cdk1-cyclin A phosphorylates the ORC

subunit Orc1 during mitosis, thus preventing its interaction with chromatin (Li et al., 2004).

1.3.2.3 Cdk substrates at the M phase

Progression through mitosis is governed mainly by cdk1-cyclin B. It is inactivated during the late mitosis in order for cell cycle exit. APC/C (anaphase-promoting complex/cyclosome) ubiquitin ligase is a major target of cdk1-cyclin B. Activation of APC/C by phosphorylation is initiated in late mitosis by cdk1-cyclin B (Kraft et al., 2003) and this allows subsequent interaction between the phosphorylated protein with one of two activator proteins, cdc20 or cdh1 (cdc20 homologue 1). Once activated, APC/C^{cdc20} (complex formed between APC/C and cdc20) initiates the ubiquitination and proteasomal degradation of securin, an anaphase inhibitor protein that blocks sister chromatid separation and activation of separase, an enzyme that allows the separation of the two sister chromatids (Hagting et al., 2002; Hauf et al., 2001). A third substrate of APC/C^{cdc20} is the cdk1-cyclin B, that will result in phosphorylation and subsequent proteasomal degradation of this cdk complex in the late anaphase, thus relieving the phosphorylation of cdh1 by cdk1-cyclin B (King et al., 1995). This allows cdh1 to interact with APC/C forming the APC/C^{cdh1} which is responsible for spindle assembly and spindle elongation and subsequent cytokinesis (Floyd et al., 2008).

Cdk1-cyclin B can also phosphorylate and activate Emil (early mitotic inhibitor) which interacts with cdc20 and inhibits APC/C, resulting in mitotic arrest (Reimann et al., 2001). Emil accumulates before mitosis and will be ubiquitinated and degraded during mitosis by the SCF (Skp, Cullin, F-box containing complex) ubiquitin ligase complex (Margottin-Goguet et al., 2003).

1.3.3 Cdk inhibitors (CKIs)

The inhibition of cdk activities by CKIs constitutes an important mechanism in cell cycle control and provides an integral link to other signalling pathways during cellular proliferation, differentiation and senescence (Ju et al., 2007; Peter, 1997).

1.3.3.1 The INK4 family of CKIs

The INK4 family of CKIs specifically targets the cyclin D-dependent kinases. There are four proteins under this family; $p16^{INK4A}$ (Serrano et al., 1993), $p15^{INK4B}$ (Hannon and Beach, 1994), $p18^{INK4C}$ (Hirai et al., 1995) and $p19^{INK4D}$ (Hirai et al., 1995); all of which compete for binding with cyclin D to cdk4 and cdk6 (McConnell et al., 1999; Sherr and Roberts, 1999). The association between the INK4 family of proteins for cdk4 and cdk6 is very specific and is dependent on the presence of pRb in the cell. In the absence of pRb, cyclin E expression and inhibition of cdk4-cyclin D complexes does not arrest cell cycle progression at the S phase (Lukas et al., 1997).

Among the INK4 family of proteins, $p16^{INK4A}$ forms a strong association with $p14^{ARF}$, $p14^{ARF}$ protein inhibits cell cycle progression by stabilising the complex between p53 and MDM2 (Weber et al., 1999) . Expression of $p14^{ARF}$ is regulated by E2F, a transcription factor controlled by pRb. E2F is also the transcription factor for *cyclins E* and *A*, whose proteins are key proteins in S phase. Loss of $p16^{INK4A}$ is functionally equivalent to loss of pRb whereas the loss of $P14^{ARF}$ is analogous to loss of p53 (James and Peters, 2000; Sherr, 2001). Both pRb and p53, being tumour suppressors are critical proteins in the regulation of cell division and apoptosis.

$p15^{INK4B}$ regulates cell cycle at the G1 phase by inhibiting cdk4/6-cyclin D in response to cytokines like transforming growth factor-β (TGF-β) (Hannon and Beach, 1994). The $p15^{INK4B}$-mediated G1/S cell arrest is often deregulated in numerous human cancers like prostate cancer, melanoma, pituitary adenoma, acute myeloid leukaemia and gastric cancer (Shima et al., 2005; Solomon et al., 2008) .

The remaining INK4 family members, $p18^{INK4C}$ and $p19^{INK4D}$ are expressed during foetal development and play key roles in terminal cellular differentiation (Zindy et al., 1997).

1.3.3.2 The CIP/KIP family of CKIs

The CIP/KIP family of cdk inhibitors consists of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (reviewed by (Besson et al., 2008)). These member proteins bind specifically and inhibit both cyclin and cdk subunits through conserved motifs for cdk and cyclin binding in the amino termini of the inhibitors (Adams et al., 1996; Chen et al., 1996). p21, p27 and p57 expressions are up-regulated during development and differentiation; and also in response to cellular stresses. However, the elevated expression of each member is due to different anti-proliferative signals. For instance, p21 is elevated in p53 mediated cell cycle arrest in response to DNA damage, resulting in cell arrest in G1 and G2 (el-Deiry et al., 1993). p27 on the other hand is up-regulated in mitogen-deprived cells (Besson et al., 2007).

Although the CIP/KIP protein members are found to act preferentially on cdk2 complexes and inhibiting these complexes (Russo et al., 1996), they can also activate cdk4/6-cyclin D by aiding with the assembly of catalytically active

cdk4/6-cyclin D (Cheng et al., 1998). The latter activity allows the downstream activation of cdk2-cyclin E (Perez-Roger et al., 1999). However there are also reports indicating the inhibitory effects of CIP/KIP proteins on the cdk4/6/-cyclin activity (Kato et al., 1994), thus demonstrating that the effects of these CKIs on cdk activities can be modulated by other factors.

The key function of $p21^{CIP1}$ in cell cycle regulation is its ability to inhibit the activity of the cdk2-cyclin A and E which are required for G1/S transition; leading to G1cell arrest. $p21^{CIP1}$ expression is enhanced in p53-dependent DNA damage which will lead to G1 cell arrest following exposure to DNA damaging agents in wild-type p53- but not in p53- mutant expressing cells ((Dulic et al., 1994). $p21^{CIP1}$ can associate and inhibit PCNA (proliferating cell nuclear antigen), a subunit of DNA-polymerase δ and preventing DNA replication in committed cells (Luo et al., 1995). Besides its involvement in cell cycle, $p21^{CIP1}$ also acts as a negative regulator of p53-dependent apoptosis. One possible mechanism is via its inhibition of cdk like cdk2-cyclins A and E, resulting in G1 cell arrest and subsequent apoptosis (Gartel and Tyner, 2002). A second possible mechanism is via its interaction with pro-apoptotic molecules such as procaspase-3 and procaspase-8 at the amino-terminus which prevents the subsequent degradation of the procaspases to the active caspases (Suzuki et al., 1998) .

The second member of the CIP/KIP family is $p27^{KIP1}$, an inhibitor of cdk2-cyclin E and therefore plays a role as a negative regulator at the G1/S transition. The activity of $p27^{KIP1}$ is controlled by its level of expression during the cell cycle. Its concentration decreases and increases in response to mitogen stimulation and mitogen deprivation respectively (Hengst and Reed, 1998). Numerous reports indicate that $p27^{KIP1}$ through its fluctuating concentration in the cells plays a

central role in the decision by the cells to either commit to cell division or withdrawal (Coats et al., 1996). A second role of $p27^{KIP1}$ has also been reported in which it is able to exert an inhibitory effect on apoptosis in cells (Levkau et al., 1998). Numerous studies have linked the induction of the CIP/KIP proteins and subsequent downstream anti-apoptotic effect to the development of resistance to apoptosis induced by cytotoxic drugs and irradiation (De la Cueva et al., 2006; St Croix et al., 1996).

The last member of the CIP/KIP protein, $p57^{KIP2}$ plays a key role in embryonic development; and down-regulation of its expression leads to developmental disorder (Yan et al., 1997; Zhang et al., 1997).

1.3.4 Cell cycle checkpoints

Mammalian cells are committed to cell division during mid G1, termed the restriction checkpoint, following phosphorylation and inactivation of retinoblastoma (pRb) protein and the release of E2F (Beijersbergen and Bernards, 1996). Free E2F, a transcription factor of *cyclins E and A* brings about S phase cell progression (Harbour and Dean, 2000b; Obeyesekere et al., 1995). To allow the cell to progress in an orderly manner through S phase and to initiate the G2 phase, E2F must be inactivated. This is brought about by cdk2-cyclin A binding to and phosphorylating the E2F-DP complex, in the process inactivating its DNA binding ability (Xu et al., 1994). There is a checkpoint at S phase termed the replication checkpoint. This checkpoint will monitor DNA synthesis and prevent cells from progressing to the G2 phase and M phase if the newly synthesised DNA was found to be defective. This checkpoint is controlled by a group of

proteins namely ATM, ATR, Chk1 and Chk2 (Falck et al., 2001; Zhou and Elledge, 2000).

The same group of enzymes (ATM, ATR, Chk1 and Chk2) also controlsanother checkpoint mediated by p53. p53 is a regulator that responds to stress signals like DNA damage and subsequent cell cycle arrest (Levine, 1997). The level of p53 in normal cells is kept low, owing to rapid ubiquitin-dependent degradation mediated by the E3 ubiquitin ligase MDM2 (Brooks and Gu, 2004). Various stresses like DNA damage caused by UV exposure can inhibit MDM2 degradation of p53. This is mediated by phosphorylation of p53 catalysed by ATM, ATR, Chk1 and Chk2 at different amino acid sites (Banin et al., 1998; Chehab et al., 2000; Urist et al., 2004). Once stabilised p53 can elicit several different cellular responses including cell cycle arrest via up-regulation of p21 (Choisy-Rossi et al., 1998) and the induction of apoptosis via up-regulation of pro-apoptotic protein, Bax (Miyashita and Reed, 1995).

1.3.5 Deregulation of the cell cycle and cancer development

Cell cycle progression is an ordered and tightly-regulated process. It involves multiple pathways and checkpoints to assess both the extracellular and intracellular signals that a cell receives. These signals govern the different cell cycle phases which will influence the cell's decision to proliferate or to arrest cellular growth, to undergo DNA repair or apoptosis. Hence these signals in the form of proteins can be positive or negative regulators of the cell cycle progression. When a cell is unable to respond to signals due to genetic instability, it will lead to aberrant cell proliferation and perpetuate its genetic instability further. Tumourigenesis will initiate when there is abnormal expression and

activation of positive regulators and suppression of negative regulators. Hence, understanding the molecular mechanisms leading to the deregulation of the cell cycle progression in cancer can provide vital and important insights into how normal cells become tumourigenic in the hope that new cancer treatment strategies can be formulated.

Numerous reports have demonstrated that tumourigenesis is frequently associated with mutation and or abnormalities in the expression of the cyclins and their associated cdks and CKIs. Over-expression of cdk4 although less frequent than the over-expression of cyclins have been identified in cell lines of myeloma (Menu et al., 2008), glioblastoma (Michaud et al., 2010) and pharyngeal squamous cell carcinoma (Koontongkaew et al., 2000). Over-expressions of cdk1 and cdk2 have also been reported in other studies (Kim et al., 1999; Liao et al., 2004; Zhou et al., 2003).

One of the best explored examples of a cyclin that contributes towards tumourigenesis is the over-expression of cyclin D in cancers of the breast, oesophageal, bladder, lung and squamous cell carcinomas (Chou et al., 2008; Hall and Peters, 1996; Koontongkaew et al., 2000; Landberg, 2002). Over-expression of cyclin E has been demonstrated in breast and colon cancer; nasopharyngeal carcinoma and in acute lymphoblastic and acute myeloid leukaemia (Chou et al., 2008; Iida et al., 1997; Kitahara et al., 1995; Nielsen et al., 1997; Scuderi et al., 1996). The up-regulation of cyclin A is not only observed in cancer but it is also an indicator of poor prognosis (Bukholm et al., 2001; Ekberg et al., 2005; Husdal et al., 2006).

Cdc25 family of cdk activators acts as a phosphatase, dephosphorylating cdk. Cdc25A activates the cdk at the G1/S transition phase, cdc25B at the S

phase and the last member of the family, cdc25C activates cdk1-cyclin B during entry into mitosis. Deregulation of the cdc25 proteins through over-expression has resulted in tumour formation (Cangi et al., 2008; Kristjansdottir and Rudolph, 2004; Loffler et al., 2003; Xing et al., 2008).

CKIs are able to inhibit the cdk activities and subsequent growth inhibition through pRb activation. Mutation to $p16^{INK4A}$ has led to the development of numerous human cancers (Demirhan et al., 2010; Krasinskas et al., 2010; Panani et al., 2009; Wiesner et al., 2010). Cells with mutated $p16^{INK4A}$ would proceed through G1 without any restraint. Both *p16* and *p15* gene loci are located closed to each other on chromosome 9 of the human genome and deletion of *p16* gene leads to *p15* deletion as well (Bostrom et al., 2001; Hallor et al., 2008; Southgate et al., 1995). Thus similar effect that one observed after *p16* deletion will also be observed after *p15* deletion. Mutation to *p19*, another member of the INK4 family of CKIs leads to subsequent deregulation of p53 (Debies et al., 2008; Moore et al., 2003).

Disruption to and subsequent loss of $p27^{KIP1}$ expression has been reported in human cancer of the lung (Pateras et al., 2006), breast (Chappuis et al., 2000) and bladder (Adachi et al., 2003) and is also an indicator of poor prognosis and tumour aggressiveness (Hommura et al., 2000; Zhu et al., 2004). Numerous reports have also demonstrated the implication of $p21^{CIP1}$ down-regulation to tumourigenesis caused by mutation in p53 (Kiyosaki et al., 2010; Liu et al., 2010; Wilson et al., 2008).

pRb is the most important cdk substrate at the G1 phase and numerous reports have linked mutation of this gene to cancer. The family members of pRb have been linked to cell cycle events like clonal expansion, terminal cell cycle

exit, maintenance of the post-mitotic state and the induction of tissue-specific gene expression and the regulation of apoptotic events (Classon and Dyson, 2001). They play a central role in cell cycle regulation by controlling key proteins like $p16^{INK4A}$, $p21^{CIP1}$, $p27^{KIP1}$, cdk2, cdk4/6, cyclins A, E and D. Mutations and deletions of the *Rb* genes result in the inactivation of their biological function as tumour suppressors and thus lead to an increased susceptibility to tumourigenesis (Dunn et al., 1988). As a direct consequence of the mutation and deletion of the *Rb* genes, E2F transcription factors are liberated from pRb control, leading to deregulation of the cell cycle (reviewed by (Scambia et al., 2006)). Binding between pRb and E2F can also be disrupted by DNA tumour virus oncoprotein like papilloma virus E7 which binds to pRb and consequently releases E2F as observed in cervical cancer and mesothelioma (Helt and Galloway, 2003; Kalejta, 2004; Shah, 2004).

Inappropriate pRb phosphorylation due to enhanced cdk4/6-cyclin D activity (over-expression of cyclin D) contributes towards the development of parathyroid adenomas, B-cell lymphomas and squamous cell carcinoma (Brizova et al., 2008; Fernandez et al., 2005; Rydzanicz et al., 2006). pRb activity is affected indirectly by $p16^{INK4A}$ as the latter binds and inhibits cdk4/6-cyclin D and thus maintains the tumour suppressor activity of pRb through the inhibition of E2F. The loss of $p16^{INK4A}$ will inhibit Rb activity, and thus result in tumourigenesis as well (Mitchell et al., 2003).

A second tumour suppressor gene that has high frequency of mutation in cancers is the *p53 g*ene (reviewed by (Brosh and Rotter, 2009; Brown et al., 2009)). p53 is a sequence-specific DNA-binding protein that is up-regulated in the presence of DNA damage and is able to induce cell cycle arrest or apoptosis at

the checkpoints of the cell cycle. Point and missense mutations lead to conformational changes and subsequent inactivation of this protein (Gannon et al., 1990; Milner, 1991). Binding of p53 to viral oncoproteins like SV40 T antigen, HPV E6 and adenovirus E1B-55K can also inactivate p53 function (Chen and Defendi, 1992; Crook and Vousden, 1994; Yi et al., 2009)

One can conclude that a breakdown of cell cycle due to a deregulation brought about by oncogenes encoding cyclins and cdks; tumour suppressor genes like *Rb* and *p53*; and genes encoding for CKIs whose function tether on the activation of the *E2F* genes can lead to tumourigenesis.

1.4 Apoptosis

1.4.1 Introduction

Apoptosis is a highly regulated physiological process of cell death, critical in the maintenance of tissue homeostasis in multicellular organisms. It is triggered by a variety of extrinsic and intrinsic signals. One of the major causes of tumourigenesis is the deregulation of apoptotic mechanisms in cells, which leads to cell accumulation and the loss of ability of multicellular organisms to maintain cell turnover.

1.4.2 Morphological and biochemical features in apoptotic cells

Apoptosis can be captured in a sequence of morphological changes observed in cells including chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing and eventually the formation of membrane-enclosed particles termed apoptotic bodies which contain intact organelles as well as a

portion of the nucleus (Rich et al., 1999). These apoptotic bodies are subsequently ingested and degraded by phagocytes and neighbouring cells, preventing inflammation or tissue scarring. Hence apoptosis is well suited to maintain normal cell turnover during embryogenesis and in adult tissues (Jacobson et al., 1997). This is in contrast to necrosis, which is a pathological mode of cell death brought about by irreversible swelling of the cytoplasm and distortion of the organelles like mitochondria. Cell death ensues when the cell loses its membrane integrity resulting in cell lysis. Due to the release of cellular contents to the extracellular space, inflammation will develop in the surrounding tissue. Necrosis is seen when cells are exposed to toxic stimuli such as hyperthermia, metabolic poisons and direct cell trauma (Kanduc et al., 2002; Proskuryakov and Gabai, 2010). The decision of the cell to die by necrosis or apoptosis is thought to depend mainly on the severity of the damage to cells (Ankarcrona et al., 1995) and intracellular ATP concentration (Leist et al., 1997). Other forms of cell death have also been reported in recent years, including autophagy, paraptosis, necroptosis and oncosis (Degterev et al., 2005; Leist and Jaattela, 2001; Okada and Mak, 2004; Yang and Klionsky, 2009).

Biochemical changes observed in cells undergoing apoptosis include the externalisation of phosphatidylserine (PS) residues, activation of caspases and nuclear DNA fragmentation (Hengartner, 2000). However, cell death by apoptosis independent of caspases has also been observed in some cells (Leist and Jaattela, 2001).

There are two main pathways of caspase-mediated cell death and they are the extrinsic or death receptor-mediated pathway and the intrinsic or mitochondria-dependent pathway. The former plays a fundamental role in the

maintenance of tissue homeostasis and the latter is used extensively by cells in response to extracellular signals and internal stimuli like DNA damage.

1.4.3 Caspases

Caspases belong to a group of cysteine-dependent aspartate-specific proteases which are involved in the initiation and execution of apoptosis (Thornberry and Lazebnik, 1998). The human genome encodes for caspases designated caspase-1 to caspase-10 and caspase-14 (Alnemri et al., 1996). Caspases are synthesised as inactive zymogens termed procaspases containing a prodomain, a p20 large subunit and a p10 small subunit (Fig 1.5).

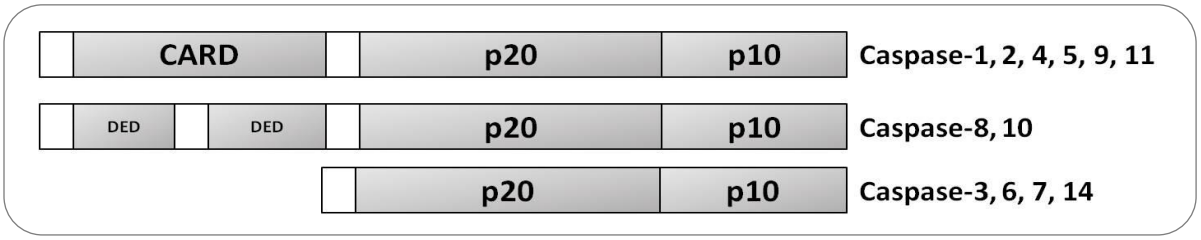


Fig 1.5: Domain organisation of caspases (adapted from (Li and Yuan, 2008))

Caspases are classified into two groups based on the lengths of their prodomains, which also correspond to their position within the apoptotic signalling pathway. These two groups include the initiator caspases (caspase -1, -2, -4, -5, -8, -9, -10, and -11) and effector caspases (caspase -3, -6, and -7). Initiator caspases possess long prodomains that may either contain the death effector domain (DED) or the caspase recruitment domain (CARD) (Fig 1.5) (Hofmann et al., 1997). These initiator caspases generally act upstream of the small prodomain-containing effector caspases (Ashkenazi and Dixit, 1998; Nicholson and Thornberry, 1997). Effector caspases with their short prodomains

perform downstream execution steps of apoptosis by causing proteolysis of cellular substrates.

In the procaspase forms, they exhibit low intrinsic enzymatic activity. Cleavage of the procaspase leads to the formation of three fragments; one large (p20) and one small (p10) catalytic subunits and the prodomain. The two protease subunits associate with each other to form a heterodimer and two such heterodimers further complex to form a tetramer, which now possesses the active form of caspases (Walker et al., 1994).

The induction of apoptosis results in the activation of initiator caspases, and once activated, they will activate the downstream effector caspases in a cascade-like pattern (Slee et al., 1999). The effector caspases subsequently act on a series of substrates including inhibitor of caspase-activated DNase (ICAD), poly(ADP-ribose)polymerase (PARP) and other proteins that bring about cellular, morphological and biochemical changes of apoptosis (Cohen, 1997).

1.4.4 The extrinsic apoptotic pathway

Cell surface death receptors (DRs) belong to the tumour necrosis factor receptor (TNFR) superfamily. They initiate the apoptotic biochemical signalling pathway following binding of death ligands. DR proteins are characterised by the presence of multiple cysteine-rich repeats in the extracellular domain and the death domain (DD) found at the cytoplasmic tails of the receptors. The members of the DR family include Fas (also known as DR2, APO-1 and CD95), TNFR1, DR3 (also known as APO-3, LARD, TRAMP and WSL1), TRAIL1 (TNF-related apoptosis-inducing ligand receptor 1) (also known as DR4 and APO-2), TRAIL2

(also known as DR5, KILLER and TRICK2), DR6, EDAR (ectodysplasin A receptor) and nerve growth factor receptor (Lavrik et al., 2005).

One of the best studied DR is Fas, a 319-amino acid transmembrane glycoprotein (Itoh et al., 1991). Each receptor possesses three extracellular regions with cysteine-rich repeats domains and a conserved 80-amino acid long region located in the carboxyl-terminus which is found among the DRs. This region is termed death domain (DD) and plays critical role in the downstream signalling pathway as well as its role in the activation of the transcription factor nuclear factor NF-κB (Ashkenazi and Dixit, 1998; Tartaglia et al., 1993). NF-κB however prevents apoptosis in cells as it promotes expression of survival factors like the IAP (inhibitor of apoptosis) family of proteins.

Binding between Fas-FasL leads to receptor oligomerisation (Ashkenazi and Dixit, 1998; Wallach et al., 1999). This is followed by binding of the adaptor protein to Fas via the DDs on both Fas and FasL. FADD (Fas-associated DD) protein is an example of an adaptor protein which possesses a DD at its carboxyl-terminus and a second protein-protein interaction domain, called the death-effector domain (DED) at its amino-terminus (Chinnaiyan et al., 1996; Muzio et al., 1996). FADD subsequently recruits procaspase-8 to its DED domain when the DED or prodomain on procaspase-8 binds to its DED, resulting in the formation of the death-inducing signalling complex (DISC) (Fig 1.6). The recruitment and oligomerisation of procaspase-8 to DISC leads to its autocatalytic activation; and subsequent activation of a series of downstream caspases as well as structural and regulatory proteins, which all culminate in cellular apoptosis (Kischkel et al., 1995).

Binding of Fas-FasL leads to two distinct phenomenal events in two groups of cells. In one group of cells, termed Type I cells activation of caspase-8 is followed by the activation of caspase -3 and -7 (Muzio et al., 1996; Srinivasula et al., 1996). In the second group of cells, designated Type II cells, limited activation of caspase-8 results in an amplification loop mediated by mitochondrial activation (Scaffidi et al., 1999b). In this case, caspase-8 cleaves Bid, a cytosolic BH (Bcl-2 homology)-3-only proapoptotic Bcl (B-cell lymphoma)-2 (Bcl-2) family protein member resulting in an active but truncated Bid (tBid) which is now able to translocate to the mitochondria to activate the downstream signalling proteins of the mitochondrial apoptotic pathway (Li et al., 1998; Luo et al., 1998) (Fig 1.6). This demonstrates a co-operation between the extrinsic and intrinsic apoptotic pathways (Fig 1.6).

In type I cells, the activation of caspase-8 triggers the cleavage of downstream caspases such as caspase-3 whose target is the inhibitor of caspase-activator deoxyribonuclease (CAD) (ICAD) (Enari et al., 1998). CAD is an endogenous endonuclease and is kept in an inactive form in the cytosol by its inhibitor, ICAD. Active caspase-3 cleaves and inactivates ICAD and in the process, releasing CAD from its inhibitory partner (Sakahira et al., 1998). The liberated CAD subsequently enters the nucleus and degrades the cell's chromosomal DNA, leading to DNA fragmentation and cell death; both events being end-point of apoptosis.

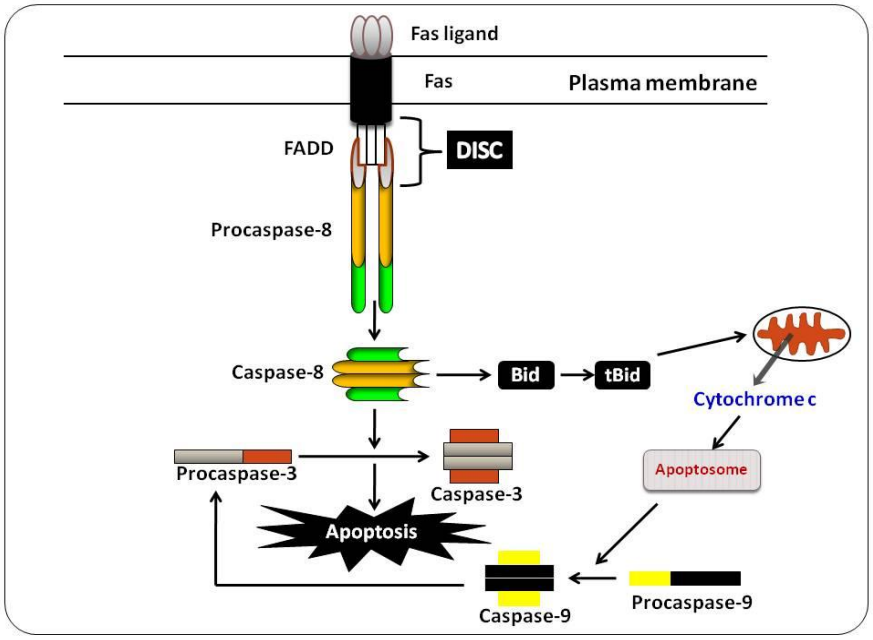


Fig 1.6: The Fas signalling pathway (adapted from (Ashe and Berry, 2003))

A protein inhibitor present in cells is able to inhibit the extrinsic apoptotic pathway. This protein, a 55-kDa inhibitor termed cellular Fas-associated DD-like interleukin (IL)-1-converting enzyme inhibitory protein (c-FLIP), is an inactive homologue of caspase-8. It possesses two DEDs and is able to bind to the DED of FADD and caspase-8 to block signal transduction of the Fas-induced death signal resulting in prolonged cell survival (Irmler et al., 1997; Scaffidi et al., 1999a). A homologous viral protein, designated v-FLIP can moderate DR-initiated apoptotic pathway that involves caspase-8 (Glykofrydes et al., 2000). In some cell types, Fas-induced apoptosis is regulated by mitochondria-associated caspases and apoptosis–promoting members of the Bcl-2 family (Scaffidi et al., 1998) (Fig 1.7).

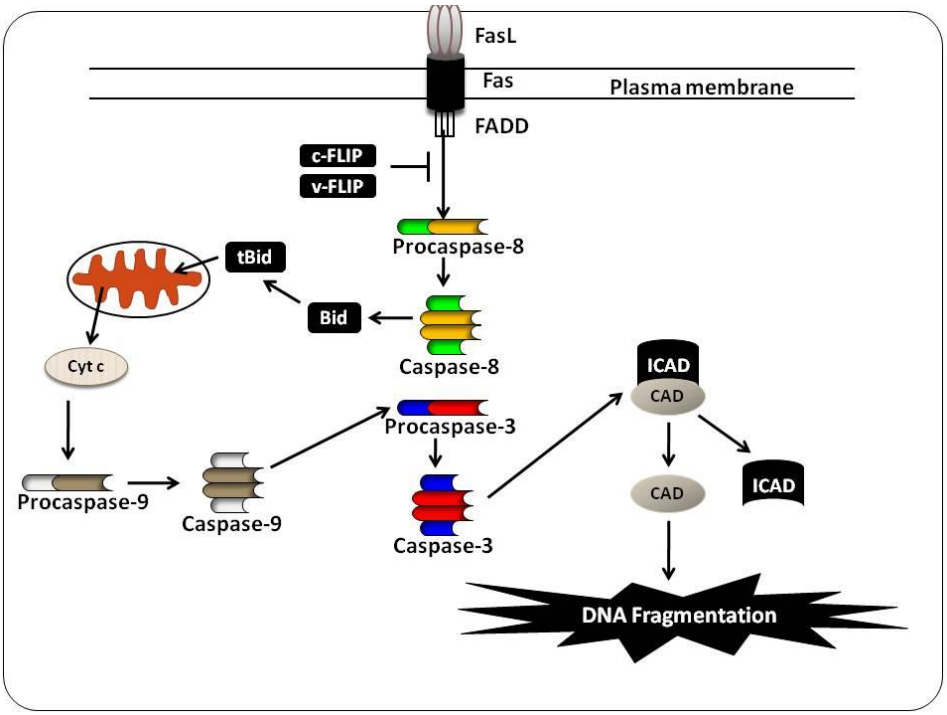


Fig 1.7: Cooperation between the extrinsic and intrinsic apoptotic pathway and the negative regulation by ICAD-CAD complex (adapted from (Ashe and Berry, 2003))

1.4.5 The intrinsic (mitochondria-associated) pathway

Mitochondria are the site of eucaryotic oxidative metabolism, used by cells for the synthesis of adenosine triphosphate (ATP) via a series of oxidative phosphorylation process and cytochrome c. Mitochondria have also been identified to play a critical role in apoptosis. A number of apoptotic proteins is located and compartmentalised within the mitochondrion and following signals transduced to the mitochondrion, these proteins are released into the cytosol to trigger the intrinsic signal transduction pathway leading to apoptosis.

The intrinsic pathways are controlled by the Bcl-2 family of proteins, a group of highly conserved proteins which consists of pro-apoptotic and anti-apoptotic members whose presence at the mitochondrial membrane determine the fate of the cells towards cell death or survival (reviewed by (Youle and Strasser,

2008). The two distinct functional groups of Bcl-2 proteins can be differentiated by their structural features. The anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-X_L, Mcl-1, Bcl-W and A1 all share two to four conserved regions within the polypeptide chains termed the BH (Bcl-2 homology) domains (BH1 to 4) (Danial and Korsmeyer, 2004; Strasser, 2005). They prevent apoptosis by binding and inactivating pro-apoptotic proteins through sequestration. The pro-apoptotic group of proteins can be divided into two sub-groups based on their structural domains. The first sub-group is made up of two protein members, namely Bax and Bak. They possess three BH domains (BH1 to 3) and will induce apoptosis when over-expressed (Danial and Korsmeyer, 2004). The second sub-group of pro-apoptotic proteins consists of Bad, Bim, Bik, Bid, Hrk, Noxa and Puma. They all possess the conserved BH3 domain. These BH-3 only proteins are regulators rather than executioners of cell death and therefore act upstream of Bax and Bak proteins (Cheng et al., 2001; Zong et al., 2001). Their activities are regulated either at the transcriptional level in the case of Noxa and Puma, or by post-translational modification as demonstrated in Bad, Bim and Bid (Strasser, 2005).

In the direct method of activation, BH-3 pro-apoptotic proteins bind and activate Bax and Bak. In this model, BH-3 proteins are sub-divided into two groups namely activators or sensitisers based on their functions (Letai et al., 2002). The activator proteins include tBid and Bim and the sensitiser proteins include Bad, Noxa and Puma. tBid and Bim bind directly to Bax and Bak and activate these proteins, whereas the sensitiser proteins bind to their anti-apoptotic Bcl-2 protein members, liberating Bax and Bak and consequently activating Bax-

and Bak-induced apoptosis (Chipuk et al., 2008; Kim et al., 2006; Walensky et al., 2006) (Fig 1.8).

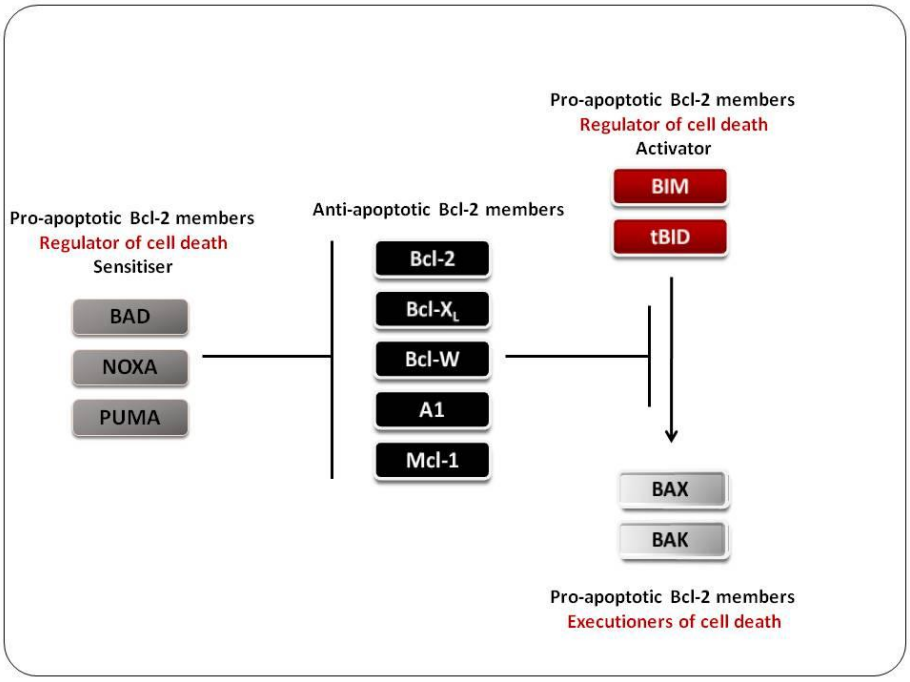


Fig 1.8: Model depicting the direct activation of Bax and Bak (adapted from (Brenner and Mak, 2009))

In the indirect activation model, Bax and Bak are kept inactive by sequestration mediated by binding to anti-apoptotic Bcl-2 family members (Willis et al., 2005; Willis et al., 2007). The binding of BH3-only proteins to anti-apoptotic Bcl-2 proteins releases Bax and Bak from the inhibitory action of these Bcl-2 proteins (Fig 1.9). This allows for the subsequent initiation of apoptosis by the active Bax and Bak proteins (Uren et al., 2007; Willis et al., 2007).

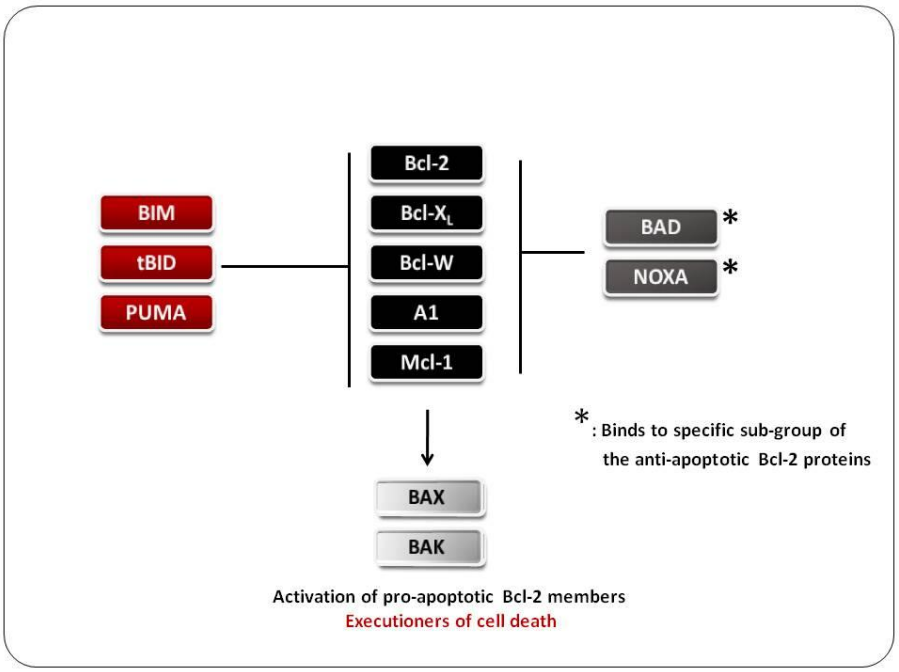


Fig 1.9: Model depicting the indirect activation of Bax and Bak (adapted from (Brenner and Mak, 2009))

Once the inhibition of Bax is lifted, it translocates from the cytosol to the OMM. Inactive Bak is found at the outer mitochondrial membrane (complex with Mcl-1 and Bcl-X_L) (Hsu et al., 1997; Willis et al., 2005; Wolter et al., 1997). The active form of Bak together with the recently translocated Bax initiates the disruption of the OMM leading to mitochondrial outer membrane permeabilisation (MOMP). The change in MOMP leads to the release of cytochrome c and other pro-apoptotic proteins from the mitochondrial inter-membrane space (IMS) into the cytosol.

Cytochrome c is found in the intermembrane space of the mitochondrion and most of them are loosely attached to the outer surface of the inner mitochrondrial membrane. Besides its role in oxidation phosphorylation, cytochrome c is also critical in the caspase activation cascade (Liu et al., 1996). Upon its release from the mitochondrion, cytochrome c interacts with the apoptotic-activating factor-1 (Apaf-1), ATP/dATP, and caspase-9 to form the

apoptosome (Hao et al., 2005; Li et al., 1997b). Apaf-1 possesses a caspase recruitment domain (CARD) which mediates its interaction with caspase-9, and a WD-40 repeat domain that maintains Apaf-1 in its inactive form in the absence of cytochrome c (Hu et al., 1998). In the presence of cytochrome c and ATP/dATP, Apaf-1 undergoes a conformational change that leads to its aggregation consisting of seven Apaf-1 molecule, each bound to one molecule of cytochrome c and one caspase-9 (Acehan et al., 2002) and exposure of CARD for subsequent recruitment of the initiator caspase, procaspase-9 to it domain. Unlike other caspases, procaspase-9 is not activated by proteolytic cleavage but, instead, must be complexed to Apaf-1 for activation (Rodriguez and Lazebnik, 1999). The apoptosome recruits and activates caspase -3 and -7 by proteolytic cleavage and subsequent cell death in an orderly manner through controlled proteolytic processing of downstream target proteins (Fig 1.10).

Caspases are kept in the inactive state in the cytosol when they are complexed with specific proteins termed inhibitors of apoptosis (IAPs). Members of the IAP family in human include X-linked inhibitor of apoptosis (XIAP), cIAP1, cIAP2, ILP2, ML-IAP, NAIP, survivin and BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme) (Srinivasula and Ashwell, 2008). These proteins bear one or more baculovirus IAP repeat (BIR) motifs, a sequence of about 70 amino acids with a RING finger zinc-binding domain at the carboxyl terminal of the BIR repeats (Liston et al., 1996; Vaux and Silke, 2005). In addition, near the RING finger of c-IAP1 and c-IAP2 is a CARD, suggesting that these IAPS may directly or indirectly regulate the processing of caspases via CARD interactions (Hofmann et al., 1997). IAPs complex with and inhibit active caspase -3 and -7 but not caspase -1, -6, -8, -10 (Deveraux et al., 1997; Roy et al.,

1997). This binding and inhibition is mediated by the BIR domains within the IAPs. IAPs can also inhibit caspase-9 but via a different mechanism; they bind to inactive procaspase-9 and interfere with the processing of procaspase-9 (Deveraux et al., 1998; Takahashi et al., 1998). IAPs thus put a brake on the apoptotic process by binding and inhibiting caspases, thereby prolonging cell survival.

IAPs in turn are subjected to regulation mediated by a second mitochondrial activator of caspases/direct IAP protein-binding protein of low pI (Smac/DIABLO). These proteins are released together with cytochrome c from the mitochondria. They bind to and antagonise the function of IAPs, freeing the caspases from the inhibitory action of these IAPs. Smac/DIABLOs do so by binding to the BIR repeats on IAPs (Wu et al., 2000). Omi/HTRA2, is also released together with Smac/DIABLO and cytochrome c can inactivate IAPs as well (Srinivasula et al., 2003; Yang et al., 2003) (Fig 1.10).

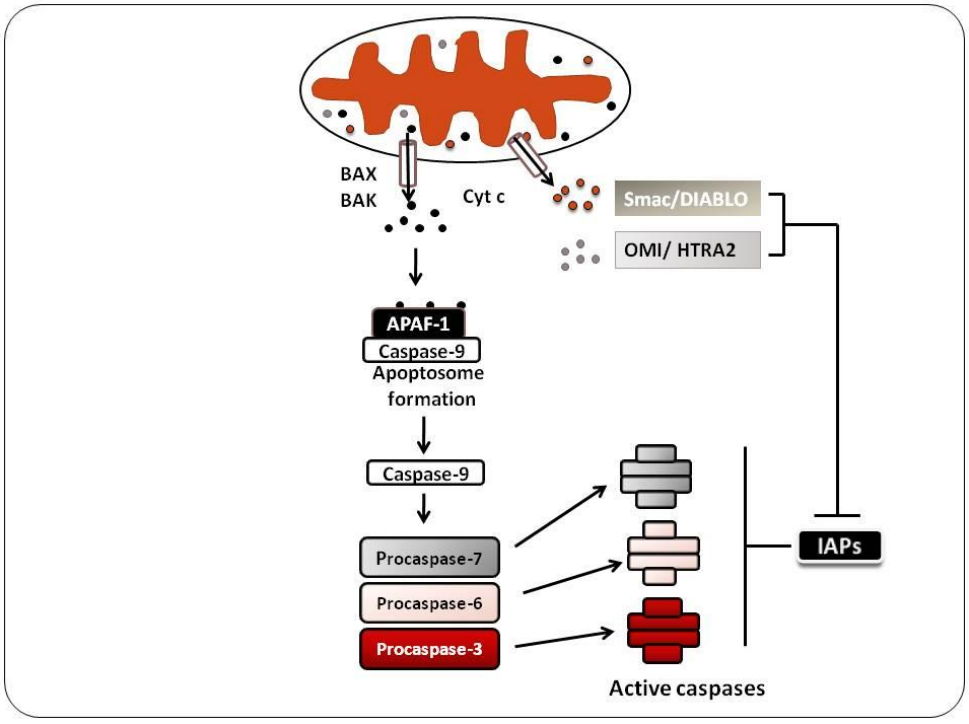


Fig 1.10: Caspase activation by cytochrome c from a mitochondrion (adapted from (Budihardjo et al., 1999))

There is cooperation between the intrinsic and extrinsic apoptotic pathways and is mediated by the Bid protein. This protein is cleaved by caspase-8 (activated by the extrinsic pathway) to generate the active pro-apoptotic protein tBid (Fig 1.7). tBid helps to amplify the signal induced by the death receptor stimulation in certain cell types (Gross et al., 1999). Activated BH3-only pro-apoptotic proteins translocate to the outer mitochondrial membrane (OMM) and exert their pro-apoptotic functions. Currently, there are two conflicting models; the direct and indirect methods of activation to explain how BH3-only proteins activate Bax and Bak.

1.5 PI3K-Akt pathway

PI3Ks, a family of lipid kinases have key regulatory roles in many cellular processes which include cell survival, cell proliferation, metabolism and motility (Engelman et al., 2006; Hietakangas and Cohen, 2009; Kolsch et al., 2008; Martelli et al., 2010; Morello et al., 2009; Oudit and Penninger, 2009). As major effectors downstream of receptor tyrosine kinases (RTKs) as well as the G protein-coupled receptors (GPCRs), PI3Ks transduce signals upon growth factors and cytokines binding to these receptors which result in activation of key proteins downstream, including the serine-threonine protein kinase Akt (also known as protein kinase B (PKB)) .

Akt was first isolated from an AKR thymonia and subsequently found to be an oncogene transduced by the acute transforming retrovirus (Staal, 1987). Since then, three members of the Akt family have been identified as Akt1, Akt2 and Akt3; and they are also referred as PKBα, PKBβ and PKBγ respectively. Although each of these classes of Akt is encoded by distinct gene, the three

proteins share more than 80% homology at the amino acid level (Datta et al., 1999; Nicholson and Anderson, 2002). All Akt isoforms share similar structures, including a pleckstrin homology (PH) domain at the amino-terminal, a central serine-threonine catalytic domain and a carboxyl-terminal that possesses regulatory domain for the induction and maintenance of its kinase activity (reviewed by (Chan et al., 1999)). Activation of Akt requires two phosphorylation events; one at Tyr^{308} and the second at Ser^{473}, catalysed by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2 respectively (Alessi et al., 1997; Scheid and Woodgett, 2001). These events occur in response to growth factors and other extracellular stimuli (Alessi et al., 1996; Brazil et al., 2004).

Akt are ubiquitously expressed, but expression levels vary depending on the tissue types. The PH domain of Akt binds to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) formed from the phosphorylation of membrane phospholipid, phosphatidylinositol-4, 5-bisphosphate (PIP_2) by PI3K (Cantley, 2002) (Fig 1.11). Activated Akt subsequently translocates to the cytosol and nucleus to phosphorylate its substrates (Arden and Biggs, 2002; Franke, 2008; Huang and Chen, 2005). Activation of Akt leads to cell cycle progression, survival, metabolism and migration via serine / threonine phosphorylation (Dahia, 2000; Downward, 2004; Kandel and Hay, 1999; Vivanco and Sawyers, 2002) (Fig 1.11).

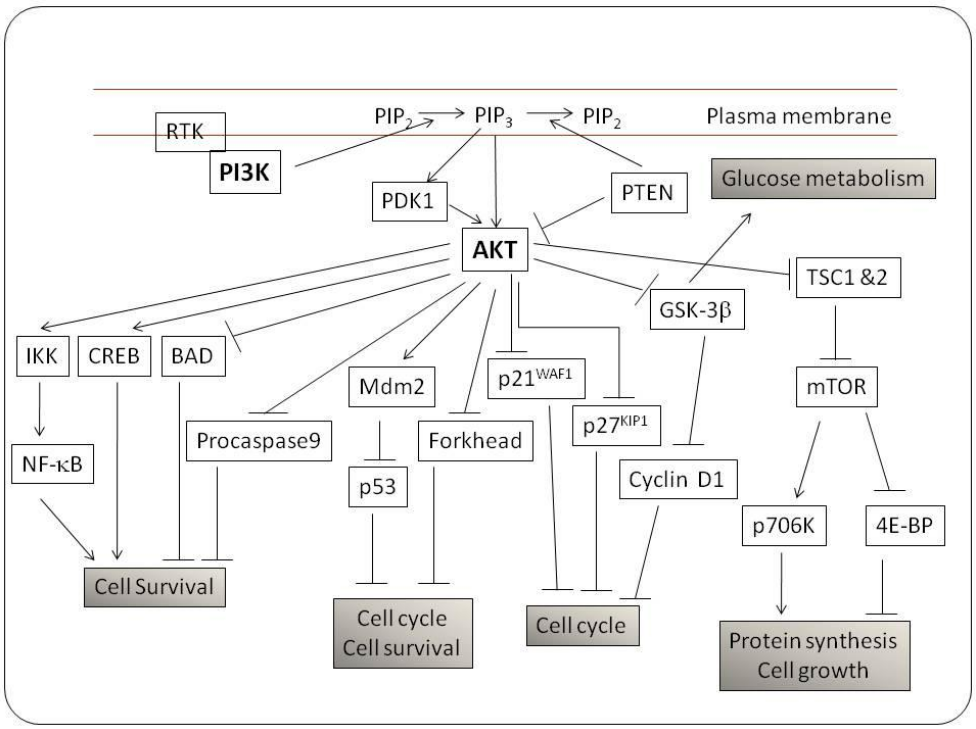


Fig 1.11: The phosphoinositide 3-kinase-Akt signalling cascade (adapted from (Tokunaga et al., 2008))

As Akt activation is initiated by Akt binding to PIP_3 which is generated downstream by PI3K, the cell has a regulator in the form of a tumour suppressor, phosphate and tensin homolog (PTEN). The latter proteins antagonise PI3K activity. PTEN possesses lipid phosphatase activity and reduces the cellular pool of PIP_3 by dephosphorylating it to PIP_2, thus negatively regulating Akt activity (Maehama and Dixon, 1998; Stambolic et al., 1998).

1.5.1 Akt in cell survival

Akt is critical for cell survival as it exerts its effect by direct phosphorylation of transcription factors involved in pro- and anti-apoptotic gene expressions and pro-apoptotic proteins (Datta et al., 1999). Akt negatively regulates the transcription factors that promote the expression of death genes encoding for the forkhead transcription factors FKHR, FKHRL1 and AFX (Biggs

et al., 1999; Brunet et al., 1999; Rena et al., 1999) (Fig 1.11). It can also prevent apoptosis by activating IκB kinase (IKK) (Romashkova and Makarov, 1999) and cyclic-AMP response element-binding protein (CREB) (Du and Montminy, 1998) (Fig 1.11). Akt also promotes cell survival by directly phosphorylating Bad and procaspase-9, key proteins in apoptosis (Datta et al., 1999) (Fig 1.11).

One must not overlook the effects of Akt on p53, a tumour suppressor that regulates cell cycle and apoptosis (Levine, 1997). One of the substrates of Akt phosphorylation is MDM2, which leads to subsequent proteasomal degradation of p53 (Mayo and Donner, 2002; Zhou et al., 2001). By enhancing the degradation of p53, Akt negatively regulate apoptosis. Akt also negatively regulate apoptosis through other key proteins of apoptosis like Bad. Akt phosphorylates Bad, a pro-apoptotic Bcl2 family member at Ser^{136} which promotes cell survival by inhibiting its interaction with the anti-apoptotic Bcl2 protein members like Bcl-xL and preventing the release of cytochrome c (Datta et al., 1997). Akt is able to phosphorylate procaspase-9 at Ser^{196} resulting in a conformational change to the protein and inhibiting its proteolytic activity (Cardone et al., 1998). The forkhead transcription factor, FKHRL1 whose target genes include *FasL*, *Bim*, *IGFBP1* and *Puma* is inhibited when phosphorylated by Akt and thus prevents transcription of these target genes whose gene products are regulatory apoptotic proteins (Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; You et al., 2006).

Akt enhances the expression of cell survival genes like *Bcl-xL*, *Bcl2*, *c-IAPs*, *c-FLIP* by phosphorylating and activating IKKα (Kane et al., 1999; Romashkova and Makarov, 1999), which in turn acts on IκB, freeing NF-κB from its complex formation with IκB. NF-κB is a transcription factor for these genes (Catz and Johnson, 2001; Lee et al., 1999; Wang et al., 1998). Similar to the

indirect effects of Akt on NF-κB, CREB is also affected but directly by Akt phosphorylation as phosphorylated CREB will up-regulate the transcription of pro-survival genes like *Bcl2*, *Mcl-1* and *Akt* itself (Pugazhenthi et al., 2000; Reusch and Klemm, 2002; Wang et al., 1999).

1.5.2 Akt in cell cycle progression and cell proliferation

PI3/Akt pathway plays a key role in the G1/S cell cycle progression (Liang and Slingerland, 2003) by phosphorylating key proteins involved in protein synthesis and cell cycle regulation. These include glycogen synthase kinase-3β (GSK-3β), the forkhead transcription factors, cyclin-dependent kinase inhibitor $p21^{CIP1}$ and $p27^{KIP1}$; and the mammalian target of rapamycin (mTOR) (Fig 1.11) (Li et al., 2002; Liang et al., 2002; Rossig et al., 2002; Shin et al., 2002).

GSK-3β phosphorylates cyclin D1 and c-Myc which are key proteins in the G1 phase of the cell cycle (Blagosklonny and Pardee, 2002) and in the process promotes their degradation resulting in cell cycle arrest (Alt et al., 2000; Diehl et al., 1998). Akt is able to promote cellular division by phosphorylating and inhibiting GSK-3β, thus preventing the breakdown of cyclin D1 and c-Myc (Diehl et al., 1998; Takahashi-Yanaga and Sasaguri, 2008).

Akt also phosphorylates and inhibits the forkhead transcription factor which leads to enhanced cyclin D1 expression and at the same time, represses $p27^{KIP1}$ and p130Rb2 expressions (Burgering and Medema, 2003; Kops et al., 2002; Medema et al., 2000; Schmidt et al., 2002). Akt can also phosphorylate and inhibit $p21^{CIP1}$ and $p27^{KIP1}$ directly; enhancing their proteasomal degradation and promoting cellular division (Li et al., 2002; Liang et al., 2002; Rossig et al., 2001; Shin et al., 2002). Akt also enhances the degradation of $p21^{CIP1}$ and $p27^{KIP1}$ by

up-regulating the expression of SKP2, a key component of the SCF/SKP2 ubiquitin ligase that mediates $p21^{CIP1}$ and $p27^{KIP1}$ degradation (Bashir et al., 2004).

1.5.3 The role of Akt in translational regulation

Translation in cells is controlled by many factors amongst them nutrient availability and growth factor stimuli which are regulated by the mammalian target of rapamycin (mTOR) (Schmelzle and Hall, 2000). Akt phosphorylates and inhibits tuberous sclerosis protein 2 (TSC2), a part of the tuberous sclerosis complex (Pan et al., 2004). Once phosphorylated, the complex is no longer able to suppress the activity of GTP-binding Rheb (Ras homology enriched in brain), a protein that phosphorylates and activates mTOR (Huang and Manning, 2009). mTOR will subsequently stimulate cell proliferation by activating the ribosomal protein S6 kinase (p70S6K) and inhibiting the elongation-initiation factor 4E-binding protein (4E-BP) (Nave et al., 1999; Wendel et al., 2004).

1.5.4 Activation of PI3K-Akt pathway and cancer development

Many human cancers exhibit excessive Akt activity and these include carcinoma, prostate, gastric, lung, ovary, pancreas and thyroid; glioblastoma and various haematological malignancies and nasopharyngeal carcinoma (Altomare and Testa, 2005; Carnero, 2010; Courtney et al., 2010; Liu et al., 2009; Morrison et al., 2004). Studies have also demonstrated a close association between Akt activation and the clinicopathological characteristics such as advanced stage, poor prognosis and histological grade (Altomare and Testa, 2005).

Akt activation in human cancer is caused by various mechanisms ranging from alteration of PI3K expression and activity (Bertelsen et al., 2006; Kadota et al., 2009; Murugan et al., 2008); Akt amplification and over-expression (Carpten et al., 2007; Davies et al., 2008; Pedrero et al., 2005) and down-regulation of PTEN via gene mutation, deletion or promoter methylation (Byun et al., 2003; Li et al., 1997a; Oki et al., 2006).

1.6 Flavonoids

1.6.1 Introduction

It has been estimated that 75 – 85% of all chronic illnesses and diseases are associated with lifestyle and cannot be explained by differences in genetic makeup alone (Wong et al., 2005). Studies have indicated a strong linkage between fat and red meat and the development of colorectal adenomas and at the same time, an inverse association between dietary fibres, fruit and vegetable intake with this disease (Mathew et al., 2004; Robertson et al., 2005). Moreover, epidemiological studies have demonstrated that consumption of food rich in fruits and vegetables results in low incidence of cancers (Block et al., 1992; Key et al., 2004; Linos and Willett, 2007; Reddy et al., 2003; Willett, 2000). A review of 206 human epidemiological studies and 22 animal studies conducted by Steinmetz & Potter demonstrated an inverse relationship between consumption of fruits and vegetables; and the risk of developing cancers of the stomach, oesophagus, lung, oral cavity pharynx, endometrium, pancreas and colon (Steinmetz and Potter, 1996).

Although it is not clear which components in fruits and vegetables are responsible for this preventive anti-cancer property, evidences point towards the presence of fibres, vitamins, minerals, polyphenols, terpenes, alkaloids and phenolics in fruits and vegetables as the contributing factors. These dietary agents can suppress the inflammatory processes that lead to transformation, proliferation and initiation of carcinogenesis.

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavour and colour to fruits and vegetables. In plants, these polyphenolic compounds are critical in plant physiology. They are involved in plant growth and reproduction, provide resistance to plant pathogens and predators, protection against diseases and pre-harvest seed germination (Bravo, 1998). When consumed in our daily diet, flavonoids have been found to exert beneficial effects like anti-oxidative (Burda and Oleszek, 2001), anti-viral (Guo et al., 2007; Liu et al., 2008; Roschek et al., 2009), anti-tumour (Cardenas et al., 2006), anti-inflammatory (Gonzalez-Gallego et al., 2007; Tunon et al., 2009). They are also able to prevent cardiovascular diseases (Tijburg et al., 1997) and exhibit hepato-protective activities (Yao et al., 2007).

1.6.2 Structures of flavonoids and their bioavailability

Flavonoids have a common diphenylpropane structure (C6-C3-C6) consisting of two aromatic rings joined by a three-carbon cyclic ring (Fig 1.12). Based on the variation to the heterocyclic C-ring, flavonoids are sub-divided into six major sub-classes. They include flavones, flavonols, flavonones, catechins, anthocyanidins and isoflavones (Fig 1.13). There are over 4000 naturally occurring flavonoids.

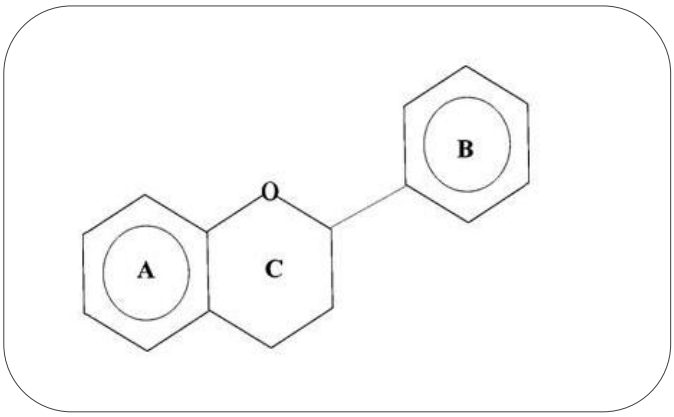


Fig 1.12: Basic structure of flavonoid (adapted from (Ross and Kasum, 2002))

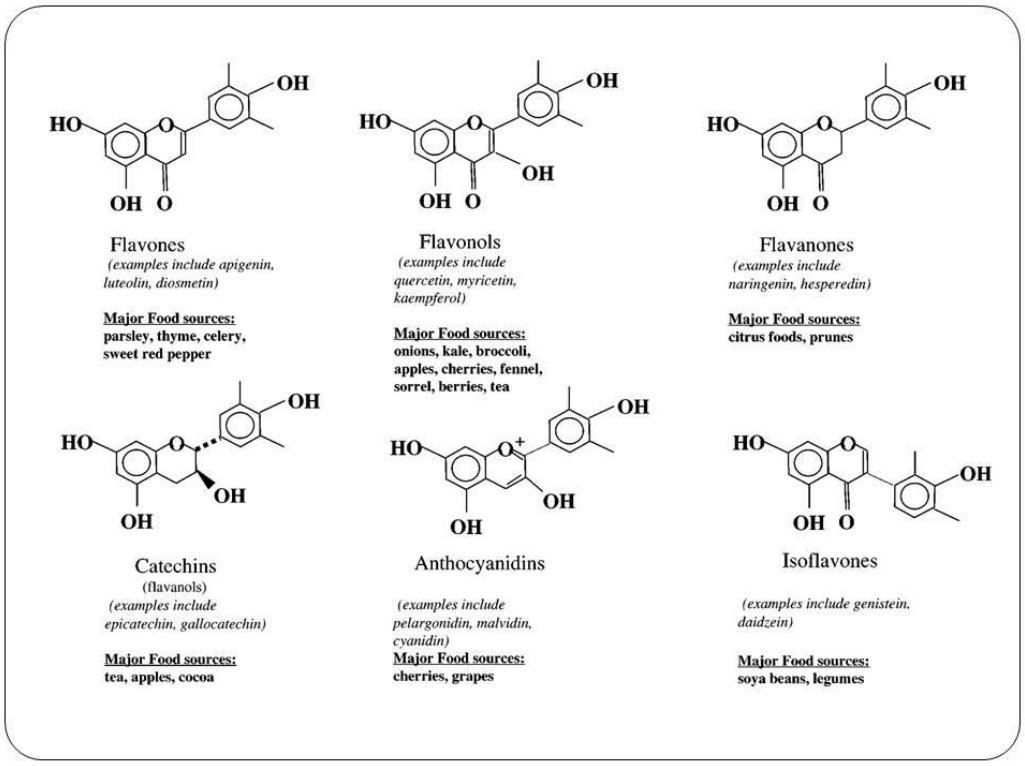


Fig 1.13: Chemical structures of the six major sub-classes of flavonoids (adapted from (Ross and Kasum, 2002))

Flavonoids present in human diet are mainly in the glycoside form. The biological fate of dietary flavonoid glycosides has always been an elusive and controversial issue. Some reports have demonstrated that the flavonoid glycosides cannot be absorbed in this form but need to be hydrolysed to their aglycone by bacterial enzymes in the lower part of the intestine before being

partially absorbed or may undergo further bio-transformation by bacteria (Bokkenheuser et al., 1987). This was further verified by studies with quercetin glycosides which indicated that they were not absorbed intact in humans and therefore not found in the systemic circulation (Erlund et al., 2000; Mullen et al., 2006). Similar findings have also been made with the glycosides of diosmetin (Cova et al., 1992), isoflavones (Williamson et al., 2005) and luteolin (Shimoi et al., 1998).

1.6.3 Anti-oxidant activity of flavonoids

The beneficial effects of flavonoids in chemopreventive therapy have been linked to their anti-oxidant activity which includes their redox potential and reactive oxygen species (ROS) –scavenging capabilities (Duthie and Crozier, 2000). Contrary to general belief, recent studies showed flavonoids exerting their anti-oxidant role through their effects on protein kinase and lipid kinase signalling pathways and not as conventional hydrogen-donating anti-oxidants (Williams et al., 2004). Although flavonoids exhibit anti-oxidant activity, however they are not likely to act as major anti-oxidants *in vivo* as endogenous anti-oxidants like ascorbic acids are present at a higher concentration in the body and therefore will be the major player as anti-oxidant instead. Flavonoid concentrations *in vivo* however may be high enough to mediate receptor or enzyme activity leading to the inhibition or up-regulation of various signalling pathways like tyrosine kinases, protein kinase C and mitogen-activated protein kinase.

Although the chemopreventive properties of flavonoids are linked with their ability to scavenge endogenous ROS, however new studies have emerged suggesting that these dietary agents may protect free-radical-induced damage to

DNA by a mechanism other than direct free-radical scavenging. Flavonoids can reduce the incidence of single-strand breaks in double-stranded DNA as well as residual base damage through fast chemical repair (Anderson et al., 2000).

There are also reports indicating flavonoids as pro-oxidative in their action and it may be this action which gives rise to the anti-cancer property of flavonoids instead of their anti-oxidant activity (Hadi et al., 2000; Rahman et al., 1990). Some anti-cancer agents cause apoptotic DNA fragmentation mediated by ROS (Kaufmann, 1989). Certain dietary phenolic compounds like quercetin can also bind and cleave DNA and at the same time generate ROS in the presence of transition metal ions like copper (Rahman et al., 1990); a mechanism similar to those of known anti-cancer drugs.

A second mechanism for the anti-cancer and tumour cell apoptosis-inducing properties of flavonoids based on their pro-oxidant activity is their effects on mitochondria. Certain flavonoids have been demonstrated to cause the collapse of the mitochondrial membrane potential causing apoptosis in tumour cells (Chung et al., 2001; Morin et al., 2001; Surh et al., 1999).

1.6.4 Anti-oestrogenic (and oestrogenic) activity of flavonoids

Exposure to both endogenous and exogenous oestrogens has been associated with an increased risk factor for some hormone-dependent cancers like breast cancers. Phyto-oestrogens are phenolic non-steroidal plant compounds with oestrogen-like biological activity. Isoflavones like genistein and other flavonoids like apigenin, kaempferol and resveratrol exhibit both anti-oestrogenic as well as oestrogenic activities associated with oestrogen-receptor binding (Cos et al., 2003). There are evidences to demonstrate that in countries where the

average intake of phyto-oestrogens is higher compared to some other countries, there is lower incidence rate of cancers associated with oestrogen exposure such as breast and prostate cancers (Jian, 2009; Messina and Hilakivi-Clarke, 2009; Perabo et al., 2008).

1.6.5 Anti-tumour property of flavonoids

Tumourigenesis is a multi-step and multi-factorial event. It can be triggered off by environmental pollutants like cigarette smoke, industrial emissions, petroleum vapours, inflammatory agents and tumour promoter chemicals like phorbol esters. These chemicals are known to modulate transcription factors involved in cell survival and cell division like NF-κB, AP-1 (activator protein-1), STAT3 (signal transducer and activator of transcription 3), anti-apoptotic proteins like Akt, Bcl-2 and Bcl-X_L; pro-apoptotic proteins like the caspases and PARP; protein kinases like IKK, EGFR (epidermal growth factor receptor), HER2 (human epidermal growth factor receptor 2), JNK (Jun N-terminal kinase) and MAPK; cell cycle proteins like cyclins and their associated cdks; cell adhesion molecules, COX-2A and growth factors (reviewed by (Aggarwal and Shishodia, 2006; Pan and Ho, 2008).

1.6.5.1 Effects of flavonoids on NF-κB

NF-κB is a transcription factor that translocates to the nucleus and induces the transcription of over 200 genes that suppress apoptosis and induce cellular transformation, proliferation, invasion, metastasis and inflammation (Luqman and Pezzuto, 2010). Many of these genes whose proteins play key roles in the establishment of early and late stage of aggressive cancers include cyclin D1, Bcl-

2, Bcl-X_L, matrix metalloproteases (MMP) and vascular endothelial growth factor (VGEF).

Numerous flavonoids have been identified to inhibit NF-κB activity and these include curcumin (Mackenzie et al., 2008; Singh and Aggarwal, 1995), diosgenin (Shishodia and Aggarwal, 2006), ellagic acid (Edderkaoui et al., 2008) , emodin (Kumar et al., 1998), gingerol (Ishiguro et al., 2007), epigallocatechin-2-gallate (EGCG) (Yang et al., 2001a), luteolin (Kim and Jobin, 2005), lycopene (Kim et al., 2004), chrysin (Li et al., 2010b) and resveratrol (Roy et al., 2009). These dietary agents inhibit the NF-κB pathway in one or more ways such as inhibiting the activation of NF-κB, translocation of this transcription factor to the nucleus, binding to the DNA and interactions with the machinery of DNA transcription.

Thus by inhibiting one or more steps in the NF-κB pathway which is a critical pathway in ensuring cell survival and growth, flavonoids are able to act as natural anti-cancer agents.

1.6.5.2 Effects of flavonoids on cell cycle

Cell cycle progression is a very tightly controlled and highly regulated process in a cell (reviewed in Chapter 1.3 of this thesis); and loss of this control and regulation lead to cancer. Critical proteins to control cells in G1, S, G2 and M phases are activated at each phase to ensure a smooth transition of cells through the phases. Accumulation of ribosomes, the translational machinery in G1 phase is also a key event in cell growth and this involves the protein S6 kinase (S6K). Negative and positive regulators of S6K play a key role in determining the level of ribosomal S6 protein and thereby the ribosomal pool (Thomas, 2000). S6K can

be activated by mitogenic stimulation in the form of EGFR and IGFR (insulin-like growth factor receptor) and the Ras/ERK (extracellular signal regulated kinase) well as the PI3K/Akt signal transduction machinery. These will lead to down-stream activation of proteins involved in cell division including cyclin D1 and c-Myc (Malumbres and Barbacid, 2001; Thomas, 2000). Many flavonoids are able to inhibit the EGFR and IGFR mitogenic signalling at the receptor level in numerous cancer cell lines, leading to cell growth inhibition and proliferation (Aggarwal, 2000; Lin et al., 1999; Zi and Agarwal, 1999).

Cancer development can occur when there is aberration to the G1/S checkpoint resulting in the deregulation of the cell cycle. Key proteins include the cyclins and their associated cdks; and the CKIs. Several dietary agents like curcumin (Mukhopadhyay et al., 2002), resveratrol (Estrov et al., 2003), genistein (Li et al., 2005), apigenin (Takagaki et al., 2005) and silibinin (Tyagi et al., 2002) are able to reverse the deregulation of cell cycle in cancer cell lines, causing cell growth arrest and apoptosis.

The Rb family proteins play central role in cell cycle progression as they are the prime targets for phosphorylation by the activated cdk-cyclin complexes. Upon phosphorylation, pRbs release E2Fs which are transcription factors of genes necessary for cell cycle progression through the G1 and S phases. There is also evidence that demonstrates pRb's role in repressing gene expression, in part mediated by chromatin condensation and thus inhibiting gene transcription downstream (Harbour and Dean, 2000a). Silibinin has been reported to increase the total as well as under-phosphorylated form of pRb in human prostate carcinoma LNCaP cells. Cyclin D1 is over-expressed in numerous cancers. Curcumin is able to inhibit cell cycle progression by down-regulating the

transcriptional and post-translational expression of cyclin D1 (Bharti et al., 2003; Mukhopadhyay et al., 2002). Numerous studies have demonstrated that different flavonoids regulate critical proteins in the cell cycle of cancer cell lines and mediate cell cycle arrest at either G1/S or G2/M. Thus these dietary agents are potential candidates for cancer management in the form of chemo-preventive therapy in cancer patients after conventional cancer treatment.

1.6.5.3 Effects of flavonoids on Akt

Akt is critical in mammalian cell survival and many cancer cell lines possess abnormal activated level of Akt (review in Chapter 1.5 of this thesis). Studies have identified several phytochemicals that are able to suppress the activation of Akt directly or via other critical proteins upstream of Akt (Fig 1.11). These include genistein (Li and Sarkar, 2002), indole-3-carbinol (Chinni and Sarkar, 2002), diosgenin (Shishodia and Aggarwal, 2006) and EGCG (Tang et al., 2003). Some flavonoids like curcumin (Aggarwal et al., 2006) and luteolin (Ong et al., 2010b) are also able to inactivate Akt.

These studies provide evidence of one of many molecular mechanisms of flavonoids as anti-cancer chemicals to suppress tumourigenesis.

1.6.5.4 Effects of flavonoids on tumour suppressor p53

p53 a tumour-suppressor and transcription factor, is a key protein in regulating many cellular processes such as the cellular response to DNA damage, genomic stability during cell cycle, cell cycle control and apoptosis. Lack of p53 favours the development of cancer (Chène, 2003). Moreover, half of the human cancer carries the mutated form of p53 and this is associated with poor prognosis

(Chène, 2003; Taylor and Stark, 2001). Activated p53 consequently leads to the up-regulation of CKI, $p21^{CIP1}$ and pro-apoptotic proteins like Bad and Bax; resulting in cell cycle arrest and apoptosis. Flavonoids like curcumin (Han et al., 1999), resveratrol (Huang et al., 1999), EGCG (Gupta et al., 2000), silibinin (Katiyar et al., 2005), quercetin (Ong et al., 2004) and luteolin (Shi et al., 2007) can modulate p53 activity, leading to cell cycle arrest and apoptosis.

1.6.5.5 Activation of apoptosis by flavonoids

Cell cycle arrest is one of the important events in sensitising cells to apoptosis. Numerous reports have demonstrated that flavonoids such as EGCG, genistein, quercetin, luteolin and silibinin lead to apoptosis which is a downstream event associated with G1 or G2/M cell cycle arrest in different types of cancers. In addition, stress signals elicited by flavonoids can also trigger apoptosis via NF-κB, AP-1, Bcl-2 and Bcl-X_L (de Kok et al., 2008; Nishino et al., 2007; Ramos, 2008; Thomasset et al., 2007) (Fig 1.14). Apoptosis can be mediated by either an extrinsic signal between death receptors and their ligands or intrinsic signal via mitochondrial induced pathway. Many flavonoids trigger apoptosis in different cancers through the mitochondrial-mediated pathway including retinoic acid (Noy, 2010), curcumin (Pesakhov et al., 2010), EGCG (Lee et al., 2010b), apigenin (Lu et al., 2010) , quercetin (Ong et al., 2004; Suh et al., 2010), chrysin (Li and Sarkar, 2002), silibinin (Pesakhov et al., 2010), silymarin (Ramakrishnan et al., 2009) and resveratrol (Bai et al., 2010).

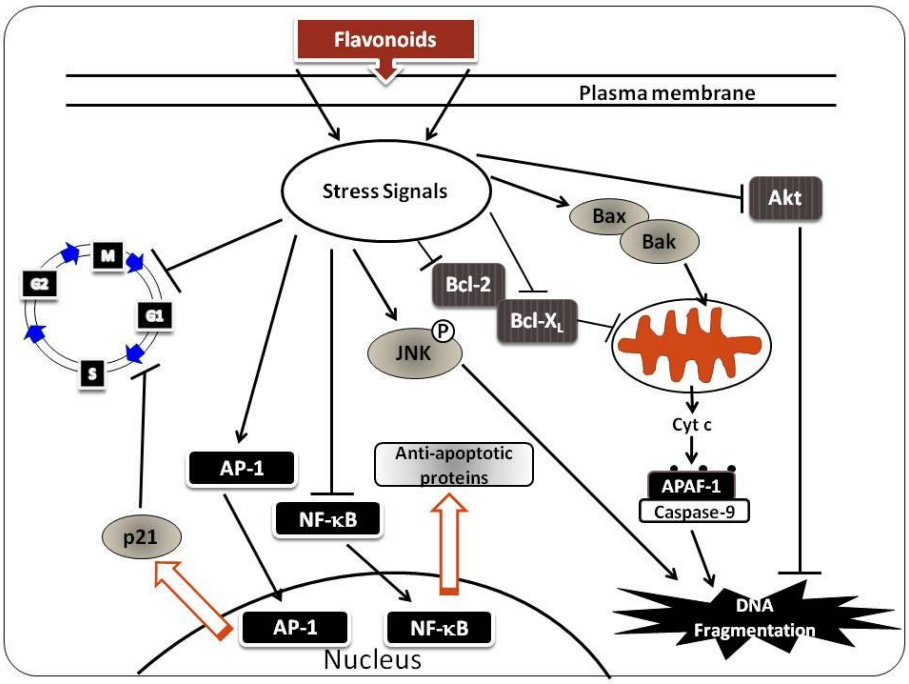


Fig 1.14: Induction of apoptosis by dietary flavonoids (adapted from (Guo et al., 2009))

A second potential target site of flavonoids that will result in apoptosis of cancer cells is the inhibition of the growth factor-mediated PI3K-Akt pathway (Liang et al., 1997). There are numerous reports that demonstrate flavonoids mediated apoptosis of cancer cells via this pathway (Adams et al., 2010; Lin et al., 2008; Sun et al., 2010).

These studies provide evidence that flavonoids act on cancer cells by exerting their effects on multiple signalling pathways in cells.

1.7 Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is a flavonol-type flavonoid ubiquitously present in plant-derived foods. It is found in a variety of fruits like red onions, grapes, apples, berries, cherries, broccoli, citrus fruits and tea. Average daily uptake of quercetin in a human diet varies between 10 – 100 mg

depending on eating habits (Erlund et al., 2000). Quercetin is commonly found as ortho-glycosides in which at least one hydroxyl group is substituted by various types of sugars (Fig 1.15). The sugar group can be attached to C3, giving rise to its glycoside derivatives like quercitrin, isoquercitrin, hyperoside and rutin (Fig 1.15). Alternatively, the sugar group can also be bound to C4 of the parent quercetin chain generating quercetin-4′-*O*-*β*-D-glucoside and quercetin-3,4′-*O*-β-D-glucoside. Their water-solubility property increases with increasing number of sugar groups. Quercetin exhibits anti-oxidant activity due to its electron-donating property which can be attributed to the presence of a phenolic hydroxyl group on its chain (Bors et al., 1990; Vargas and Burd, 2010).

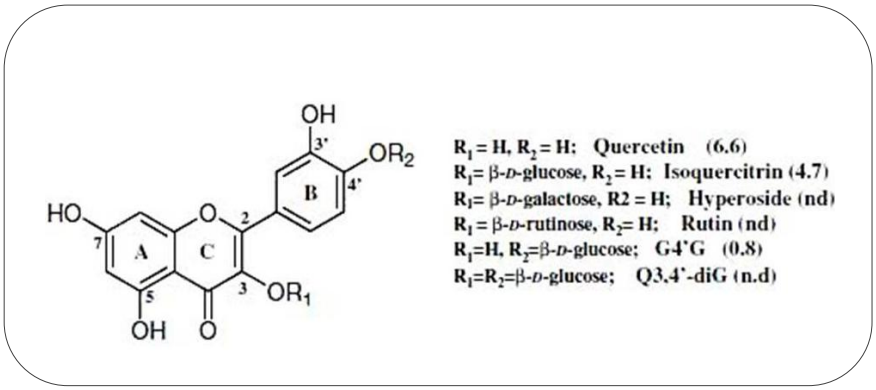


Fig 1.15: Chemical structure of quercetin and its glycosides (adapted from (Murakami et al., 2008))

Studies have provided evidence to indicate that dietary quercetin can be absorbed from the digestive tract and subsequently undergoes metabolic conversion (Murota et al., 2000). Quercetin glycosides are absorbed in the small intestine as quercetin aglycone following hydrolysis by lactose phorizin hydrolase (LPH) in the brush border membranes (Walle et al., 2000) of cells in the small intestines. Once quercetin is absorbed by the intestinal epithelium as aglycone derivative and subsequently entered the blood stream, it can be glucorinated,

methylated or sulphated and then bound to transport proteins. The plasma protein-bound quercetin glucoronides enter tissues and this is then followed by the separation of the quercetin aglycone from the plasma protein inside cells (O'Leary et al., 2003; Spencer et al., 2003).

Many biological effects of quercetin on cells and tissues have been reported (reviewed by (Bischoff, 2008)). Of interest in this thesis is its anti-cancer property. There are numerous reports to demonstrate the effects of quercetin on signal transductions associated with tumourigenesis and these include cell cycle regulation, apoptosis, pro-inflammatory protein induction and angiogenesis (Hirpara et al., 2009; Murakami et al., 2008). In our study, quercetin was able to inhibit cell growth of nasopharyngeal carcinoma cells by inhibiting cell cycle progression to S phase mediated by the inhibition of E2F function; and apoptosis (Ong et al., 2004).

1.8 Luteolin

Luteolin (3′,4′,5,7-tetrahydroxylflavone) is a flavonol-type flavonoid ubiquitously present in plant-derived foods. Luteolin-rich vegetables and fruits include celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins and chrysanthemum flowers (Miean and Mohamed, 2001; Sun et al., 2007; Xie et al., 2009). In these vegetables and fruits, luteolin is present as aglycone (without sugar moiety) and glycosides (aglycone with one or more sugar moieties) (Fig 1.16). Like quercetin, luteolin is hydrolysed to its aglycone form before being absorbed by the intestinal epithelium (Shimoi et al., 1998).

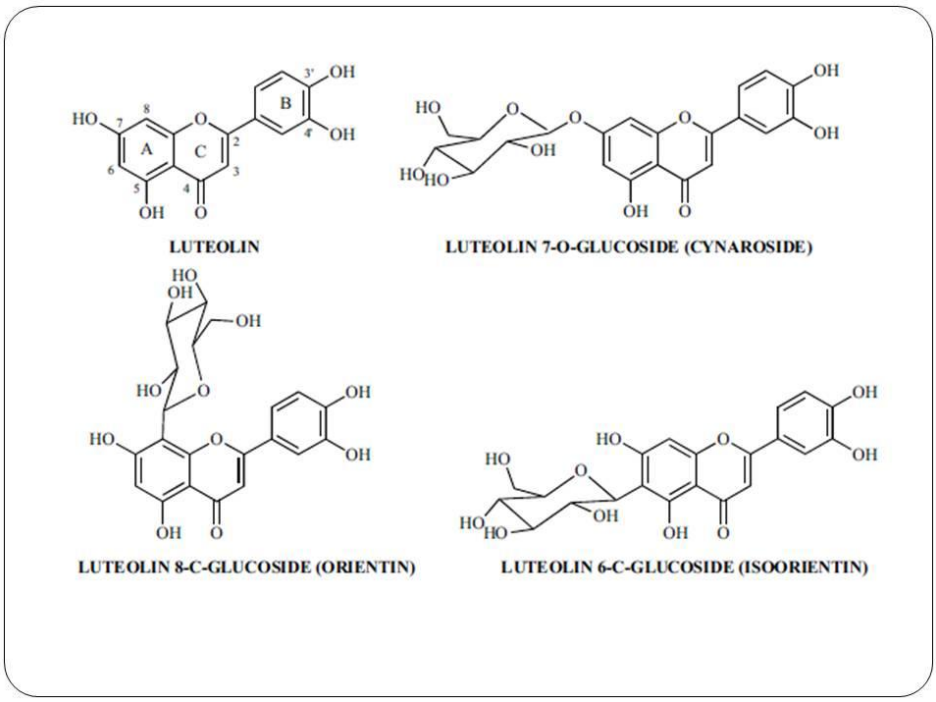


Fig 1.16: Chemical structures of luteolin and its glycosides (adapted from (Lopez-Lazaro, 2009))

Luteolin exhibits a wide range of biological activities in the prevention and treatment of chronic diseases due to their anti-oxidant, anti-inflammatory, anti-microbial and anti-cancer activities (reviewed by (Lopez-Lazaro, 2009). Of particular interest in this thesis is the anti-cancer property of luteolin. In our laboratory, luteolin has been found to enhance TNF-α-induced apoptosis in human cell lines like colorectal cancer COLO205, HCT116; and cervical cancer Hela cells via suppression of NF-κB (Shi et al., 2004). In a second study, pre-treatment of TRAIL-sensitive cancer cells like Hela and TRAIL-resistant cancer cells such as CNE1, HT29 and Hep G2 with a non-cytotoxic concentration of luteolin was able to enhance TRAIL-induced apoptosis mediated by caspase -8 and -3 activation (Shi et al., 2005). In a separate study, we have also demonstrated that luteolin was able to induce G1 cell cycle arrest in nasopharyngeal carcinoma cells and this was brought about by the suppression of

Akt activity that led to the proteasomal degradation of cyclin D1 and subsequently inhibition of E2F, a transcription factor critical in the cell progression from G1 to S phase (Ong et al., 2010a). This last study will be elaborated under Chapter 3 of this thesis.

1.9 Objective of this study

Flavonoids as chemo-preventive chemicals provide an attractive and viable form of cancer management as they are able to inhibit tumourigenesis via signal transduction involved in cell survival, cell growth and cell death. To achieve its role in chemo-prevention, molecular actions of these flavonoids alone and in combinations on different cancer types must be elucidated to enhance efficacy and reduced toxicity. A clear understanding in this area will definitely provide a platform for future development of flavonoids in cancer management as well as in other diseases such as cardiovascular, inflammatory and neurological diseases. Moreover, it will also elevate our understanding of the overall health benefit of flavonoids which are consumed in our human diet.

Thus, the objectives of this study are:

1. To investigate the effect of quercetin on the cell growth inhibition and apoptosis of nasopharyngeal carcinoma cells and
2. To examine the effect of luteolin on cell cycle arrest on the same type of cancer; and
3. To examine the sensitisation effect of luteolin and quercetin on apoptosis induced by cancer chemotherapeutics.

Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions

Chapter 2

2.1 Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy of epithelial origin occurring with a high incidence rate in Southern China and South-East Asia. In Southern China, it is the third most common form of malignancy amongst men, with incidence rate between 15 and 50 per 100, 000 (Ho, 1978). In Singapore, the incidence rates are 18.4 per 100 000 in males and 7.3 per 100,000 in females (Chia et al., 1996). Independent studies by Serin et al (Serin et al., 1999), Cheng et al (Cheng et al., 2000) and Wei and Kwong (Wei and Kwong, 2010) have demonstrated that the combination of radiotherapy and chemotherapy in both early and late stage cases have helped to improve the prognosis of NPC patients. Patients were treated with cisplatin (Serin et al., 1999) and cis-diamine-dichloroplatinum and fluorouracil (Cheng et al., 2000) in addition to radiotherapy, and they exhibited improved prognosis. However toxicities were observed in some patients after chemotherapy which included mucositis (Wong et al., 2006), grade III/IV neutropenia (Chua et al., 2004; McCarthy et al., 2002), grade III/IV anaemia, granulocytopenia and thrombocytopenia (Leong et al., 2005; Ngan et al., 2002). The use of flavonoids with anti-tumour property to control cancer growth may help to circumvent some of these toxicities experienced by patients as these are found in fruits and vegetables; and are consumed in our daily diet.

Humans ingest about 1 g of flavonoids daily in their diet (Scalbert and Williamson, 2000). The most common flavonoid aglycones found in the diet are quercetin, rutin and robinin (Kuhnau, 1976). Quercitrin and rutin are hydrolysed to quercetin by obligate anaerobes in the gastrointestinal tract (Bokkenheuser et al., 1987). Quercetin is also widely distributed in the edible portion of most dietary plants like citrus fruits, berries, leafy vegetables, roots, tubers and bulbs;

and legumes. These flavonoids are increasingly been associated with cytoprotective anti-tumour properties against cancers in various animal models (Steinmetz and Potter, 1996). The molecular actions responsible for these effects have not been fully elucidated but may involve interaction with xenobiotic metabolising enzymes that are capable of altering the metabolic activation of potential carcinogens (Eaton et al., 1996; Obermeier et al., 1995; Polyak et al., 2010; Siess et al., 1989). Quercetin has been found to inhibit growth of human cancers like leukaemia (Kang and Liang, 1997; Russo et al., 2010), breast carcinoma (Chien et al., 2009; Choi et al., 2008; Choi et al., 2001), colon adenocarcinoma (Murtaza et al., 2006; Salucci et al., 2002), prostate cancer (Aalinkeel et al., 2008; Jung et al., 2010) and endometrial cancer (Kaneuchi et al., 2003). It can inhibit the growth of malignant tumour cells through various molecular actions which include cell cycle arrest (Choi et al., 2001; Kaneuchi et al., 2003; Salucci et al., 2002), and apoptosis (Chien et al., 2009; Choi et al., 2001; Iwao and Tsukamoto, 1999). However, the exact mechanism that leads to cell cycle and/or apoptosis in most cancers remains unclear.

Although quercetin exhibits anti-proliferative effects in numerous cancers (Chien et al., 2009; Choi et al., 2008; Jung et al., 2010; Kang and Liang, 1997; Russo et al., 2010), there is no report on its anti-proliferative activity on NPC. In this study, we demonstrate that two NPC lines, CNE2 and HK1 exhibit different degree of susceptibility to quercetin; with CNE2 cells less susceptible to the cytotoxic effect of quercetin compared to HK1 cells. Quercetin induced growth inhibition in these cells by entrapping cells in the G2/M and G0/G1 phases and hence preventing cell cycle progression to the S phase. It also induced cell death through apoptosis and necrosis.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Dimethyl sulphoxide (DMSO), Igepal CA-630, quercetin, antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), Triton X-100 and trypsin-EDTA (ethylenediaminetetraacetic acid) (0.25% trypsin and 6.8 mM EDTA) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Quercetin was dissolved in DMSO at a concentration of 296 mM (50.0 mg/ml) stock. RPMI-1640 and foetal bovine serum (FBS) were purchased from Gibco Ltd. (Grand Island, NY, USA). Reagents used for Western blot analysis were purchased from Bio-Rad (Hercules, CA, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Rabbit anti-Bad, rabbit anti-cleaved caspase-3, rabbit anti-cleaved caspase-7, rabbit anti-PARP, rabbit anti-cleaved PARP, rabbit anti-phosphorylated-p53 (Ser^{9}) antibodies, horseradish peroxidise-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-Bax, mouse anti-p53, mouse anti-$p21^{CIP1}$ and mouse anti-α-tubulin antibodies were purchased from Neomarkers Inc (Fremont, CA, USA). Mouse anti-Rb and mouse anti-hypophosphorylated Rb antibodies were purchased from Becton-Dickinson (BD) Pharmingen (Palo Alto, CA, USA).

2.2.2 Cell lines and cell culture

CNE2, a poorly differentiated line from a 68-year –old Chinese male (Sizhong et al., 1983) and HK1, a well differentiated NPC line from a Chinese male (Huang et al., 1980) were kindly provided by Professor KM Hui, National

Cancer Centre, Singapore. They were grown and maintained at 37^{o}C in a humidified 5% CO_2 and 95% air atmosphere in RPMI-1640 medium, supplemented with 10% FBS and antibiotics. Cells for experiments were trypsinised in trypsin-EDTA.

2.2.3 Proliferation assay

Cell inhibition/proliferation assays were carried out by seeding cells between 0.5 – 1.0 x 10^4 cells to each well of 96-well microtitre plates and incubated overnight to allow for cell adherence. This was followed by adding quercetin at 14.8 (5.0 μg/ml), 29.6 (10.0 μg/ml) and 59.2 (20.0 μg/ml) μM in serum-free RPMI-1640 medium to the cells and incubating for 24 and 48 h. The incorporation of bromodeoxyuridine (BrdU) and subsequent enzyme linked immunosorbent assay (ELISA) were carried out as specified by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). All results were presented as means ± standard error from four independent experiments, each measured in quadruple.

2.2.4 Cell cycle and apoptosis analysis assays

Cell cycle analysis of DNA content was performed using propidium iodide (PI) provided in the cell cycle test kit (BD Biosciences, Palo Alto, CA, USA). Briefly, cells were grown in 100 mm dishes until 70% confluence before being treated with 14.8, 29.6 and 59.2 μM quercetin in serum-free RPMI-1640 medium for 24 and 48 h. Treated cells were trypsinised, harvested and subjected to treatment as described by the manufacturer. Apoptosis in untreated and quercetin treated cells were determined by using the Annexin V-FITC (fluorescein

isothiocyanate) apoptosis detection kit (BD Biosciences, Palo Alto, CA, USA) and the data analysed using the FACSort flow cytometer (BD).

2.2.5 Protein extraction and western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 0.3 M NaCl, 20 mM Tris pH 7.4, 2 mM EDTA, 0.5 mM sodium vanadate, 1% Igepal CA-630 and protease inhibitor cocktail). The extracted cellular proteins were subjected to gel electrophoresis at 100.0 μg/ml and western blot analysis as previously described (Huynh et al., 1995). Blots were incubated with indicated primary antibodies (all the antibodies were used at a final concentration of 1.0 μg/ml) and horseradish peroxidise-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:1500). Blots were visualised with a chemiluminescent detection system as described by the manufacturer (Perkin Elmer, Boston, MA, USA).

2.3 Results and discussion

2.3.1 Quercetin inhibits the growth of CNE2 and HK1 cells

The growth inhibitory effect of quercetin on CNE2 and HK1 cells were examined by exposing these cells to different concentrations of quercetin ranging from 14.8 – 59.2 μM, for 24 h. The effect on cell growth was monitored by BrdU incorporation and subsequently quantified by ELISA. Growth of these cells was inhibited by quercetin in a dose-dependent manner (Fig 2.1). By applying linear progression on Fig 2.1, IC_{50} (inhibitory concentration resulting in 50% inhibition of cell growth) at 24 h were estimated to be 35.0 and 54.5 μM for HK1 and CNE2

cells respectively. This demonstrates that HK1 cells were more susceptible than CNE2 cells to the anti-proliferative activity of quercetin.

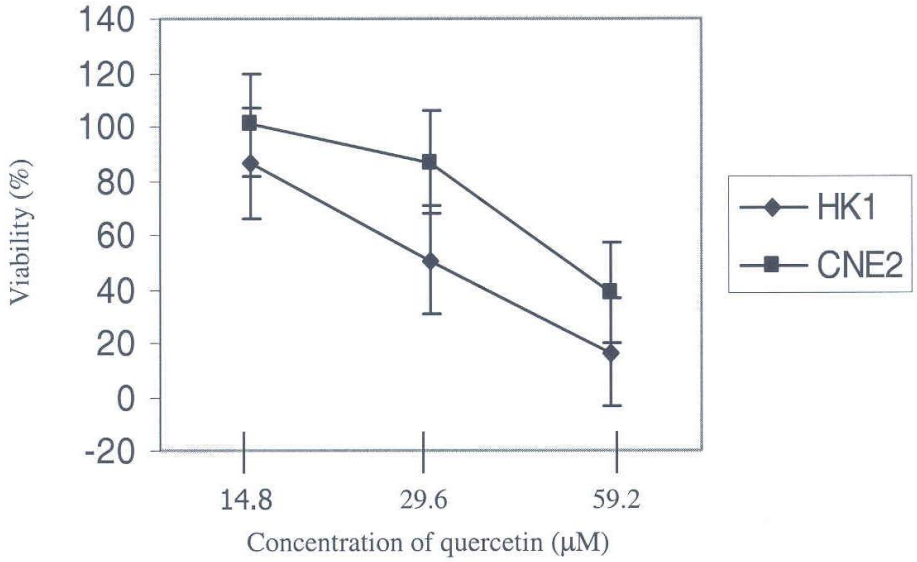


Fig 2.1: Survival curves of quercetin treated CNE2 and HK1 cells
CNE2 (5.0 x 10^3/well) and HK1 (1.0 x 10^4 /well) were plated in 96-well microtitre plates in RPMI-1640 medium supplemented with 10% FBS overnight. Cells were treated with 0, 14.8, 29.6 and 59.2 μM of quercetin in serum-free RPMI-1640 medium for 24 h. Cell proliferation was determined by the amount of BrdU incorporated into the cells. These values were expressed in percentage with untreated cells as 100%. All results were presented as means ± standard error from four independent experiments, each measured in quadruple. By applying linear regression on the graph, IC_{50} for CNE2 and HK1 were subsequently determined.

A possible reason to explain the different susceptibility of CNE2 and HK1 cells to quercetin could be attributed to an increase in Bad and Bax protein levels in quercetin treated HK1 as demonstrated by Western blot analysis (Fig 2.6 F and G). Bad and Bax proteins reside in the cytosol but translocate to the mitochondria following death signalling, thereby promoting the release of cytochrome c. The consequence of this activity is cell death by apoptosis. Hence cell death of HK1 cells by apoptosis was triggered by the increase in Bad and Bax. In CNE2 cells, quercetin treatment led to an increase in Bad but not Bax expression (Fig 2.5 F and G). The increase level of both pro-apoptotic proteins Bad and Bax in quercetin treated HK1 cells compared to only Bad increase in quercetin treated

CNE2 could have made the former more susceptible to quercetin compared to the latter. This was verified by a lower IC_{50} value observed in quercetin treated CNE2 cells compared to HK1. A possible explanation could be linked to the type of p53 expressed by these cells. HK1 cells express wild-type p53 whereas CNE2 cells express the mutant form of p53. Since p53 is a transcription factor for Bax, the different form of p53 may play a critical role in the level of Bax expression. This would need further verification. The mutant form of p53 in CNE2 cells may cause the cells to be less responsive to quercetin treatment compared to the wild-type bearing p53 HK1 cells, thus making CNE2 cells more resistant to the anti-proliferative activity of quercetin compared to HK1 cells.

2.3.2 Cell cycle arrest at G2/M and G0/G1 phases in quercetin treated CNE2 and HK1 cells

Treating CNE2 and HK1 cells with 14.8 μM quercetin for 24 h markedly increased the accumulation of cells in the G2/M phase equivalent to 36.9% and 31.3% of their cell population respectively (Fig 2.2). However, in the presence of higher doses of quercetin, there was a shift in the cell cycle pattern. At 29.6 μM of quercetin, 56.4% of CNE2 and 44.8% of HK1 cell populations were in the G0/G1 phase (Fig 2.2 C and G). A similar cell cycle profile was also observed when both cell lines were exposed to quercetin for 48 h (data not shown). Since p53 and pRb have been found to regulate cell cycle progression, we next examined the expression of the two proteins by western blot analysis. As demonstrated in Fig 2.3 A and B, expression of p53 and the phosphorylated form of p53 Ser^9 were not significantly increased by treatment with quercetin, hence it is unlikely that p53 was involved in cell cycle regulation following this treatment.

Detection of other phosphorylated forms of p53 at Ser^{6}, Ser^{20} and Ser^{392} were also performed but did not yield any band (data not shown). The expression of the hypophosphorylated form of pRb however was significantly increased in a dose-dependent manner from 1.0-fold in untreated CNE2 cells to 3.2-fold when these cells were treated with 29.6 μM quercetin for 24 h (Fig 2.3). Similarly in HK1 cells, the amount of hypophosphorylated form also increased from 1.0-fold for untreated cells to 2.6-fold when they were exposed to 29.6 μM of quercetin for 24 h (Fig 2.3A). The increase in total pRb in quercetin treated CNE2 cells was more significant, ranging from 2.0- to 3.5- fold increase compared to the untreated cells (Fig 2.3B), with most of the proteins existing as the hypophosphorylated form. This helped to explain the cell cycle arrest of CNE2 and HK1 cells by quercetin as the hypophosphorylated form of Rb blocks the activity of the E2F-1 family of transcription factors, consequently preventing cells from entering the S phase.

This argument is supported by our flow cytometry data which demonstrated that quercetin treated HK1 and CNE2 cells were all trapped in either the G2/M or G/G1 phase (Fig 2.2)

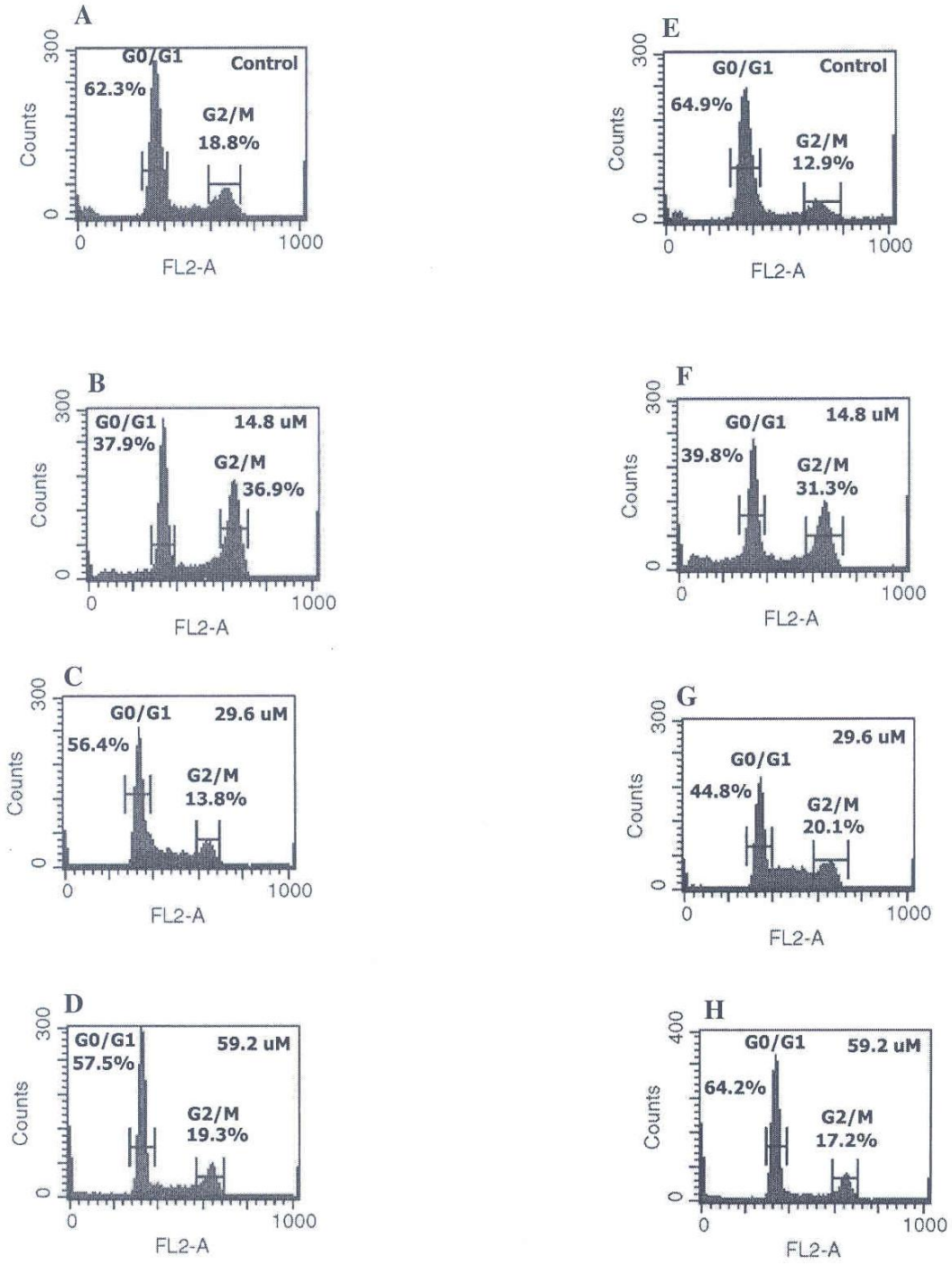


Fig 2.2: Cell analysis of quercetin treated and untreated CNE2 (A-D) and HK1 (E-H) cells

Actively dividing cells were incubated with quercetin in serum-free RPMI-1640 medium at 0, 14.8, 29.6 and 59.2 μM for 24 h, followed by flow cytometry analysing PI. The data were analysed as described in Materials and Methods.

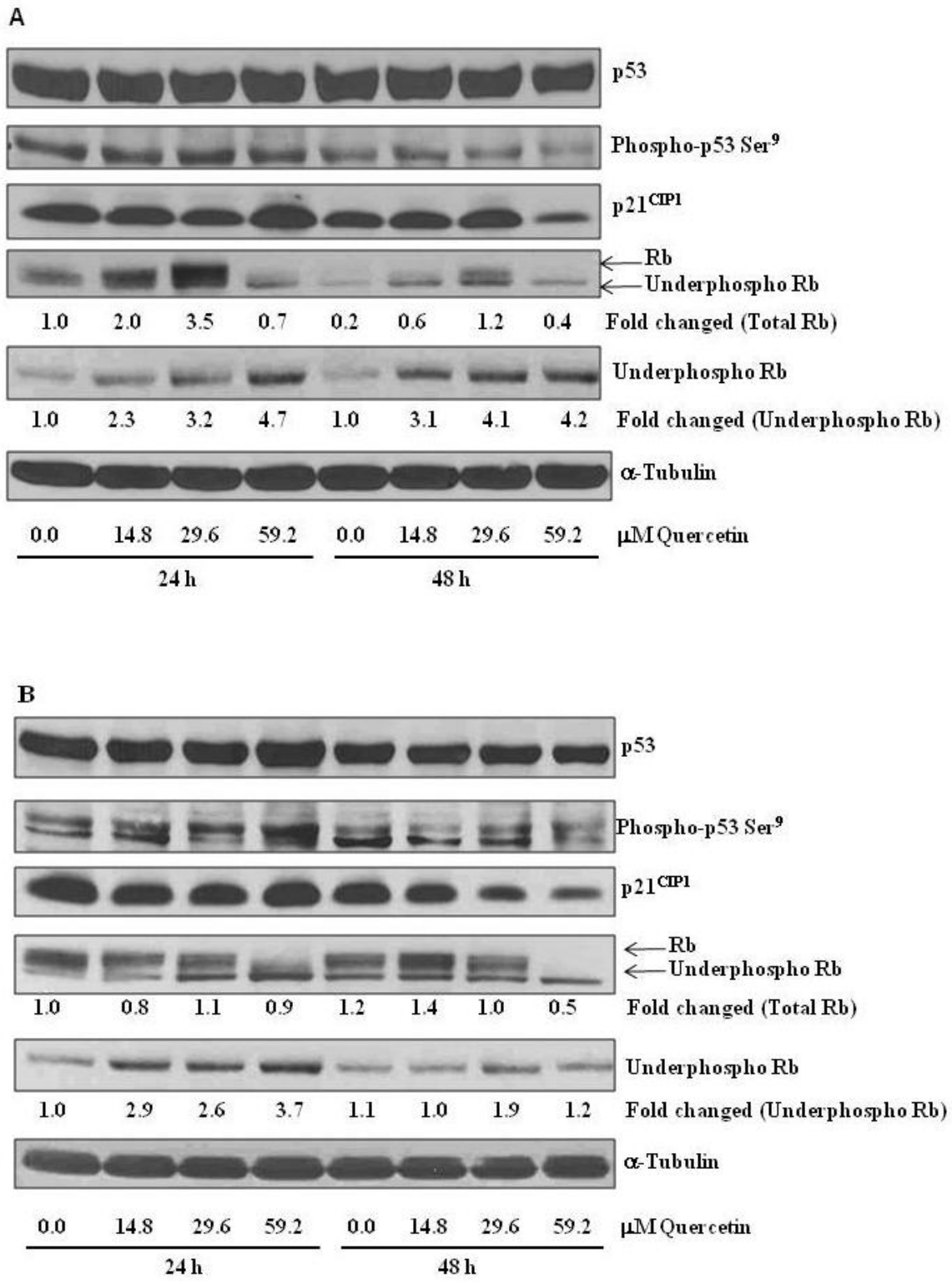


Fig 2.3: Quercetin up-regulates pRb and underphospho form of Rb in NPC cells

(A) CNE2 cells were treated with different concentrations of quercetin for 24 and 48 h. Total cellular protein extracts were prepared and subjected to immunoblotting. (B) HK1 cells were treated as in (A) and the total cellular protein extracts were prepared and subjected to immunoblotting. Band intensity was quantified using the Bio-Rad Quantity-One programme and normalised against α-tubulin. The fold change/difference was computed against the intensity of the band obtained from untreated cells at 24 h.

2.3.3 Induction of cell death via apoptosis and necrosis in quercetin treated cells

We next examined the cytotoxic activity of quercetin on CNE2 and HK1 cells. Data from Figs 2.4 and 2.5 demonstrated that quercetin was able to induce apoptosis in both cell lines in a dose- and time-dependent manner. Taking into consideration the basal level of apoptosis seen in untreated HK1 cells, at 14.8 μM, quercetin was able to induce 6.42% of the cell population to undergo apoptosis at 24 h incubation and almost quadrupled to 24.34% with prolonged incubation up to 48 h demonstrating the apoptotic effect of quercetin on HK1 cells in a time dependent manner. When quercetin concentration was increased from 14.8 to 29.6 μM, the percentage of apoptotic cells at 24 h incubation were 6.42% and 16.36% respectively, showing a 2.5X increase in a dose-dependent manner. Interestingly, CNE2 cells exhibited a different cytotoxic response to quercetin compared to HK1. At 14.8 μM, quercetin was able to induce 11.14% of the cells to undergo apoptosis at 24 h. However with prolonged exposure to quercetin at 48 h incubation, a different cytotoxic effect was observed; at the same concentration of quercetin, 25.30% of CNE2 cells became necrotic and the percentage of apoptosis was decreased to almost basal level (as what was observed in control cells). The necrotic effect of quercetin on CNE2 cells was also dose- as well as time- dependent as the percentage of necrotic cells increased with increased in quercetin concentration as observed in Fig 2.4I. However, the increase in apoptotic cells with increase in quercetin concentration and time exposure was not observed. Thus this study demonstrates that different nasopharyngeal carcinoma cell lines respond to quercetin in different manner. At a lower concentration (14.8 μM) of quercetin and 24 h incubation, both HK1 and

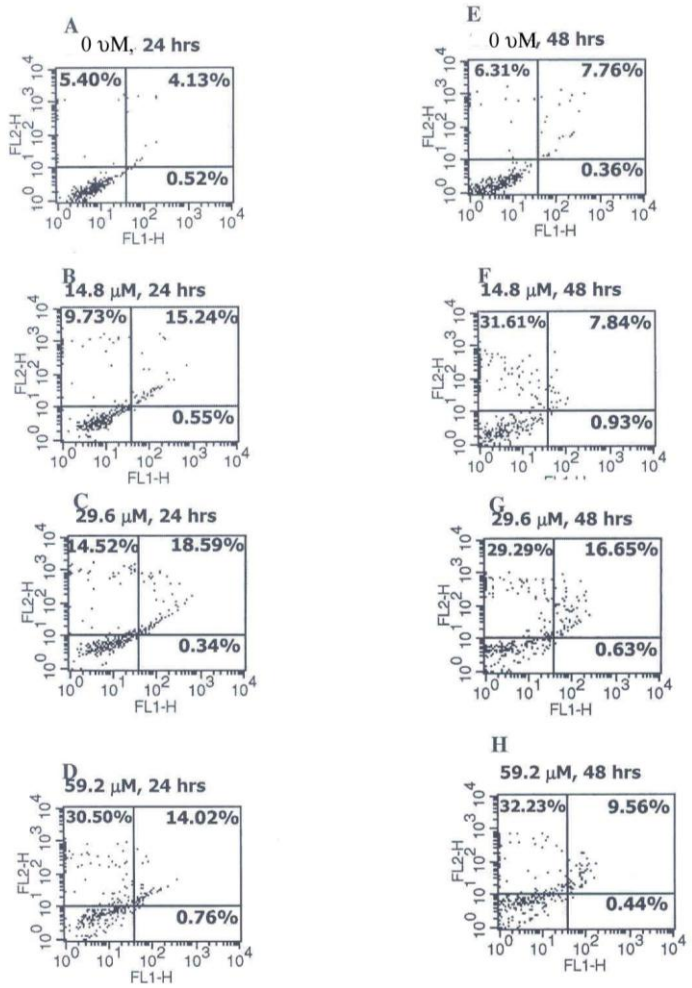
CNE2 cells were susceptible to quercetin and cell death by apoptosis was observed in both cell lines. HK1 cells continued to exhibit susceptibility to quercetin and cell death by apoptosis was observed in a dose- and time-dependent manner. However, when the concentration of quercetin was increased from 29.6 μM to 59.2 μM, the percentage of apoptotic cells remained consistent at 24 h exposure (Fig 2.5I) but, the percentage of necrotic cells increased from 0.26% to 14.46%. In contrast, the cytotoxic response of CNE2 cells to quercetin was more necrotic and typically it followed a dose- and time-dependent manner. At 24 h incubation, CNE2 cells was responsive to apoptosis brought about by quercetin but not in a dose-dependent manner as the percentage of apoptotic cells (10.13% to 14.28%) did not vary much between 14.8, 29.6 and 59.2 μM quercetin (Fig 2.4I). However, the percentage of necrosis became apparent in a dose- and time-dependent manner; at 24 h, the percentage of necrosis increased from 4.33% to 25.10% when quercetin concentration was increased from 14.8 μM to 59.2 μM. Moreover, at 14.8 μM, the percentage of necrotic cells was 4.33% and 25.30% at 24 and 48 h incubation respectively demonstrating a time-dependent response of CNE2 cells to the necrotic effect of quercetin.

Quercetin causes apoptosis in numerous cancer cells (Choi et al., 2001; Kang and Liang, 1997; Kobayashi et al., 2002; Salucci et al., 2002). Separate studies by Choi et al (Choi et al., 2001) and Moon et al (Moon et al., 2003) have indicated that quercetin induced $p21^{CIP1}$ expression but p53 level remained the same level, demonstrating that $p21^{CIP1}$ may play an essential role in the induction of apoptosis in response to quercetin in a p53-independent pathway. However, our experimental data together with those from Kobayashi et al (Kobayashi et al., 2002) and Kaneuchi et al (Kaneuchi et al., 2003) did not show a significant

increase in $p21^{CIP1}$ expression when both cell lines, CNE2 and HK1 were treated with quercetin (Figs 2.3 A and B). We therefore examined other mechanisms leading to apoptosis. Mitochondria play a crucial role in the control of cell death as they provide major intracellular apoptotic signals in the form of cytochrome c (Huynh et al., 1995). The release of cytochrome c and subsequent apoptosis can be regulated by the Bcl-2 family of proteins (Green and Reed, 1998; Moon et al., 2003). These proteins are divided into two groups; pro-apoptotic proteins such as Bad and Bax and anti-apoptotic proteins like Bcl-2 and Bcl-X_L. Bad and Bax proteins reside in the cytosol but translocate to the mitochondria following death signalling, where they promote the release of cytochrome c. In our experiments, quercetin treatment on HK1 cells was able to up-regulate the expressions of Bad and Bax (Fig 2.6B). However, when CNE2 cells were treated with quercetin, only Bad but not Bax expression was up-regulated (Fig 2.6B).

The release of cytochrome c into the cytosol of cell plays an important role in apoptosis through the activation of caspase proteins, amongst them caspase -3 and -7 (Moon et al., 2003). Our Western blot analyses (Figs 2.6 A and B) demonstrated that quercetin caused the cleavage of both caspase -3 and -7 to their respective active fragments in CNE2 as well as HK1 cells. Once activated, active caspase-3 cleaves many substrate proteins downstream including PARP, resulting in DNA fragmentation and finally apoptosis. Experimental results in Figs 2.6 A and B demonstrated that quercetin treated CNE2 and HK1 cells indeed exhibited an increased expression of the cleaved form of PARP. Our findings suggest that in HK1 cells, quercetin induced apoptosis via the mitochondrial-initiated pathway through the recruitment of Bad and Bax proteins which would subsequently led to the activation of caspase proteins such as caspase -3 and -7; and PARP. For

CNE2 cells, apoptosis was induced via increased Bad but not Bax expression. Furthermore at a higher concentration of quercetin, cell death was mediated by necrosis.

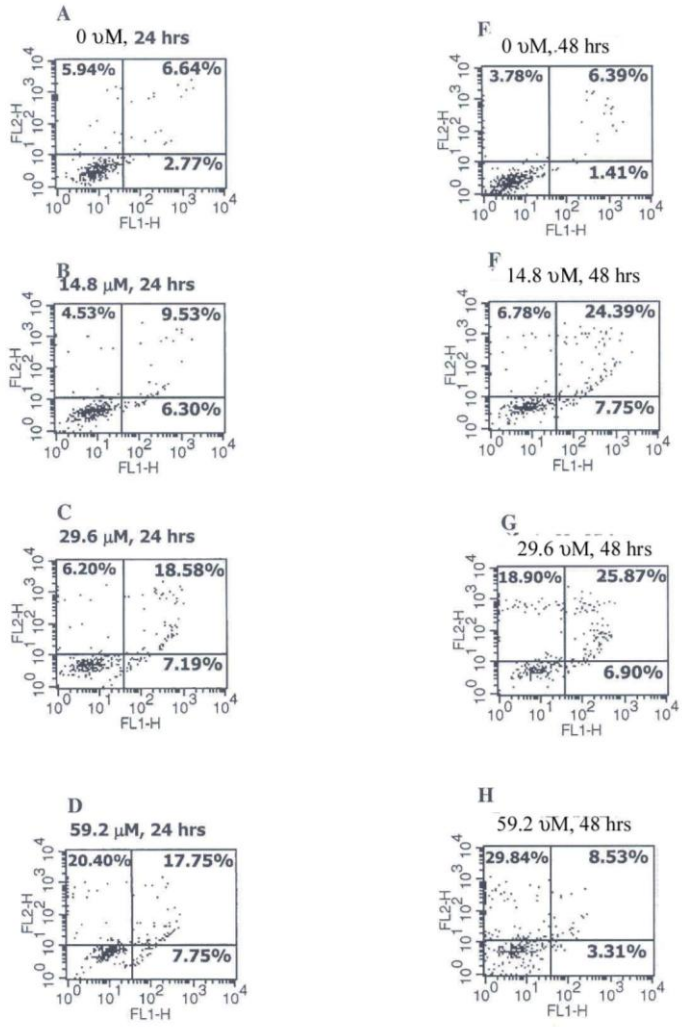


I

Concentration of quercetin (μM)	Percentage of CNE2 undergoing			
	Apoptosis (24 h)	Necrosis (24h)	Apoptosis (48h)	Necrosis (48h)
0	0.00	0.00	0.00	0.00
14.8	11.14	4.33	0.65	25.30
29.6	14.28	9.12	9.16	22.98
59.2	10.13	25.10	1.80	25.92

Fig 2.4: Annexin V-FITC/PI double staining flow cytometric analysis of CNE2 cells

Actively dividing cells were incubated with quercetin in serum-free RPMI-1640 medium at 0, 14.8, 29.6 and 59.2 μM for 24 and 48 h, followed by flow cytometry analysis using Annexin V-FITC/PI . The data above were representative of experiments conducted in triplicates. A - D, Cells were treated with 0, 14.8, 29.6 and 59.2 μM for 24 h. E – H, Cells were treated with 0, 14.8, 29.6 and 59.2 μM for 48 h. I: All values for the cells in apoptosis and necrosis were computed against the control (0 μM). The lower right and upper right quadrants of each represent cells undergoing apoptosis. The upper left quadrant represents cells undergoing necrosis. FL1-H: Annexin V-FITC, FL2-H: PI.



I

Concentration of quercetin (μM)	Percentage of HK1 undergoing			
	Apoptosis (24 h)	Necrosis (24h)	Apoptosis (48h)	Necrosis (48h)
0	0.00	0.00	0.00	0.00
14.8	6.42	0.00	24.34	3.00
29.6	16.36	0.26	24.77	12.12
59.2	16.09	14.46	4.04	23.06

Fig 2.5: Annexin V-FITC/PI double staining flow cytometric analysis of HK cells

Actively dividing cells were incubated with quercetin in serum-free RPMI-1640 medium at 0, 14.8, 29.6 and 59.2 μM for 24 and 48 h, followed by flow cytometry analysis using Annexin V-FITC/PI . The data above were representative of experiments conducted in triplicates. A - D, Cells were treated with 0, 14.8, 29.6 and 59.2 μM for 24 h. E – H, Cells were treated with 0, 14.8, 29.6 and 59.2 μM for 48 h. I: All values for the cells in apoptosis and necrosis were computed against the control (0 μM). The lower right and upper right quadrants of each represent cells undergoing apoptosis. The upper left quadrant represents cells undergoing necrosis. FL1-H: Annexin V-FITC, FL2-H: PI.

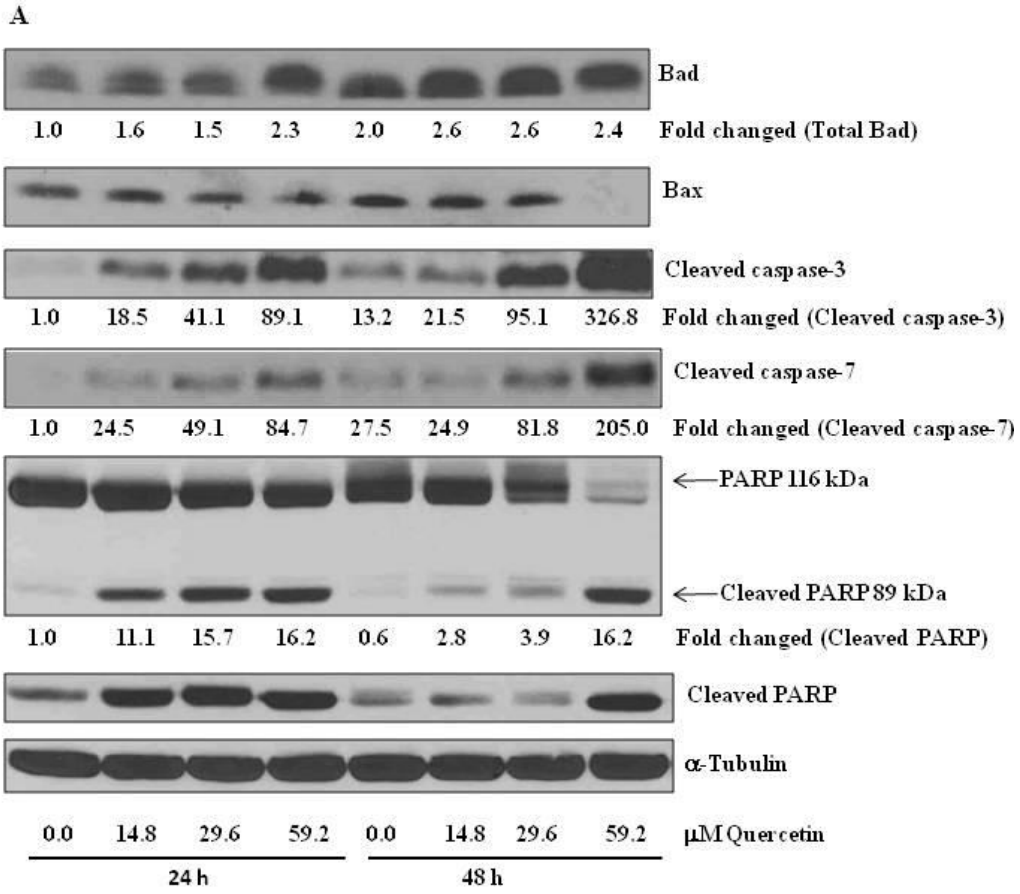


Fig 2.6A: Quercetin mediates apoptosis via the intrinsic mitochondrial signalling pathway in CNE2 cells

Cells were treated with different concentrations of quercetin for 24 and 48 h. Total cellular protein extracts were prepared and subjected to immunoblotting. Band intensity was quantified using the Bio-Rad Quantity-One programme and normalised against α-tubulin. The fold change/difference was computed against the intensity of the band obtained from untreated cells at 24 h.

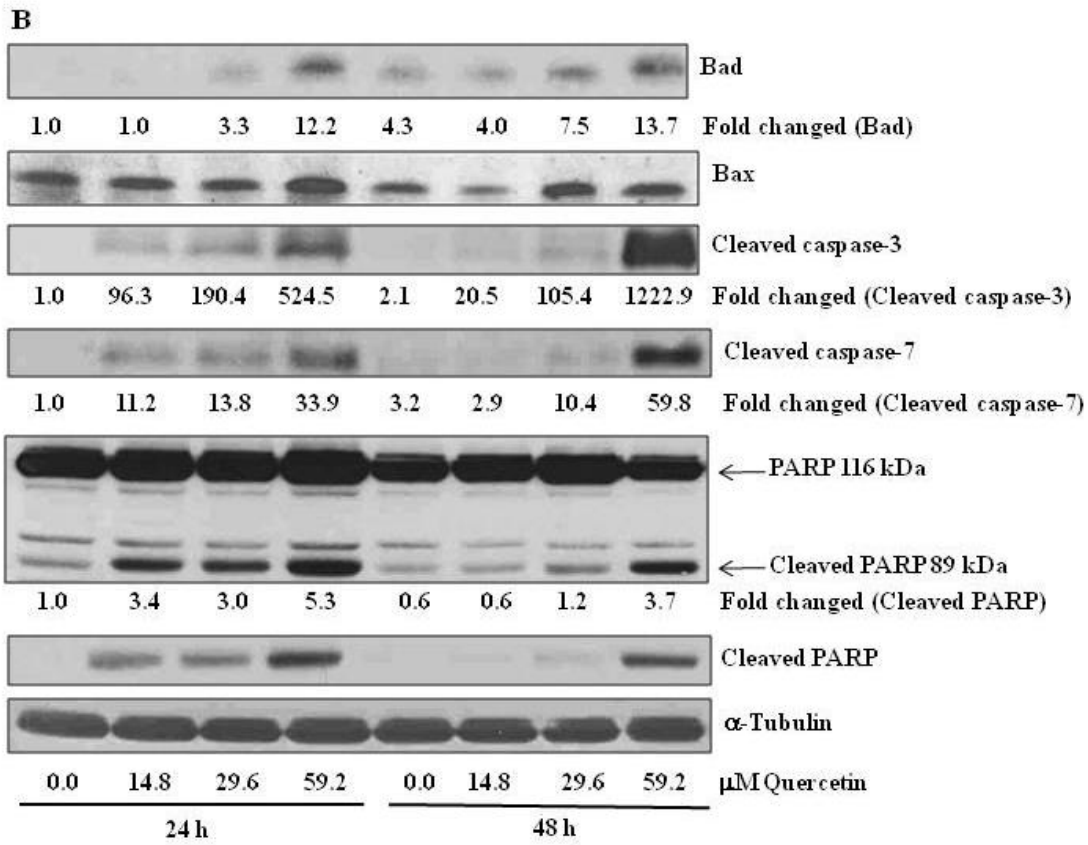


Fig 2.6B: Quercetin mediates apoptosis via the intrinsic mitochondrial signalling pathway in HK1 cells

Cells were treated with different concentrations of quercetin for 24 and 48 h. Total cellular protein extracts were prepared and subjected to immunoblotting. Band intensity was quantified using the Bio-Rad Quantity-One programme and normalised against α-tubulin. The fold change/difference was computed against the intensity of the band obtained from untreated cells at 24 h.

2.4 Conclusions

In summary, our data presented in this report indicate that the flavonoid quercetin was able to induce cell growth inhibition in two nasopharyngeal carcinoma cell lines, CNE2 and HK1 through two different mechanisms; one by preventing cell cycle progression into the S phase through cell cycle arrest at the G2/M and G0/G1 phases. The second mechanism is by inducing cell death and in the case of HK1 cells, mediated by apoptosis though a p53-independent

mitochondrial-initiated pathway. Cell death is also observed in CNE2 cells and at low concentration of quercetin, cell death is also mediated by apoptosis. At a higher concentration and with prolonged incubation, quercetin induces necrotic cell death in CNE2 cells. It remains to be investigated how quercetin at higher concentration causes necrosis in CNE2 but not in HK1 cells.

Thus, our results together with findings from others, suggest that quercetin can be a potential agent for chemopreventive or therapy against nasopharyngeal carcinoma.

Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3β-cyclin D1 pathway

Chapter 3

3.1 Introduction

Flavonoids are polyphenolic compounds ubiquitously present in plants including fruits and vegetables. There is growing evidence of the health benefits of flavonoids due to their biological activities such as anti-oxidant, anti-inflammatory and anti-cancer (Middleton et al., 2000; Ross and Kasum, 2002). Among these activities, the anti-cancer effect of flavonoids has been extensively studied (Li et al., 2007; Lopez-Lazaro, 2002). Many types of dietary flavonoids are able to inhibit cancer cell proliferation, induce cancer cell death by apoptosis and cell cycle arrest by targeting key intracellular molecules and pathways (Kale et al., 2008; Ramos, 2007). For instance, the anti-proliferative activity of flavonoids on tumour cell growth has been linked to their effects on numerous intracellular biochemical pathways including the cyclins-cyclin-dependent kinases (CDKs) network (Singh and Agarwal, 2006).

Cyclins are essential components of the cell cycle machinery; each binds and activates specific types of cyclin-dependent kinases (CDKs). Progression through the G1 phase of the cell cycle requires both cyclin D and cyclin E to activate CDK4/6 and CDK2 respectively (Obaya and Sedivy, 2002). The cyclin D1-CDK4/6 complexes formed during G1 phase phosphorylate retinoblastoma (Rb) protein and activate the transcriptional factor E2F-1 which initiates the transcription of key cell cycle regulators such as cyclins E and A and in the process, driving cells into the S phase (Genovese et al., 2006; Giacinti and Giordano, 2006). Therefore, it has been well established that cyclin D plays a crucial role in the progression of cell cycle from G1 to S phase and the down-regulation of cyclin D will lead to cell cycle arrest at G1 (Blain, 2008; Malumbres and Barbacid, 2009).

The phosphoinositide 3-kinase (PI3K)/Akt pathway is known to play a major role in cell cycle progression during the G1/S transition (Liang and Slingerland, 2003). Amongst various substrates of Akt, several of them are involved in cell cycle regulation, including GSK-3β, the forkhead transcription factors, CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (Blume-Jensen and Hunter, 2001). Akt is capable of phosphorylating GSK-3β at Ser^{9} and subsequently inhibiting its kinase activity. Active GSK-3β phosphorylates cyclin D1 at Thr^{286} that triggers its subsequent ubiquitination and degradation by proteasomes (Diehl et al., 1998; Diehl et al., 1997). Therefore, the Akt-GSK-3β-cyclin D1 signalling pathway appears to be crucial in regulating the cell cycle at G1/S transition.

Luteolin (3', 4', 5', 7'-tetrahydroxyflavone), a member of the flavonoid family which usually exists in the glycosylated forms, is commonly found in celery, green peppers, perilla leaf, camomile and chrysanthemum tea (Lopez-Lazaro, 2009). It exhibits a wide spectrum of pharmacologic properties ranging from anti-cancer, anti-oxidant, anti-inflammatory and anti-allergic properties (Lin et al., 2008; Seelinger et al., 2008a; Seelinger et al., 2008b). At present, the anti-cancer property of luteolin has been evaluated mainly on its ability to induce apoptosis (Lin et al., 2008). For instance, luteolin is capable of directly inducing apoptotic cell death in numerous human cancer cells (Cheng et al., 2005; Fang et al., 2007b; Horinaka et al., 2005; Lee et al., 2005; Selvendiran et al., 2006; Xavier et al., 2009) and sensitising cancer cells to chemotherapeutics or biotherapeutic agents (Horinaka et al., 2005; Shi et al., 2007; Shi et al., 2004, 2005; Wu et al., 2008). However, relatively little is known about the anti-proliferative activity of luteolin. Thus, in this study, we focussed on the effect of luteolin on cell cycle regulation. Data from this study demonstrate that luteolin induces G1 arrest in

human nasopharyngeal carcinoma cells by down-regulating cyclin D1, which subsequently leads to suppression of the E2F-1 transcriptional activity. We further identified the molecular mechanism in which luteolin down-regulates cyclin D1 through the inhibition of the Akt-GSK-3β signalling pathway. Data from this study thus expand the spectrum of the anti-cancer potential of luteolin and support its potential application in cancer prevention and therapy

3.2 Materials and methods

3.2.1 Chemicals and reagents

Luteolin, insulin, lithium chloride (LiCl), DMSO, camptothecin, MG132 as well as other chemicals were purchased from Sigma (St Louis, MO, USA). Cycloheximide (CHX), anti-cyclin D1 and anti-α-tubulin were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-cyclin A, anti-cyclin E, anti-Rb, anti-pRb Ser^{780}, anti-Akt, anti-pAkt Ser^{473}, anti-pcyclin D1 Thr^{286}, anti-GSK-3β, anti-pGSK-3β Ser^{9}, anti-ubiquitin, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). Protease inhibitors cocktail was purchased from Roche (Mannheim, Germany). RPMI-1640 and trypsin-EDTA (0.25% porcine trypsin and 0.02% EDTA-2Na) were purchased from Gibco Ltd (Grand Island NY, USA). FBS was purchased from JRH Biosciences (Lenexa, KS, USA). Reagents used for western blot analysis were purchased from Bio-Rad (Hercules, CA, USA). The cell cycle-flow cytometry kit and apoptosis kit were from BD Biosciences (Palo Alto, CA, USA). Enhanced chemiluminescent substrate was purchased from Pierce (Rockford, USA). The ubiquitinated protein enrichment

kit was purchased from Calbiochem (San Diego, USA). RT-PCR experiment was conducted using the Qiagen Onestep RT-PCR kit (Qiagen, Valencia, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

3.2.2 Cell culture and treatment

Nasopharyngeal carcinoma cell lines HK1 and CNE2 were kindly provided by Dr. KM Hui, Singapore, National Cancer Centre. Cells were grown and maintained at 37^{o}C in a humidified 5% CO_2 and 95% air atmosphere in RPMI-1640 supplemented with 10% FBS. Equal number of cells were seeded in RPMI-1640 supplemented with 10% FBS. In order to synchronise cell cycle, the medium was switched to FBS-free overnight before treatment with luteolin in RPMI-1640 supplemented with 10% FBS. As luteolin was dissolved in DMSO, the same concentration of DMSO was always applied to the control group. For the pre-treatment experiments, cells were pre-treated with 1.0 μM MG132, 200 nM insulin or 30 mM LiCl for one h before luteolin treatment. For pre-treatment with CHX, cells were pre-treated with 0.5 μg/ml of CHX for 30 mins.

3.2.3 Cell cycle analysis

Cell cycle analysis by flow cytometry was performed using the bromodeoxyuridine – 7-amino-actinomycin D (BrdU-7-AAD) kit. Briefly, treated and control cells were first labelled with BrdU (final concentration of 1 mM) in culture for 15 mins and subsequently harvested. These cells were subjected to a second round of labelling with FITC-conjugated antibody to BrdU and 7-AAD as described by the instruction manual. This was followed by flow cytometry

analysis. With this combination of BrdU and 7-AAD, a two-colour flow cytometric analysis permits the enumeration and characterisation of cells in terms of their cell cycle position including G0/G1, S and G2/M phases.

3.2.4 Apoptosis analysis

Flow cytometry to detect and quantify the presence of apoptotic cells was first conducted using an active caspase-3-FITC antibody apoptosis kit (BD Biosciences). Cells were harvested and permeabilised using saponin buffer followed by incubation with anti-active caspase-3-FITC conjugated monoclonal antibodies. Apoptotic cells were detected and quantified by flow cytometry.

3.2.5 Immunoblot analysis

Cells were first lysed in cell lysis (20 mM Tris, pH 7.5, 150 mM NaCl, 1mM PMSF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA, 1mM EGTA, 1% Triton-X100, 1mM sodium vanadate, 1mM PMSF (Phenylmethanesulfonylfluoride) and a protease inhibitor cocktail). Equal amount of proteins were fractionated on SDS-PAGE gel using the Mini-PROTEAN II system (Bio-Rad) before being transferred to nitrocellulose membrane (Bio-Rad). The membrane was first blocked with 5% fat-free milk in TBST (10 mm Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween-20), followed by probing with the various primary antibodies and developed using the enhanced chemiluminescent reagents (Pierce). Detection of specific bands could be viewed using the Kodak Image Station 4000MM Pro.

3.2.6 Immunoprecipitation of ubiquitinated enriched proteins

Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton® X-100 , 10 mM N-ethylmaleimide and a protease inhibitor cocktail). Cell extracts were added to polyubiquitin affinity beads (Calbiochem) and incubated at 4^{o}C for 2 – 4 h with constant mixing to allow the binding of ubiquitinated proteins to beads. Ubiquitinated bound proteins were subsequently detached from the beads by boiling in the presence of SDS gel loading buffer, separated by SDS PAGE and blotted onto nitrocellulose membrane for detection of both cyclin D1 and ubiquitin.

3.2.7 RT-PCR

mRNA was extracted using the Qiagen Onestep RT-PCR kit and RT-PCR performed using the following primers (Qi et al., 2007) . Forward and reverse primer sequences for cyclin D1 were 5′TAG CAG CAA ACA ATG TGA AAG AG3′ and 5′CTT ACA TCA TAG CAA CAC GGA CTT3′ respectively. Forward and reverse primer sequences for cyclin E were 5′AGA AGC CAA CCA CAG TCT ATA CCA3′ and 5′TTA CGA CAC CGA GGA AGG ATT GA3′ respectively. Forward and reverse primer sequences for β-actin were 5′CCA AGG CCA ACC GCG AGA AGA TGA C3′ and 5′CAG ACC GCC GTG GTG GTA CAT GGG A3′ respectively.

3.2.8 Luciferase reporter gene assay

The transient transfection of E2F-1 luciferase vector (Promega, Madison, WI, USA) was performed in HK1 cells using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies) according to the manufacturer's protocols.

Renilla luciferase vector, which acts as a transfection control, was also cotransfected. Luciferase activity was measured in the cellular extracts using a Dual-Luciferase Reporter Assay System (Promega) based on the protocol provided by the manufacturer. Briefly, following the treatments, cell lysate was collected from each well of the 24-well plate after the addition of cell lysis reagent. After the addition of the luciferase assay substrate, firefly luciferase activity was determined using a luminometer (Promega).

3.3 Results

3.3.1 Luteolin induces cell cycle arrest at G1 phase in a dose- and time-dependent manner

The effects of luteolin on the cell cycle progression in two NPC lines, HK1 and CNE2 were determined by flow cytometry with anti-BrdU-FITC and 7-AAD staining. In HK1 cells, treatment with various concentrations of luteolin for 24 h resulted in a dose-dependent increase in the percentage of cells in G0/G1 phase and a concomitant reduction of cell numbers in S phase (Fig 3.1A, upper panel). Higher concentrations of luteolin (50 and 100 μM) almost completely abolished the S phase in these cells (Fig 3.1A, lower panel). CNE2 also exhibited a similar pattern when treated with luteolin: it underwent G1 arrest in a dose-dependent manner (Fig 3.1B). Consistent with the occurrence of G1 cell cycle arrest, there was a dose-dependent reduction in total number of HK1cells counted per well with 24 h of luteolin treatment (Fig 3.1C, lower panel). This was further verified by microscopic examination that showed a decrease of viable cell number in treated samples (Fig 3.1C, upper panel).

Using HK1 cells for subsequent studies, we next investigated the temporal pattern of G1 arrest induced by 50 μM luteolin. An increase of G0/G1 cells was observed from 3 h onwards, with concomitant reduction of cells in S phase (Fig 3.1D) in a time-dependent manner. Taken together, these data indicate that luteolin arrests cell cycle progression at G1 in both HK1 and CNE2 in a dose- and time- dependent manner.

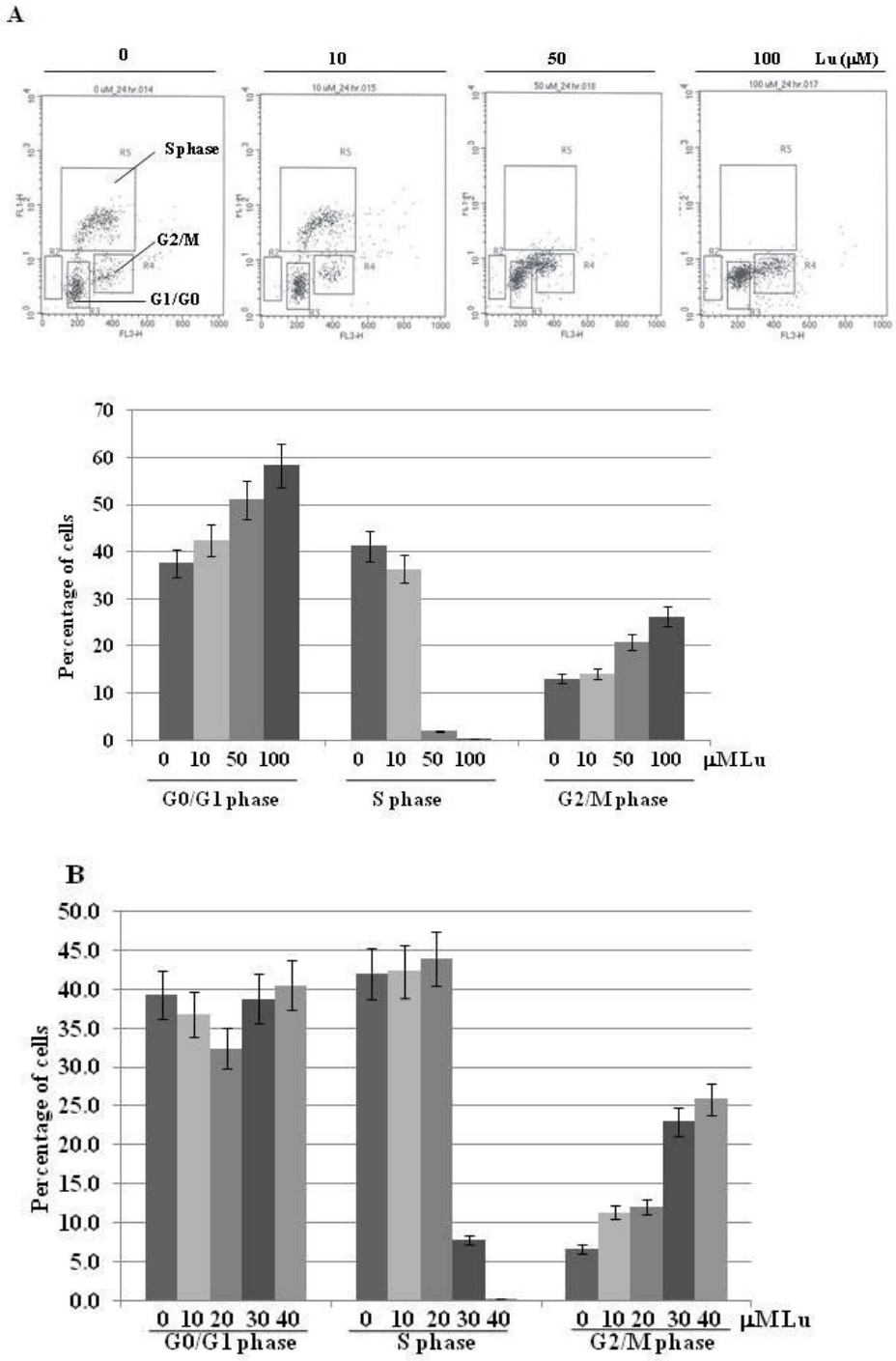


Fig 3.1 A & B: Luteolin induces cell cycle arrest at G1 in a dose- and time-dependent manner in HK1 and CNE2 cells

(A) HK1 cells were first synchronised by maintaining the cells in FBS-free medium overnight before treatment with luteolin in RPMI-1640 supplemented with 10% FBS for 24 h and labelled with BrdU for 15 mins. Cells were subsequently harvested and stained with anti-BrdU-FITC and 7-AAD and subjected to flow cytometry assay as described under Section 3.2. The percentage of cells in G0/G1, S and G2/M phases based on BrdU incorporation (anti-BrdU-FITC) (FL1) and 7-AAD staining on DNA (FL3) was quantified by manual gating of the dot plots. Values are presented as means ± SD from three independent experiments. (B) A similar experiment as that in (A) was performed on CNE2 cells. Cells were treated with designated concentrations of luteolin for 24 h.

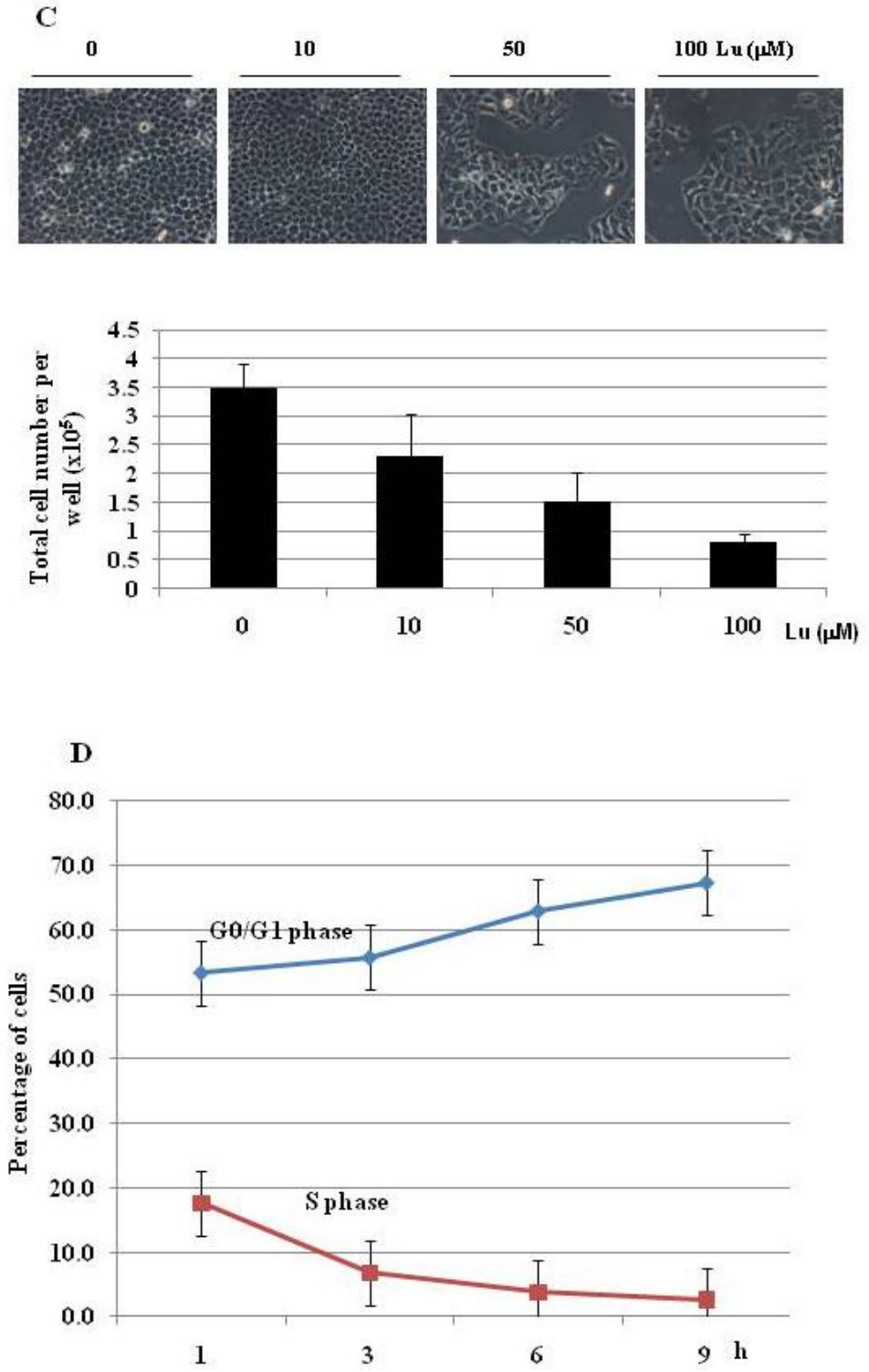


Fig 3.1C & D: Luteolin induces cell cycle arrest at G1 in a dose- and time-dependent manner in HK1 and CNE2 cells

HK1 cells were treated as in (A), harvested, stained with trypan blue and counted using a haemocytometer. Values are presented as means ± SD from three independent experiments (lower panel). Micrographs of luteolin treated and untreated cells (100X) (upper panel). (D) HK1 was treated with 50 μM luteolin for the indicated period using experiment procedure to (A) and cell analysis by flow cytometry conducted for the percentage of cells in G1and S phases. All numeric data are presented as means ± SD from three independent experiments.

3.3.2 Luteolin does not induce apoptosis in HK1 and CNE2 cells

Luteolin has been found to induce apoptosis in a number of studies (Lin et al., 2008). In our study, we also determined whether luteolin was capable of inducing apoptosis in both HK1 and CNE2 cells. Apoptotic cell death was quantified using the active caspase-3 apoptotic kit coupled with flow cytometry. Interestingly, there was no apoptotic cell death in HK1 treated with luteolin when treated for 24 h (Fig 3.2 A – D), while treatment with 20 μM camptothecin induced significant increase of caspase-3 activation (Fig 3.2E). No caspase-3 activation was found even when treatment for up to 48 h (Fig 3.2 F – I). Similar negative results were also obtained with other apoptotic markers such as PARP cleavage as determined by Western blot. Luteolin was also unable to cause apoptosis in CNE2 when treated for up to 48 h, similar to the effect in HK1 cells (Fig 3.3). Since earlier work in our laboratory has demonstrated luteolin-induced apoptosis in other human cancers (Shi et al., 2007; Shi et al., 2004, 2005), it is possible that the effect of luteolin on cell cycle is cell specific, pertaining to NPC cells.

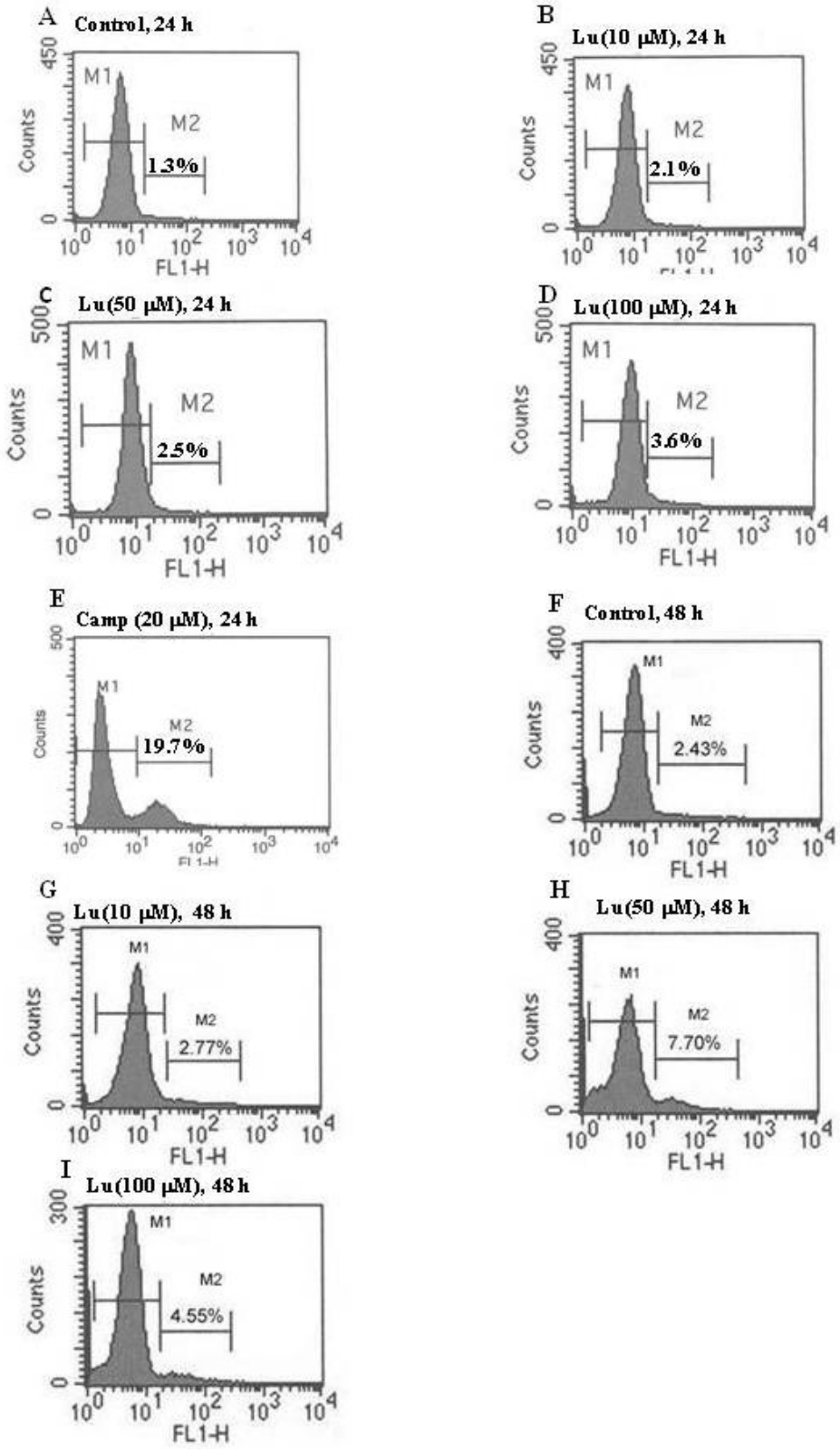


Fig 3.2: Luteolin fails to induce apoptosis in HK1 cells

Cells were treated with luteolin for 24 h with designated concentrations. Cells treated with 20 μM camptothecin for 24 were used as a positive control. Cells were harvested and stained with antibody to active caspase-3-FITC, followed by analysis with flow cytometry. Gates were configured manually to measure the percentage of apoptotic cells (M2) versus non-apoptotic cells (M1). Presented histograms were representatives from three independent experiments.

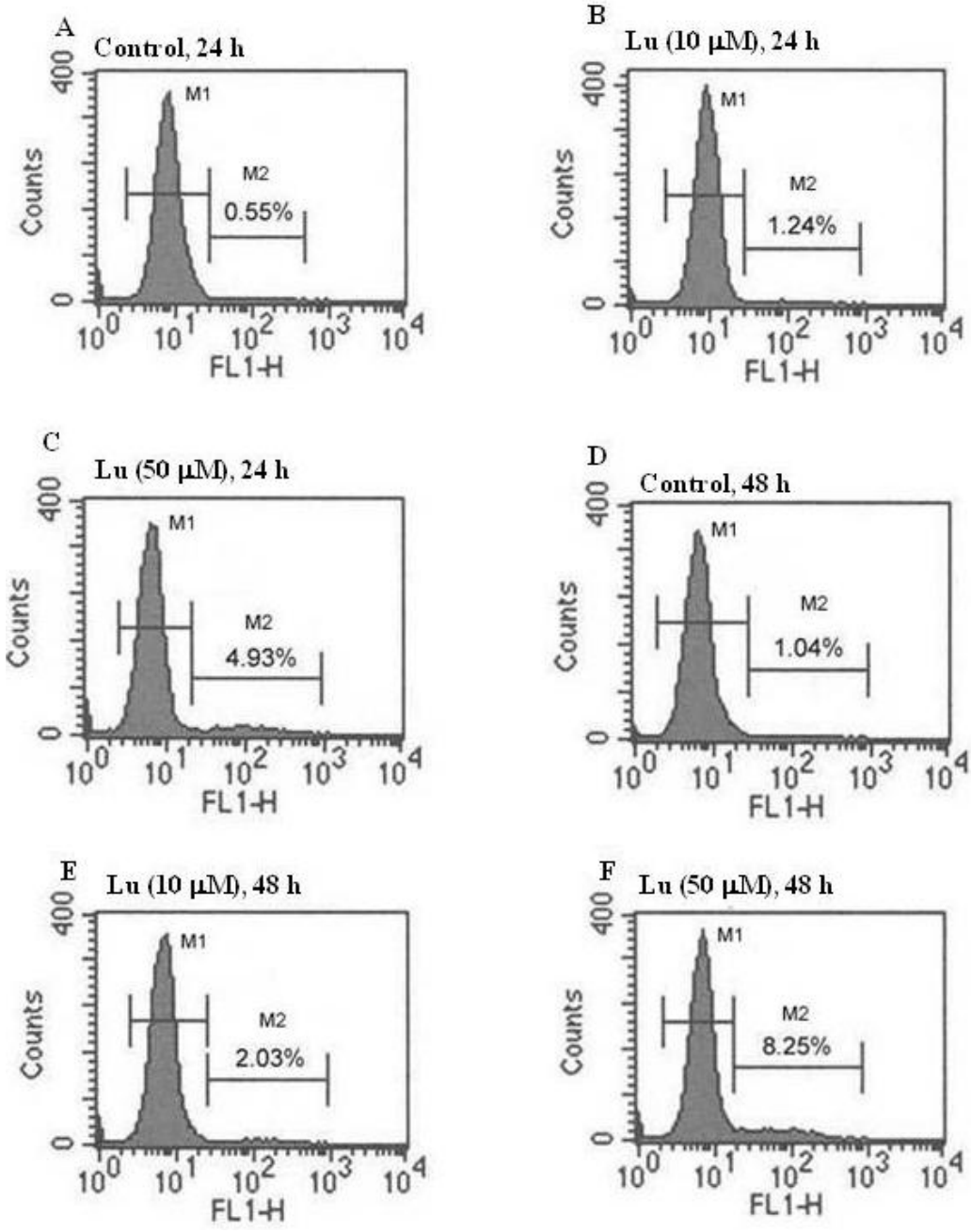


Fig 3.3: Luteolin fails to induce apoptosis in CNE2 cells

Cells were treated with luteolin for 24 h with designated concentrations. Cells were harvested and stained with antibody to active caspase-3-FITC, followed by analysis with flow cytometry. Gates were configured manually to measure the percentage of apoptotic cells (M2) versus non-apoptotic cells (M1). Presented histograms were representatives from three independent experiments.

3.3.3 Luteolin induces cell cycle arrest at G1 phase by down-regulation of cyclin D1 and subsequent suppression of E2F-1 transcription activity

In order to understand the molecular mechanisms underlying the G1 cell cycle arrest in HK1 cells induced by luteolin, we examined the level of cyclin D1, the main cyclin controlling the G1/S checkpoint (Obaya and Sedivy, 2002). It was found that luteolin treatment led to rapid reduction of cyclin D1 protein level (Fig 3.4A). It is well established that cyclin D1 is required for CDK4/6 to phosphorylate Rb and subsequent release of the transcriptional factor E2F-1 to initiate cell cycle progression to S phase (Genovese et al., 2006; Giacinti and Giordano, 2006). Here, we also observed a significant reduction of Rb phosphorylation level (Fig 3.4A), indicating the compromised activation of the CDK4/6. Consistently, the protein levels of cyclin A and E, the two main transcriptional targets of E2F-1 were also decreased (Fig 3.4A). We further measured the mRNA level of cyclin E using RT-PCR and it was evident that luteolin treatment resulted in suppression of cyclin E gene transcription (Fig 3.4B). To further confirm that luteolin inhibits the transcriptional activity of E2F-1, we utilised the E2F-1 luciferase reporter vector and the results in Fig 3.4C clearly demonstrate that luteolin is capable of suppressing the E2F-1 transcription activity.

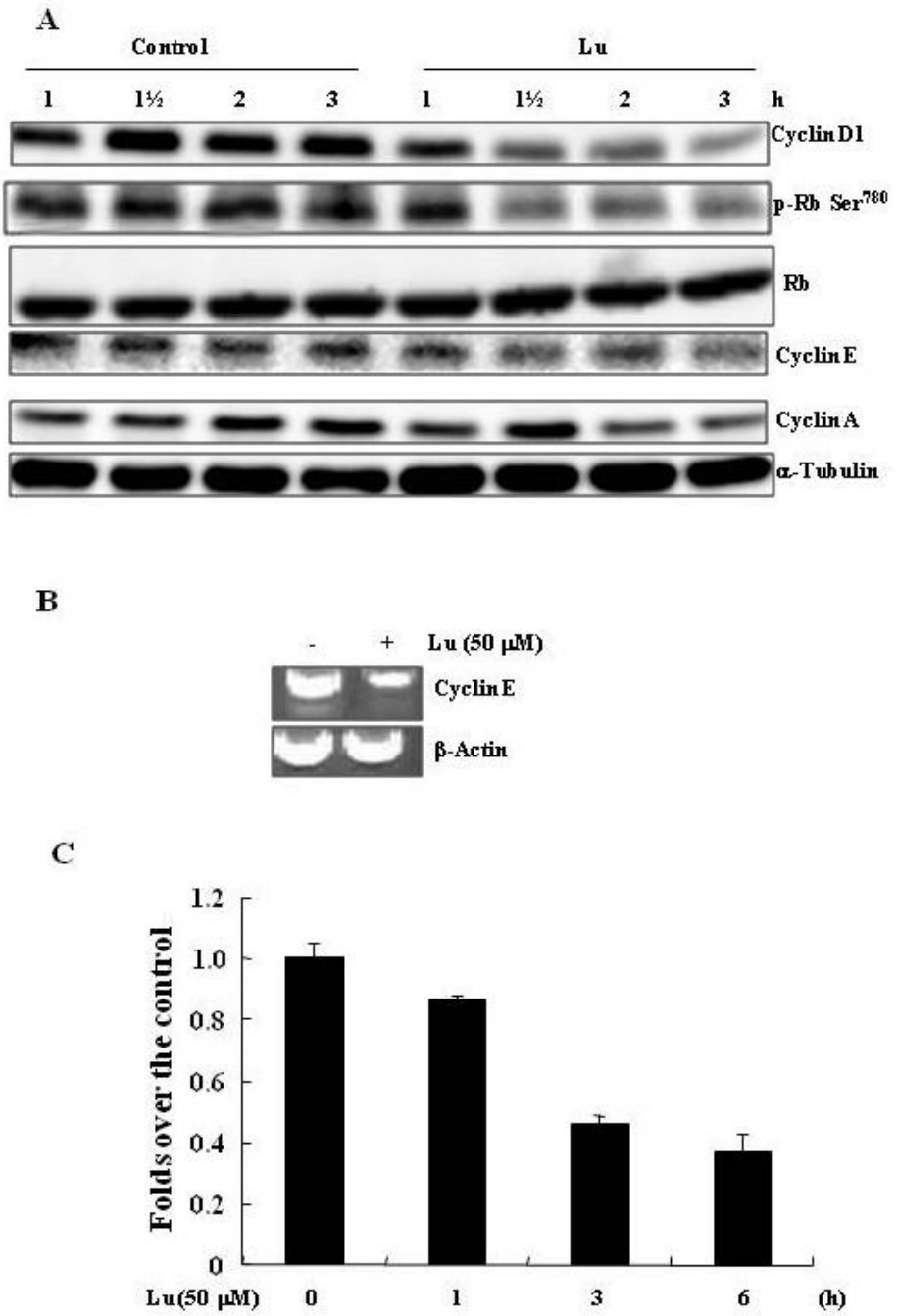


Fig 3.4: Luteolin down-regulates cyclin D1 and suppresses Rb phosphorylation and E2F-1 transcription activity in HK1 cells

(A) Cells were treated with 50 μM luteolin for the indicated periods. Total cellular protein extracts were prepared and subjected to immunoblotting. (B) Detection of cyclin E mRNA level using RT-PCR, as described in Section 3.2. β-actin was used as an internal control. (C) Changes of E2F-1 transcription activity. Cells were first transfected with the E2F-1 luciferase construct together with the *Renilla* vector using Lipofectamine 2000. Transfected cells were subsequently treated with luteolin and their luciferase activity measured using a Dual-Luciferase Reporter Assay System (Promega). Values are presented as means ± SD from three experiments.

3.3.4 Luteolin promotes phosphorylation and subsequent proteasomal degradation of cyclin D1

After establishing the critical role of cyclin D1 and its downstream events in luteolin-induced cell cycle arrest, here we sought to investigate the underlying molecular mechanism leading to cyclin D1 down-regulation in luteolin-treated HK1 cells. To address whether the reduction of cyclin D1 protein level was due to the suppression of gene expression, we first checked the mRNA level of cyclin D1 using RT-PCR. As shown in Fig 3.5A, the cyclin D1 mRNA level remained unchanged when cells were treated with luteolin for 3 h, suggesting that luteolin could down-regulate cyclin D1 via translational and post-translational regulations.

We next examined whether luteolin affects the protein stability of cyclin D1 using CHX to inhibit *de novo* protein synthesis. As shown in Fig 3.5B, in the presence of CHX, luteolin led to further reduction of cyclin D1 protein level, indicating that luteolin is likely to down-regulate cyclin D1 protein level via promotion of protein degradation.

We next tested the two important post-translational modifications of cyclin D1: phosphorylation and ubiquitination. As shown in Fig 3.5C, in the presence of MG132, a specific proteasome inhibitor, treatment with luteolin significantly enhanced the phosphorylation of cyclin D1. The low level of phosphorylated cyclin D1 in the control group (without MG132) is most probably due to the rapid degradation of the phosphorylated protein. Consistently, MG132 prevented the reduction of cyclin D1 protein level in cells treated with luteolin (Fig 3.5C). Moreover, in order to test the effect of luteolin on ubiquitination of cyclin D1, we performed immunoprecipitation (IP) using polyubiquitin affinity beads to pull down the ubiquitinated protein. As shown in Fig 3.5D (upper panel), treatment with luteolin enhanced the level of ubiquitinated proteins, especially in the

presence of MG132. Consistently, luteolin markedly promoted ubiquitination of cyclin D1 in the presence of MG132 (middle panel). The total cyclin D1 protein level was also detected in the whole lysate (lower panel), with similar findings as shown earlier in Fig 3.5C. One intriguing finding from both Figs 3.5 B and D is that the basal level of cyclin D1 was not increased in the presence of MG132 whereas the phospho-cyclin D1 level enhanced markedly. One possible explanation is that the basal phosphorylation of cyclin D1 does not trigger significant proteasomal degradation. Taken together, these data indicate that luteolin-induced down-regulation of cyclin D1 is mediated by enhanced protein phosphorylation, ubiquitination and subsequent proteasomal degradation.

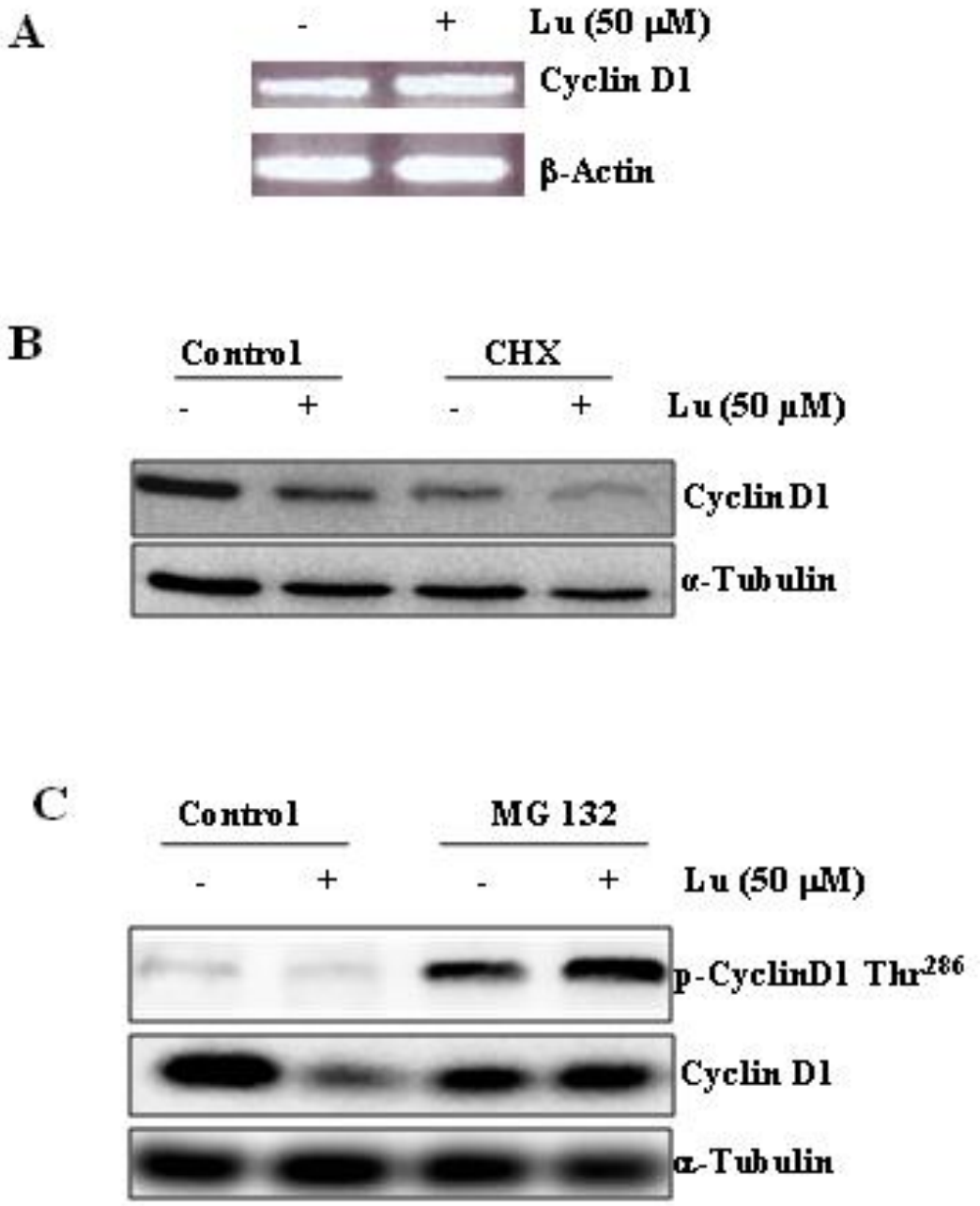


Fig 3.5 A – C: Luteolin enhances cyclin D1 ubiquitination and proteasomal degradation in HK1 cells

(A) Detection of cyclin D1 mRNA level using RT-PCR in HK1 cells after treatment with luteolin for 3 h. β-actin was used as an internal control. (B) Cyclin D1 protein stability assay. Cells were pre-treated with 0.5 μg/ml CHX for 30 mins, followed by luteolin treatment for 1 h. Total cellular protein extracts were prepared and subjected to immunoblotting. (C) Detection of phospho-cyclin D1. Cells were pre-treated with 1.0 μM MG132 for 1 h followed by luteolin treatment. Total cellular protein extracts were prepared and subjected to immunoblotting. α-Tubulin was used as a loading control.

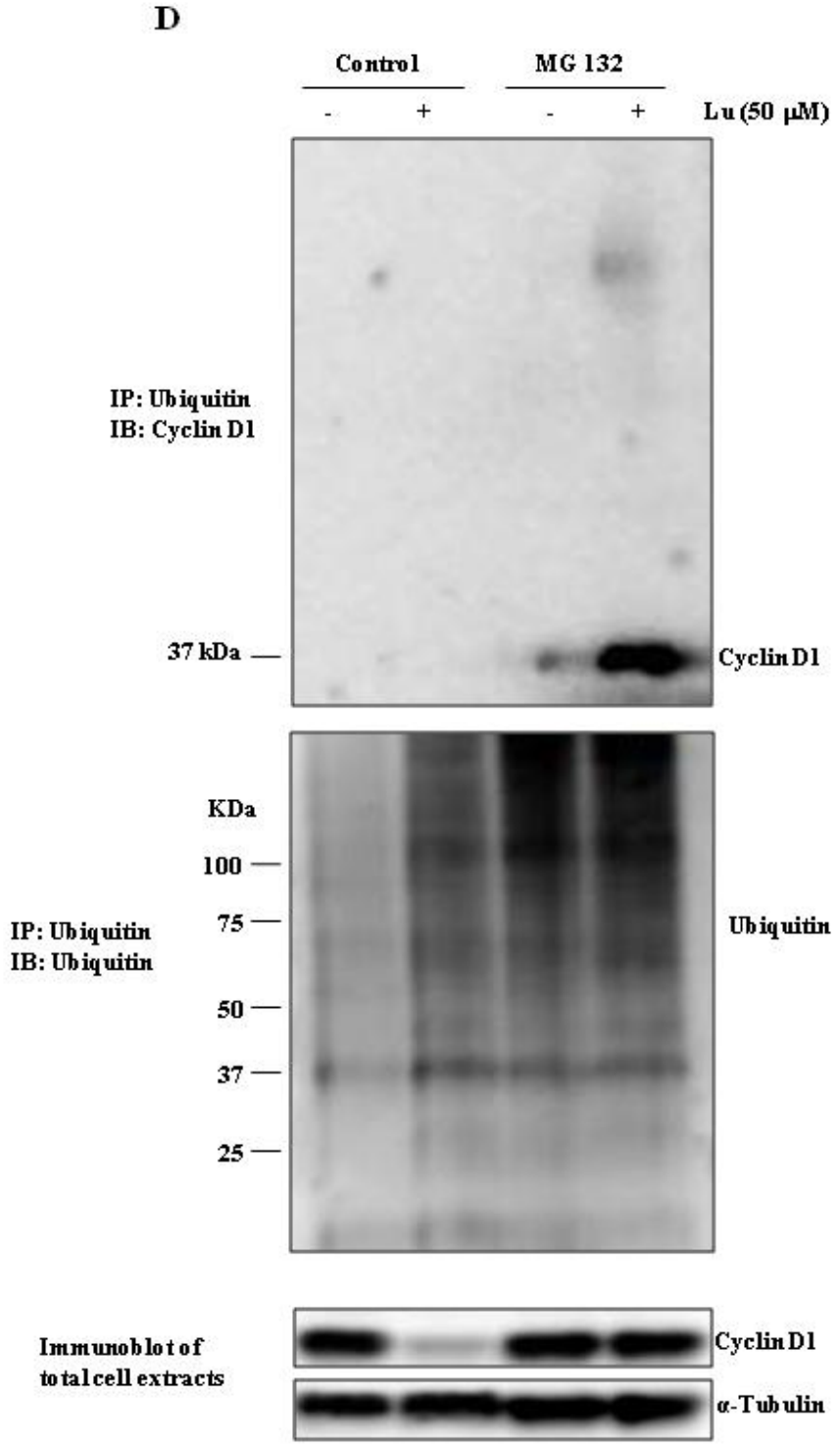


Fig 3.5D: Luteolin enhances cyclin D1 ubiquitination and proteasomal degradation in HK1 cells

(D) Detection of cyclin D1ubiquitination. Cell lysates from cells treated using the same experimental condition as in (C) was used for immunoprecipitation using polyubiquitin affinity beads, as described in Section 3.2, followed by immunoblotting with anti-ubiquitin and anti-cyclin D1. IB: Immunoblotting: IP: Immunoprecipitation

3.3.5 Luteolin inhibits the Akt-GSK-3β signalling pathway upstream of cyclin D1

It has been established that cyclin D1 phosphorylation is mediated by the Akt-GSK-3β pathway (Diehl et al., 1998; Diehl et al., 1997). Activated Akt phosphorylates and inhibits GSK-3β function, leading to the de-phosphorylation and stabilisation of cyclin D1. We therefore examined whether the Akt-GSK-3β-cyclin D1 axis was involved in luteolin-induced degradation of cyclin D1 in HK1. In our experiment, we first found that luteolin rapidly and markedly suppressed Akt phosphorylation, as early as 15 mins (Fig 3.6). Consistent with the inhibition of Akt activity, phosphorylation of GSK-3β, a target of Akt kinase, was also reduced (Fig 3.6), suggesting that luteolin likely promotes cyclin D1 phosphorylation via suppression of Akt and activation of GSK-3β.

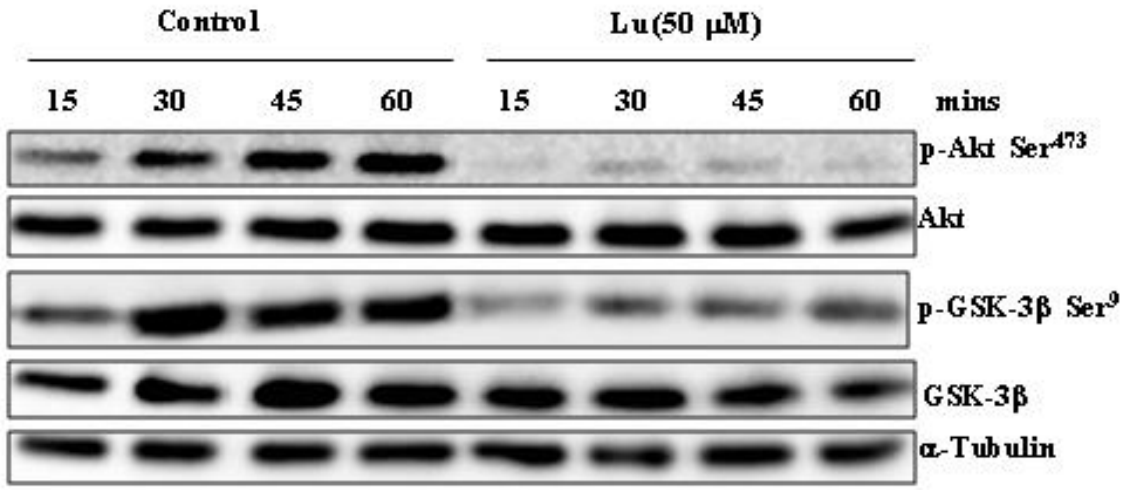


Fig 3.6: Luteolin suppresses Akt and GSK-3β phosphorylation in HK1 cells
Cells were treated with luteolin for the indicated periods. Total cellular protein extracts were prepared for immunoblotting to detect the levels of phospho-Akt $Ser^{473,}$ total Akt, phosphor-GSK-3β Ser^{9} and total GSK-3β. α-Tubulin was used as a loading control.

To further examine the involvement of Akt-GSK-3β signalling pathway, we pre-treated HK1 cells with LiCl, an inhibitor of GSK-3β before luteolin treatment. As shown in Fig 3.7A, pre-treatment with LiCl could reverse the effect of luteolin on GSK-3β phosphorylation and when HK1 cells were treated with insulin, insulin could nullify the inactivation of Akt and subsequent activation of GSK-3β in luteolin-treated cells (Fig 3.7A). As a result, both insulin and LiCl were capable of reducing the phosphorylation of cyclin D1 (Fig 3.7B), and subsequently the down-regulation of cyclin D1 protein level induced by luteolin (Fig 3.7B). In order to detect the presence of the phosphorylated form of cyclin D1 as this protein is rapidly in cells, cells must first be pre-treated with MG132 as illustrated in Fig 3.7B. Finally, we verified the effect of insulin on the cell cycle profile in luteolin-treated cells. As shown in Fig 7C, pre-treatment with insulin significantly increased the percentage of cells in S phase in HK1 cells treated with luteolin. Similar results were also obtained when the experiment was repeated using CNE2 cells (Fig 3.7D). Taken together, these findings suggest that luteolin suppresses the Akt-GSK-3β pathway, leading to enhanced cyclin D1 phosphorylation, proteasomal degradation and eventually cell cycle arrest at G1.

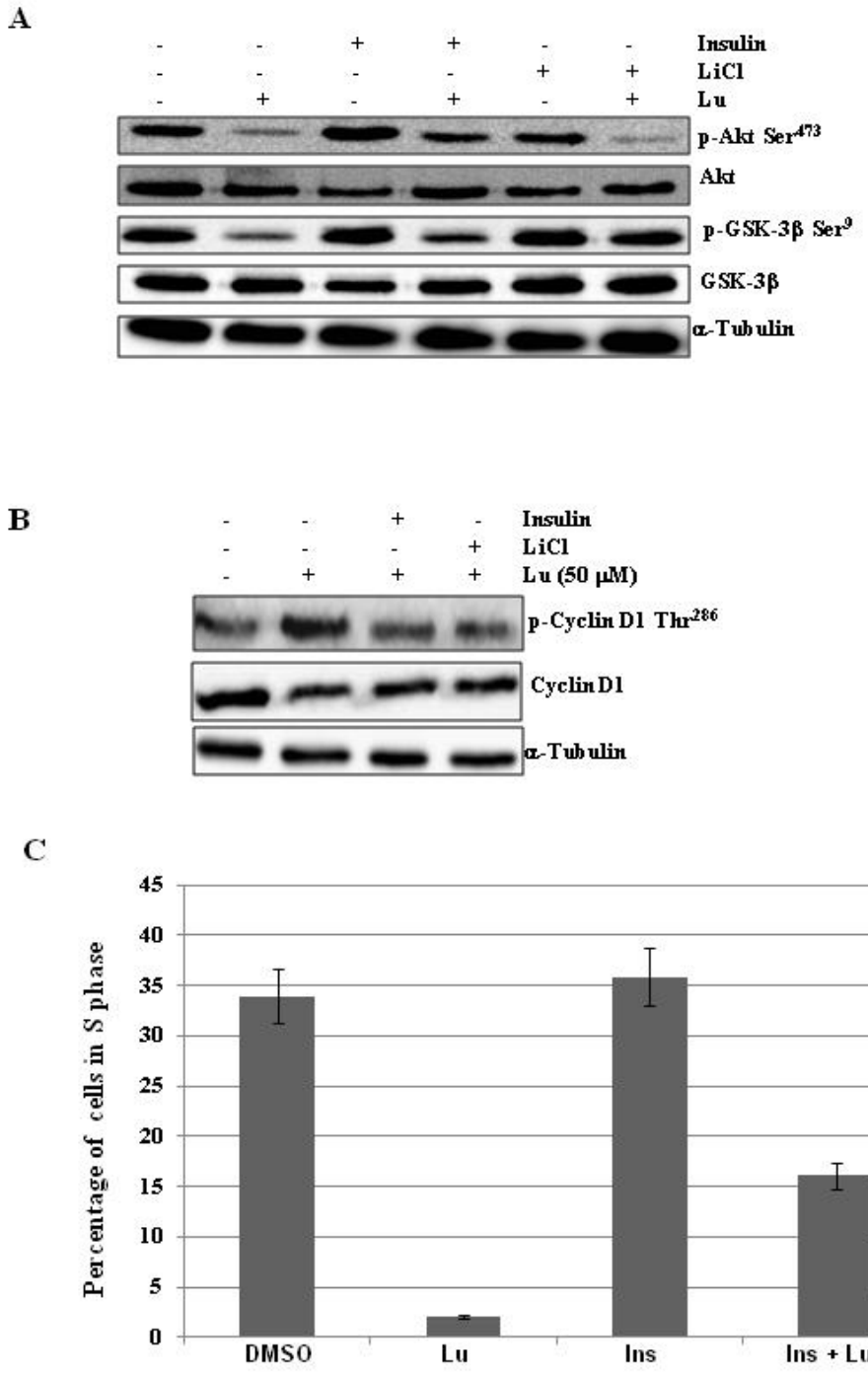


Fig 3.7 A – C: Insulin and LiCl prevent down-regulation of cyclin D1 induced by luteolin in HK1 cells

(A) HK1 cells were pre-treated with either 200 nM insulin or 30 mM LiCl, followed by 50 μM luteolin treatment for 3 h. Total cellular protein extracts were prepared for immunoblotting. α-Tubulin was used as a loading control. (B) HK1 cells were treated using the same experimental condition as (A) and in the presence of MG132. Total cellular protein extracts were prepared for immunoblotting. α-Tubulin was used as a loading control. (C) HK1 cells were first synchronised in FBS-free medium overnight before pre-treatment with 200 nM insulin followed by 50 μM luteolin treatment for 3 h. BrdU was added 15 mins before the end of incubation. Cells were subsequently harvested and stained with anti-BrdU-FITC and 7-AAD, followed by flow cytometry analysis to quantify the percentage of cells in G0/G1 and S phase, as described in Fig 3.1A. Data in C are presented as means ± SD from three independent experiments.

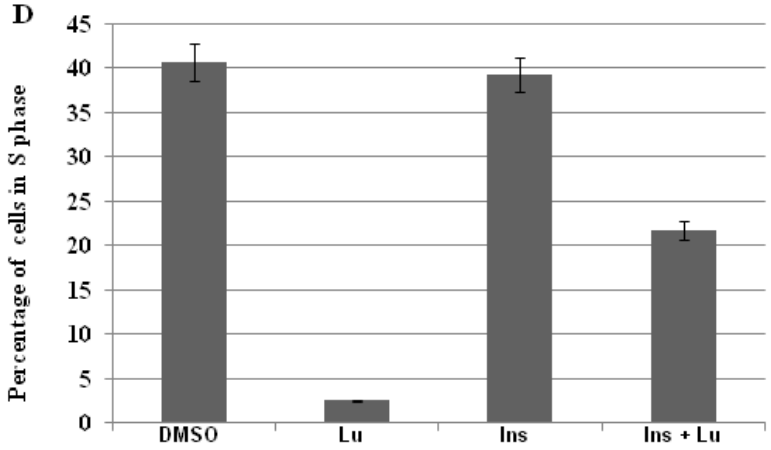


Fig 3.7D: Insulin and LiCl abrogate the effects of luteolin on CNE2 cells
Cells were first synchronised in FBS-free medium overnight before pre-treatment with 200 nM insulin followed by 40 μM luteolin treatment for 3 h. BrdU was added 15 mins before the end of incubation. Cells were subsequently harvested and stained with anti-BrdU-FITC and 7-AAD. Data are presented as means ± SD from three independent experiments.

3.4 Discussion

At present, the anti-cancer potential of luteolin is mainly based on its ability to induce apoptosis in cancer cells (Lin et al., 2008). However, relatively little is known about the effect of luteolin on cell cycle progression. Several earlier reports have found that luteolin induces cell cycle arrest either at G1 by down-regulating cellular protein levels of cdk4 and cdk6 (Casagrande and Darbon, 2001; Lim do et al., 2007) or G2/M arrest by the inhibition of cdc2 and up-regulation of $p21^{CIP1}$ (Wu et al., 2008). In the present study, we identified the molecular mechanism in luteolin-induced G1 arrest: luteolin inhibits the Akt-GSK-3β-cyclin D1 signalling pathway, promotes cyclin D1 phosphorylation and proteasomal degradation, and subsequently causing hypophosphorylation of Rb and suppression of the E2F-1 transcriptional activity.

Progression from G1 to S phase of the cell cycle is controlled by cyclin Ds and their kinases, namely cdk4 and cdk6, which act by phosphorylating and

inactivating Rb, thus liberating E2F-1 transcriptional activity to drive the cells into S phase (Genovese et al., 2006; Malumbres and Barbacid, 2009). In this study, we first defined the critical role of cyclin D1 in luteolin-induced cell cycle arrest. It is well known that cyclin D1 is important in the development and progression of numerous cancers (Gladden and Diehl, 2005; Tashiro et al., 2007). Moreover, cyclin D1 over-expression is a common event in cancer and is usually a result of defective regulation at the post-translational level (Kim and Diehl, 2009). Therefore, regulation of cyclin D1 protein level is one of the critical aspects in cell proliferation and tumour development. An earlier study by Diehl et al (Diehl et al., 1997) demonstrated that cyclin D1 degradation is dependent on Thr^{286} phosphorylation by GSK-3β and ubiquitin-dependent proteasomal degradation. Interestingly, we found that luteolin acts on this signalling pathway in HK1 cells, resulting in the induction of cell cycle arrest. An increase in the phospho form of cyclin D1 Thr^{286} was observed but due to the rapid turnover of this protein, it could only be detected when cells were pre-treated with MG132 before luteolin treatment (Fig 3.5C). As GSK-3β regulates cyclin D1 degradation, a GSK-3β-specific inhibitor LiCl was able to suppress luteolin-induced down-regulation of this protein (Fig 3.7B), suggesting the involvement of the GSK-3β pathway in luteolin-mediated cell cycle arrest.

Frequent activation of Akt has been reported in many human cancers (Altomare and Testa, 2005; Tokunaga et al., 2008) and GSK-3β has been identified as one of Akt's molecular targets. Akt inactivates GSK-3β kinase activity by site-specific phosphorylation at Ser^{9} which leads to subsequent reduction in cyclin D1 phosphorylation and an increase in its protein stability (Diehl, 2002). In this study, we found that luteolin is capable of inhibiting Akt

phosphorylation and activation. It remains to be investigated how luteolin inhibits Akt enzymatic activity. One possibility is that luteolin may target phosphoinositide 3′-kinase (PI3K), as suggested by earlier reports (Bagli et al., 2004; Lee et al., 2006).

It has been extensively studied that luteolin induces apoptotic cell death in many cancer cells (Lin et al., 2008). Interestingly, in our study, luteolin was unable to induce apoptosis in HK1 and CNE2 cells, as verified by the absence of active caspase-3 in luteolin-treated cells by flow cytometry (Figs 3.2 and 3.3) and cleaved PARP proteins by Western blot (data not shown). Since these cells are capable of undergoing apoptosis as demonstrated by camptothecin treatment (Fig 3.2), the exact reason for the lack of apoptotic response to luteolin in HK1 and CNE2 cells is not clear. One possible explanation is that this group of NPC cells exhibit a high basal level of Akt activation (as shown in Fig 3.7A), thus making them more susceptible to the inhibitory effect of luteolin on cell cycle via the Akt-GSK-3β-cyclin D1 pathway. It would be of interest to elucidate the underlying mechanisms responsible for the different response to luteolin by different types of cancer cells.

NPC is one of the common cancers in the regions of East Asia, especially among the Chinese (Chan et al., 2003; Hanley et al., 1995; Yu, 1991). The main modalities for NPC are chemotherapy and radiotherapy. Our study demonstrates that luteolin is able to suppress NPC cell proliferation via cell cycle arrest by targeting the Akt-GSK-3β-cyclin D1 signalling axis. Since Akt is often over-activated in many human cancers including NPC (Chou et al., 2008; Tokunaga et al., 2008), it is thus believed data from this study support the potential application of luteolin as a chemotherapeutic or chemopreventive agent in human cancer.

Luteolin and quercetin sensitise NPC cells to the cytotoxic effects of chemotherapeutics

Chapter 4

4.1 Introduction

The traditional treatment for NPC includes radiation and chemotherapy. There are two major limitations with these conventional therapies: resistance and toxic side-effects (Chua et al., 2005; Ngan et al., 2002; Wong et al., 2006). Many tumours exhibit a good response when they are first exposed to chemotherapeutic drugs, however the majority of patients eventually develop resistance to these agents. Therefore, acquired resistance to chemotherapy is a major obstacle to successful cancer treatment. Understanding the molecular mechanisms in which tumours become resistant to a particular therapeutic is thus critical.

Several mechanisms of drug resistance have been identified, including (i) changes to membrane transporters that result in reduced drug accumulation, (ii) an enhanced DNA damage repair mechanism; and (iii) multiple deficiencies in apoptosis induction in tumour (Abrams et al., 2010; Goto and Takano, 2009; Pauwels et al., 2007; Zhou et al., 2008). In addition, alteration to critical signalling pathways such as the PI3K/Akt pathway has also been associated with drug resistance (Huang and Hung, 2009; Li et al., 2010a; Liang et al., 2009). PI3K/Akt pathway is known to play a central role in many cellular physiological functions and in numerous cancers this pathway is up-regulated (reviewed in Section 1.2 of this thesis). This has generated an increasing interest in designing drugs that specifically target the PI3K/Akt pathway as single agent or in combination to other chemotherapeutic agents to overcome drug resistance.

Quercetin, a flavonoid has been identified to possess inhibitory activity against the PI3K/Akt pathway (Matter et al., 1992). Subsequently, more specific and potent inhibitors like wortmannin (Arcaro and Wymann, 1993) and LY294002 (Vlahos et al., 1994) were developed. However, the use of these

inhibitors as potential candidates in tumour growth inhibition was dampened by the fact that it is impractical to use as *in vivo* pharmacological agents due to their toxicity and insolubility. This leads to the hunt for other novel agents to overcome chemoresistance (Ihle and Powis, 2009). Identified inhibitory agents work on different aspects of the PI3K/Akt pathway. These include inhibitors that (i) inhibit catalytic activity of PI3K via its PH domain-PtdIns(3,4,5)P_3 interaction for example inositol pentakisphosphate (Piccolo et al., 2004), (ii) interfere with Akt translocation to the plasma membrane like perifosine (Kondapaka et al., 2003) and (iii) inhibit Akt activation like phosphatidylinositol ether lipid analogues and GSK690693 (Gills et al., 2006; Rhodes et al., 2008).

Microtubules are components of the cytoskeleton with important roles in intracellular trafficking of vesicles and organelles, maintenance of cell shape and polarity; cellular motility; cell signalling and mitotic chromosome segregation (Nogales, 2001). During mitosis, microtubules form the mitotic spindle separate daughter chromosomes to the two poles of a dividing cell. Any agents that interfere with microtubule dynamics will inhibit the ability of cells to successfully complete mitosis, thus making the microtubules a desirable target for the development of chemotherapeutics against rapidly dividing cancer cells. Microtubule inhibitors like taxanes (De Dosso and Berthold, 2008; Nishiyama and Wada, 2009; Rodriguez-Antona, 2010), vinca alkaloids (Chan and Verrill, 2009; Eden et al., 2010; Lin et al., 2010) and epothilones (Frye, 2010; Hurtig, 2010; Larkin and Kaye, 2006) alone or in combinations with other chemotherapeutics have been used against many solid and haematologic malignancies. Taxanes and epothilones interact with polymerised tubulin and prevent depolymerisation, while vinca alkaloids bind to monomeric tubulin and prevent polymerisation. The

success of microtubule inhibitors as cancer therapeutics has been plagued with the development of acquired drug resistance in tumour cells (Fojo and Menefee, 2007; McGrogan et al., 2008). One strategy to overcome this drug resistance is to use a multiple drug treatment approach. Each agent in the multiple drug regime is administered at sub-cytotoxic dose (less toxic side-effect and lower the chance of developing drug resistance) and yet able to achieve the same efficacy at a higher dosage as each agent works on targeting a different site, thus leading to an overall additive or synergistic cytotoxic effects on tumours. There are reports where microtubule inhibitors like docetaxel (Gomez et al., 2006; Motwani et al., 2003) and epothilone B (Wittmann et al., 2003) when combined with flavonoids like flavopiridol (semi-synthetic flavonoid) were able to improve the overall efficacy of treatment on cancer cells like prostate cancer, breast cancer and gastric cancer compared to the single agents.

In Chapter 3 of this thesis, we have identified luteolin, a natural plant flavonoid that is able to inhibit Akt very efficiently by preventing Akt phosphorylation at ser^{473}. In the first part of this study, a panel of conventional chemotherapeutics used for NPC treatment in patients was selected. This includes 5-Fluorouracil (5-FU) (Azli et al., 1992), docetaxel (DTX) (Ngeow et al., 2010), paclitaxel (PTX) (Chan et al., 2004) and vincristine (VCR) (Kwong et al., 2004) used singly or in combination with sub-cytotoxic dose of luteolin or quercetin were screened to identify paired combinations that were able induced cell death (microscopic examination of cell death) in two nasopharyngeal carcinoma cell lines, HK1 and CNE2. DTX, PTX and VCR are mitotic inhibitors that affect microtubule formation during cell division. 5-FU is an anti-metabolite that inhibits thymidylate synthase and thus prevents the synthesis of thymidine. In the

second part of the study, the identified combinations of either luteolin or quercetin and chemotherapeutics were used on the same cell lines and cell death by apoptosis determined by flow cytometry. The data obtained clearly demonstrate that luteolin and quercetin were able to sensitise HK1 and CNE2 cells to VCR-induced cell death when VCR was used at sub-cytotoxic concentration. Cell death was mediated by caspase-3-dependent apoptosis.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Luteolin, quercetin, DMSO, VCR, 5-FU, docetaxel, paclitaxel as well as other chemicals were purchased from Sigma (St Louis, MO, USA). zVAD-fmk was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Anti-α-tubulin was purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Akt, anti-pAkt Ser^{473}, anti-caspase-3, anti-PARP, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). Protease inhibitors cocktail was purchased from Roche (Mannheim, Germany). RPMI-1640 and trypsin-EDTA (0.25% porcine trypsin and 0.02% EDTA-2Na were purchased from Gibco Ltd (Grand Island NY, USA). FBS was purchased from JRH Biosciences (Lenexa, KS, USA). Reagents used for Western blot analysis were purchased from Bio-Rad (Hercules, CA, USA). The active caspase-3 apoptosis kit was purchased from BD Biosciences (Palo Alto, CA, USA). Enhanced chemiluminescent substrate was purchased from Pierce (Rockford, USA).

4.2.2 Cell culture and treatment

Nasopharyngeal carcinoma cell lines HK1 and CNE2 were used in this study. Cells were grown and maintained at 37^{o}C in a humidified 5% CO_2 and 95% air atmosphere in RPMI-1640 supplemented with 10% FBS. Equal number of cells were seeded in RPMI-1640 supplemented with 10% FBS. As luteolin, quercetin and all test chemotherapeutics were dissolved in DMSO, the same concentration of DMSO was always applied to the control group. All treatments on cells were conducted in RPMI-1640 supplemented with 1% FBS.

4.2.3 Apoptosis analysis

Flow cytometry to detect and quantify the presence of apoptotic cells was performed using an active caspase-3-FITC antibody apoptosis kit (BD Biosciences). Cells were harvested and permeabilised using saponin buffer followed by incubation with anti-active caspase-3-FITC conjugated monoclonal antibodies. Apoptotic cells were detected and quantified by flow cytometry (This method used is similar to the one used in Chapter 3 of this thesis).

4.2.4 Immunoblot analysis

Cells were first lysed in cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1mM PMSF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA, 1mM EGTA, 1% Triton-X100, 1mM sodium vanadate, 1mM PMSF (Phenylmethanesulfonylfluoride) and a protease inhibitor cocktail). Equal amount of proteins were fractionated on SDS-PAGE gel using the Mini-PROTEAN II system (Bio-Rad) before being transferred to nitrocellulose membrane (Bio-Rad). The membrane was first blocked with 5% fat-free milk in TBST (10 mm Tris-

HCl, pH 7.5, 100 mM NaCl and 0.1% Tween-20), followed by probing with the various primary antibodies and developed using the enhanced chemiluminescent reagents (Pierce). Detection of specific bands could be viewed using the Kodak Image Station 4000MM Pro.

4.2.5 Statistical analysis

The apoptotic effects of flavonoids and vincristine singly and in combination on cells as quantified by flow cytometry (active caspase-3) were analysed using the statistical analysis software from Statgraphics® Centurion XVI.

4.3 Results

4.3.1 Luteolin sensitises CNE2 cells to the cytotoxic effect of VCR

In this study, sub-cytotoxic concentration of luteolin at 10 μM was selected. A total of four chemotherapeutics namely 5-fluorouracil (5-FU), docetaxel (DTX), vincristine (VCR) and paclitaxel (PTX) was used singly or in combination with 10 μM luteolin in CNE2 cells for 24 h or 48 h. Experiments were performed in which these cells were first pre-treated with 10 μM luteolin in RPMI-1640 supplemented with 1% FBS for two hours, followed by the addition of various concentrations of chemotherapeutics for the remaining 24 h. These cells were subsequently observed under the microscope for morphological changes (Fig 4.1). Based on morphological examination of cells treated for 24 h, it was observed that 5-FU alone and in combination with luteolin did not lead to cell death of CNE2 cells (Fig 4.1A). Luteolin in combination with DTX, VCR or

PTX was able to augment cell death in CNE2 cells (Fig 4.1 B – D). With the exception of 5-FU, DTX, VCR and PTX were used in nanomolar range (Fig 4.1).

The cytotoxic effect of luteolin plus VCR was observed to be time dependent as with prolonged incubation to 48 h, cell death was more apparent compared to 24 h (Fig 4.1C and Fig 4.2).

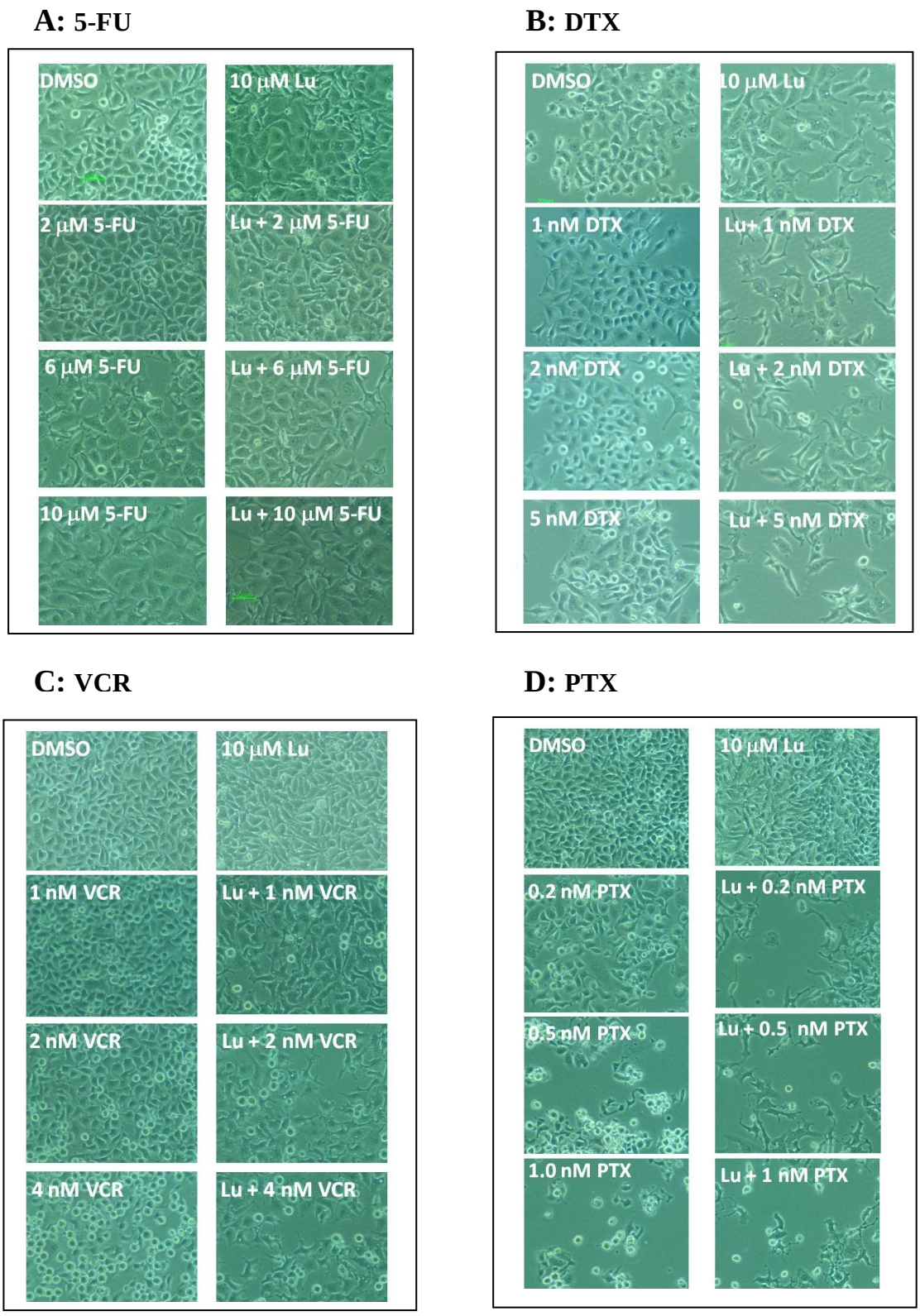


Fig 4.1: Combined effect of luteolin (Lu) and chemotherapeutics on CNE2 cells

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 10 μM Lu for 2 h before the addition of chemotherapeutics for 24 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Micrographs of cells were captured at total magnification of 100X. (A) 5-Fluorouracil (5-FU) at 0 to 10 μM. (B) Docetaxel (DTX) at 0 to 5 nM. (C) Vincristine (VCR) at 0 to 4 nM. (D) Paclitaxel (PTX) at 0.2 to 1 nM.

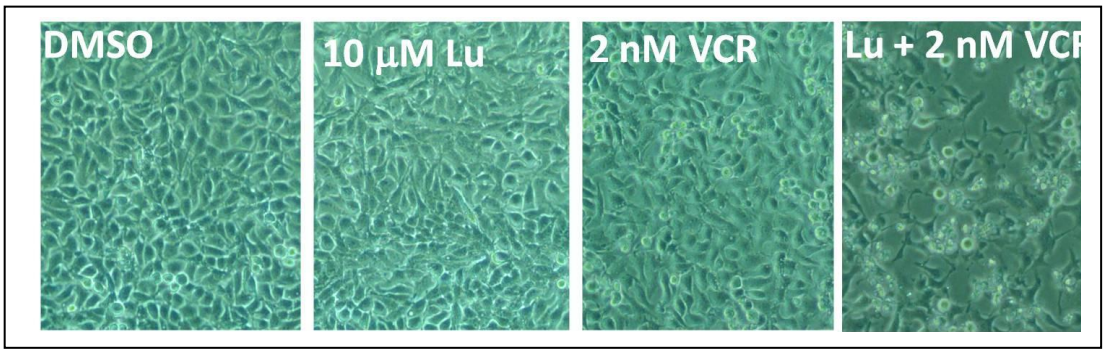


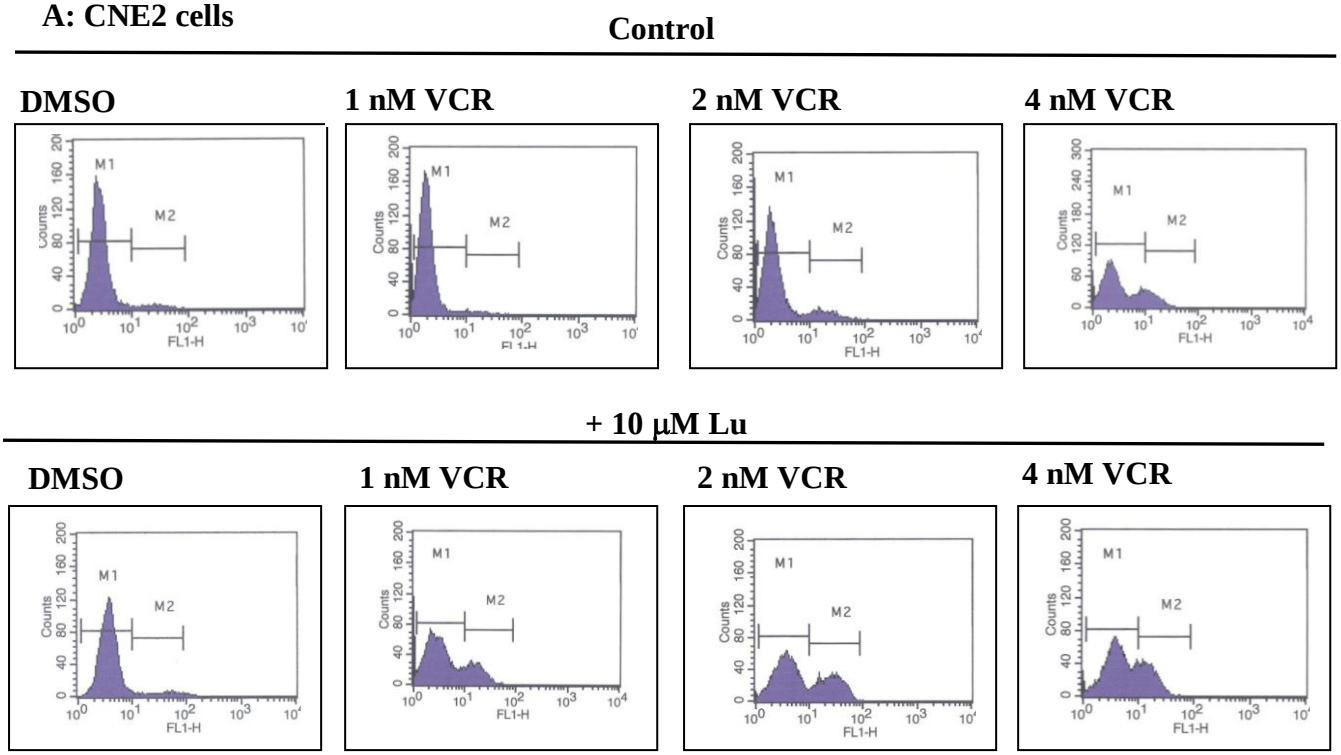
Fig 4.2: Combined effect of 10 μM luteolin (Lu) and 2 nM VCR on CNE2 cells for 48 h

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 10 μM Lu for 2 h before the addition of 2 nM VCR for up to 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Micrographs of cells were captured at total magnification of 100X.

As morphological study indicated that sub-cytotoxic combination of luteolin and VCR together was able to induce cell death in CNE2 cells, we next performed flow cytometry to quantify the cytotoxic effect of this combination.

It was observed that the percentage of apoptotic cells in CNE2 cells after luteolin, 1 or 2 nM VCR- treated were 3.77%, 4.17% and 5.25% respectively with untreated cells exhibiting a base level of apoptosis at 2.12% (Fig 4.3) after 48 h of treatment. These values of apoptosis after these treatments were considered negligible when compared to untreated cells, thus indicating that luteolin and VCR alone could not induce apoptotic cell death in these cells. However, when luteolin was combined with 1 or 2 nM VCR, the percentage of cell death by apoptosis rose to 17.23 and 22.70% respectively (Fig 4.3). Based on these percentages, one could observe that luteolin sensitised CNE2 cells to the cytotoxic effect of VCR that resulted in a caspase-3-dependent apoptotic cell death.

A: CNE2 cells



B: CNE2 cells

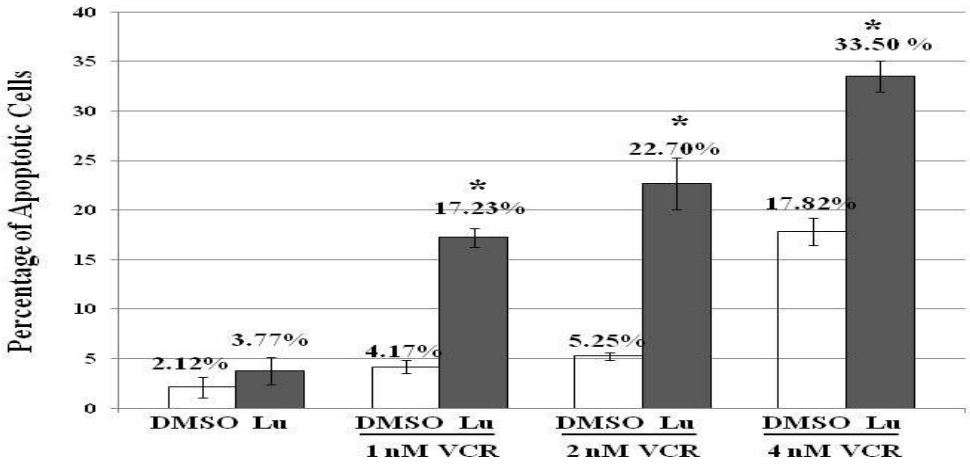


Fig 4.3: Quantification of the combined cytotoxic effect of Lu and VCR on CNE2 cells

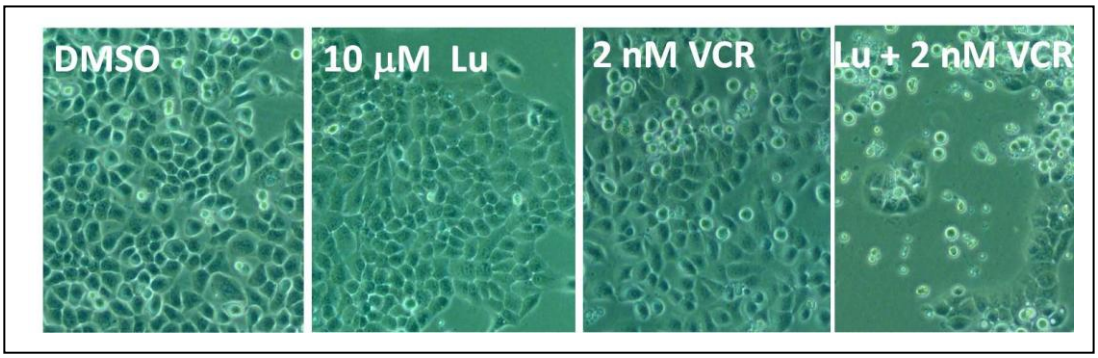
Cells were pre-treated with 10 μM Lu for 2 h before the addition of VCR up to 48 h. They were subsequently harvested and stained with antibody to active caspase-3-FITC, followed by flow cytometry analysis to quantify the percentage of apoptotic cells as described in Materials and Methods). (A) Histogram plots of the results obtained from the flow cytometry experiments. In the upper panel, the results were obtained from cells treated with VCR alone. Results in the lower panel were obtained when cells were treated with Lu and VCR. Gates were configured manually to measure the percentage of apoptotic cells (M2) versus non-apoptotic cells (M1). (B) The percentage of apoptotic cells (M2) is presented as means ± SD from three independent experiments and represented by bar chart. Data are presented as means ± SD from three independent experiments and represented by bar chart. *: Data compared to the control group (DMSO, Lu or VCR alone) based on One-way ANOVA with Scheffe's test exhibits a statistically significant difference.

4.3.2 Luteolin sensitises HK1 cells to the cytotoxic effect of VCR

We next performed preliminary screening using 5-FU, DTX and VCR together with luteolin on HK1 cells. Luteolin did not sensitise these cells to the growth inhibitory effect of 5-FU and DTX (data not shown). Like CNE2, HK1 cells could be sensitised by luteolin to the cytotoxic effect of VCR and in a time-dependent manner since there was visually more cell death at 48 h compared to the same treatment for 24 h (Fig 4.4). The concentration of VCR used was also similar to that for CNE2 treatment, which was 1, 2 and 4 nM.

The percentage of cell death mediated by the combined luteolin and VCR treatment on HK1 cells for 48 h was quantified by flow cytometry. A value equivalent to 6.53%, 2.43% and 5.23% of apoptotic cell death was observed in HK1 cells treated with 10 μM luteolin, 1 nM VCR and 2 nM VCR respectively, with the base level of apoptosis observed in untreated cells at 3.29% (Fig 4.5). When these cells were treated with luteolin in combination with either 1 or 2 nM VCR, the percentage of cell death by apoptosis increased to 28.17% and 31.93% respectively (Fig 4.5). These data demonstrated that the combined treatment of luteolin and VCR on HK1 cells was able to augment a cytotoxic effect resulting in cell death. Based on the percentage of cell death, it was observed that luteolin exerted the same effect on HK1cells as observed in CNE2 cells; it sensitised HK1 cells to VCR, resulting in a caspase-3-dependent apoptotic cell death.

A: 24 h (HK1 cells)



B: 48 h (HK1 cells)

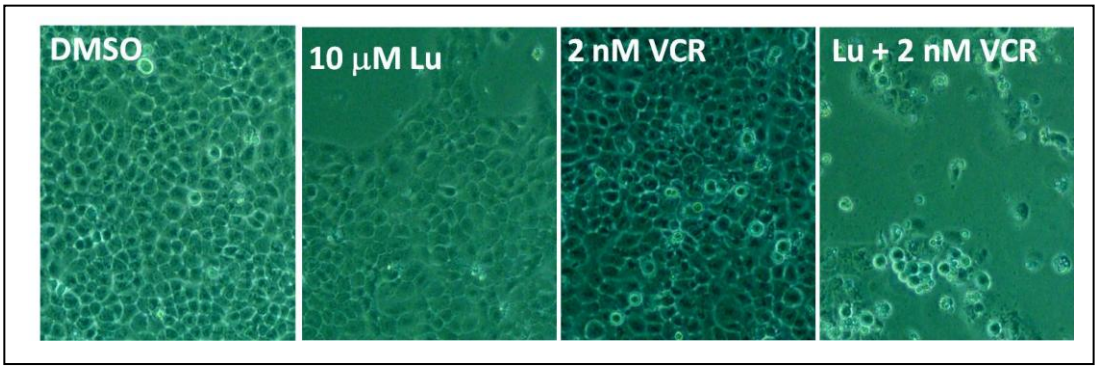
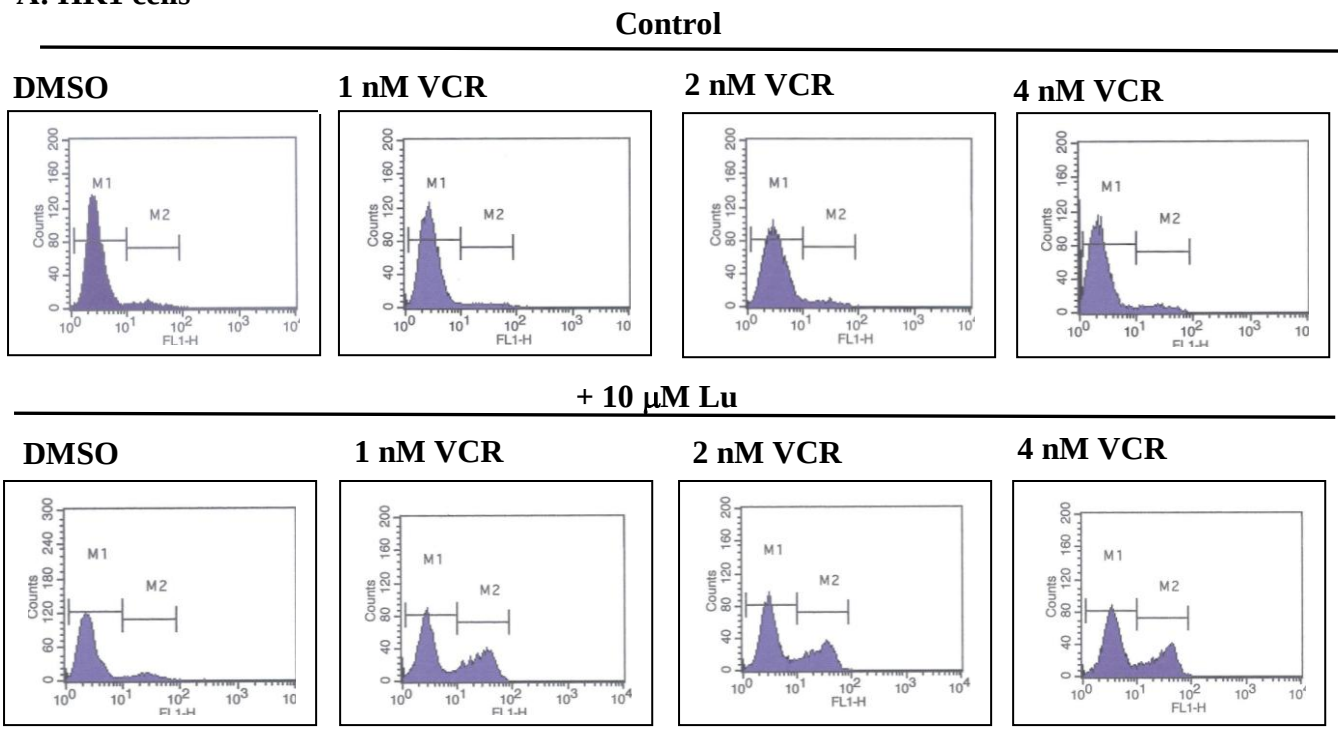


Fig 4.4: Combined effect of 10 μM luteolin (Lu) and 2 nM VCR on HK1 cells for (A) 24 h and (B) 48 h

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 10 μM Lu for 2 h before the addition of 2 nM VCR for up to 24 or 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Micrographs of cells were captured at total magnification of 100X.

A: HK1 cells



B: HK1 cells

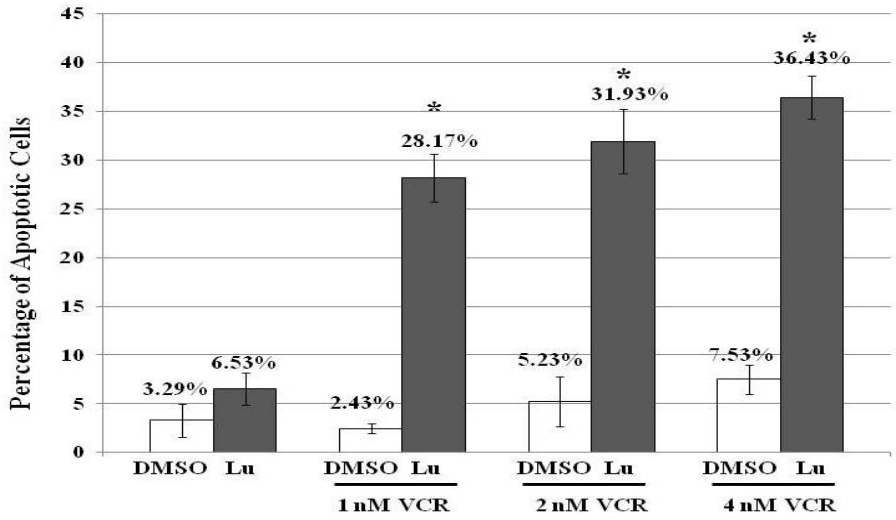


Fig 4.5: Quantification of the combined cytotoxic effect of Lu and VCR on HK1 cells

Cells were pre-treated with 10 μM Lu for 2 h before the addition of VCR up to 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. They were subsequently harvested and stained with antibody to active caspase-3-FITC, followed by flow cytometry analysis to quantify the percentage of apoptotic cells. (A) Histogram plots of the results obtained from the flow cytometry experiments. In the upper panel, the results were obtained from cells treated with VCR alone. Results in the lower panel were obtained when cells were treated with Lu and VCR. Gates were configured manually to measure the percentage of apoptotic cells (M2) versus non-apoptotic cells (M1). (B) The percentage of apoptotic cells (M2) is presented as means ± SD from three independent experiments and represented by bar chart. Data are presented as means ± SD from three independent experiments and represented by bar chart. *: Data compared to the control group (DMSO, Lu or VCR alone) based on One-way ANOVA with Scheffe's test exhibits a statistically significant difference.

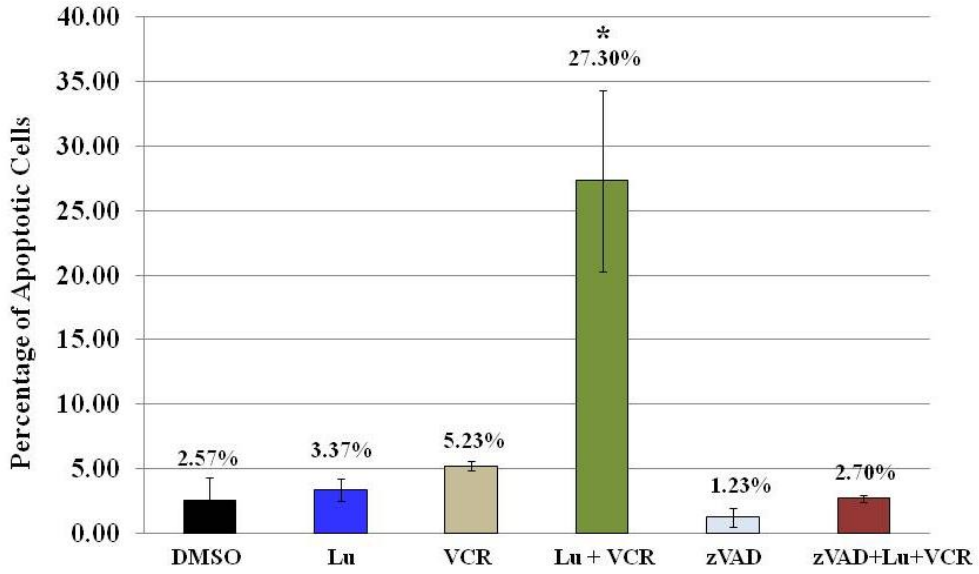
4.3.3 zVAD-fmk abrogates the cytotoxic effects of luteolin and VCR on CNE2 and HK1 cells

To verify whether the cytotoxic effect of luteolin and VCR on CNE2 was due to an increase in active caspase-3 expression, cells were pre-treated with luteolin and or 40 μM zVAD-fmk, a pan-caspase inhibitor for 2 h before being treated with VCR at 2 nM up to 48 h before the cells were subjected to flow cytometry. Luteolin sensitised CNE2 cells to 2 nM VCR resulting in cell death equivalent to 27.30% (Fig 4.6A). The presence of zVAD-fmk completely blocked cell death induced by luteolin and VCR as verified by reduction of apoptotic cell death equivalent to 2.70% (basal level of apoptosis observed in control cells was 2.57%).

The same experiments were also performed on HK1 cells. As the data in Fig 4.6B indicated, zVAD-fmk was able to abrogate luteolin and VCR induced cell death from 35.90% to 3.20%.

Based on these results, it seems that augmentation of cell death by the combined treatment of luteolin and VCR on CNE2 and HK1 cells was mediated by a caspase-3-dependent apoptotic cell death since zVAD-fmk could abrogate the cytotoxic effect.

A



B

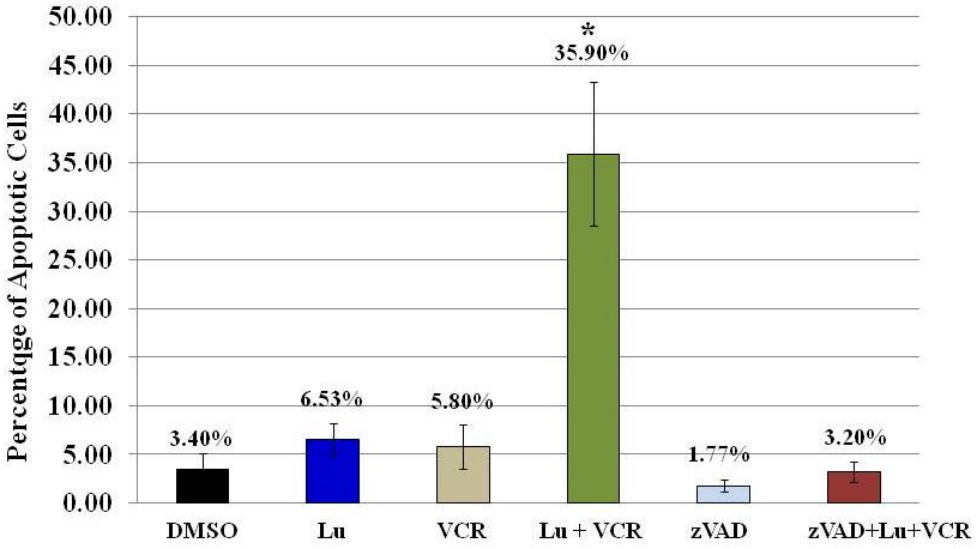


Fig 4.6: Cytotoxic effect of Lu and VCR on CNE2 (A), and HK1 (B) cells could be abrogated by zVAD-fmk

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 10 μM Lu and/ or 40 μM zVAD-fmk for 2 h before the addition of VCR for 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Cells were harvested and stained with antibody to active caspase-3-FITC, followed by flow cytometry analysis to quantify the percentage of apoptotic cells. Data are presented as means ± SD from three independent experiments. *: Data compared to the control group (DMSO, Lu or VCR alone) based on One-way ANOVA with Scheffe's test exhibits a statistically significant difference.

4.3.4 Quercetin sensitises HK1 cells to the cytotoxic effect of VCR and this effect can be abrogated by zVAD-fmk

We next examined the cytotoxic effect of quercetin and VCR on HK1. Quercetin at 5 μM was used in these experiments as this concentration did not cause cell death (based on morphological examination of cells). This concentration was subsequently used together with 2 nM VCR where cells were incubated for 48 h with both reagents. Quercetin, like luteolin could sensitise HK1 cells to 2 nM VCR, resulting in cell death (Fig 4.7). This cytotoxic effect was also time-dependent as more cell death was visually observed in the 48 h treated cell sample compared to the 24 h sample (data not shown for the 24 h sample).

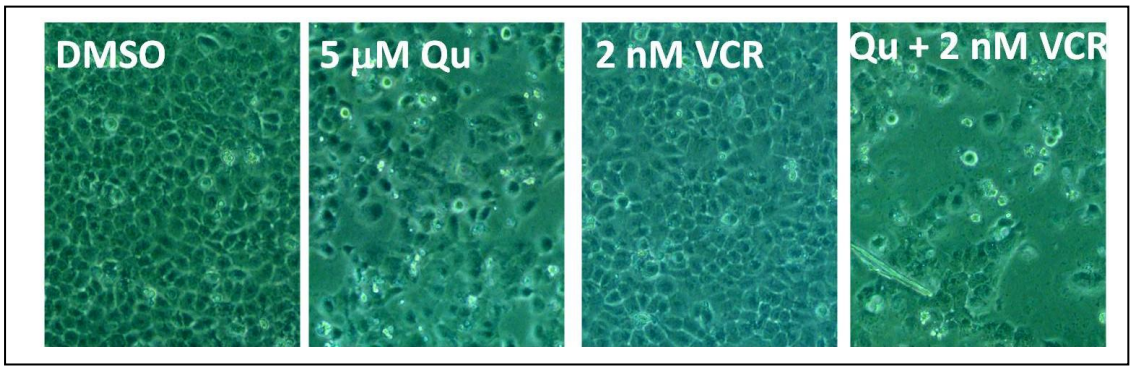


Fig 4.7: Combined effect of 5 μM quercetin (Qu) and 2 nM VCR on HK1 cells for 48 h
Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with5 μM for 2 h before the addition of 2 nM VCR for the remaining 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Micrographs of cells were captured at total magnification of 100X.

Cell death was further quantified by flow cytometry. In this experiment, cells were treated like in previous experiment with luteolin and VCR. Experimental data illustrated that VCR alone was unable to induce cell death by apoptosis (percentage of apoptotic cells of VCR and DMSO treated cells was 4.43% and 2.30% respectively). HK1 cells were more susceptible to quercetin

compared to luteolin as at 5 μM quercetin, there was 12.20% apoptotic cell death compared to 6.53% in 10 μM luteolin treated sample. When HK1 cells were treated with both quercetin and VCR, the percentage of apoptotic cell death increased to 37.03% which was considered to be statistically significant compared to the control and single agents when these data were analysed by One-way ANOVA with Scheffe's test (Fig 4.8). Like in luteolin treated HK1 cells, quercetin was able to sensitise the cells to the cytotoxic effect of VCR. As illustrated in Fig 4.8, cell death was mediated by the caspase-3-dependent pathway since zVAD-fmk could block cell death (the percentage of apoptotic cell death in sample pre-treated with zVAD-fmk and luteolin followed by VCR was 4.13% with basal level of apoptosis in the control sample at 2.30%).

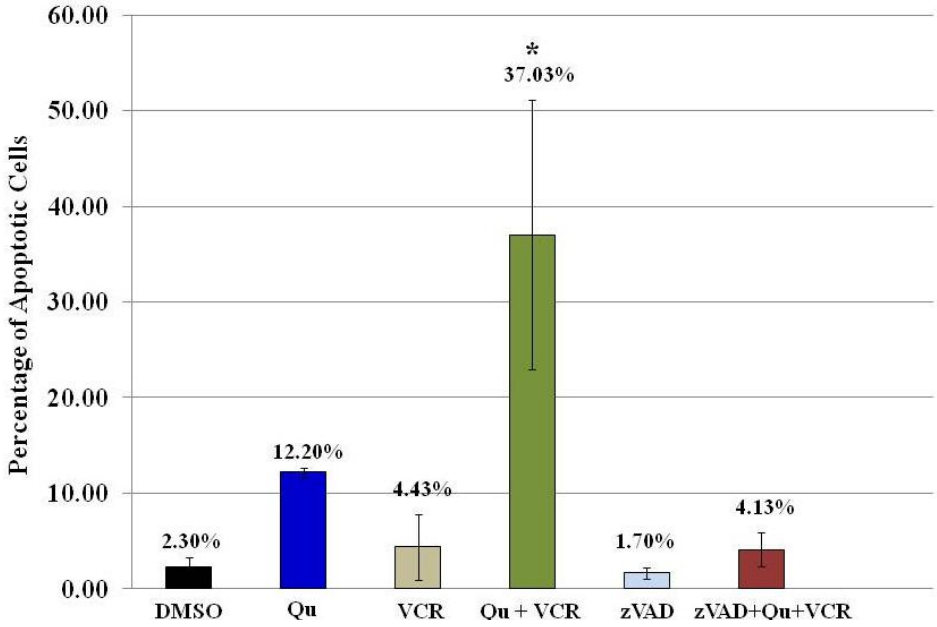


Fig 4.8: Cytotoxic effect of Qu and VCR on HK1 cells could be abrogated by zVAD-fmk

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 5 μM Qu and/ or 40 μM zVAD-fmk for 2 h before the addition of VCR for 48 h. Cells were treated in RPMI-1640 supplemented with 1% FBS. Cells were harvested and stained with antibody to active caspase-3-FITC, followed by flow cytometry analysis to quantify the percentage of apoptotic cells. The percentage of apoptotic cells is presented as means ± SD from three independent experiments. . *: Data compared to the control group based on One-way ANOVA with Scheffe's test exhibits a statistically significant difference.

4.3.5 Sensitisation effect of flavonoids on VCR-induced cell death is mediated by caspase-3-dependent apoptosis

To verify that the sensitisation of cells by luteolin or quercetin to the cytotoxic effect of VCR is mediated by a caspase-3-dependent apoptotic pathway, western blot experiments were performed to detect for the presence of proteins like active caspase-3 and PARP. CNE2 and HK1 cells were pre-treated with 10 μM luteolin and/or 40 μM zVAD-fmk for 2 h before the addition of 2 nM VCR for 48 h. Cellular extracts were subsequently obtained from these treated cells and used for western blot experiments. Similarly HK1 cells were also pre-treated with 5 μM quercetin and/or 40 μM zVAD-fmk before the addition of 2 nM VCR

for 48 h. Cellular extracts of these treated cells were also obtained and used for western blot experiments.

Luteolin and VCR alone did not induce apoptosis in CNE2 and HK1 cells as no cleavage of caspase-3 and PARP was observed (Fig 4.9 A and B). However, cleaved caspase-3 and PARP proteins were observed in cellular extracts from luteolin and VCR as well as quercetin and VCR treated cells (Fig 4.9 A and B).

In the previous flow cytometry experiment, 5 μM quercetin induced 12.20% of apoptosis in HK1 cells (Fig 4.8) and this could be verified by western blot experiment where there was slight increase in active caspase-3 (Fig 4.9C). However the intensity of the cleaved caspase-3 in sample obtained from quercetin and VCR treated HK1 cells was more apparent (Fig 4.9C). PARP cleavage followed closely the pattern of cleaved caspase-3.

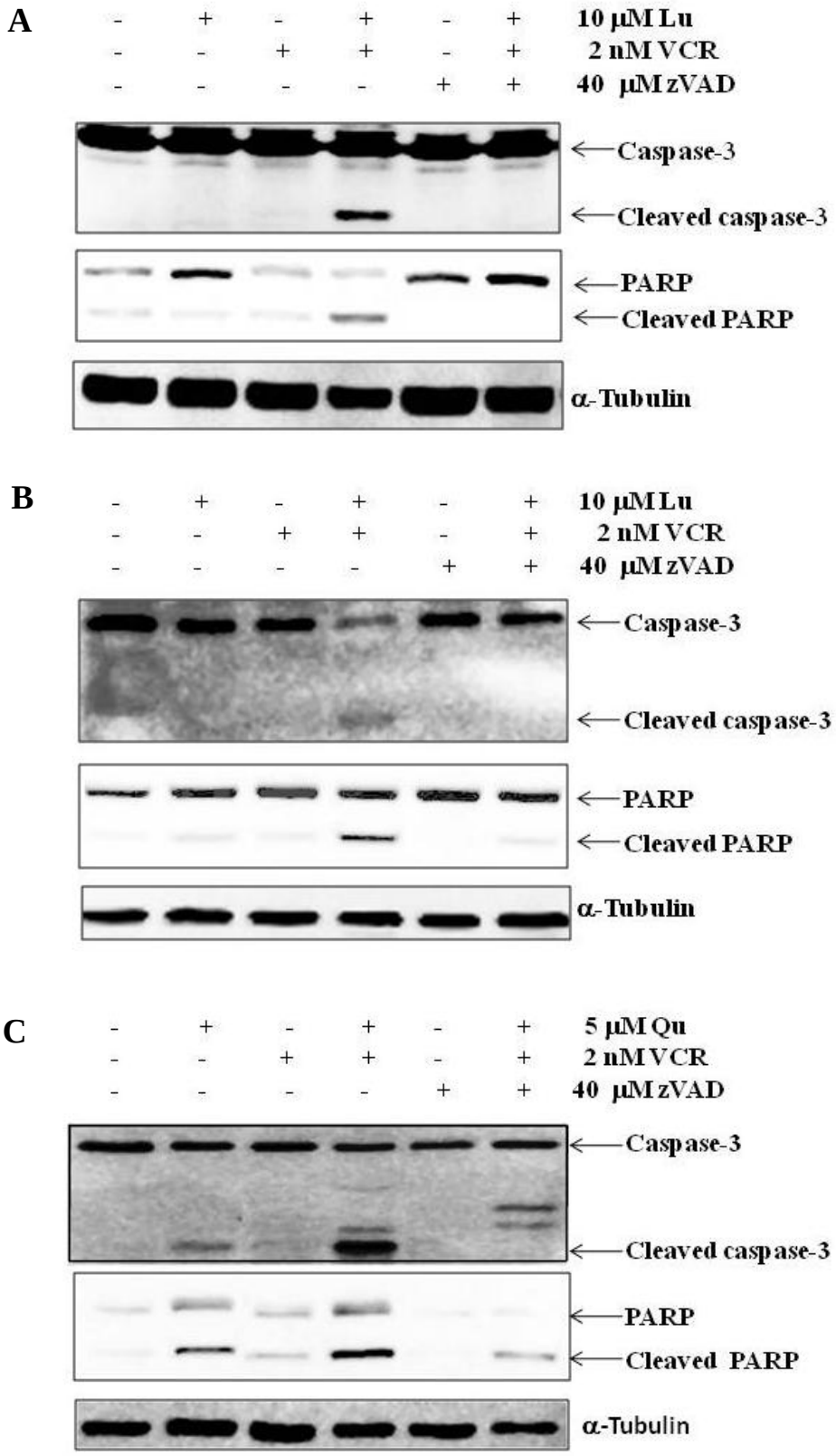


Fig 4.9: The combined effects of either Lu or Qu with VCR led to an increase in cleaved and active caspase-3 and PARP in CNE2 and HK1 cells
(A) Lu and VCR on CNE2. (B) Lu and VCR on HK1. (C) Qu and VCR on HK1.

4.4 Discussion

There are numerous ways to limit cancer cell growth; one of which is chemotherapy: the use of chemical agents which interferes with the rapid division of cells. The untargeted nature of the chemotherapeutics inevitably causes toxic side effects on healthy cells especially those that divide quickly. In recent years, the necessity for more effective treatment with reduced toxic side effects has led to the development of targeted therapy where the anti-cancer agent interferes with macromolecules in cancer cells. Such targeted therapy has resulted in a dramatic improvement in cancer treatments. However problems continue to persist. One of the major setbacks is the development of drug resistance despite the fact that many tumours show an initial good response when they are first exposed to these chemotherapeutics but patients eventually developed resistance to these agents.

Several mechanisms of drug resistance have been identified which include membrane transporters that lead to a decrease in drug accumulation, enhanced DNA damage repair mechanism and multiple deficiencies in the activation of apoptosis (Pauwels et al., 2007; Zhou et al., 2008). In addition, alteration to critical signalling pathways such as the PI3K/Akt pathway has also been associated with drug resistance (Huang and Hung, 2009; Li et al., 2010a). PI3K/Akt pathway is known to play central role in many cellular physiological functions and in many cancers, this pathway is up-regulated. There are also reports that reveal its key role in drug resistance (Huang and Hung, 2009; Li et al., 2010a; Liang et al., 2009). Therefore there is an increasing interest in designing drugs that specifically target this pathway as single agent or in combination to other chemotherapeutic agents to overcome drug resistance.

In this study, we demonstrated that flavonoids like luteolin and quercetin sensitised Akt-expressing cells like nasopharyngeal carcinoma cell lines, HK1 and CNE2 to VCR-induced apoptosis. Our results showed that pre-treatment of HK1 and CNE2 cells with luteolin or quercetin, followed by sub-cytotoxic concentration of VCR was able to induce a significant increase in apoptosis and growth inhibition compared with cells treated with each agent alone. The sensitisation effect by the flavonoid was associated with the activation of caspase-3 which could be abrogated with zVAD-fmk, pan-caspase inhibitor. Hence, the combination of flavonoids like luteolin and quercetin with VCR could potentially be useful in cancer therapy of the nasopharynx. However this study does not answer queries on the molecular interaction of these two chemicals inside cells. Therefore the challenge ahead is to understand the underlying mechanism leading to the augmentation of cell death brought about by flavonoids like luteolin or quercetin together with VCR.

General Discussion and Conclusions

Chapter 5

5.1 Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions

Nasopharyngeal carcinoma (NPC), a head and neck cancer of epithelial origin has a high incidence rate in Southern China and South-East Asia with an incidence rates of between 15 and 50 per 100 000 in man (Ho, 1978). In Singapore, the incidence rates are 18.4 per 100 000 in males and 7.3 per 100 000 in females (Chia et al., 1996). NPC is responsive to radiotherapy for which there is a high local control rate after radical radiotherapy (RT) (Fang et al., 2007a; Lu and Yao, 2008). Studies have illustrated that concurrent radiotherapy and chemotherapy (chemoradiotherapy) results in a statistically significant reduction in failure and cancer-specific deaths compared with radiotherapy alone (Lee et al., 2010a). Chemotherapeutics used in chemoradiotherapy include cisplatin, 5-fluorouracil, vincristine, bleomycin and methotrexate (Airoldi et al., 2010; Kwong et al., 2004; Lin et al., 2003). However, toxicities were observed in some patients after chemotherapy which led to mucositis (Wong et al., 2006), grade III/IV neutropenia (Chua et al., 2004; McCarthy et al., 2002) , grade III/IV anaemia, granulocytopenia thrombocytopenia (Leong et al., 2005; Ngan et al., 2002). The potential use of natural products like flavonoids may circumvent some of these toxicities experienced by patients.

Quercetin (3, 3′, 4′, 5, 7-pentahydroxylflavone) is a flavonol-type flavonoid ubiquitously present in plant-derived foods and is the most abundant amongst the flavonoid family. It is found in a variety of fruits like red onion, grapes, apples, berries, cherries, broccoli, citrus fruits and tea. Many biological effects of quercetin on cells and tissues have been reported (reviewed by (Bischoff, 2008). Of interest in this report is its anti-cancer property. There are

numerous studies that demonstrate the effects of quercetin on signal transductions associated with tumourigenesis and these include cell cycle regulation, apoptosis, pro-inflammatory protein induction and angiogenesis (Hirpara et al., 2009; Murakami et al., 2008).

In this study (experimental details and data are presented in Chapter 2 of this thesis), we demonstrate that quercetin was able to induce cell growth inhibition in two nasopharyngeal carcinoma cell lines, CNE2 and HK1 through two different mechanisms; one by preventing cell cycle progression into the S phase through cell cycle arrest at the G2/M and G0/G1 phases. This was mediated by the inhibition of E2F-1 function via complex formation with the hypophosphorylated form of Rb (Fig 2.3). The second mechanism is the induction of cell death by apoptosis via a p53-independent mitochondrial-initiated pathway. This was mediated by the presence of pro-apoptotic proteins like Bad and Bax in quercetin treated cells which led to subsequent activation of caspase -7 and -3 (Fig 2.6). Another interesting finding is that at a higher concentration and with prolonged incubation quercetin induces necrotic cell death in CNE2 but not in HK1 cells (Fig 2.4 and 2.5). It remains to be investigated how quercetin at higher concentration causes necrosis in CNE2 but not in HK1 cells.

In conclusion, this study (Chapter 2 of this thesis) demonstrates that quercetin is able to arrest cell growth by preventing quercetin treated CNE2 and HK1 cells to progress to the S phase. At low concentration, it is able to induce apoptosis and at higher concentration, necrosis ensued. The molecular mechanism that results in the cell cycle arrest and apoptosis by quercetin is mediated by the inactivation of E2F-1 by Rb and the increase in the presence of

pro-apoptotic proteins like Bad and Bax which leads to the cleavage and activation of caspase -3 and -7.

5.2 Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3β-cyclin D1 pathway

Luteolin (3′, 4′, 5, 7- tetrahydroxylflavone) is a flavonol-type flavonoid ubiquitously present in plant-derived foods. Luteolin-rich vegetables and fruits include celery, parsley, broccoli, onion leaves, carrots, peppers, cabbages, apple skins and chrysanthemum flowers (Miean and Mohamed, 2001; Sun et al., 2007; Xie et al., 2009).

Luteolin exhibits a wide range of biological activities in the prevention and treatment of chronic diseases due to their anti-oxidant, anti-inflammatory, anti-microbial and anti-cancer activities (reviewed by (Lopez-Lazaro, 2009). In our laboratory, luteolin has been found to enhance TNF-α-induced apoptosis in human colorectal cancer COLO205, HCT116 and cervical cancer Hela cells via suppression of NF-κB (Shi et al., 2004). In a second study, pre-treatment of TRAIL-sensitive cancer cells like Hela and TRAIL-resistant cancer cells like CNE1, HT29 and Hep G2 with a non-cytotoxic concentration of luteolin was able to enhance TRAIL-induced apoptosis mediated by caspase -8 and -3 activation (Shi et al., 2005).

At present, the anti-cancer potential of luteolin is mainly based on its ability to induce apoptosis in cancer cells (Lin et al., 2008). However, relatively little is known about the effects of luteolin on cell cycle regulation. Several earlier reports have demonstrated that luteolin induces cell cycle arrest either at G1 by down-regulating cellular protein levels of CDK4 and CDK2 (Casagrande

and Darbon, 2001; Lim do et al., 2007) or G2/M arrest by the inhibition of cdc2 and up-regulation of $p21^{CIP1}$ (Wu et al., 2008).

In this study, we also focussed on the effect of luteolin on cell cycle regulation in human nasopharyngeal carcinoma cells. We identified a different molecular mechanism leading to cell cycle arrest by luteolin. Luteolin inhibits the Akt-GSK-3β-cyclin D1 signalling pathway in the NPC line, HK1 by promoting cyclin D1 phosphorylation and subsequent proteasomal degradation. In the absence of cyclin D1, pRb is maintained in the hypophosphorylated form and this prevents the activation of E2F-1 transcription activity.

Progression from G1 to S phase of the cell cycle is controlled by cyclin Ds and their kinases, namely CDK4 and CDK6, which act by phosphorylating and inactivating Rb, thus liberating E2F-1 transcriptional activity to drive the cells into S phase (Genovese et al., 2006; Malumbres and Barbacid, 2009). In this study, we first defined the critical role of cyclin D1 in luteolin-induced cell cycle arrest. It is well known that cyclin D1 is important in the development of numerous cancers including NPC (Gladden and Diehl, 2005; Tashiro et al., 2007; Xie et al., 2000) (reviewed in Chapter 1.3 of this thesis). Moreover, cyclin D1 over-expression is a common event in cancer and is usually a result of defective regulation at the post-translational level (Kim and Diehl, 2009). Therefore, regulation of the cyclin D1 protein level is one of the critical aspects in cell proliferation and tumour development. An earlier study by Diehl et al (Diehl et al., 1997) demonstrated that cyclin D1 degradation is dependent on Thr^{286} phosphorylation by GSK-3β and ubiquitin-dependent proteasomal degradation. Interestingly, we found that luteolin acts on this signalling pathway in HK1, resulting in the induction of cell cycle arrest. An increase in the phospho form of

cyclin D1 Thr^{286} was observed when HK1 was treated with luteolin. As GSK-3β regulates cyclin D1 degradation, a GSK-3β-specific inhibitor, LiCl, suppressed luteolin-induced down-regulation of this protein, suggesting the involvement of the GSK-3β pathway in luteolin-mediated cell cycle arrest.

Frequent activation of Akt has been reported in many human cancers (Altomare and Testa, 2005; Tokunaga et al., 2008) and GSK-3β has been identified as one of Akt's molecular targets. Akt inactivates GSK-3β kinase activity by site-specific phosphorylation at Ser^{9} which leads to subsequent reduction in cyclin D1 phosphorylation and an increase in its protein stability (Diehl, 2002). In this study, we found that luteolin is capable of inhibiting Akt phosphorylation and activation. It remains to be investigated how luteolin may target PI3K, as suggested by earlier reports (Bagli et al., 2004; Lee et al., 2006).

Interestingly in our study luteolin was unable to induce apoptosis in HK1 and CNE2 (although these cells are responsive to camptothecin-induced apoptosis) when many studies have demonstrated the apoptotic effects of luteolin on numerous cancer cell lines. The exact reason for this lack of apoptotic response to luteolin by HK1 and CNE2 is not clear. One possible explanation is that this group of cells have higher basal level of Akt activation (as demonstrated in Chapter 3 of this thesis), thus making them more susceptible to the inhibitory effect by luteolin on cell cycle via the Akt-GSK-3β-cyclin D1 pathway. It would be of interest to elucidate the underlying mechanisms responsible of the different response by different types of cancer cells.

Thus, this study (Chapter 3 of this thesis) demonstrates that luteolin is able to suppress NPC cells proliferation via cell cycle arrest by targeting the Akt-GSK-3β-cyclin D1 signalling axis. Since Akt is often over-activated in many human

cancers including NPC, it is thus believed that the data from this study support the potential application of luteolin as a chemotherapeutic or chemopreventive agent in human cancer.

5.3 Luteolin and quercetin sensitise NPC cells to the cytotoxic effect of chemotherapeutics

The traditional treatment for NPC includes radiotherapy or chemotherapy or a combination of both (chemoradiotherapy). Concurrent chemotherapy together with radiotherapy is able to reduce failure and cancer-specific deaths when compared with radiotherapy alone in patients (Lee et al., 2010a). However, cumulative incidence of acute toxicity increases with chemotherapy as observed in numerous studies (Lee et al., 2010a; Lu et al., 2009). Moreover many patients eventually develop resistance to anti-cancer agents. Thus, acquired resistance to chemotherapy is another major hurdle to successful cancer treatment. Understanding the underlying mechanisms in which tumours become resistance to particular therapeutics is therefore critical.

Several mechanisms of drug resistance have been associated with major signal transduction pathways including the PI3K/Akt pathway (Li et al., 2010a; Liang et al., 2009). There is now an increasing interest in designing drugs that specifically target this pathway as single agent or in combination with other chemotherapeutics to overcome drug resistance.

In this study, we combined the effects of VCR, a chemotherapeutic that affect microtubule dynamics in cells with flavonoids like luteolin and quercetin which can inhibit the PI3K/Akt pathway. Both agents were added to cells at sub-cytotoxic concentrations.

Microscopic examination illustrated that the combined treatment of flavonoid (either luteolin or quercetin) and vincristine led to cell death but not for cells treated with either flavonoid or vincristine alone. The percentage of cell death by caspase-3-dependent apoptosis was quantified by flow cytometry and verified by western blot experiments that detected apoptotic markers like active caspase-3 and PARP. The presence of zVAD-fmk, a pan-caspase inhibitor was able to abrogate the combined cytotoxic effect of flavonoid and vincristine on both cell lines.

The data obtained from this study was also analysed based on analysis variance by One-way ANOVA using the statistical analysis software from Statgraphics® Centurion XVI. Results indicated that luteolin and quercetin were able to sensitise HK1 and CNE2 cells to VCR, resulting in apoptotic cell death, whereas the individual reagents were unable to kill the cells.

This preliminary study however, does not answer queries on how the two chemicals interact with each other to enhance cell death by apoptosis. It will be interesting to elucidate the underlying cell signalling pathways leading to the sensitisation of NPC cells to vincristine induced by luteolin and quercetin which subsequently resulted in the augmentation of cell death in these cells.

5.4 Future studies

It has been estimated that 75 – 85% of all chronic illness and diseases are associated with lifestyle and cannot be explained by differences in genetic makeup alone (Wong et al., 2005). A review of 206 human epidemiological studies and 22 animal studies conducted by Steinmetz & Potter demonstrated an inverse relationship between consumption of fruits and vegetables; and the risk of

developing cancers of the stomach, oesophagus, lung, oral cavity, pharynx, endometrium, pancreas and colon (Steinmetz and Potter, 1996).

While fruits and vegetables are recommended for the prevention of cancer as well as other diseases, the active components and their underlying mechanisms leading to cancer cell growth and inhibition are not well understood. Although extensive research over the past decades has identified various molecular targets that these bio-active reagents can potentially inhibit and prevent cancer, however lack of success with targeted monotherapy using these agents has redirected researchers to employ either combination therapy or agents that interfere with multiple cell signalling pathways. Aggarwal and Shishodia have presented a review identifying known bio-active agents in fruits and vegetables and their effects on several cell signalling pathways (Aggarwal and Shishodia, 2006). Such agents include curcumin, genistein, lycopene, capsaicin, diosgenin, luteolin, quercetin, catechins and beta carotene to name a few. Major cell signalling pathways affected by these bio-active agents include the NK-κB, AP-1, STAT3, Akt, Bcl-2, Bcl-X_L, caspases, PARP, IKK, EGFR, HER2, JNK, MAPK and COX2 (reviewed by (Aggarwal and Shishodia, 2006).

To translate the successful and meaningful data obtained from studies performed in *in vitro* cell culture systems and animal models to human with beneficial pharmacological effects, several challenges and obstacles need to be overcome. One most immediate challenge will be the ability to deliver and accumulate concentration of bio-active agents in tissues and organs high enough to achieve chemopreventive effect. Most preclinical mechanistic studies on dietary bio-active agents performed in cell lines and animal models may have adopted significantly higher doses than the amounts that are consumed in our

daily diet. Unlike therapeutics like tamoxifen used for breast cancer prevention, dietary chemopreventive bio-active agents might not possess the optimum pharmacokinetics and toxicology profiles. Due to their natural chemical properties some of them may have poor absorption in the gut upon oral ingestion, extensive metabolic breakdown leading to rapid clearance from the body which render them unavailable or make them available in a pharmacologically inactive form. If ever a high blood concentration of the bio-active agent is achieved, cytotoxicity to normal cells may be observed. To overcome some of these inherent problems associated with bio-active agents, potential agents can be chemically modified to make them less resistant to metabolism, more stable in the blood and therefore can reach target tissues at a concentration that is toxic to cancer cells but not to the normal cells.

Another practical strategy is to explore the use of bio-active agents together with current therapeutics whereby a cytotoxic synergism can be reached to augment cell death with lower concentrations of both chemicals and yet achieve the same efficacy *in vivo* when patients are exposed to a higher but toxic concentration of the individual agents. In our study, we have reported a preliminary but promising result in which flavonoids like luteolin and quercetin are able to sensitise NPC cells to sub-cytotoxic concentration of VCR and yet achieve cell death. However to bring this work to fruition with the ultimate aim of using the combination of flavonoids with other therapeutics, it will require more work to be done to truly understand and map the molecular targets of luteolin and quercetin which ultimately results in cell death, establishing animal models to verify the efficacy of these treatments *in vivo* and at the same time,

study the various mechanism to deliver these bio-active agents to target sites at concentration high enough to bring about cytotoxic effect on cancer cells.

5.5 Conclusions

In this study, we have systemically investigated the anti-cancer property of flavonoids like quercetin and luteolin on human nasopharyngeal carcinoma cells and we have:

1. identified the mechanism leading to quercetin-mediated cell cycle arrest in S phase was due to the inhibition of E2F-1 activity. In addition, quercetin induced apoptosis through Bad and Bax.
2. demonstrated that luteolin inhibited cell cycle progression at G1 phase was via the Akt-GSK-3β-cyclin D1 pathway, which resulted in enhanced protein phosphorylation of cyclin D1 and subsequent proteasomal degradation of this protein. Luteolin, however was unable to induce apoptosis in NPC cells.
3. illustrated the sensitisation effect of luteolin and quercetin on apoptosis induced by vincristine.

Our findings provide evidence to support the potential application of quercetin and luteolin as chemopreventive and chemotherapeutic chemicals or when used in combination with other chemotherapeutics as chemosensitiser in cancer treatment and management.

One must also be cautioned that data obtained from *in vitro* cell culture system do not translate into successful results in an *in vivo* environment. Thus *in*

vivo studies using animal models will be required to verify the potential applications of these flavonoids in cancer prevention and management.

In conclusion, this work reaffirms what Hippocrates said 2500 years ago, I quote ***"Let food by thy medicine and medicine be thy food'*** unquote.

References

Aalinkeel, R., Bindukumar, B., Reynolds, J.L., Sykes, D.E., Mahajan, S.D., Chadha, K.C., and Schwartz, S.A. (2008). The dietary bioflavonoid, quercetin, selectively induces apoptosis of prostate cancer cells by down-regulating the expression of heat shock protein 90. Prostate *68*, 1773-1789.
Abrams, S.L., Steelman, L.S., Shelton, J.G., Wong, E.W., Chappell, W.H., Basecke, J., Stivala, F., Donia, M., Nicoletti, F., Libra, M.*, et al.* (2010). The Raf/MEK/ERK pathway can govern drug resistance, apoptosis and sensitivity to targeted therapy. Cell Cycle *9*, 1781-1791.
Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X., and Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell *9*, 423-432.
Adachi, H., Igawa, M., Shiina, H., Urakami, S., Shigeno, K., and Hino, O. (2003). Human bladder tumors with 2-hit mutations of tumor suppressor gene TSC1 and decreased expression of p27. J Urol *170*, 601-604.
Adams, L.S., Phung, S., Yee, N., Seeram, N.P., Li, L., and Chen, S. (2010). Blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway. Cancer Res *70*, 3594-3605.
Adams, P.D. (2001). Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. Biochim Biophys Acta *1471*, M123-133.
Adams, P.D., Sellers, W.R., Sharma, S.K., Wu, A.D., Nalin, C.M., and Kaelin, W.G., Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol Cell Biol *16*, 6623-6633.
Aggarwal, B.B. (2000). Apoptosis and nuclear factor-[kappa]b: a tale of association and dissociation. Biochemical Pharmacology *60*, 1033-1039.
Aggarwal, B.B., and Shishodia, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol *71*, 1397-1421.
Aggarwal, S., Ichikawa, H., Takada, Y., Sandur, S.K., Shishodia, S., and Aggarwal, B.B. (2006). Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IkappaBalpha kinase and Akt activation. Mol Pharmacol *69*, 195-206.
Airoldi, M., Gabriele, P., Gabriele, A.M., Garzaro, M., Raimondo, L., Pedani, F., Beatrice, F., Pecorari, G., and Giordano, C. (2010). Induction chemotherapy with carboplatin and taxol followed by radiotherapy and concurrent weekly carboplatin + taxol in locally advanced nasopharyngeal carcinoma. Cancer Chemother Pharmacol.
Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J *15*, 6541-6551.
Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D.*, et al.* (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol *7*, 776-789.
Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. Cell *87*, 171.
Alt, J.R., Cleveland, J.L., Hannink, M., and Diehl, J.A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev *14*, 3102-3114.
Altomare, D.A., and Testa, J.R. (2005). Perturbations of the AKT signaling pathway in human cancer. Oncogene *24*, 7455-7464.
Anderson, R.F., Amarasinghe, C., Fisher, L.J., Mak, W.B., and Packer, J.E. (2000). Reduction in free-radical-induced DNA strand breaks and base damage through fast chemical repair by flavonoids. Free Radic Res *33*, 91-103.

Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A., and Nicotera, P. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron *15*, 961-973.
Arcaro, A., and Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J *296 (Pt 2)*, 297-301.
Arden, K.C., and Biggs, W.H., 3rd (2002). Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signaling. Arch Biochem Biophys *403*, 292-298.
Armstrong, R.W., Imrey, P.B., Lye, M.S., Armstrong, J.A., Yu, M.C., and Sani, S. (1998). Nasopharyngeal carcinoma in Malaysian Chinese: salted fish and other dietary exposures. Int J Cancer *77*, 228-235.
Ashe, P.C., and Berry, M.D. (2003). Apoptotic signaling cascades. Prog Neuropsychopharmacol Biol Psychiatry *27*, 199-214.
Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science *281*, 1305-1308.
Attwooll, C., Lazzerini Denchi, E., and Helin, K. (2004). The E2F family: specific functions and overlapping interests. EMBO J *23*, 4709-4716.
Azli, N., Armand, J.P., Rahal, M., Wibault, P., Boussen, H., Eschwege, F., Schwaab, G., Gasmi, J., Bachouchi, M., and Cvitkovic, E. (1992). Alternating chemo-radiotherapy with cisplatin and 5-fluorouracil plus bleomycin by continuous infusion for locally advanced undifferentiated carcinoma nasopharyngeal type. Eur J Cancer *28A*, 1792-1797.
Bagli, E., Stefaniotou, M., Morbidelli, L., Ziche, M., Psillas, K., Murphy, C., and Fotsis, T. (2004). Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity. Cancer Res *64*, 7936-7946.
Bai, Y., Mao, Q.Q., Qin, J., Zheng, X.Y., Wang, Y.B., Yang, K., Shen, H.F., and Xie, L.P. (2010). Resveratrol induces apoptosis and cell cycle arrest of human T24 bladder cancer cells in vitro and inhibits tumor growth in vivo. Cancer Sci *101*, 488-493.
Balmain, A., Gray, J., and Ponder, B. (2003). The genetics and genomics of cancer. Nat Genet *33 Suppl*, 238-244.
Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y*., et al.* (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.
Bashir, T., Dorrello, N.V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004). Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature *428*, 190-193.
Beijersbergen, R.L., and Bernards, R. (1996). Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochim Biophys Acta *1287*, 103-120.
Bell, D.W. (2010). Our changing view of the genomic landscape of cancer. J Pathol *220*, 231-243.
Bertelsen, B.I., Steine, S.J., Sandvei, R., Molven, A., and Laerum, O.D. (2006). Molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia: frequent PIK3CA amplification and AKT phosphorylation. Int J Cancer *118*, 1877-1883.
Bertram, J.S. (2000). The molecular biology of cancer. Mol Aspects Med *21*, 167-223.
Besson, A., Dowdy, S.F., and Roberts, J.M. (2008). CDK inhibitors: cell cycle regulators and beyond. Dev Cell *14*, 159-169.
Besson, A., Hwang, H.C., Cicero, S., Donovan, S.L., Gurian-West, M., Johnson, D., Clurman, B.E., Dyer, M.A., and Roberts, J.M. (2007). Discovery of an oncogenic activity in p27Kip1 that causes stem cell expansion and a multiple tumor phenotype. Genes Dev *21*, 1731-1746.

Bharti, A.C., Donato, N., Singh, S., and Aggarwal, B.B. (2003). Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood *101*, 1053-1062.
Biggs, W.H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W.K., and Arden, K.C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A *96*, 7421-7426.
Bignold, L.P. (2004). Chaotic genomes make chaotic cells: the mutator phenotype theory of carcinogenesis applied to clinicopathological relationships of solid tumors. Cancer Invest *22*, 338-343.
Bischoff, S.C. (2008). Quercetin: potentials in the prevention and therapy of disease. Curr Opin Clin Nutr Metab Care *11*, 733-740.
Blagosklonny, M.V., and Pardee, A.B. (2002). The restriction point of the cell cycle. Cell Cycle *1*, 103-110.
Blain, S.W. (2008). Switching cyclin D-Cdk4 kinase activity on and off. Cell Cycle *7*, 892-898.
Blake, M.C., and Azizkhan, J.C. (1989). Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol Cell Biol *9*, 4994-5002.
Block, G., Patterson, B., and Subar, A. (1992). Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer *18*, 1-29.
Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.
Bokkenheuser, V.D., Shackleton, C.H., and Winter, J. (1987). Hydrolysis of dietary flavonoid glycosides by strains of intestinal Bacteroides from humans. Biochem J *248*, 953-956.
Bors, W., Heller, W., Michel, C., and Saran, M. (1990). Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods Enzymol *186*, 343-355.
Bostrom, J., Meyer-Puttlitz, B., Wolter, M., Blaschke, B., Weber, R.G., Lichter, P., Ichimura, K., Collins, V.P., and Reifenberger, G. (2001). Alterations of the tumor suppressor genes CDKN2A (p16(INK4a)), p14(ARF), CDKN2B (p15(INK4b)), and CDKN2C (p18(INK4c)) in atypical and anaplastic meningiomas. Am J Pathol *159*, 661-669.
Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev *56*, 317-333.
Brazil, D.P., Yang, Z.Z., and Hemmings, B.A. (2004). Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci *29*, 233-242.
Brenner, D., and Mak, T.W. (2009). Mitochondrial cell death effectors. Curr Opin Cell Biol *21*, 871-877.
Brizova, H., Kalinova, M., Krskova, L., Mrhalova, M., and Kodet, R. (2008). Quantitative monitoring of cyclin D1 expression: a molecular marker for minimal residual disease monitoring and a predictor of the disease outcome in patients with mantle cell lymphoma. Int J Cancer *123*, 2865-2870.
Brooks, C.L., and Gu, W. (2004). Dynamics in the p53-Mdm2 ubiquitination pathway. Cell Cycle *3*, 895-899.
Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.
Brown, C.J., Lain, S., Verma, C.S., Fersht, A.R., and Lane, D.P. (2009). Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer *9*, 862-873.
Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol *15*, 269-290.
Buell, P. (1974). The effect of migration on the risk of nasopharyngeal cancer among Chinese. Cancer Res *34*, 1189-1191.
Bukholm, I.R., Bukholm, G., and Nesland, J.M. (2001). Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast carcinomas. Int J Cancer *93*, 283-287.
Burda, S., and Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. J Agric Food Chem *49*, 2774-2779.
Burgering, B.M., and Medema, R.H. (2003). Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol *73*, 689-701.
Burgos, J.S. (2005). Involvement of the Epstein-Barr virus in the nasopharyngeal carcinoma pathogenesis. Med Oncol *22*, 113-121.
Byun, D.S., Cho, K., Ryu, B.K., Lee, M.G., Park, J.I., Chae, K.S., Kim, H.J., and Chi, S.G. (2003). Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma. Int J Cancer *104*, 318-327.
Cangi, M.G., Piccinin, S., Pecciarini, L., Talarico, A., Dal Cin, E., Grassi, S., Grizzo, A., Maestro, R., and Doglioni, C. (2008). Constitutive overexpression of CDC25A in primary human mammary epithelial cells results in both defective DNA damage response and chromosomal breaks at fragile sites. Int J Cancer *123*, 1466-1471.
Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.
Cardenas, M., Marder, M., Blank, V.C., and Roguin, L.P. (2006). Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. Bioorg Med Chem *14*, 2966-2971.
Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed, J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.
Carnero, A. (2010). The PKB/AKT pathway in cancer. Curr Pharm Des *16*, 34-44.
Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, S.L., Robbins, C.M., Hostetter, G., Boguslawski, S., Moses, T.Y., Savage, S.*, et al.* (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439-444.
Casagrande, F., and Darbon, J.M. (2001). Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem Pharmacol *61*, 1205-1215.
Catz, S.D., and Johnson, J.L. (2001). Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene *20*, 7342-7351.
Chan, A., and Verrill, M. (2009). Capecitabine and vinorelbine in metastatic breast cancer. Eur J Cancer *45*, 2253-2265.
Chan, A.T., Ma, B.B., Lo, Y.M., Leung, S.F., Kwan, W.H., Hui, E.P., Mok, T.S., Kam, M., Chan, L.S., Chiu, S.K.*, et al.* (2004). Phase II study of neoadjuvant carboplatin and paclitaxel followed by radiotherapy and concurrent cisplatin in patients with locoregionally advanced nasopharyngeal carcinoma: therapeutic monitoring with plasma Epstein-Barr virus DNA. J Clin Oncol *22*, 3053-3060.
Chan, A.T., Teo, P.M., and Johnson, P.J. (2003). Nasopharyngeal cancer. Cancer Treat Res *114*, 275-293.
Chan, C.M., Ma, B.B., Wong, S.C., and Chan, A.T. (2005). Celecoxib induces dose dependent growth inhibition in nasopharyngeal carcinoma cell lines independent of cyclooxygenase-2 expression. Biomed Pharmacother *59 Suppl 2*, S268-271.
Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem *68*, 965-1014.

Chappuis, P.O., Kapusta, L., Begin, L.R., Wong, N., Brunet, J.S., Narod, S.A., Slingerland, J., and Foulkes, W.D. (2000). Germline BRCA1/2 mutations and p27(Kip1) protein levels independently predict outcome after breast cancer. J Clin Oncol *18*, 4045-4052.
Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev *14*, 278-288.
Chen, J., Saha, P., Kornbluth, S., Dynlacht, B.D., and Dutta, A. (1996). Cyclin-binding motifs are essential for the function of p21CIP1. Mol Cell Biol *16*, 4673-4682.
Chen, M.K., Lee, H.S., Chang, J.H., and Chang, C.C. (2004). Expression of p53 protein and primary tumour volume in patients with nasopharyngeal carcinoma. J Otolaryngol *33*, 304-307.
Chen, T.M., and Defendi, V. (1992). Functional interaction of p53 with HPV18 E6, c-myc and H-ras in 3T3 cells. Oncogene *7*, 1541-1547.
Chène, P. (2003). Inhibiting the p53–MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer *3*, 102-109.
Cheng, A.C., Huang, T.C., Lai, C.S., and Pan, M.H. (2005). Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. Eur J Pharmacol *509*, 1-10.
Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell *8*, 705-711.
Cheng, M., Sexl, V., Sherr, C.J., and Roussel, M.F. (1998). Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc Natl Acad Sci U S A *95*, 1091-1096.
Cheng, S.H., Tsai, S.Y., Yen, K.L., Jian, J.J., Chu, N.M., Chan, K.Y., Tan, T.D., Cheng, J.C., Hsieh, C.Y., and Huang, A.T. (2000). Concomitant radiotherapy and chemotherapy for early-stage nasopharyngeal carcinoma. J Clin Oncol *18*, 2040-2045.
Chia, K.K., Lee, H.P., and Seow, A. (1996). Treands in cancer incidence in Singapore 1968-1992. Singapore Cancer Registry Report *4*, 70-71.
Chien, S.Y., Wu, Y.C., Chung, J.G., Yang, J.S., Lu, H.F., Tsou, M.F., Wood, W.G., Kuo, S.J., and Chen, D.R. (2009). Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. Hum Exp Toxicol *28*, 493-503.
Chinnaiyan, A.M., Tepper, C.G., Seldin, M.F., O'Rourke, K., Kischkel, F.C., Hellbardt, S., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996). FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem *271*, 4961-4965.
Chinni, S.R., and Sarkar, F.H. (2002). Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. Clin Cancer Res *8*, 1228-1236.
Chipuk, J.E., Fisher, J.C., Dillon, C.P., Kriwacki, R.W., Kuwana, T., and Green, D.R. (2008). Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. Proc Natl Acad Sci U S A *105*, 20327-20332.
Choi, E.J., Bae, S.M., and Ahn, W.S. (2008). Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. Arch Pharm Res *31*, 1281-1285.
Choi, J.A., Kim, J.Y., Lee, J.Y., Kang, C.M., Kwon, H.J., Yoo, Y.D., Kim, T.W., Lee, Y.S., and Lee, S.J. (2001). Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int J Oncol *19*, 837-844.
Choisy-Rossi, C., Reisdorf, P., and Yonish-Rouach, E. (1998). Mechanisms of p53-induced apoptosis: in search of genes which are regulated during p53-mediated cell death. Toxicol Lett *102-103*, 491-496.

Chou, J., Lin, Y.C., Kim, J., You, L., Xu, Z., He, B., and Jablons, D.M. (2008). Nasopharyngeal carcinoma--review of the molecular mechanisms of tumorigenesis. Head Neck *30*, 946-963.
Chua, D.T., Sham, J.S., and Au, G.K. (2004). A concurrent chemoirradiation with cisplatin followed by adjuvant chemotherapy with ifosfamide, 5-fluorouracil, and leucovorin for stage IV nasopharyngeal carcinoma. Head Neck *26*, 118-126.
Chua, D.T., Sham, J.S., and Au, G.K. (2005). A phase II study of docetaxel and cisplatin as first-line chemotherapy in patients with metastatic nasopharyngeal carcinoma. Oral Oncol *41*, 589-595.
Chung, L.Y., Cheung, T.C., Kong, S.K., Fung, K.P., Choy, Y.M., Chan, Z.Y., and Kwok, T.T. (2001). Induction of apoptosis by green tea catechins in human prostate cancer DU145 cells. Life Sci *68*, 1207-1214.
Classon, M., and Dyson, N. (2001). p107 and p130: versatile proteins with interesting pockets. Exp Cell Res *264*, 135-147.
Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer *2*, 910-917.
Coats, S., Flanagan, W.M., Nourse, J., and Roberts, J.M. (1996). Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science *272*, 877-880.
Cohen, G.M. (1997). Caspases: the executioners of apoptosis. Biochem J *326 (Pt 1)*, 1-16.
Cos, P., De Bruyne, T., Apers, S., Vanden Berghe, D., Pieters, L., and Vlietinck, A.J. (2003). Phytoestrogens: recent developments. Planta Med *69*, 589-599.
Courtney, K.D., Corcoran, R.B., and Engelman, J.A. (2010). The PI3K pathway as drug target in human cancer. J Clin Oncol *28*, 1075-1083.
Cova, D., De Angelis, L., Giavarini, F., Palladini, G., and Perego, R. (1992). Pharmacokinetics and metabolism of oral diosmin in healthy volunteers. Int J Clin Pharmacol Ther Toxicol *30*, 29-33.
Crook, T., and Vousden, K.H. (1994). Interaction of HPV E6 with p53 and associated proteins. Biochem Soc Trans *22*, 52-55.
Dahia, P.L. (2000). PTEN, a unique tumor suppressor gene. Endocr Relat Cancer *7*, 115-129.
Dales, J.P., Beaufils, N., Silvy, M., Picard, C., Pauly, V., Pradel, V., Formisano-Treziny, C., Bonnier, P., Giusiano, S., Charpin, C*., et al.* (2010). Hypoxia inducible factor 1alpha gene (HIF-1alpha) splice variants: potential prognostic biomarkers in breast cancer. BMC Med *8*, 44.
Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.
Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. Genes Dev *13*, 2905-2927.
Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.
Davies, M.A., Stemke-Hale, K., Tellez, C., Calderone, T.L., Deng, W., Prieto, V.G., Lazar, A.J., Gershenwald, J.E., and Mills, G.B. (2008). A novel AKT3 mutation in melanoma tumours and cell lines. Br J Cancer *99*, 1265-1268.
De Dosso, S., and Berthold, D.R. (2008). Docetaxel in the management of prostate cancer: current standard of care and future directions. Expert Opin Pharmacother *9*, 1969-1979.
de Kok, T.M., van Breda, S.G., and Manson, M.M. (2008). Mechanisms of combined action of different chemopreventive dietary compounds: a review. Eur J Nutr *47 Suppl 2*, 51-59.

De la Cueva, E., Garcia-Cao, I., Herranz, M., Lopez, P., Garcia-Palencia, P., Flores, J.M., Serrano, M., Fernandez-Piqueras, J., and Martin-Caballero, J. (2006). Tumorigenic activity of p21Waf1/Cip1 in thymic lymphoma. Oncogene *25*, 4128-4132.
Debies, M.T., Gestl, S.A., Mathers, J.L., Mikse, O.R., Leonard, T.L., Moody, S.E., Chodosh, L.A., Cardiff, R.D., and Gunther, E.J. (2008). Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19Arf/p53 pathway lesions but not p16 Ink4a loss. J Clin Invest *118*, 51-63.
Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., and Yuan, J. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol *1*, 112-119.
Demirhan, O., Tastemir, D., Hasturk, S., Kuleci, S., and Hanta, I. (2010). Alterations in p16 and p53 genes and chromosomal findings in patients with lung cancer: Fluorescence in situ hybridization and cytogenetic studies. Cancer Epidemiol.
Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J *17*, 2215-2223.
Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.
Diehl, J.A. (2002). Cycling to cancer with cyclin D1. Cancer Biol Ther *1*, 226-231.
Diehl, J.A., Cheng, M., Roussel, M.F., and Sherr, C.J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev *12*, 3499-3511.
Diehl, J.A., Zindy, F., and Sherr, C.J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev *11*, 957-972.
Dou, Q.P., Zhao, S., Levin, A.H., Wang, J., Helin, K., and Pardee, A.B. (1994). G1/S-regulated E2F-containing protein complexes bind to the mouse thymidine kinase gene promoter. J Biol Chem *269*, 1306-1313.
Downward, J. (2004). PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol *15*, 177-182.
Draetta, G., and Eckstein, J. (1997). Cdc25 protein phosphatases in cell proliferation. Biochim Biophys Acta *1332*, M53-63.
Draviam, V.M., Orrechia, S., Lowe, M., Pardi, R., and Pines, J. (2001). The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. J Cell Biol *152*, 945-958.
Du, K., and Montminy, M. (1998). CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem *273*, 32377-32379.
Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J., and Reed, S.I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell *76*, 1013-1023.
Dunn, J.M., Phillips, R.A., Becker, A.J., and Gallie, B.L. (1988). Identification of germline and somatic mutations affecting the retinoblastoma gene. Science *241*, 1797-1800.
Duthie, G., and Crozier, A. (2000). Plant-derived phenolic antioxidants. Curr Opin Lipidol *11*, 43-47.
Eaton, E.A., Walle, U.K., Lewis, A.J., Hudson, T., Wilson, A.A., and Walle, T. (1996). Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase. Potential role in drug metabolism and chemoprevention. Drug Metab Dispos *24*, 232-237.
Edderkaoui, M., Odinokova, I., Ohno, I., Gukovsky, I., Go, V.L., Pandol, S.J., and Gukovskaya, A.S. (2008). Ellagic acid induces apoptosis through inhibition of nuclear factor kappa B in pancreatic cancer cells. World J Gastroenterol *14*, 3672-3680.
Eden, T.O., Pieters, R., and Richards, S. (2010). Systematic review of the addition of vincristine plus steroid pulses in maintenance treatment for childhood acute

lymphoblastic leukaemia - an individual patient data meta-analysis involving 5,659 children. Br J Haematol *149*, 722-733.
Ekberg, J., Holm, C., Jalili, S., Richter, J., Anagnostaki, L., Landberg, G., and Persson, J.L. (2005). Expression of cyclin A1 and cell cycle proteins in hematopoietic cells and acute myeloid leukemia and links to patient outcome. Eur J Haematol *75*, 106-115.
el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.
Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43-50.
Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet *7*, 606-619.
Erlund, I., Kosonen, T., Alfthan, G., Maenpaa, J., Perttunen, K., Kenraali, J., Parantainen, J., and Aro, A. (2000). Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. Eur J Clin Pharmacol *56*, 545-553.
Estrov, Z., Shishodia, S., Faderl, S., Harris, D., Van, Q., Kantarjian, H.M., Talpaz, M., and Aggarwal, B.B. (2003). Resveratrol blocks interleukin-1beta-induced activation of the nuclear transcription factor NF-kappaB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. Blood *102*, 987-995.
Etzioni, R., Urban, N., Ramsey, S., McIntosh, M., Schwartz, S., Reid, B., Radich, J., Anderson, G., and Hartwell, L. (2003). The case for early detection. Nat Rev Cancer *3*, 243-252.
Ezhevsky, S.A., Ho, A., Becker-Hapak, M., Davis, P.K., and Dowdy, S.F. (2001). Differential regulation of retinoblastoma tumor suppressor protein by G(1) cyclin-dependent kinase complexes in vivo. Mol Cell Biol *21*, 4773-4784.
Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature *410*, 842-847.
Fang, F.M., Tsai, W.L., Chen, H.C., Hsu, H.C., Hsiung, C.Y., Chien, C.Y., and Ko, S.F. (2007a). Intensity-modulated or conformal radiotherapy improves the quality of life of patients with nasopharyngeal carcinoma: comparisons of four radiotherapy techniques. Cancer *109*, 313-321.
Fang, J., Zhou, Q., Shi, X.L., and Jiang, B.H. (2007b). Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells. Carcinogenesis *28*, 713-723.
Fernandez, V., Hartmann, E., Ott, G., Campo, E., and Rosenwald, A. (2005). Pathogenesis of mantle-cell lymphoma: all oncogenic roads lead to dysregulation of cell cycle and DNA damage response pathways. J Clin Oncol *23*, 6364-6369.
Ferreira, R., Naguibneva, I., Mathieu, M., Ait-Si-Ali, S., Robin, P., Pritchard, L.L., and Harel-Bellan, A. (2001). Cell cycle-dependent recruitment of HDAC-1 correlates with deacetylation of histone H4 on an Rb-E2F target promoter. EMBO Rep *2*, 794-799.
Floyd, S., Pines, J., and Lindon, C. (2008). APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. Curr Biol *18*, 1649-1658.
Fojo, T., and Menefee, M. (2007). Mechanisms of multidrug resistance: the potential role of microtubule-stabilizing agents. Ann Oncol *18 Suppl 5*, v3-8.
Franke, T.F. (2008). PI3K/Akt: getting it right matters. Oncogene *27*, 6473-6488.
Friedberg, E.C., McDaniel, L.D., and Schultz, R.A. (2004). The role of endogenous and exogenous DNA damage and mutagenesis. Curr Opin Genet Dev *14*, 5-10.
Frye, D.K. (2010). Advances in breast cancer treatment: the emerging role of ixabepilone. Expert Rev Anticancer Ther *10*, 23-32.

Furnari, B., Blasina, A., Boddy, M.N., McGowan, C.H., and Russell, P. (1999). Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. Mol Biol Cell *10*, 833-845.
Gannon, J.V., Greaves, R., Iggo, R., and Lane, D.P. (1990). Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J *9*, 1595-1602.
Garcia, M., Jemal, A., Ward, E., Center, M., Hao, Y., and Siegel, R. (2007). Global Cancer Facts & Figures. The Americal Cancer Society.
Gartel, A.L., and Tyner, A.L. (2002). The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther *1*, 639-649.
Geng, Y., Eaton, E.N., Picon, M., Roberts, J.M., Lundberg, A.S., Gifford, A., Sardet, C., and Weinberg, R.A. (1996). Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. Oncogene *12*, 1173-1180.
Genovese, C., Trani, D., Caputi, M., and Claudio, P.P. (2006). Cell cycle control and beyond: emerging roles for the retinoblastoma gene family. Oncogene *25*, 5201-5209.
Giacinti, C., and Giordano, A. (2006). RB and cell cycle progression. Oncogene *25*, 5220-5227.
Gills, J.J., Holbeck, S., Hollingshead, M., Hewitt, S.M., Kozikowski, A.P., and Dennis, P.A. (2006). Spectrum of activity and molecular correlates of response to phosphatidylinositol ether lipid analogues, novel lipid-based inhibitors of Akt. Mol Cancer Ther *5*, 713-722.
Gladden, A.B., and Diehl, J.A. (2005). Location, location, location: the role of cyclin D1 nuclear localization in cancer. J Cell Biochem *96*, 906-913.
Glykofrydes, D., Niphuis, H., Kuhn, E.M., Rosenwirth, B., Heeney, J.L., Bruder, J., Niedobitek, G., Muller-Fleckenstein, I., Fleckenstein, B., and Ensser, A. (2000). Herpesvirus saimiri vFLIP provides an antiapoptotic function but is not essential for viral replication, transformation, or pathogenicity. J Virol *74*, 11919-11927.
Goldsmith, D.B., West, T.M., and Morton, R. (2002). HLA associations with nasopharyngeal carcinoma in Southern Chinese: a meta-analysis. Clin Otolaryngol Allied Sci *27*, 61-67.
Gomez, L.A., de Las Pozas, A., and Perez-Stable, C. (2006). Sequential combination of flavopiridol and docetaxel reduces the levels of X-linked inhibitor of apoptosis and AKT proteins and stimulates apoptosis in human LNCaP prostate cancer cells. Mol Cancer Ther *5*, 1216-1226.
Gonzalez-Gallego, J., Sanchez-Campos, S., and Tunon, M.J. (2007). Anti-inflammatory properties of dietary flavonoids. Nutr Hosp *22*, 287-293.
Goto, T., and Takano, M. (2009). Transcriptional role of FOXO1 in drug resistance through antioxidant defense systems. Adv Exp Med Biol *665*, 171-179.
Grana, X., and Reddy, E.P. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene *11*, 211-219.
Green, D.R., and Reed, J.C. (1998). Mitochondria and apoptosis. Science *281*, 1309-1312.
Gross, A., Yin, X.M., Wang, K., Wei, M.C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S.J. (1999). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem *274*, 1156-1163.
Guo, Q., Zhao, L., You, Q., Yang, Y., Gu, H., Song, G., Lu, N., and Xin, J. (2007). Anti-hepatitis B virus activity of wogonin in vitro and in vivo. Antiviral Res *74*, 16-24.
Guo, S., Rena, G., Cichy, S., He, X., Cohen, P., and Unterman, T. (1999). Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. J Biol Chem *274*, 17184-17192.

Guo, W., Kong, E., and Meydani, M. (2009). Dietary polyphenols, inflammation, and cancer. Nutr Cancer *61*, 807-810.
Gupta, S., Ahmad, N., Nieminen, A.L., and Mukhtar, H. (2000). Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicol Appl Pharmacol *164*, 82-90.
Hadi, S.M., Asad, S.F., Singh, S., and Ahmad, A. (2000). Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. IUBMB Life *50*, 167-171.
Haenszel, W., and Kurihara, M. (1968). Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. J Natl Cancer Inst *40*, 43-68.
Hagting, A., Den Elzen, N., Vodermaier, H.C., Waizenegger, I.C., Peters, J.M., and Pines, J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. J Cell Biol *157*, 1125-1137.
Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol *9*, 680-689.
Hall, M., and Peters, G. (1996). Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv Cancer Res *68*, 67-108.
Hallor, K.H., Staaf, J., Jonsson, G., Heidenblad, M., Vult von Steyern, F., Bauer, H.C., Ijszenga, M., Hogendoorn, P.C., Mandahl, N., Szuhai, K.*, et al.* (2008). Frequent deletion of the CDKN2A locus in chordoma: analysis of chromosomal imbalances using array comparative genomic hybridisation. Br J Cancer *98*, 434-442.
Hamlin, J.L., Mosca, P.J., and Levenson, V.V. (1994). Defining origins of replication in mammalian cells. Biochim Biophys Acta *1198*, 85-111.
Han, S.S., Chung, S.T., Robertson, D.A., Ranjan, D., and Bondada, S. (1999). Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B, and p53. Clin Immunol *93*, 152-161.
Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
Hanley, A.J., Choi, B.C., and Holowaty, E.J. (1995). Cancer mortality among Chinese migrants: a review. Int J Epidemiol *24*, 255-265.
Hannon, G.J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature *371*, 257-261.
Hao, Z., Duncan, G.S., Chang, C.C., Elia, A., Fang, M., Wakeham, A., Okada, H., Calzascia, T., Jang, Y., You-Ten, A.*, et al.* (2005). Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. Cell *121*, 579-591.
Harbour, J.W., and Dean, D.C. (2000a). Chromatin remodeling and Rb activity. Current Opinion in Cell Biology *12*, 685-689.
Harbour, J.W., and Dean, D.C. (2000b). Rb function in cell-cycle regulation and apoptosis. Nat Cell Biol *2*, E65-67.
Harbour, J.W., Luo, R.X., Dei Santi, A., Postigo, A.A., and Dean, D.C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell *98*, 859-869.
Hateboer, G., Wobst, A., Petersen, B.O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol *18*, 6679-6697.
Hauf, S., Waizenegger, I.C., and Peters, J.M. (2001). Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. Science *293*, 1320-1323.
Helt, A.M., and Galloway, D.A. (2003). Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. Carcinogenesis *24*, 159-169.

Hengartner, M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.
Hengst, L., and Reed, S.I. (1998). Inhibitors of the Cip/Kip family. Curr Top Microbiol Immunol *227*, 25-41.
Hietakangas, V., and Cohen, S.M. (2009). Regulation of tissue growth through nutrient sensing. Annu Rev Genet *43*, 389-410.
Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A., and Sherr, C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol *15*, 2672-2681.
Hirpara, K.V., Aggarwal, P., Mukherjee, A.J., Joshi, N., and Burman, A.C. (2009). Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. Anticancer Agents Med Chem *9*, 138-161.
Ho, J.H.C. (1978). An epidemiologic and clinical study of nasopharyngeal carcinoma Int J Radiat Oncol Biol Phys *4*, 183-205.
Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J *12*, 53-63.
Hofmann, K., Bucher, P., and Tschopp, J. (1997). The CARD domain: a new apoptotic signalling motif. Trends Biochem Sci *22*, 155-156.
Hommura, F., Dosaka-Akita, H., Mishina, T., Nishi, M., Kojima, T., Hiroumi, H., Ogura, S., Shimizu, M., Katoh, H., and Kawakami, Y. (2000). Prognostic significance of p27KIP1 protein and ki-67 growth fraction in non-small cell lung cancers. Clin Cancer Res *6*, 4073-4081.
Horinaka, M., Yoshida, T., Shiraishi, T., Nakata, S., Wakada, M., Nakanishi, R., Nishino, H., Matsui, H., and Sakai, T. (2005). Luteolin induces apoptosis via death receptor 5 upregulation in human malignant tumor cells. Oncogene *24*, 7180-7189.
Hsu, Y.T., Wolter, K.G., and Youle, R.J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A *94*, 3668-3672.
Hu, Y., Ding, L., Spencer, D.M., and Nunez, G. (1998). WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. J Biol Chem *273*, 33489-33494.
Huang, C., Ma, W.Y., Goranson, A., and Dong, Z. (1999). Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinogenesis *20*, 237-242.
Huang, D.P., Ho, J.H., Poon, Y.F., Chew, E.C., Saw, D., Lui, M., Li, C.L., Mak, L.S., Lai, S.H., and Lau, W.H. (1980). Establishment of a cell line (NPC/HK1) from a differentiated squamous carcinoma of the nasopharynx. Int J Cancer *26*, 127-132.
Huang, J., and Manning, B.D. (2009). A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans *37*, 217-222.
Huang, W.C., and Chen, C.C. (2005). Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. Mol Cell Biol *25*, 6592-6602.
Huang, W.C., and Hung, M.C. (2009). Induction of Akt activity by chemotherapy confers acquired resistance. J Formos Med Assoc *108*, 180-194.
Hurtig, J. (2010). Managing patients with advanced and metastatic breast cancer. Clin J Oncol Nurs *14*, 313-323.
Husdal, A., Bukholm, G., and Bukholm, I.R. (2006). The prognostic value and overexpression of cyclin A is correlated with gene amplification of both cyclin A and cyclin E in breast cancer patient. Cell Oncol *28*, 107-116.
Huynh, H.T., Larsson, C., Narod, S., and Pollak, M. (1995). Tumor suppressor activity of the gene encoding mammary-derived growth inhibitor. Cancer Res *55*, 2225-2231.
Hwang, C.F., Cho, C.L., Huang, C.C., Wang, J.S., Shih, Y.L., Su, C.Y., and Chang, H.W. (2002). Loss of cyclin D1 and p16 expression correlates with local recurrence in nasopharyngeal carcinoma following radiotherapy. Ann Oncol *13*, 1246-1251.

Hwang, C.F., Su, C.Y., Huang, S.C., Huang, C.C., Fang, F.M., Lui, C.C., Chang, H.W., and Cho, C.L. (2003). Low expression levels of p27 correlate with loco-regional recurrence in nasopharyngeal carcinoma. Cancer Lett *189*, 231-236.
Ihle, N.T., and Powis, G. (2009). Take your PIK: phosphatidylinositol 3-kinase inhibitors race through the clinic and toward cancer therapy. Mol Cancer Ther *8*, 1-9.
Iida, H., Towatari, M., Tanimoto, M., Morishita, Y., Kodera, Y., and Saito, H. (1997). Overexpression of cyclin E in acute myelogenous leukemia. Blood *90*, 3707-3713.
Ioachim, E., Peschos, D., Goussia, A., Mittari, E., Charalabopoulos, K., Michael, M., Salmas, M., Vougiouklakis, T., Assimakopoulos, D., and Agnantis, N.J. (2004). Expression patterns of cyclins D1, E in laryngeal epithelial lesions: correlation with other cell cycle regulators (p53, pRb, Ki-67 and PCNA) and clinicopathological features. J Exp Clin Cancer Res *23*, 277-283.
Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C.*, et al.* (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.
Ishiguro, K., Ando, T., Maeda, O., Ohmiya, N., Niwa, Y., Kadomatsu, K., and Goto, H. (2007). Ginger ingredients reduce viability of gastric cancer cells via distinct mechanisms. Biochem Biophys Res Commun *362*, 218-223.
Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233-243.
Iwao, K., and Tsukamoto, I. (1999). Quercetin inhibited DNA synthesis and induced apoptosis associated with increase in c-fos mRNA level and the upregulation of p21WAF1CIP1 mRNA and protein expression during liver regeneration after partial hepatectomy. Biochim Biophys Acta *1427*, 112-120.
Izumi, M., Yokoi, M., Nishikawa, N.S., Miyazawa, H., Sugino, A., Yamagishi, M., Yamaguchi, M., Matsukage, A., Yatagai, F., and Hanaoka, F. (2000). Transcription of the catalytic 180-kDa subunit gene of mouse DNA polymerase alpha is controlled by E2F, an Ets-related transcription factor, and Sp1. Biochim Biophys Acta *1492*, 341-352.
Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.
James, M.C., and Peters, G. (2000). Alternative product of the p16/CKDN2A locus connects the Rb and p53 tumor suppressors. Prog Cell Cycle Res *4*, 71-81.
Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010. CA Cancer J Clin *60*, 277-300.
Jian, L. (2009). Soy, isoflavones, and prostate cancer. Mol Nutr Food Res *53*, 217-226.
Johnson, D.G., and Walker, C.L. (1999). Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol *39*, 295-312.
Ju, Z., Choudhury, A.R., and Rudolph, K.L. (2007). A dual role of p21 in stem cell aging. Ann N Y Acad Sci *1100*, 333-344.
Jung, Y.H., Heo, J., Lee, Y.J., Kwon, T.K., and Kim, Y.H. (2010). Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. Life Sci *86*, 351-357.
Kadota, M., Sato, M., Duncan, B., Ooshima, A., Yang, H.H., Diaz-Meyer, N., Gere, S., Kageyama, S., Fukuoka, J., Nagata, T.*, et al.* (2009). Identification of novel gene amplifications in breast cancer and coexistence of gene amplification with an activating mutation of PIK3CA. Cancer Res *69*, 7357-7365.
Kaldis, P., Russo, A.A., Chou, H.S., Pavletich, N.P., and Solomon, M.J. (1998). Human and yeast cdk-activating kinases (CAKs) display distinct substrate specificities. Mol Biol Cell *9*, 2545-2560.
Kale, A., Gawande, S., and Kotwal, S. (2008). Cancer phytotherapeutics: role for flavonoids at the cellular level. Phytother Res *22*, 567-577.

Kalejta, R.F. (2004). Human cytomegalovirus pp71: a new viral tool to probe the mechanisms of cell cycle progression and oncogenesis controlled by the retinoblastoma family of tumor suppressors. J Cell Biochem *93*, 37-45.
Kandel, E.S., and Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res *253*, 210-229.
Kanduc, D., Mittelman, A., Serpico, R., Sinigaglia, E., Sinha, A.A., Natale, C., Santacroce, R., Di Corcia, M.G., Lucchese, A., Dini, L*., et al.* (2002). Cell death: apoptosis versus necrosis (review). Int J Oncol *21*, 165-170.
Kane, L.P., Shapiro, V.S., Stokoe, D., and Weiss, A. (1999). Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol *9*, 601-604.
Kaneuchi, M., Sasaki, M., Tanaka, Y., Sakuragi, N., Fujimoto, S., and Dahiya, R. (2003). Quercetin regulates growth of Ishikawa cells through the suppression of EGF and cyclin D1. Int J Oncol *22*, 159-164.
Kang, T.B., and Liang, N.C. (1997). Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. Biochem Pharmacol *54*, 1013-1018.
Katiyar, S.K., Roy, A.M., and Baliga, M.S. (2005). Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome c release, and caspase activation. Mol Cancer Ther *4*, 207-216.
Kato, J.Y., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C.J. (1994). Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell *79*, 487-496.
Kaufmann, S.H. (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res *49*, 5870-5878.
Kerkhoff, E., and Rapp, U.R. (1998). Cell cycle targets of Ras/Raf signalling. Oncogene *17*, 1457-1462.
Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A., and Travis, R.C. (2004). Diet, nutrition and the prevention of cancer. Public Health Nutr *7*, 187-200.
Kim, G.Y., Kim, J.H., Ahn, S.C., Lee, H.J., Moon, D.O., Lee, C.M., and Park, Y.M. (2004). Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. Immunology *113*, 203-211.
Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., and Cheng, E.H. (2006). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol *8*, 1348-1358.
Kim, J.H., Kang, M.J., Park, C.U., Kwak, H.J., Hwang, Y., and Koh, G.Y. (1999). Amplified CDK2 and cdc2 activities in primary colorectal carcinoma. Cancer *85*, 546-553.
Kim, J.K., and Diehl, J.A. (2009). Nuclear cyclin D1: an oncogenic driver in human cancer. J Cell Physiol *220*, 292-296.
Kim, J.S., and Jobin, C. (2005). The flavonoid luteolin prevents lipopolysaccharide-induced NF-kappaB signalling and gene expression by blocking IkappaB kinase activity in intestinal epithelial cells and bone-marrow derived dendritic cells. Immunology *115*, 375-387.
Kimbro, K.S., and Simons, J.W. (2006). Hypoxia-inducible factor-1 in human breast and prostate cancer. Endocr Relat Cancer *13*, 739-749.
King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279-288.
Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J *14*, 5579-5588.

Kitahara, K., Yasui, W., Kuniyasu, H., Yokozaki, H., Akama, Y., Yunotani, S., Hisatsugu, T., and Tahara, E. (1995). Concurrent amplification of cyclin E and CDK2 genes in colorectal carcinomas. Int J Cancer *62*, 25-28.
Kiyosaki, K., Nakada, C., Hijiya, N., Tsukamoto, Y., Matsuura, K., Nakatsuka, K., Daa, T., Yokoyama, S., Imaizumi, M., and Moriyama, M. (2010). Analysis of p53 mutations and the expression of p53 and p21WAF1/CIP1 protein in 15 cases of sebaceous carcinoma of the eyelid. Invest Ophthalmol Vis Sci *51*, 7-11.
Kobayashi, T., Nakata, T., and Kuzumaki, T. (2002). Effect of flavonoids on cell cycle progression in prostate cancer cells. Cancer Lett *176*, 17-23.
Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., and Roberts, J.M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science *257*, 1689-1694.
Kolonel, L.N., Altshuler, D., and Henderson, B.E. (2004). The multiethnic cohort study: exploring genes, lifestyle and cancer risk. Nat Rev Cancer *4*, 519-527.
Kolsch, V., Charest, P.G., and Firtel, R.A. (2008). The regulation of cell motility and chemotaxis by phospholipid signaling. J Cell Sci *121*, 551-559.
Kondapaka, S.B., Singh, S.S., Dasmahapatra, G.P., Sausville, E.A., and Roy, K.K. (2003). Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther *2*, 1093-1103.
Koontongkaew, S., Chareonkitkajorn, L., Chanvitan, A., Leelakriangsak, M., and Amornphimoltham, P. (2000). Alterations of p53, pRb, cyclin D(1) and cdk4 in human oral and pharyngeal squamous cell carcinomas. Oral Oncol *36*, 334-339.
Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L., and Burgering, B.M. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature *398*, 630-634.
Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W., and Burgering, B.M. (2002). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol *22*, 2025-2036.
Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J.M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. EMBO J *22*, 6598-6609.
Krasinskas, A.M., Bartlett, D.L., Cieply, K., and Dacic, S. (2010). CDKN2A and MTAP deletions in peritoneal mesotheliomas are correlated with loss of p16 protein expression and poor survival. Mod Pathol *23*, 531-538.
Kristjansdottir, K., and Rudolph, J. (2004). Cdc25 phosphatases and cancer. Chem Biol *11*, 1043-1051.
Kuhnau, J. (1976). The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev Nutr Diet *24*, 117-191.
Kumar, A., Dhawan, S., and Aggarwal, B.B. (1998). Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) inhibits TNF-induced NF-kappaB activation, IkappaB degradation, and expression of cell surface adhesion proteins in human vascular endothelial cells. Oncogene *17*, 913-918.
Kwong, D.L., Sham, J.S., Au, G.K., Chua, D.T., Kwong, P.W., Cheng, A.C., Wu, P.M., Law, M.W., Kwok, C.C., Yau, C.C*., et al.* (2004). Concurrent and adjuvant chemotherapy for nasopharyngeal carcinoma: a factorial study. J Clin Oncol *22*, 2643-2653.
Kwong, L.N., and Dove, W.F. (2009). APC and its modifiers in colon cancer. Adv Exp Med Biol *656*, 85-106.
Landberg, G. (2002). Multiparameter analyses of cell cycle regulatory proteins in human breast cancer: a key to definition of separate pathways in tumorigenesis. Adv Cancer Res *84*, 35-56.

Larkin, J.M., and Kaye, S.B. (2006). Epothilones in the treatment of cancer. Expert Opin Investig Drugs *15*, 691-702.
Lavrik, I., Golks, A., and Krammer, P.H. (2005). Death receptor signaling. J Cell Sci *118*, 265-267.
Lee, A.W., Tung, S.Y., Chua, D.T., Ngan, R.K., Chappell, R., Tung, R., Siu, L., Ng, W.T., Sze, W.K., Au, G.K.*, et al.* (2010a). Randomized trial of radiotherapy plus concurrent-adjuvant chemotherapy vs radiotherapy alone for regionally advanced nasopharyngeal carcinoma. J Natl Cancer Inst *102*, 1188-1198.
Lee, H.H., Dadgostar, H., Cheng, Q., Shu, J., and Cheng, G. (1999). NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc Natl Acad Sci U S A *96*, 9136-9141.
Lee, H.J., Wang, C.J., Kuo, H.C., Chou, F.P., Jean, L.F., and Tseng, T.H. (2005). Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicol Appl Pharmacol *203*, 124-131.
Lee, J.H., Jeong, Y.J., Lee, S.W., Kim, D., Oh, S.J., Lim, H.S., Oh, H.K., Kim, S.H., Kim, W.J., and Jung, J.Y. (2010b). EGCG induces apoptosis in human laryngeal epidermoid carcinoma Hep2 cells via mitochondria with the release of apoptosis-inducing factor and endonuclease G. Cancer Lett *290*, 68-75.
Lee, W.J., Wu, L.F., Chen, W.K., Wang, C.J., and Tseng, T.H. (2006). Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K-Akt pathways. Chem Biol Interact *160*, 123-133.
Leist, M., and Jaattela, M. (2001). Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol *2*, 589-598.
Leist, M., Single, B., Castoldi, A.F., Kuhnle, S., and Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med *185*, 1481-1486.
Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643-649.
Leong, S.S., Wee, J., Tay, M.H., Toh, C.K., Tan, S.B., Thng, C.H., Foo, K.F., Lim, W.T., Tan, T., and Tan, E.H. (2005). Paclitaxel, carboplatin, and gemcitabine in metastatic nasopharyngeal carcinoma: a Phase II trial using a triplet combination. Cancer *103*, 569-575.
Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S., and Korsmeyer, S.J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183-192.
Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.
Levkau, B., Koyama, H., Raines, E.W., Clurman, B.E., Herren, B., Orth, K., Roberts, J.M., and Ross, R. (1998). Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. Mol Cell *1*, 553-563.
Li, C.J., Vassilev, A., and DePamphilis, M.L. (2004). Role for Cdk1 (Cdc2)/cyclin A in preventing the mammalian origin recognition complex's largest subunit (Orc1) from binding to chromatin during mitosis. Mol Cell Biol *24*, 5875-5886.
Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.
Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R.*, et al.* (1997a). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.
Li, J., and Yuan, J. (2008). Caspases in apoptosis and beyond. Oncogene *27*, 6194-6206.

Li, L., Wei, X.H., Pan, Y.P., Li, H.C., Yang, H., He, Q.H., Pang, Y., Shan, Y., Xiong, F.X., Shao, G.Z*., et al.* (2010a). LAPTM4B: A novel cancer-associated gene motivates multidrug resistance through efflux and activating PI3K/AKT signaling. Oncogene.
Li, M., Zhang, Z., Hill, D.L., Chen, X., Wang, H., and Zhang, R. (2005). Genistein, a dietary isoflavone, down-regulates the MDM2 oncogene at both transcriptional and posttranslational levels. Cancer Res *65*, 8200-8208.
Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997b). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.
Li, X., Huang, Q., Ong, C.N., Yang, X.F., and Shen, H.M. (2010b). Chrysin sensitizes tumor necrosis factor-alpha-induced apoptosis in human tumor cells via suppression of nuclear factor-kappaB. Cancer Lett *293*, 109-116.
Li, Y., Dowbenko, D., and Lasky, L.A. (2002). AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. J Biol Chem *277*, 11352-11361.
Li, Y., Fang, H., and Xu, W. (2007). Recent advance in the research of flavonoids as anticancer agents. Mini Rev Med Chem *7*, 663-678.
Li, Y., and Sarkar, F.H. (2002). Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via Akt signaling pathway. Clin Cancer Res *8*, 2369-2377.
Liang, J., Ge, F., Guo, C., Luo, G., Wang, X., Han, G., Zhang, D., Wang, J., Li, K., Pan, Y*., et al.* (2009). Inhibition of PI3K/Akt partially leads to the inhibition of PrP(C)-induced drug resistance in gastric cancer cells. FEBS J *276*, 685-694.
Liang, J., and Slingerland, J.M. (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle *2*, 339-345.
Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R*., et al.* (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med *8*, 1153-1160.
Liang, Y.C., Lin-shiau, S.Y., Chen, C.F., and Lin, J.K. (1997). Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. J Cell Biochem *67*, 55-65.
Liao, C., Li, S.Q., Wang, X., Muhlrad, S., Bjartell, A., and Wolgemuth, D.J. (2004). Elevated levels and distinct patterns of expression of A-type cyclins and their associated cyclin-dependent kinases in male germ cell tumors. Int J Cancer *108*, 654-664.
Liao, X., Siu, M.K., Au, C.W., Chan, Q.K., Chan, H.Y., Wong, E.S., Ip, P.P., Ngan, H.Y., and Cheung, A.N. (2009). Aberrant activation of hedgehog signaling pathway contributes to endometrial carcinogenesis through beta-catenin. Mod Pathol *22*, 839-847.
Lim do, Y., Jeong, Y., Tyner, A.L., and Park, J.H. (2007). Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. Am J Physiol Gastrointest Liver Physiol *292*, G66-75.
Lin, J.-K., Liang, Y.-C., and Lin-Shiau, S.-Y. (1999). Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. Biochemical Pharmacology *58*, 911-915.
Lin, J.C., Jan, J.S., Hsu, C.Y., Jiang, R.S., and Wang, W.Y. (2003). Outpatient weekly neoadjuvant chemotherapy followed by radiotherapy for advanced nasopharyngeal carcinoma: high complete response and low toxicity rates. Br J Cancer *88*, 187-194.
Lin, P.H., Lu, Y.S., Lin, C.H., Chang, D.Y., Huang, C.S., Cheng, A.L., and Yeh, K.H. (2010). Vinorelbine plus 24-hour infusion of high-dose 5-fluorouracil and leucovorin as effective palliative chemotherapy for breast cancer patients with acute disseminated intravascular coagulation. Anticancer Res *30*, 3087-3091.
Lin, S.Y., Tsang, N.M., Kao, S.C., Hsieh, Y.L., Chen, Y.P., Tsai, C.S., Kuo, T.T., Hao, S.P., Chen, I.H., and Hong, J.H. (2001). Presence of Epstein-Barr virus latent membrane

protein 1 gene in the nasopharyngeal swabs from patients with nasopharyngeal carcinoma. Head Neck *23*, 194-200.
Lin, Y., Shi, R., Wang, X., and Shen, H.M. (2008). Luteolin, a flavonoid with potential for cancer prevention and therapy. Curr Cancer Drug Targets *8*, 634-646.
Linos, E., and Willett, W.C. (2007). Diet and breast cancer risk reduction. J Natl Compr Canc Netw *5*, 711-718.
Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J.E., MacKenzie, A.*, et al.* (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature *379*, 349-353.
Liu, A.L., Wang, H.D., Lee, S.M., Wang, Y.T., and Du, G.H. (2008). Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities. Bioorg Med Chem *16*, 7141-7147.
Liu, F., Stanton, J.J., Wu, Z., and Piwnica-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. Mol Cell Biol *17*, 571-583.
Liu, L.T., Peng, J.P., Chang, H.C., and Hung, W.C. (2003). RECK is a target of Epstein-Barr virus latent membrane protein 1. Oncogene *22*, 8263-8270.
Liu, P., Cheng, H., Roberts, T.M., and Zhao, J.J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov *8*, 627-644.
Liu, T.Y., Wu, S.J., Huang, M.H., Lo, F.Y., Tsai, M.H., Tsai, C.H., Hsu, S.M., and Lin, C.W. (2010). EBV-positive Hodgkin lymphoma is associated with suppression of p21cip1/waf1 and a worse prognosis. Mol Cancer *9*, 32.
Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.
Lo, H.W., Zhu, H., Cao, X., Aldrich, A., and Ali-Osman, F. (2009). A novel splice variant of GLI1 that promotes glioblastoma cell migration and invasion. Cancer Res *69*, 6790-6798.
Lobjois, V., Jullien, D., Bouche, J.P., and Ducommun, B. (2009). The polo-like kinase 1 regulates CDC25B-dependent mitosis entry. Biochim Biophys Acta *1793*, 462-468.
Loeb, L.A. (1991). Mutator phenotype may be required for multistage carcinogenesis. Cancer Res *51*, 3075-3079.
Loeb, L.A., Bielas, J.H., and Beckman, R.A. (2008). Cancers exhibit a mutator phenotype: clinical implications. Cancer Res *68*, 3551-3557; discussion 3557.
Loffler, H., Syljuasen, R.G., Bartkova, J., Worm, J., Lukas, J., and Bartek, J. (2003). Distinct modes of deregulation of the proto-oncogenic Cdc25A phosphatase in human breast cancer cell lines. Oncogene *22*, 8063-8071.
Lopez-Lazaro, M. (2002). Flavonoids as anticancer agents: structure-activity relationship study. Curr Med Chem Anticancer Agents *2*, 691-714.
Lopez-Lazaro, M. (2009). Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem *9*, 31-59.
Lu, H., Peng, L., Yuan, X., Hao, Y., Lu, Z., Chen, J., Cheng, J., Deng, S., Gu, J., Pang, Q.*, et al.* (2009). Concurrent chemoradiotherapy in locally advanced nasopharyngeal carcinoma: a treatment paradigm also applicable to patients in Southeast Asia. Cancer Treat Rev *35*, 345-353.
Lu, H., and Yao, M. (2008). The current status of intensity-modulated radiation therapy in the treatment of nasopharyngeal carcinoma. Cancer Treat Rev *34*, 27-36.
Lu, H.F., Chie, Y.J., Yang, M.S., Lee, C.S., Fu, J.J., Yang, J.S., Tan, T.W., Wu, S.H., Ma, Y.S., Ip, S.W.*, et al.* (2010). Apigenin induces caspase-dependent apoptosis in human lung cancer A549 cells through Bax- and Bcl-2-triggered mitochondrial pathway. Int J Oncol *36*, 1477-1484.
Lukas, J., Herzinger, T., Hansen, K., Moroni, M.C., Resnitzky, D., Helin, K., Reed, S.I., and Bartek, J. (1997). Cyclin E-induced S phase without activation of the pRb/E2F pathway. Genes Dev *11*, 1479-1492.

Lundberg, A.S., and Weinberg, R.A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol *18*, 753-761.
Luo, J., Xiao, J., Tao, Z., and Li, X. (1997). Detection of c-myc gene expression in nasopharyngeal carcinoma by nonradioactive in situ hybridization and immunohistochemistry. Chin Med J (Engl) *110*, 229-232.
Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell *94*, 481-490.
Luo, Y., Chia, K.S., Chia, S.E., Reilly, M., Tan, C.S., and Ye, W. (2007). Secular trends of nasopharyngeal carcinoma incidence in Singapore, Hong Kong and Los Angeles Chinese populations, 1973-1997. Eur J Epidemiol *22*, 513-521.
Luo, Y., Hurwitz, J., and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature *375*, 159-161.
Luqman, S., and Pezzuto, J.M. (2010). NFkappaB: a promising target for natural products in cancer chemoprevention. Phytother Res *24*, 949-963.
Mackenzie, G.G., Queisser, N., Wolfson, M.L., Fraga, C.G., Adamo, A.M., and Oteiza, P.I. (2008). Curcumin induces cell-arrest and apoptosis in association with the inhibition of constitutively active NF-kappaB and STAT3 pathways in Hodgkin's lymphoma cells. Int J Cancer *123*, 56-65.
Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-13378.
Makitie, A.A., MacMillan, C., Ho, J., Shi, W., Lee, A., O'Sullivan, B., Payne, D., Pintilie, M., Cummings, B., Waldron, J.*, et al.* (2003). Loss of p16 expression has prognostic significance in human nasopharyngeal carcinoma. Clin Cancer Res *9*, 2177-2184.
Malumbres, M., and Barbacid, M. (2001). Milestones in cell division : To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer *1*, 222-231.
Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends Biochem Sci *30*, 630-641.
Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer *9*, 153-166.
Margottin-Goguet, F., Hsu, J.Y., Loktev, A., Hsieh, H.M., Reimann, J.D., and Jackson, P.K. (2003). Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev Cell *4*, 813-826.
Martelli, A.M., Chiarini, F., Evangelisti, C., Grimaldi, C., Ognibene, A., Manzoli, L., Billi, A.M., and McCubrey, J.A. (2010). The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin signaling network and the control of normal myelopoiesis. Histol Histopathol *25*, 669-680.
Mathers, J.C., Strathdee, G., and Relton, C.L. (2010). Induction of epigenetic alterations by dietary and other environmental factors. Adv Genet *71*, 3-39.
Mathew, A., Peters, U., Chatterjee, N., Kulldorff, M., and Sinha, R. (2004). Fat, fiber, fruits, vegetables, and risk of colorectal adenomas. Int J Cancer *108*, 287-292.
Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell *71*, 323-334.
Matter, W.F., Brown, R.F., and Vlahos, C.J. (1992). The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. Biochem Biophys Res Commun *186*, 624-631.
Maynard, S., Schurman, S.H., Harboe, C., de Souza-Pinto, N.C., and Bohr, V.A. (2009). Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis *30*, 2-10.

Mayo, L.D., and Donner, D.B. (2002). The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem Sci *27*, 462-467.
McCarthy, J.S., Tannock, I.F., Degendorfer, P., Panzarella, T., Furlan, M., and Siu, L.L. (2002). A Phase II trial of docetaxel and cisplatin in patients with recurrent or metastatic nasopharyngeal carcinoma. Oral Oncol *38*, 686-690.
McConnell, B.B., Gregory, F.J., Stott, F.J., Hara, E., and Peters, G. (1999). Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. Mol Cell Biol *19*, 1981-1989.
McGrogan, B.T., Gilmartin, B., Carney, D.N., and McCann, A. (2008). Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta *1785*, 96-132.
Medema, R.H., Kops, G.J., Bos, J.L., and Burgering, B.M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782-787.
Menu, E., Garcia, J., Huang, X., Di Liberto, M., Toogood, P.L., Chen, I., Vanderkerken, K., and Chen-Kiang, S. (2008). A novel therapeutic combination using PD 0332991 and bortezomib: study in the 5T33MM myeloma model. Cancer Res *68*, 5519-5523.
Messina, M., and Hilakivi-Clarke, L. (2009). Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. Nutr Cancer *61*, 792-798.
Meyerson, M., and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol Cell Biol *14*, 2077-2086.
Michaud, K., Solomon, D.A., Oermann, E., Kim, J.S., Zhong, W.Z., Prados, M.D., Ozawa, T., James, C.D., and Waldman, T. (2010). Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. Cancer Res *70*, 3228-3238.
Michor, F., Iwasa, Y., Vogelstein, B., Lengauer, C., and Nowak, M.A. (2005). Can chromosomal instability initiate tumorigenesis? Semin Cancer Biol *15*, 43-49.
Middleton, E., Jr., Kandaswami, C., and Theoharides, T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev *52*, 673-751.
Miean, K.H., and Mohamed, S. (2001). Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. J Agric Food Chem *49*, 3106-3112.
Milner, J. (1991). A conformation hypothesis for the suppressor and promoter functions of p53 in cell growth control and in cancer. Proc Biol Sci *245*, 139-145.
Mitchell, P.J., Perez-Nadales, E., Malcolm, D.S., and Lloyd, A.C. (2003). Dissecting the contribution of p16(INK4A) and the Rb family to the Ras transformed phenotype. Mol Cell Biol *23*, 2530-2542.
Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-299.
Moon, S.K., Cho, G.O., Jung, S.Y., Gal, S.W., Kwon, T.K., Lee, Y.C., Madamanchi, N.R., and Kim, C.H. (2003). Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. Biochem Biophys Res Commun *301*, 1069-1078.
Moore, L., Venkatachalam, S., Vogel, H., Watt, J.C., Wu, C.L., Steinman, H., Jones, S.N., and Donehower, L.A. (2003). Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. Oncogene *22*, 7831-7837.
Morello, F., Perino, A., and Hirsch, E. (2009). Phosphoinositide 3-kinase signalling in the vascular system. Cardiovasc Res *82*, 261-271.
Morin, D., Barthelemy, S., Zini, R., Labidalle, S., and Tillement, J.P. (2001). Curcumin induces the mitochondrial permeability transition pore mediated by membrane protein thiol oxidation. FEBS Lett *495*, 131-136.

Morrison, J.A., Gulley, M.L., Pathmanathan, R., and Raab-Traub, N. (2004). Differential signaling pathways are activated in the Epstein-Barr virus-associated malignancies nasopharyngeal carcinoma and Hodgkin lymphoma. Cancer Res *64*, 5251-5260.
Morrison, J.A., and Raab-Traub, N. (2005). Roles of the ITAM and PY motifs of Epstein-Barr virus latent membrane protein 2A in the inhibition of epithelial cell differentiation and activation of {beta}-catenin signaling. J Virol *79*, 2375-2382.
Motoyama, N., and Naka, K. (2004). DNA damage tumor suppressor genes and genomic instability. Curr Opin Genet Dev *14*, 11-16.
Motwani, M., Rizzo, C., Sirotnak, F., She, Y., and Schwartz, G.K. (2003). Flavopiridol enhances the effect of docetaxel in vitro and in vivo in human gastric cancer cells. Mol Cancer Ther *2*, 549-555.
Mukhopadhyay, A., Banerjee, S., Stafford, L.J., Xia, C., Liu, M., and Aggarwal, B.B. (2002). Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. Oncogene *21*, 8852-8861.
Mullen, W., Edwards, C.A., and Crozier, A. (2006). Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. Br J Nutr *96*, 107-116.
Murakami, A., Ashida, H., and Terao, J. (2008). Multitargeted cancer prevention by quercetin. Cancer Lett *269*, 315-325.
Murota, K., Shimizu, S., Chujo, H., Moon, J.H., and Terao, J. (2000). Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Arch Biochem Biophys *384*, 391-397.
Murtaza, I., Marra, G., Schlapbach, R., Patrignani, A., Kunzli, M., Wagner, U., Sabates, J., and Dutt, A. (2006). A preliminary investigation demonstrating the effect of quercetin on the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism in human CO115 colon-adenocarcinoma cells using DNA microarray. Biotechnol Appl Biochem *45*, 29-36.
Murugan, A.K., Hong, N.T., Fukui, Y., Munirajan, A.K., and Tsuchida, N. (2008). Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. Int J Oncol *32*, 101-111.
Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R.*, et al.* (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell *85*, 817-827.
Nagaraj, N.S., and Datta, P.K. (2010). Targeting the transforming growth factor-beta signaling pathway in human cancer. Expert Opin Investig Drugs *19*, 77-91.
Nambiar, M., Kari, V., and Raghavan, S.C. (2008). Chromosomal translocations in cancer. Biochim Biophys Acta *1786*, 139-152.
Nave, B.T., Ouwens, M., Withers, D.J., Alessi, D.R., and Shepherd, P.R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J *344 Pt 2*, 427-431.
Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. (2010). Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol *11*, 220-228.
Ngan, R.K., Yiu, H.H., Lau, W.H., Yau, S., Cheung, F.Y., Chan, T.M., Kwok, C.H., Chiu, C.Y., Au, S.K., Foo, W.*, et al.* (2002). Combination gemcitabine and cisplatin chemotherapy for metastatic or recurrent nasopharyngeal carcinoma: report of a phase II study. Ann Oncol *13*, 1252-1258.
Ngeow, J., Lim, W.T., Leong, S.S., Ang, M.K., Toh, C.K., Gao, F., Chowbay, B., and Tan, E.H. (2010). Docetaxel is effective in heavily pretreated patients with disseminated nasopharyngeal carcinoma. Ann Oncol.

Nicholson, D.W., and Thornberry, N.A. (1997). Caspases: killer proteases. Trends Biochem Sci *22*, 299-306.
Nicholson, K.M., and Anderson, N.G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal *14*, 381-395.
Nielsen, N.H., Emdin, S.O., Cajander, J., and Landberg, G. (1997). Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. Oncogene *14*, 295-304.
Nigg, E.A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol *2*, 21-32.
Nishino, H., Satomi, Y., Tokuda, H., and Masuda, M. (2007). Cancer control by phytochemicals. Curr Pharm Des *13*, 3394-3399.
Nishiyama, M., and Wada, S. (2009). Docetaxel: its role in current and future treatments for advanced gastric cancer. Gastric Cancer *12*, 132-141.
Noe, V., Chen, C., Alemany, C., Nicolas, M., Caragol, I., Chasin, L.A., and Ciudad, C.J. (1997). Cell-growth regulation of the hamster dihydrofolate reductase gene promoter by transcription factor Sp1. Eur J Biochem *249*, 13-20.
Nogales, E. (2001). Structural insight into microtubule function. Annu Rev Biophys Biomol Struct *30*, 397-420.
Noy, N. (2010). Between death and survival: retinoic acid in regulation of apoptosis. Annu Rev Nutr *30*, 201-217.
Nutting, P.A., Freeman, W.L., Risser, D.R., Helgerson, S.D., Paisano, R., Hisnanick, J., Beaver, S.K., Peters, I., Carney, J.P., and Speers, M.A. (1993). Cancer incidence among American Indians and Alaska Natives, 1980 through 1987. Am J Public Health *83*, 1589-1598.
O'Leary, K.A., Day, A.J., Needs, P.W., Mellon, F.A., O'Brien, N.M., and Williamson, G. (2003). Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol *65*, 479-491.
Obaya, A.J., and Sedivy, J.M. (2002). Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci *59*, 126-142.
Obermeier, M.T., White, R.E., and Yang, C.S. (1995). Effects of bioflavonoids on hepatic P450 activities. Xenobiotica *25*, 575-584.
Obeyesekere, M.N., Herbert, J.R., and Zimmerman, S.O. (1995). A model of the G1 phase of the cell cycle incorporating cyclin E/cdk2 complex and retinoblastoma protein. Oncogene *11*, 1199-1205.
Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol *15*, 2612-2624.
Okada, H., and Mak, T.W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer *4*, 592-603.
Oki, E., Kakeji, Y., Baba, H., Tokunaga, E., Nakamura, T., Ueda, N., Futatsugi, M., Yamamoto, M., Ikebe, M., and Maehara, Y. (2006). Impact of loss of heterozygosity of encoding phosphate and tensin homolog on the prognosis of gastric cancer. J Gastroenterol Hepatol *21*, 814-818.
Okuda, M., Horn, H.F., Tarapore, P., Tokuyama, Y., Smulian, A.G., Chan, P.K., Knudsen, E.S., Hofmann, I.A., Snyder, J.D., Bove, K.E*., et al.* (2000). Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell *103*, 127-140.
Ong, C.S., Tran, E., Nguyen, T.T., Ong, C.K., Lee, S.K., Lee, J.J., Ng, C.P., Leong, C., and Huynh, H. (2004). Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. Oncol Rep *11*, 727-733.

Ong, C.S., Zhou, J., Ong, C.N., and Shen, H.M. (2010a). Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3beta-Cyclin D1 pathway. Cancer Lett.
Ong, C.S., Zhou, J., Ong, C.N., and Shen, H.M. (2010b). Luteolin induces G1 arrest in human nasopharyngeal carcinoma cells via the Akt-GSK-3beta-Cyclin D1 pathway. Cancer Lett *298*, 167-175.
Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ *10*, 431-442.
Ortega, S., Malumbres, M., and Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta *1602*, 73-87.
Oudit, G.Y., and Penninger, J.M. (2009). Cardiac regulation by phosphoinositide 3-kinases and PTEN. Cardiovasc Res *82*, 250-260.
Ozyar, E., Ayhan, A., Korcum, A.F., and Atahan, I.L. (2004). Prognostic role of Ebstein-Barr virus latent membrane protein-1 and interleukin-10 expression in patients with nasopharyngeal carcinoma. Cancer Invest *22*, 483-491.
Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J *11*, 961-971.
Pan, D., Dong, J., Zhang, Y., and Gao, X. (2004). Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol *14*, 78-85.
Pan, M.H., and Ho, C.T. (2008). Chemopreventive effects of natural dietary compounds on cancer development. Chem Soc Rev *37*, 2558-2574.
Panani, A.D., Maliaga, K., Babanaraki, A., and Bellenis, I. (2009). Numerical abnormalities of chromosome 9 and p16CDKN2A gene deletion detected by FISH in non-small cell lung cancer. Anticancer Res *29*, 4483-4487.
Parker, L.L., and Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science *257*, 1955-1957.
Parkin, D.M., Whelan, S.L., Ferlay, J., Raymond, L., and Young, J. (1997). Cancer Incidence in Five Continents IARC Scientific Publications No 143, International Agency for Research on Cancer, Lyon *7*.
Pateras, I.S., Apostolopoulou, K., Koutsami, M., Evangelou, K., Tsantoulis, P., Liloglou, T., Nikolaidis, G., Sigala, F., Kittas, C., Field, J.K.*, et al.* (2006). Downregulation of the KIP family members p27(KIP1) and p57(KIP2) by SKP2 and the role of methylation in p57(KIP2) inactivation in nonsmall cell lung cancer. Int J Cancer *119*, 2546-2556.
Pauwels, E.K., Erba, P., Mariani, G., and Gomes, C.M. (2007). Multidrug resistance in cancer: its mechanism and its modulation. Drug News Perspect *20*, 371-377.
Pedrero, J.M., Carracedo, D.G., Pinto, C.M., Zapatero, A.H., Rodrigo, J.P., Nieto, C.S., and Gonzalez, M.V. (2005). Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. Int J Cancer *114*, 242-248.
Peng, C.Y., Graves, P.R., Ogg, S., Thoma, R.S., Byrnes, M.J., 3rd, Wu, Z., Stephenson, M.T., and Piwnica-Worms, H. (1998). C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. Cell Growth Differ *9*, 197-208.
Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.
Perabo, F.G., Von Low, E.C., Ellinger, J., von Rucker, A., Muller, S.C., and Bastian, P.J. (2008). Soy isoflavone genistein in prevention and treatment of prostate cancer. Prostate Cancer Prostatic Dis *11*, 6-12.
Perez-Roger, I., Kim, S.H., Griffiths, B., Sewing, A., and Land, H. (1999). Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J *18*, 5310-5320.

Pesakhov, S., Khanin, M., Studzinski, G.P., and Danilenko, M. (2010). Distinct combinatorial effects of the plant polyphenols curcumin, carnosic acid, and silibinin on proliferation and apoptosis in acute myeloid leukemia cells. Nutr Cancer *62*, 811-824.
Peter, M. (1997). The regulation of cyclin-dependent kinase inhibitors (CKIs). Prog Cell Cycle Res *3*, 99-108.
Piccolo, E., Vignati, S., Maffucci, T., Innominato, P.F., Riley, A.M., Potter, B.V., Pandolfi, P.P., Broggini, M., Iacobelli, S., Innocenti, P.*, et al.* (2004). Inositol pentakisphosphate promotes apoptosis through the PI 3-K/Akt pathway. Oncogene *23*, 1754-1765.
Pines, J. (1995). Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J *308 (Pt 3)*, 697-711.
Pines, J., and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J Cell Biol *115*, 1-17.
Pines, J., and Hunter, T. (1994). The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J *13*, 3772-3781.
Polyak, S.J., Morishima, C., Lohmann, V., Pal, S., Lee, D.Y., Liu, Y., Graf, T.N., and Oberlies, N.H. (2010). Identification of hepatoprotective flavonolignans from silymarin. Proc Natl Acad Sci U S A *107*, 5995-5999.
Ponder, B.A. (2001). Cancer genetics. Nature *411*, 336-341.
Prives, C., and Hall, P.A. (1999). The p53 pathway. J Pathol *187*, 112-126.
Proskuryakov, S.Y., and Gabai, V.L. (2010). Mechanisms of tumor cell necrosis. Curr Pharm Des *16*, 56-68.
Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E., and Reusch, J.E. (2000). Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J Biol Chem *275*, 10761-10766.
Qi, Y., Tu, Y., Yang, D., Chen, Q., Xiao, J., Chen, Y., Fu, J., Xiao, X., and Zhou, Z. (2007). Cyclin A but not cyclin D1 is essential for c-myc-modulated cell-cycle progression. J Cell Physiol *210*, 63-71.
Raab-Traub, N. (2002). Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol *12*, 431-441.
Rahman, A., Shahabuddin, Hadi, S.M., and Parish, J.H. (1990). Complexes involving quercetin, DNA and Cu(II). Carcinogenesis *11*, 2001-2003.
Ramakrishnan, G., Lo Muzio, L., Elinos-Baez, C.M., Jagan, S., Augustine, T.A., Kamaraj, S., Anandakumar, P., and Devaki, T. (2009). Silymarin inhibited proliferation and induced apoptosis in hepatic cancer cells. Cell Prolif *42*, 229-240.
Ramos, S. (2007). Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. J Nutr Biochem *18*, 427-442.
Ramos, S. (2008). Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Mol Nutr Food Res *52*, 507-526.
Rassool, F.V., Gaymes, T.J., Omidvar, N., Brady, N., Beurlet, S., Pla, M., Reboul, M., Lea, N., Chomienne, C., Thomas, N.S.*, et al.* (2007). Reactive oxygen species, DNA damage, and error-prone repair: a model for genomic instability with progression in myeloid leukemia? Cancer Res *67*, 8762-8771.
Reddy, L., Odhav, B., and Bhoola, K.D. (2003). Natural products for cancer prevention: a global perspective. Pharmacol Ther *99*, 1-13.
Reimann, J.D., Freed, E., Hsu, J.Y., Kramer, E.R., Peters, J.M., and Jackson, P.K. (2001). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. Cell *105*, 645-655.
Rena, G., Guo, S., Cichy, S.C., Unterman, T.G., and Cohen, P. (1999). Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. J Biol Chem *274*, 17179-17183.

Reusch, J.E., and Klemm, D.J. (2002). Inhibition of cAMP-response element-binding protein activity decreases protein kinase B/Akt expression in 3T3-L1 adipocytes and induces apoptosis. J Biol Chem *277*, 1426-1432.
Rhodes, N., Heerding, D.A., Duckett, D.R., Eberwein, D.J., Knick, V.B., Lansing, T.J., McConnell, R.T., Gilmer, T.M., Zhang, S.Y., Robell, K.*, et al.* (2008). Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity. Cancer Res *68*, 2366-2374.
Rich, T., Watson, C.J., and Wyllie, A. (1999). Apoptosis: the germs of death. Nat Cell Biol *1*, E69-71.
Robertson, D.J., Sandler, R.S., Haile, R., Tosteson, T.D., Greenberg, E.R., Grau, M., and Baron, J.A. (2005). Fat, fiber, meat and the risk of colorectal adenomas. Am J Gastroenterol *100*, 2789-2795.
Robles, A.I., Larcher, F., Whalin, R.B., Murillas, R., Richie, E., Gimenez-Conti, I.B., Jorcano, J.L., and Conti, C.J. (1996). Expression of cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia. Proc Natl Acad Sci U S A *93*, 7634-7638.
Rodriguez-Antona, C. (2010). Pharmacogenomics of paclitaxel. Pharmacogenomics *11*, 621-623.
Rodriguez, J., and Lazebnik, Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev *13*, 3179-3184.
Romashkova, J.A., and Makarov, S.S. (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature *401*, 86-90.
Roschek, B., Jr., Fink, R.C., McMichael, M.D., Li, D., and Alberte, R.S. (2009). Elderberry flavonoids bind to and prevent H1N1 infection in vitro. Phytochemistry *70*, 1255-1261.
Rosell, R., Moran, T., Carcereny, E., Quiroga, V., Molina, M.A., Costa, C., Benlloch, S., and Taron, M. (2010). Non-small-cell lung cancer harbouring mutations in the EGFR kinase domain. Clin Transl Oncol *12*, 75-80.
Ross, J.A., and Kasum, C.M. (2002). Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr *22*, 19-34.
Rossig, L., Badorff, C., Holzmann, Y., Zeiher, A.M., and Dimmeler, S. (2002). Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21Cip1 degradation. J Biol Chem *277*, 9684-9689.
Rossig, L., Jadidi, A.S., Urbich, C., Badorff, C., Zeiher, A.M., and Dimmeler, S. (2001). Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. Mol Cell Biol *21*, 5644-5657.
Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J *16*, 6914-6925.
Roy, P., Madan, E., Kalra, N., Nigam, N., George, J., Ray, R.S., Hans, R.K., Prasad, S., and Shukla, Y. (2009). Resveratrol enhances ultraviolet B-induced cell death through nuclear factor-kappaB pathway in human epidermoid carcinoma A431 cells. Biochem Biophys Res Commun *384*, 215-220.
Rubin, S.M., Gall, A.L., Zheng, N., and Pavletich, N.P. (2005). Structure of the Rb C-terminal domain bound to E2F1-DP1: a mechanism for phosphorylation-induced E2F release. Cell *123*, 1093-1106.
Russo, A.A., Jeffrey, P.D., Patten, A.K., Massague, J., and Pavletich, N.P. (1996). Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature *382*, 325-331.
Russo, M., Spagnuolo, C., Volpe, S., Mupo, A., Tedesco, I., and Russo, G.L. (2010). Quercetin induced apoptosis in association with death receptors and fludarabine in cells isolated from chronic lymphocytic leukaemia patients. Br J Cancer.

Rydzanicz, M., Golusinski, P., Mielcarek-Kuchta, D., Golusinski, W., and Szyfter, K. (2006). Cyclin D1 gene (CCND1) polymorphism and the risk of squamous cell carcinoma of the larynx. Eur Arch Otorhinolaryngol *263*, 43-48.
Saif, M.W. (2010). Colorectal cancer in review: the role of the EGFR pathway. Expert Opin Investig Drugs *19*, 357-369.
Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.
Salucci, M., Stivala, L.A., Maiani, G., Bugianesi, R., and Vannini, V. (2002). Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). Br J Cancer *86*, 1645-1651.
Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J *17*, 1675-1687.
Scaffidi, C., Schmitz, I., Krammer, P.H., and Peter, M.E. (1999a). The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem *274*, 1541-1548.
Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S.J., Krammer, P.H., and Peter, M.E. (1999b). Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem *274*, 22532-22538.
Scalbert, A., and Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. J Nutr *130*, 2073S-2085S.
Scambia, G., Lovergine, S., and Masciullo, V. (2006). RB family members as predictive and prognostic factors in human cancer. Oncogene *25*, 5302-5308.
Scheid, M.P., and Woodgett, J.R. (2001). PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol *2*, 760-768.
Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253-262.
Schmidt, M., Fernandez de Mattos, S., van der Horst, A., Klompmaker, R., Kops, G.J., Lam, E.W., Burgering, B.M., and Medema, R.H. (2002). Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol *22*, 7842-7852.
Schwartz, G.K., and Shah, M.A. (2005). Targeting the cell cycle: a new approach to cancer therapy. J Clin Oncol *23*, 9408-9421.
Scuderi, R., Palucka, K.A., Pokrovskaja, K., Bjorkholm, M., Wiman, K.G., and Pisa, P. (1996). Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. Blood *87*, 3360-3367.
Seelinger, G., Merfort, I., and Schempp, C.M. (2008a). Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med *74*, 1667-1677.
Seelinger, G., Merfort, I., Wolfle, U., and Schempp, C.M. (2008b). Anti-carcinogenic effects of the flavonoid luteolin. Molecules *13*, 2628-2651.
Selvendiran, K., Koga, H., Ueno, T., Yoshida, T., Maeyama, M., Torimura, T., Yano, H., Kojiro, M., and Sata, M. (2006). Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. Cancer Res *66*, 4826-4834.
Serin, M., Erkal, H.S., and Cakmak, A. (1999). Radiation therapy and concurrent cisplatin in management of locoregionally advanced nasopharyngeal carcinomas. Acta Oncol *38*, 1031-1035.
Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704-707.
Shah, K.V. (2004). Causality of mesothelioma: SV40 question. Thorac Surg Clin *14*, 497-504.
Shanmugaratnam, K., and Sobin, L. (1991). Histologic typing of tumours of the upper respiratory tract and ear., 2nd edn (Geneva, WHO).

Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res *60*, 3689-3695.
Sherr, C.J. (2001). The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol *2*, 731-737.
Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.
Shi, R., Huang, Q., Zhu, X., Ong, Y.B., Zhao, B., Lu, J., Ong, C.N., and Shen, H.M. (2007). Luteolin sensitizes the anticancer effect of cisplatin via c-Jun NH2-terminal kinase-mediated p53 phosphorylation and stabilization. Mol Cancer Ther *6*, 1338-1347.
Shi, R.X., Ong, C.N., and Shen, H.M. (2004). Luteolin sensitizes tumor necrosis factor-alpha-induced apoptosis in human tumor cells. Oncogene *23*, 7712-7721.
Shi, R.X., Ong, C.N., and Shen, H.M. (2005). Protein kinase C inhibition and x-linked inhibitor of apoptosis protein degradation contribute to the sensitization effect of luteolin on tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in cancer cells. Cancer Res *65*, 7815-7823.
Shi, W., Bastianutto, C., Li, A., Perez-Ordonez, B., Ng, R., Chow, K.Y., Zhang, W., Jurisica, I., Lo, K.W., Bayley, A*., et al.* (2006). Multiple dysregulated pathways in nasopharyngeal carcinoma revealed by gene expression profiling. Int J Cancer *119*, 2467-2475.
Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer *3*, 155-168.
Shima, H., Hiyama, T., Tanaka, S., Ito, M., Kitadai, Y., Yoshihara, M., Arihiro, K., and Chayama, K. (2005). Loss of heterozygosity on chromosome 10p14-p15 in colorectal carcinoma. Pathobiology *72*, 220-224.
Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H., and Kinae, N. (1998). Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. FEBS Lett *438*, 220-224.
Shin, I., Yakes, F.M., Rojo, F., Shin, N.Y., Bakin, A.V., Baselga, J., and Arteaga, C.L. (2002). PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med *8*, 1145-1152.
Shishodia, S., and Aggarwal, B.B. (2006). Diosgenin inhibits osteoclastogenesis, invasion, and proliferation through the downregulation of Akt, I kappa B kinase activation and NF-kappa B-regulated gene expression. Oncogene *25*, 1463-1473.
Sieber, O.M., Heinimann, K., and Tomlinson, I.P. (2003). Genomic instability--the engine of tumorigenesis? Nat Rev Cancer *3*, 701-708.
Siess, M.H., Guillermic, M., Le Bon, A.M., and Suschetet, M. (1989). Induction of monooxygenase and transferase activities in rat by dietary administration of flavonoids. Xenobiotica *19*, 1379-1386.
Singh, R.P., and Agarwal, R. (2006). Natural flavonoids targeting deregulated cell cycle progression in cancer cells. Curr Drug Targets *7*, 345-354.
Singh, S., and Aggarwal, B.B. (1995). Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem *270*, 24995-25000.
Sizhong, Z., Xiukung, G., and Yi, Z. (1983). Cytogenetic studies on an epithelial cell line derived from poorly differentiated nasopharyngeal carcinoma. Int J Cancer *31*, 587-590.
Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S*., et al.* (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol *144*, 281-292.
Solomon, D.A., Kim, J.S., Jean, W., and Waldman, T. (2008). Conspirators in a capital crime: co-deletion of p18INK4c and p16INK4a/p14ARF/p15INK4b in glioblastoma multiforme. Cancer Res *68*, 8657-8660.
Song, G., Ouyang, G., and Bao, S. (2005a). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med *9*, 59-71.

Song, X., Tao, Y.G., Zeng, L., Deng, X.Y., Lee, L.M., Gong, J.P., Wu, Q., and Cao, Y. (2005b). Latent membrane protein 1 encoded by Epstein-Barr virus modulates directly and synchronously cyclin D1 and p16 by newly forming a c-Jun/Jun B heterodimer in nasopharyngeal carcinoma cell line. Virus Res *113*, 89-99.
Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M.F., Piwnica-Worms, H., and Cantley, L.C. (1994). Use of an oriented peptide library to determine the optimal substrates of protein kinases. Curr Biol *4*, 973-982.
Songyang, Z., Lu, K.P., Kwon, Y.T., Tsai, L.H., Filhol, O., Cochet, C., Brickey, D.A., Soderling, T.R., Bartleson, C., Graves, D.J*., et al.* (1996). A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. Mol Cell Biol *16*, 6486-6493.
Southgate, J., Proffitt, J., Roberts, P., Smith, B., and Selby, P. (1995). Loss of cyclin-dependent kinase inhibitor genes and chromosome 9 karyotypic abnormalities in human bladder cancer cell lines. Br J Cancer *72*, 1214-1218.
Spencer, J.P., Kuhnle, G.G., Williams, R.J., and Rice-Evans, C. (2003). Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. Biochem J *372*, 173-181.
Spruck, C.H., Won, K.A., and Reed, S.I. (1999). Deregulated cyclin E induces chromosome instability. Nature *401*, 297-300.
Srinivasan, J., Koszelak, M., Mendelow, M., Kwon, Y.G., and Lawrence, D.S. (1995). The design of peptide-based substrates for the cdc2 protein kinase. Biochem J *309 (Pt 3)*, 927-931.
Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E.S. (1996). Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci U S A *93*, 14486-14491.
Srinivasula, S.M., and Ashwell, J.D. (2008). IAPs: what's in a name? Mol Cell *30*, 123-135.
Srinivasula, S.M., Gupta, S., Datta, P., Zhang, Z., Hegde, R., Cheong, N., Fernandes-Alnemri, T., and Alnemri, E.S. (2003). Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. J Biol Chem *278*, 31469-31472.
St Croix, B., Florenes, V.A., Rak, J.W., Flanagan, M., Bhattacharya, N., Slingerland, J.M., and Kerbel, R.S. (1996). Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. Nat Med *2*, 1204-1210.
Staal, S.P. (1987). Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A *84*, 5034-5037.
Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.
Stark, G.R., and Taylor, W.R. (2006). Control of the G2/M transition. Mol Biotechnol *32*, 227-248.
Steinitz, R., Parkin, D.M., Young, J.L., Bieber, C.A., and Katz, L. (1989). Cancer Incidence in Jewish Migrants to Israel, 1961-1981. IARC Scientific Publications No 98, International Agency for Research on Cancer, Lyon.
Steinmetz, K.A., and Potter, J.D. (1996). Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc *96*, 1027-1039.
Strasser, A. (2005). The role of BH3-only proteins in the immune system. Nat Rev Immunol *5*, 189-200.
Stratton, M.R., Campbell, P.J., and Futreal, P.A. (2009). The cancer genome. Nature *458*, 719-724.

Suh, D.K., Lee, E.J., Kim, H.C., and Kim, J.H. (2010). Induction of G(1)/S phase arrest and apoptosis by quercetin in human osteosarcoma cells. Arch Pharm Res *33*, 781-785.
Sun, T., Xu, Z., Wu, C.T., Janes, M., Prinyawiwatkul, W., and No, H.K. (2007). Antioxidant activities of different colored sweet bell peppers (Capsicum annuum L.). J Food Sci *72*, S98-102.
Sun, Z.J., Chen, G., Hu, X., Zhang, W., Liu, Y., Zhu, L.X., Zhou, Q., and Zhao, Y.F. (2010). Activation of PI3K/Akt/IKK-alpha/NF-kappaB signaling pathway is required for the apoptosis-evasion in human salivary adenoid cystic carcinoma: its inhibition by quercetin. Apoptosis *15*, 850-863.
Surh, Y.J., Hurh, Y.J., Kang, J.Y., Lee, E., Kong, G., and Lee, S.J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett *140*, 1-10.
Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T., and Miura, M. (1998). Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. Oncogene *17*, 931-939.
Takagaki, N., Sowa, Y., Oki, T., Nakanishi, R., Yogosawa, S., and Sakai, T. (2005). Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. Int J Oncol *26*, 185-189.
Takahashi-Yanaga, F., and Sasaguri, T. (2008). GSK-3beta regulates cyclin D1 expression: a new target for chemotherapy. Cell Signal *20*, 581-589.
Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S., and Reed, J.C. (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem *273*, 7787-7790.
Takizawa, C.G., and Morgan, D.O. (2000). Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr Opin Cell Biol *12*, 658-665.
Tang, F.Y., Nguyen, N., and Meydani, M. (2003). Green tea catechins inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule. Int J Cancer *106*, 871-878.
Tao, Y., Song, X., Deng, X., Xie, D., Lee, L.M., Liu, Y., Li, W., Li, L., Deng, L., Wu, Q.*, et al.* (2005). Nuclear accumulation of epidermal growth factor receptor and acceleration of G1/S stage by Epstein-Barr-encoded oncoprotein latent membrane protein 1. Exp Cell Res *303*, 240-251.
Tartaglia, L.A., Ayres, T.M., Wong, G.H., and Goeddel, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. Cell *74*, 845-853.
Tashiro, E., Tsuchiya, A., and Imoto, M. (2007). Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression. Cancer Sci *98*, 629-635.
Taylor, W.R., and Stark, G.R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803-1815.
Thomas, G. (2000). An encore for ribosome biogenesis in the control of cell proliferation. Nat Cell Biol *2*, E71-E72.
Thomasset, S.C., Berry, D.P., Garcea, G., Marczylo, T., Steward, W.P., and Gescher, A.J. (2007). Dietary polyphenolic phytochemicals--promising cancer chemopreventive agents in humans? A review of their clinical properties. Int J Cancer *120*, 451-458.
Thornberry, N.A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-1316.
Tijburg, L.B., Mattern, T., Folts, J.D., Weisgerber, U.M., and Katan, M.B. (1997). Tea flavonoids and cardiovascular disease: a review. Crit Rev Food Sci Nutr *37*, 771-785.
Tokunaga, E., Oki, E., Egashira, A., Sadanaga, N., Morita, M., Kakeji, Y., and Maehara, Y. (2008). Deregulation of the Akt pathway in human cancer. Curr Cancer Drug Targets *8*, 27-36.

Tokuyama, Y., Horn, H.F., Kawamura, K., Tarapore, P., and Fukasawa, K. (2001). Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. J Biol Chem *276*, 21529-21537.
Tsao, S.W., Tramoutanis, G., Dawson, C.W., Lo, A.K., and Huang, D.P. (2002). The significance of LMP1 expression in nasopharyngeal carcinoma. Semin Cancer Biol *12*, 473-487.
Tse, K.P., Su, W.H., Chang, K.P., Tsang, N.M., Yu, C.J., Tang, P., See, L.C., Hsueh, C., Yang, M.L., Hao, S.P*., et al.* (2009). Genome-wide association study reveals multiple nasopharyngeal carcinoma-associated loci within the HLA region at chromosome 6p21.3. Am J Hum Genet *85*, 194-203.
Tunon, M.J., Garcia-Mediavilla, M.V., Sanchez-Campos, S., and Gonzalez-Gallego, J. (2009). Potential of flavonoids as anti-inflammatory agents: modulation of pro-inflammatory gene expression and signal transduction pathways. Curr Drug Metab *10*, 256-271.
Tyagi, A.K., Singh, R.P., Agarwal, C., Chan, D.C., and Agarwal, R. (2002). Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. Clin Cancer Res *8*, 3512-3519.
Tysnes, B.B., and Bjerkvig, R. (2007). Cancer initiation and progression: involvement of stem cells and the microenvironment. Biochim Biophys Acta *1775*, 283-297.
Uren, R.T., Dewson, G., Chen, L., Coyne, S.C., Huang, D.C., Adams, J.M., and Kluck, R.M. (2007). Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. J Cell Biol *177*, 277-287.
Urist, M., Tanaka, T., Poyurovsky, M.V., and Prives, C. (2004). p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. Genes Dev *18*, 3041-3054.
Vargas, A.J., and Burd, R. (2010). Hormesis and synergy: pathways and mechanisms of quercetin in cancer prevention and management. Nutr Rev *68*, 418-428.
Vaux, D.L., and Silke, J. (2005). IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol *6*, 287-297.
Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer *2*, 489-501.
Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem *269*, 5241-5248.
Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.
Volgelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nature Medicine *10*, 789-799.
Walensky, L.D., Pitter, K., Morash, J., Oh, K.J., Barbuto, S., Fisher, J., Smith, E., Verdine, G.L., and Korsmeyer, S.J. (2006). A stapled BID BH3 helix directly binds and activates BAX. Mol Cell *24*, 199-210.
Walker, N.P., Talanian, R.V., Brady, K.D., Dang, L.C., Bump, N.J., Ferenz, C.R., Franklin, S., Ghayur, T., Hackett, M.C., Hammill, L.D*., et al.* (1994). Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. Cell *78*, 343-352.
Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A.V., and Boldin, M.P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol *17*, 331-367.
Walle, T., Otake, Y., Walle, U.K., and Wilson, F.A. (2000). Quercetin glucosides are completely hydrolyzed in ileostomy patients before absorption. J Nutr *130*, 2658-2661.
Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S., Jr. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680-1683.

Wang, J.M., Chao, J.R., Chen, W., Kuo, M.L., Yen, J.J., and Yang-Yen, H.F. (1999). The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB. Mol Cell Biol *19*, 6195-6206.
Wasch, R., Robbins, J.A., and Cross, F.R. (2010). The emerging role of APC/CCdh1 in controlling differentiation, genomic stability and tumor suppression. Oncogene *29*, 1-10.
Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nat Cell Biol *1*, 20-26.
Wei, W.I., and Kwong, D.L. (2010). Current management strategy of nasopharyngeal carcinoma. Clin Exp Otorhinolaryngol *3*, 1-12.
Wendel, H.G., De Stanchina, E., Fridman, J.S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J., and Lowe, S.W. (2004). Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature *428*, 332-337.
Wiesner, T., Obenauf, A.C., Cota, C., Fried, I., Speicher, M.R., and Cerroni, L. (2010). Alterations of the cell-cycle inhibitors p27(KIP1) and p16(INK4a) are frequent in blastic plasmacytoid dendritic cell neoplasms. J Invest Dermatol *130*, 1152-1157.
Willett, W.C. (2000). Diet and cancer. Oncologist *5*, 393-404.
Williams, R.J., Spencer, J.P.E., and Rice-Evans, C. (2004). Flavonoids: antioxidants or signalling molecules? Free Radical Biology and Medicine *36*, 838-849.
Williamson, G., Barron, D., Shimoi, K., and Terao, J. (2005). In vitro biological properties of flavonoid conjugates found in vivo. Free Radic Res *39*, 457-469.
Willis, S.N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J.I., Adams, J.M., and Huang, D.C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev *19*, 1294-1305.
Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P.*, et al.* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.
Wilson, A.J., Byun, D.S., Nasser, S., Murray, L.B., Ayyanar, K., Arango, D., Figueroa, M., Melnick, A., Kao, G.D., Augenlicht, L.H.*, et al.* (2008). HDAC4 promotes growth of colon cancer cells via repression of p21. Mol Biol Cell *19*, 4062-4075.
Wimmer, K., and Etzler, J. (2008). Constitutional mismatch repair-deficiency syndrome: have we so far seen only the tip of an iceberg? Hum Genet *124*, 105-122.
Wittmann, S., Bali, P., Donapaty, S., Nimmanapalli, R., Guo, F., Yamaguchi, H., Huang, M., Jove, R., Wang, H.G., and Bhalla, K. (2003). Flavopiridol down-regulates antiapoptotic proteins and sensitizes human breast cancer cells to epothilone B-induced apoptosis. Cancer Res *63*, 93-99.
Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., and Youle, R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol *139*, 1281-1292.
Wong, A.H., Gottesman, II, and Petronis, A. (2005). Phenotypic differences in genetically identical organisms: the epigenetic perspective. Hum Mol Genet *14 Spec No 1*, R11-18.
Wong, A.S., Soo, R.A., Lu, J.J., Loh, K.S., Tan, K.S., Hsieh, W.S., Shakespeare, T.P., Chua, E.T., Lim, H.L., and Goh, B.C. (2006). Paclitaxel, 5-fluorouracil and hydroxyurea concurrent with radiation in locally advanced nasopharyngeal carcinoma. Ann Oncol *17*, 1152-1157.
Wu, B., Zhang, Q., Shen, W., and Zhu, J. (2008). Anti-proliferative and chemosensitizing effects of luteolin on human gastric cancer AGS cell line. Mol Cell Biochem *313*, 125-132.
Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X., and Shi, Y. (2000). Structural basis of IAP recognition by Smac/DIABLO. Nature *408*, 1008-1012.
Xavier, C.P., Lima, C.F., Preto, A., Seruca, R., Fernandes-Ferreira, M., and Pereira-Wilson, C. (2009). Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and

inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells. Cancer Lett *281*, 162-170.
Xie, L., Xu, L., He, Z., Zhou, W., Wang, L., Zhang, L., Lan, K., Ren, C., Liu, W., and Yao, K. (2000). Identification of differentially expressed genes in nasopharyngeal carcinoma by means of the Atlas human cancer cDNA expression array. J Cancer Res Clin Oncol *126*, 400-406.
Xie, Y.Y., Yuan, D., Yang, J.Y., Wang, L.H., and Wu, C.F. (2009). Cytotoxic activity of flavonoids from the flowers of Chrysanthemum morifolium on human colon cancer Colon205 cells. J Asian Nat Prod Res *11*, 771-778.
Xing, X., Chen, J., and Chen, M. (2008). Expression of CDC25 phosphatases in human gastric cancer. Dig Dis Sci *53*, 949-953.
Xu, M., Sheppard, K.A., Peng, C.Y., Yee, A.S., and Piwnica-Worms, H. (1994). Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol *14*, 8420-8431.
Yan, Y., Frisen, J., Lee, M.H., Massague, J., and Barbacid, M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes Dev *11*, 973-983.
Yang, F., Oz, H.S., Barve, S., de Villiers, W.J., McClain, C.J., and Varilek, G.W. (2001a). The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. Mol Pharmacol *60*, 528-533.
Yang, G., and Yang, X. (2010). Smad4-mediated TGF-beta signaling in tumorigenesis. Int J Biol Sci *6*, 1-8.
Yang, H.J., Cho, Y.J., Kim, H.S., Chang, M.S., Sung, M.W., and Kim, W.H. (2001b). Association of p53 and BCL-2 expression with Epstein-Barr virus infection in the cancers of head and neck. Head Neck *23*, 629-636.
Yang, J., Bardes, E.S., Moore, J.D., Brennan, J., Powers, M.A., and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes Dev *12*, 2131-2143.
Yang, Q.H., Church-Hajduk, R., Ren, J., Newton, M.L., and Du, C. (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. Genes Dev *17*, 1487-1496.
Yang, Z., and Klionsky, D.J. (2009). An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol *335*, 1-32.
Yao, P., Nussler, A., Liu, L., Hao, L., Song, F., Schirmeier, A., and Nussler, N. (2007). Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways. J Hepatol *47*, 253-261.
Yi, F., Saha, A., Murakami, M., Kumar, P., Knight, J.S., Cai, Q., Choudhuri, T., and Robertson, E.S. (2009). Epstein-Barr virus nuclear antigen 3C targets p53 and modulates its transcriptional and apoptotic activities. Virology *388*, 236-247.
You, H., Pellegrini, M., Tsuchihara, K., Yamamoto, K., Hacker, G., Erlacher, M., Villunger, A., and Mak, T.W. (2006). FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med *203*, 1657-1663.
Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*, 47-59.
Yu, K.J., Gao, X., Chen, C.J., Yang, X.R., Diehl, S.R., Goldstein, A., Hsu, W.L., Liang, X.S., Marti, D., Liu, M.Y.*, et al.* (2009). Association with human leucocyte antigens with nasopharyngeal carcinoma in high-risk multiplex families in Taiwan. Hum Immunol *70*, 910-914.
Yu, M.C. (1991). Nasopharyngeal carcinoma: epidemiology and dietary factors. IARC Sci Publ, 39-47.

Yu, M.C., and Henderson, B.E. (1987). Intake of Cantonese-style salted fish as a cause of nasopharyngeal carcinoma. IARC Sci Publ *84*, 547-549.
Yu, M.C., Mo, C.-C., Chong, W.-X., Yeh, F.-S., and Henderson, B.E. (1988). Preserved foods and nasopharyngeal carcinoma: a case-control study in Guangxi, China. Cancer Res *48*, 1954-1959.
Yuan, J.-M., Wang, X.-L., Xiang, Y.-B., Gao, Y.-T., Ross, R.K., and Yu, M.C. (2000). Preserved foods in relatio to risk of nasopharyngeal carcinoma in Shanghai, China. Int J Cancer *85*, 358-363.
Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A., and Elledge, S.J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature *387*, 151-158.
Zhao, J., Dynlacht, B., Imai, T., Hori, T., and Harlow, E. (1998). Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. Genes Dev *12*, 456-461.
Zheng, X., Luo, Y., Christensson, B., and Drettner, B. (1994). Induction of nasal and nasopharyngeal tumours in Sprague-Dawley rats fed with Chinese salted fish. Acta Otalaryngol *114*, 98-104.
Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.
Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M.C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol *3*, 973-982.
Zhou, Q., He, Q., and Liang, L.J. (2003). Expression of p27, cyclin E and cyclin A in hepatocellular carcinoma and its clinical significance. World J Gastroenterol *9*, 2450-2454.
Zhou, S.F., Wang, L.L., Di, Y.M., Xue, C.C., Duan, W., Li, C.G., and Li, Y. (2008). Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. Curr Med Chem *15*, 1981-2039.
Zhu, C.Q., Blackhall, F.H., Pintilie, M., Iyengar, P., Liu, N., Ho, J., Chomiak, T., Lau, D., Winton, T., Shepherd, F.A., *et al.* (2004). Skp2 gene copy number aberrations are common in non-small cell lung carcinoma, and its overexpression in tumors with ras mutation is a poor prognostic marker. Clin Cancer Res *10*, 1984-1991.
Zi, X., and Agarwal, R. (1999). Modulation of Mitogen-Activated Protein Kinase Activation and Cell Cycle Regulators by the Potent Skin Cancer Preventive Agent Silymarin. Biochemical and Biophysical Research Communications *263*, 528-536.
Ziegler, R.G., Hoover, R.N., Pike, M.C., Hildesheim, A., Nomura, A.M., West, D.W., Wu-Williams, A.H., Kolonel, L.N., Horn-Ross, P.L., Rosenthal, J.F., *et al.* (1993). Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst *85*, 1819-1827.
Zindy, F., Quelle, D.E., Roussel, M.F., and Sherr, C.J. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene *15*, 203-211.
Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev *15*, 1481-1486.