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 Thr286 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.
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<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
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<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-promoting complex/cyclosome</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic-activating factor-1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia-mutated</td>
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<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ATR</td>
<td>Ataxia and rad3 related</td>
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<td>B-cell lymphoma-2</td>
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<td>BH</td>
<td>Bcl-2 homology</td>
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<td>BIR</td>
<td>Baculovirus IAP repeat</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>BRUCE</td>
<td>BIR repeat-containing ubiquitin-conjugating enzyme</td>
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<tr>
<td>CAD</td>
<td>Caspase-activator deoxyribonuclease</td>
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<tr>
<td>CAK</td>
<td>Cdk-activating kinase</td>
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<td>CARD</td>
<td>Caspase recruitment domain</td>
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<td>CDH1</td>
<td>CDC20 homologue 1</td>
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<td>Cdks</td>
<td>Cyclin-dependent kinases</td>
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<tr>
<td>CHX</td>
<td>Cycloheximide</td>
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<td>CKIs</td>
<td>Cyclin-dependent kinase inhibitors</td>
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<tr>
<td>c-FLIP</td>
<td>Cellular Fas-associated DD-like interleukin (IL)-1-converting enzyme inhibitory protein</td>
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<td>CREB</td>
<td>Cyclic-AMP response element-binding protein</td>
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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
<table>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<td>NPA</td>
<td>Nuclear protein mapped at the AT locus</td>
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<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
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<td>NPM/B23</td>
<td>Nucleophosmin</td>
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<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<td>ORC</td>
<td>Origin recognition complex</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PDK1/2</td>
<td>3-phosphoinositide-dependent protein kinase 1 / 2</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIP₂</td>
<td>Phosphatidylinositol-4, 5-bisphosphate</td>
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<td>PIP₃</td>
<td>Phosphatidylinositol-3, 4, 5-triphosphate</td>
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<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>Plk</td>
<td>Polo-related kinase</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylefluoride</td>
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<tr>
<td>pRb</td>
<td>Retinoblastoma</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>PTP</td>
<td>Mitochondrial permeability transition pore</td>
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<td>PTX</td>
<td>Paclitaxel</td>
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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, 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

Presentation at scientific conferences:


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 make 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 residues 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
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).
PI3K is involved in a wide variety of cellular pathways 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 Cdns 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 (Matsushima 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\textsuperscript{216} residue (in human). However, little is known about the identity of the kinases and phosphatases that act on Ser\textsuperscript{216}. There are strong indications that Chk1 and Chk2 are possible candidates as both enzymes are able to phosphorylate cdc25 at ser\textsuperscript{216} \textit{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).
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 \textit{CDC6} (Hateboer et al., 1998); \textit{DHFR} (dihydrofolate reductase) (Blake and Azizkhan, 1989; Noe et al., 1997); \textit{TK} (thymidine kinase) (Dou et al., 1994); \textit{DNA polymerase \alpha} (Izumi et al., 2000); and \textit{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).
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\textsuperscript{INK4A} (Serrano et al., 1993), p15\textsuperscript{INK4B} (Hannon and Beach, 1994), p18\textsuperscript{INK4C} (Hirai et al., 1995) and p19\textsuperscript{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\textsuperscript{INK4A} forms a strong association with p14\textsuperscript{ARF}. p14\textsuperscript{ARF} protein inhibits cell cycle progression by stabilising the complex between p53 and MDM2 (Weber et al., 1999). Expression of p14\textsuperscript{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\textsuperscript{INK4A} is functionally equivalent to loss of pRb whereas the loss of P14\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{INK4C} and p19\textsuperscript{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\textsuperscript{CIP1}, p27\textsuperscript{KIP1} and p57\textsuperscript{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<sup>CIP1</sup> 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<sup>CIP1</sup> 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<sup>CIP1</sup> 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<sup>CIP1</sup> 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<sup>KIP1</sup>, an inhibitor of cdk2-cyclin E and therefore plays a role as a negative regulator at the G1/S transition. The activity of p27<sup>KIP1</sup> 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<sup>KIP1</sup> 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 p27KIP1 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, p57KIP2 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 controls another 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 $p53$ gene (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).

![Caspase Domain Organisation](image)

**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 pro-caspase-8 to its DED domain when the DED or prodomain on pro-caspase-8 binds to its DED, resulting in the formation of the death-inducing signalling complex (DISC) (Fig 1.6). The recruitment and oligomerisation of pro-caspase-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.
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 Bel-2 family (Scaffidi et al., 1998) (Fig 1.7).
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\textsubscript{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).

![Diagram]

**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).
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	extsubscript{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 intermembrane 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 mitochondrial 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.,
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-tri phosphate (PIP$_3$) formed from the phosphorylation of membrane phospholipid, phosphatidylinositol-4, 5-bisphosphate (PIP$_2$) by PI3K (Cantley, 2002) (Fig 1.1). 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 1.1).
As Akt activation is initiated by Akt binding to PIP₃ 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 PIP₃ by dephosphorylating it to PIP₂, 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

Fig 1.11: The phosphoinositide 3-kinase-Akt signalling cascade (adapted from (Tokunaga et al., 2008))
et al., 1999; Brunet et al., 1999; Rena et al., 1999) (Fig 1.11). It can also prevent apoptosis by activating IkB 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 p21CIP1 and p27KIP1; 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 p27KIP1 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 p21CIP1 and p27KIP1 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 p21CIP1 and p27KIP1 by
up-regulating the expression of SKP2, a key component of the SCF/SKP2 ubiquitin ligase that mediates p21\textsuperscript{CIP1} and p27\textsuperscript{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<sub>L</sub>, 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) as well as the PI3K/Akt signal transduction machinery. These will lead to downstream 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
Activated p53 consequently leads to the up-regulation of CKI, p21\(^\text{Cip1}\) 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-\(\kappa\)B, 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).
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-pentahydroxyflavone) 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 glucuronides 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-tetrahydroxyflavone) 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 (Ser9) 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-p21CIP1 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°C in a humified 5% CO₂ 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 10⁴ 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 FACSsort 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, IC₅₀ (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 \(\mu\)M 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 \(\mu\)M 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\textsuperscript{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\textsubscript{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.
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**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.
<table>
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**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 p21CIP1 and p27KIP1 (Blume-Jensen and Hunter, 2001). Akt is capable of phosphorylating GSK-3β at Ser9 and subsequently inhibiting its kinase activity. Active GSK-3β phosphorylates cyclin D1 at Thr286 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 One step 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°C in a humidified 5% CO₂ 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°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 HK1 cells 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 G1 and 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 D1 ubiquitination. 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](image)

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 p21CIP1 (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-3\(\beta\) 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-3\(\beta\) regulates cyclin D1 degradation, a GSK-3\(\beta\)-specific inhibitor LiCl was able to suppress luteolin-induced down-regulation of this protein (Fig 3.7B), suggesting the involvement of the GSK-3\(\beta\) 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-3\(\beta\) has been identified as one of Akt’s molecular targets. Akt inactivates GSK-3\(\beta\) 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
epophilones 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 ser473. 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\textsuperscript{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°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.
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 HK1 cells as observed in CNE2 cells; it sensitised HK1 cells to VCR, resulting in a caspase-3-dependent apoptotic cell death.
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.
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.
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](image)

Cells were first grown overnight in full medium (RPMI-1640 supplemented with 10% FBS) and pre-treated with 5 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<sup>CIP1</sup> (Wu et al., 2008).

In this study, we also focused 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<sup>286</sup>
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-XL, 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 \textit{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 \textit{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.
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