CHAPTER II

LITERATURE REVIEWS

Cancer is a cellular disorder characterized by progressive accumulation of a mass of cells as a result of excessive cell proliferation over cell death. Cancer cells arise when normal cells lose the normal regulatory mechanisms that control their growth and proliferation. They lose differentiation and become immortal. Many currently used anticancer agents were developed to overcome excessive cellular proliferation by causing cytostasis or cytotoxicity. These cytotoxic agents kill cancer cells by either necrosis or apoptosis.

Necrosis is an accidental cell death. It is always the outcome of severe and acute injury: i.e. abrupt anoxia, sudden shortage of nutrients such as glucose, or extreme physicochemical injury (heat, detergents, strong bases etc) **[11]**. Necrosis does not involve any regular DNA and protein degradation pattern and is accompanied by swelling of the entire cytoplasm and of the mitochondrial matrix, which occur shortly before the cell membrane ruptures **[12]** and release of cellular contents into the extracellular space. These cellular contents collectively called danger-associated molecular patterns (DAMPs) or alarmins, include the high mobility group protein B1, uric acid, certain heat shock proteins, single-stranded RNA and genomic DNA. These molecules trigger inflammation by stimulating one or more pattern-recognition receptors on neutrophils, macrophages, dendritic cells and natural killer cells **[13, 14]** and cause the information of an inflammatory process that damages the cells and surrounding tissues.

In contrast, Apoptosis, a form of cell death with critical importance for the embryonic development and for the maintenance of tissue homeostasis, was carried out in a safe and controlled manner to ensure that neighboring structures remain unaffected. Cancer cells are characterized by a deregulated proliferation, and/or an inability to undergo programmed cell death. The machinery responsible for killing and degradation of the cell via apoptosis is constitutively expressed and becomes activated through various stimuli. **[15]**. Nowadays, many currently used cytotoxic drugs kill cancer cells by apoptotic induction. Not only apoptotic induction, the induction of cell cycle

arrest is also the common activity shared by many anticancer drugs. Many stimuli such as radiation, mitotic inhibitors and topoisomerase inhibitors have been found to arrest cancer cells at specific phase of the cell cycle. Inhibition of the cell cycle can result in severe cell damage and provoke apoptosis **[16]**. The detail of apoptosis and cell cycle arrest are described below.

Programmed cell death by apoptosis

Apoptosis is characterized by cellular biochemical and morphological changes. These include cell shrinkage, chromatin condensation, DNA fragmentation, plasma membrane convolutes or blebbing, and the formation of membrane bound vesicles named as apoptotic bodies, but most organelles remain intact. These apoptotic bodies are recognized and engulfed by phagocytes without initiating any inflammatory response (Fig. 1) **[17, 18, 19]**. Many events during the processes are initiated and executed by the core effectors of apoptosis encompass proteolytic enzymes of the caspase family. The caspases constitute a family of cysteine proteases. These peptidases use their cysteine residue as the catalytic nucleophile, that share an exquisite specificity for cleaving target proteins at sites next to aspartic acid residues of their target proteins **[20]**. These caspases specifically target several hundred proteins for proteolysis in a controlled manner that minimizes damage neighboring cells and avoids the release of alarmins **[21]**.

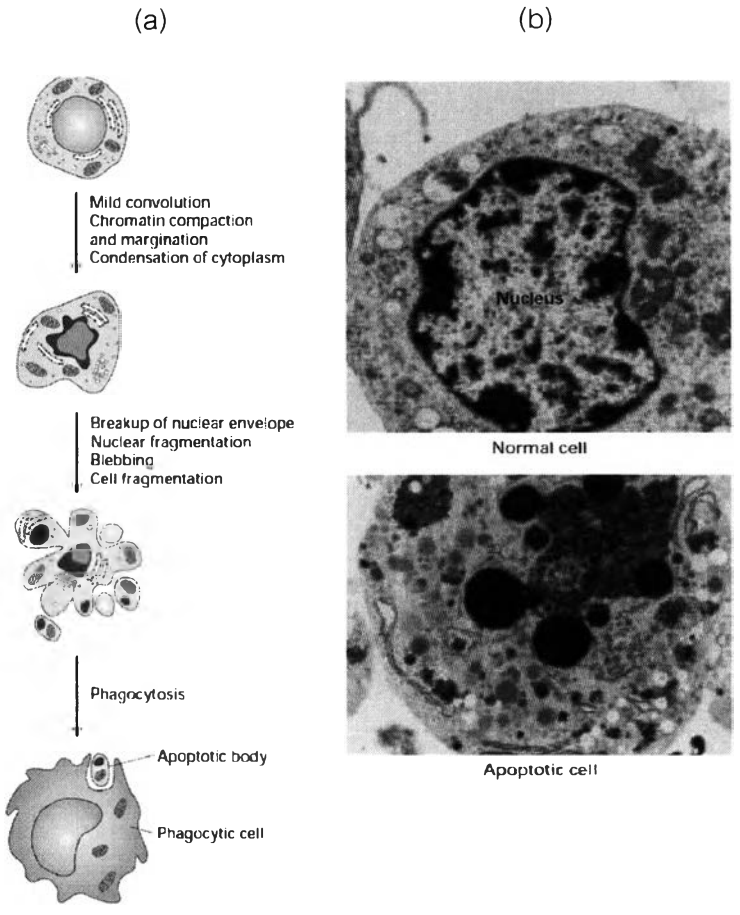

Figure 1: Characteristics of cell death by apoptosis **[22]**. a) The progression of morphologic changes observed in apoptotoc cell, b) photographs under electron microscope comparing a normal cell (top) and apoptotic cell (bottom).

Caspases

All caspases are synthesized in single polypeptide chains as pro-enzymes or inactive zymogens. These zymogens contain three domains in their molecules. There are prodomain at the NH_2-terminal end, central large internal domain (p20) containing an active site, and small C-terminal domain (p10) called small catalytic subunit (Fig. 2) **[23]**. The active form of each caspase consists of two of p20 and p10 subunits as a heterotetrameric enzyme.

Twelve human caspases (caspases 1–10, caspase 12 and caspase 14) have been identified to date **[24]**. These caspases are divided into 3 major groups based on the structure and function of prodomians (Fig. 3). Inflammatory caspases are the group I caspases with a long prodomains (caspase-1, caspase-4, caspase-5, caspase-12, caspase-13 and caspase-14). They play roles on cytokine maturation and inflammatory responses. The group II caspases are initiator caspase containing a long prodomain with a death effector domain, DED, (caspase 8 and 10) or a caspase-recruitment domain, CARD, (caspase 2 and 9). These enzymes play role as initiator caspases in apoptosis. The group III caspases are effector caspases (caspase-3, caspase-6, and caspase-7) containing a short prodomain (20–30 amino acids) **[25, 26]**. They play roles as executioner caspases during apoptotic process.

All known caspases contain an active-site cysteine and cleave substrates at Asp-Xxx bonds. The substrate specificity of each caspase is determined by the four residues amino-terminal to the cleavage site of its target protein **[27]**. These caspases are activated by proteolytic cleavage of their zymogens between the p20 and p10 domains, and also between the prodomain and the p20 domain (Fig. 2). All these cleavage sites occur at Asp-X sites which are also their substrate specificity sites. They can autocatalysis themselves as for initiator caspases as well as catalysis their following caspases as effector caspases in the caspase cascade activation. The caspase cascade is a useful method to amplify and integrate pro-apoptotic signals.

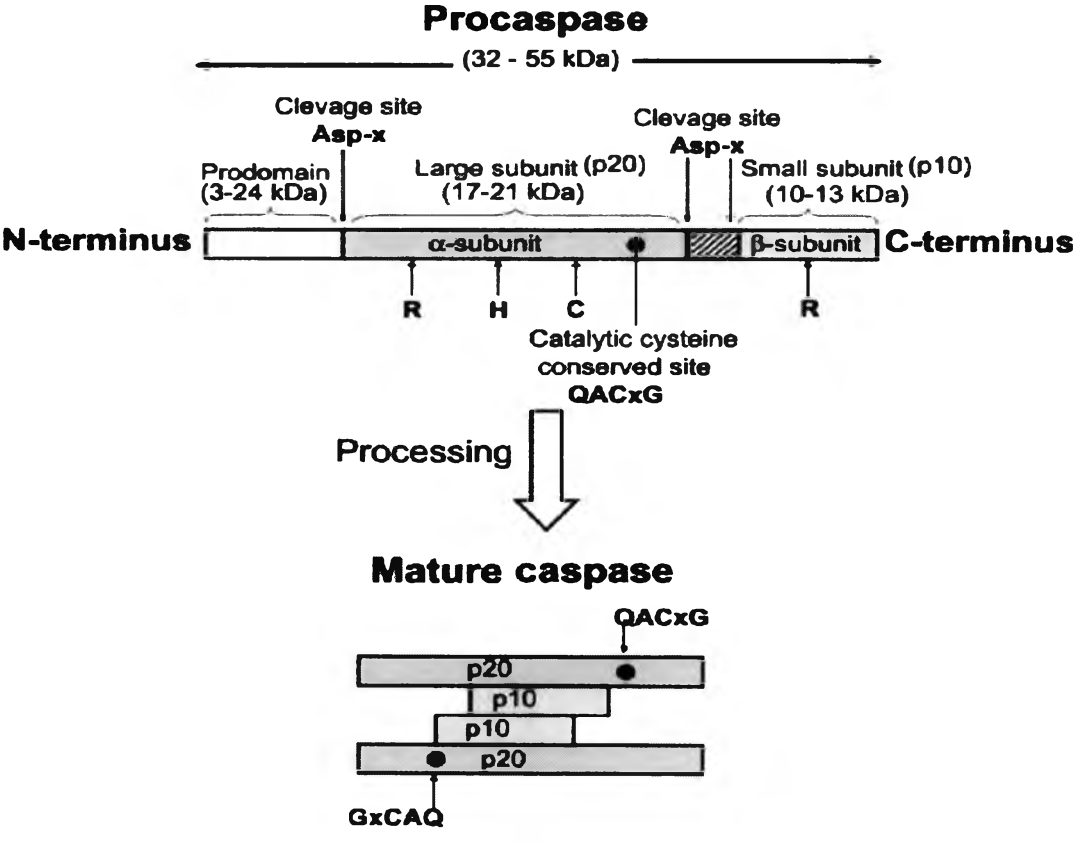

Figure 2: A schematic representation of structural features of mammalian caspases [25]. C, H and R represent the active site residues.

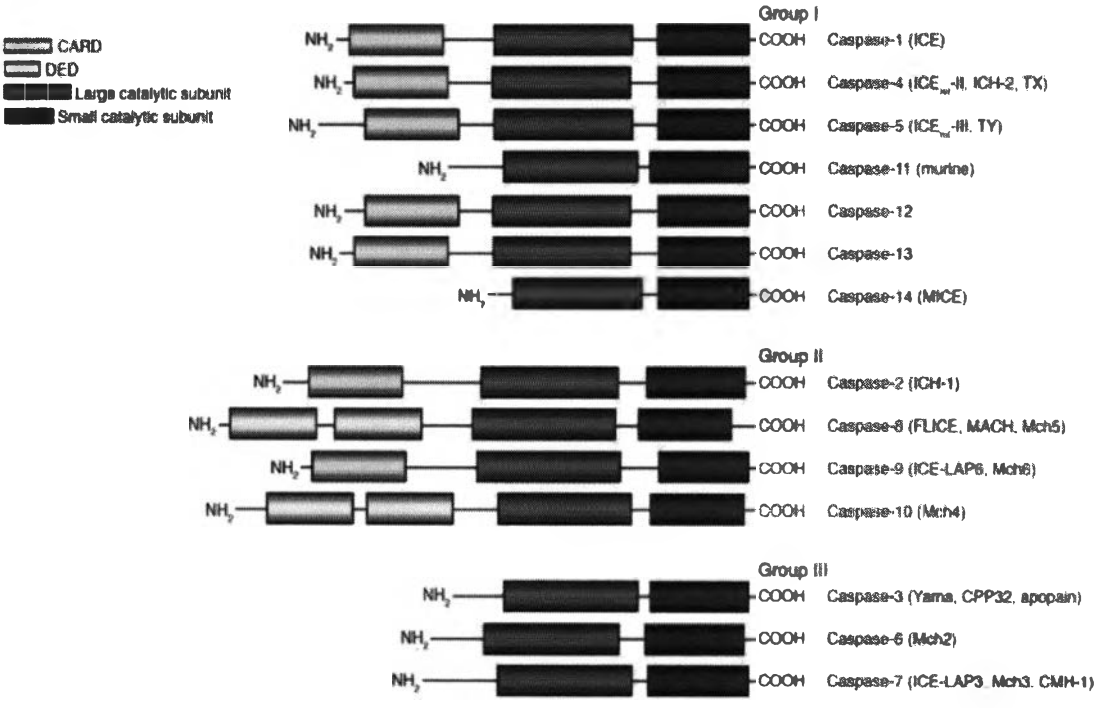

Figure 3: Three major groups of mammalian caspases [26].

Caspase signaling in apoptosis

Caspases involved in apoptosis are the initiator caspases (caspase-2, -8, -9, and -10) and the effector caspases (caspase-3, -6, and -7). By their prodomains, the initiator caspases are recruited to and activated at death inducing signaling complexes in response to either the ligation of cell surface death receptors (DRs) in the extrinsic apoptosis pathway or the intracellular signals in the intrinsic apoptosis pathway **[28]**. The induction of apoptosis through both pathways results in the auto-activation of initiator caspases which is the first step of a highly regulated, irreversible, self amplifying proteolytic pathway. Active initiator caspases then cleave effector procaspases to become active effector caspases (caspases-3, -6, and -7). Both the extrinsic and intrinsic death pathways use these common effector caspases to induce apoptosis **[29]**. Active effector caspases are in charge for the proteolytic cleavage of a numerous cellular targets, leading to cell death in apoptotic manner (Fig. 4). Caspases cleave inhibitor of caspase-activated DNase (ICAD) to release caspase-activated DNase (CAD) which can then catalyze DNA cleavage **[30-31]**. They mediate the cleavage of lamins at the nuclear lamina, causing nuclear fragmentation and proteolysis of nuclear envelope proteins **[32]**. Proteolysis of proteins at focal adhesion sites and cell–cell adhesion sites causes cell detachment and retraction. Proteolysis of the Rho effector, ROCK1, leads to actin contraction and plasma membrane blebbing as well as DNA fragmentation **[33]**. The cleavage of tubulin and microtubule-associated proteins leads to changes in the microtubule cytoskeleton and contribute to apoptotic body formation **[34-35]**. Caspases cleave the Golgi-stacking proteins causing the Golgi apparatus fragmentation **[36-37]**. They cleave the mammalian sterile-20 kinase, MST1, to an active kinase. This kinase then translocates to the nucleus and phosphorylates histone H2B to initiate chromatin condensation **[38]**. Moreover, caspase-mediated proteolysis of multiple translation initiation factors (eIFs) causing translation disruption **[39]**.

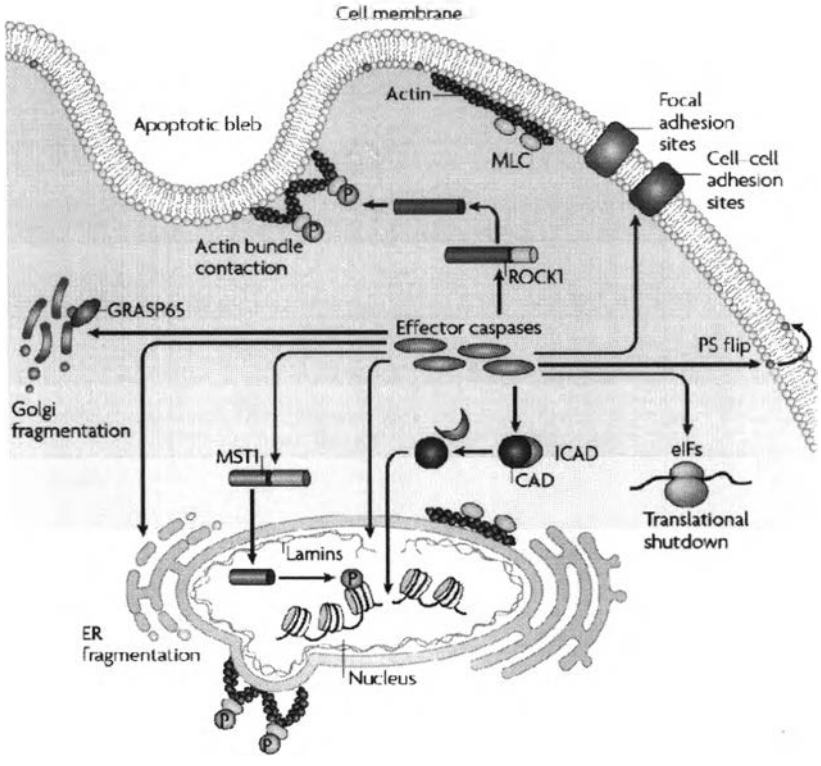

Figure 4: Effector caspases function in demolition of several cellular structures and organelles **[40]**.

Apoptotic cells and apoptotic bodies are eliminated by phagocytes. Several molecules on the cell surface of apoptotic cells are proposed to act as signals for the engulfment of these cells by phagocytes. The most likely signal is phosphatidylserine (PS) which only exposes on the cell surface of apoptotic cells. PS on apoptotic cells is recognized by a specific receptor on phagocytes and lead to the engulfment of these apoptotic cells. PS also induces opsonization of apoptotic cells by the complement factor, iC3b, which binds to its receptors (complement receptors CR3 and CR4) and promote the uptake of apoptotic cells, by integrins **[41]**.

Caspase activation pathways

There are three major pathways to caspase activation (Fig. 5); the extrinsic pathway or the death receptor-mediated pathway, the intrinsic pathway or the mitochondria-mediated pathway, and the granzyme B-mediated pathway **[40]**.

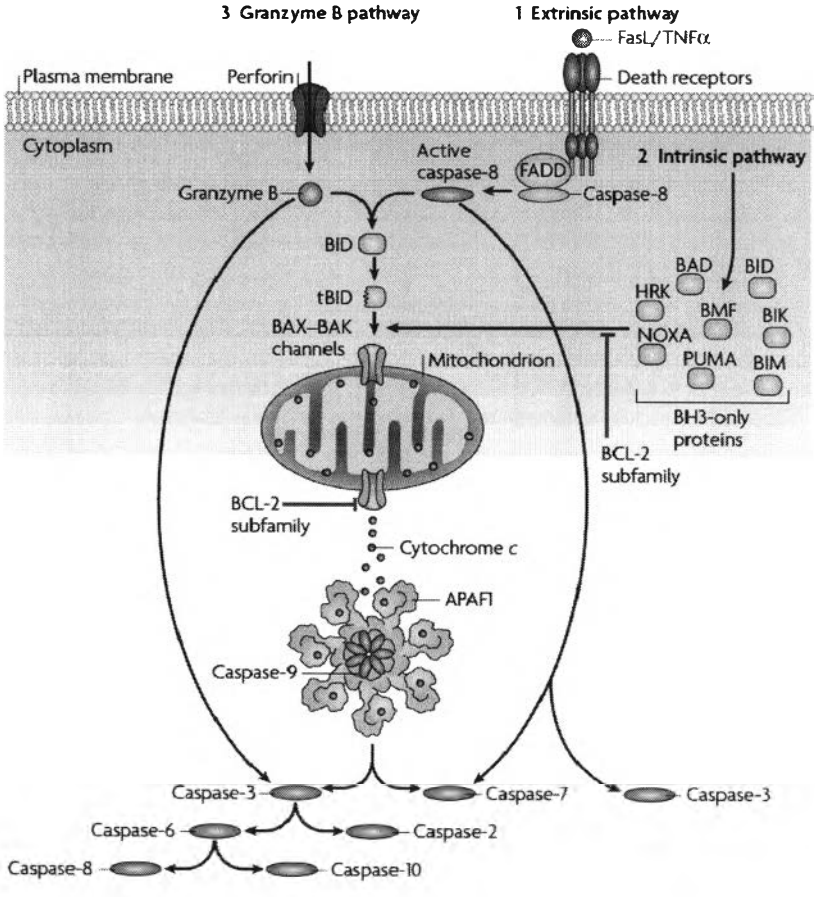

Figure 5: Caspase activation pathways **[40]**.

The extrinsic pathway of apoptosis

The extrinsic pathway is initiated by the activation of cell surface death receptors (DRs) after binding to their specific ligands. These DRs are the members of the tumor necrosis factor receptor (TNFR) superfamily. The well known members and their specific ligands include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 **[42-44]**. Members of the TNF receptor superfamily share similar extracellular cyteine-rich domains (CRD) and intracellular death domains (DDs) of about 80 amino acids at the cytoplasmic tail. The CRD are critical for receptor self-association (CRD1) and receptor–ligand interactions (CRD2 and CRD3) **[45]**. The DD plays an important role in transmitting the death signal from the cell surface to the intracellular signaling cascades. These receptors initiate apoptotic signaling cascades within seconds after binding to their specific death ligands and result in apoptosis within hours. Upon ligand binding, they undergo oligomerization and/or conformational change. They then recruit both an adaptor molecule, either Fas-associated death domain (FADD) or TNF receptor-associated death domain (TNFADD), and procaspase-8 or procaspase-10 to form the death inducing signaling complex (DISC). At the DISC, the auto-catalytic activation of procaspase-8 occurs and becomes active caspase-8. This caspase then triggers execution phase of apoptosis **[46]**.

Signaling by Fas/FasL

Fas(apo-1 or CD95) is a 45–52 kDa glycoprotein. It is a transmembrane receptor widely expressed in various tissues and constitutively present on activated T and B lymphocytes. It binds specifically to Fas ligand (FasL) which is a transmembrane protein expressed mainly in T lymphocytes and natural killer cells. FasL is also expressed on many tumor cells, including melanomas, astrocytomas, lymphomas and various carcinomas **[47]**. It's expression is induced in activated lymphocytes and can be cleaved from the cell surface by metalloprotease to become soluble FasL. This soluble FasL is found *in vivo* and can trigger apoptosis **[48]**. The Fas-FasL signaling pathway of apoptosis is shown in Fig. 6. Once ligation with FasL, Fas undergoes trimerization and recruits an adapter protein called the Fas-associated death domain (FADD), which interacts with the homologous death domain (DD) of Fas. FADD contains not only DD

but also a death effector domain (DED) which can associate with other DED-containing proteins such as pro-caspase-8 or procaspase-10. The oligomerization of Fas, FADD and procaspase-8 is termed the death-inducing signaling complex (DISC), which plays role as a platform to initiate cascade of the caspase activation. At the DISC, pro-caspase-8 undergoes autoproteolytic cleavage to generate active caspase-8. Active caspase-8 is released from the DISC and subsequently cleaves downstream pro-caspases, such procaspase-3, -6 or -7, to produce active effector caspases **[49]**, which results in the cleavage and activation of a vast number of downstream substrates eliciting the morphological and biochemical characteristics of apoptosis.

Cells using Fas signaling pathway of apoptosis have been identified into 2 types **[50]**. In type I cell, there is sufficient active caspase 8 for directly activating effector caspases including caspase-3, -6, and -7. In type II cell, the activation of effector caspases cell needs to be amplified by the mitochondrial pathway. The type II cell has limited activation of caspase-8 at the DISC due to either insufficient procaspase-8 expression or height levels of caspase-8 inhibitor FLICE inhibitory protein (FLIP). Limited active caspase-8 cannot precede apoptotic signaling through the direct pathway. Therefore, the caspase cascade needs to be amplified via the mitochondrial pathway **[51]**. The mitochondrial amplification loop is depends on the cleavage of Bid, a cytoplasmic pro-apoptotic Bcl-2 family protein, to a truncated protein, tBid, by active caspase-8 (Fig. 6). tBid translocates to the mitochondria to efficiently activates BAX, the other pro-apoptotic Bcl-2 family member. BAX initiate the release of cytochrome c and second mitochondria-derived activator of caspases (Smac/DIABLO) into the cytosol to drive the formation of the caspase-9 –activating apoptosome in the mitochondria pathway of apoptosis **[52]**.

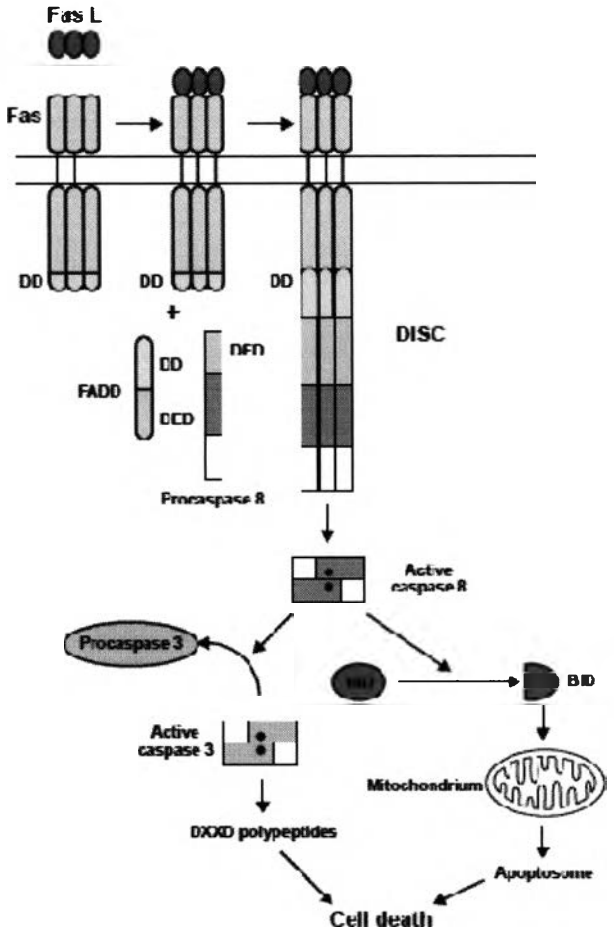

Figure 6: The Fas-FasL signaling pathway of apoptosis **[53]**.

The mitochondrial/intrinsic pathway of apoptosis

The mitochondria or intrinsic pathway originates from apoptotic signals resulting in a perturbation of intracellular homeostasis. A vast number of stimuli, such as ultraviolet radiation, reactive oxygen and reactive nitrogen species, chemotherapeutic agents, heat shock, growth factor withdrawal and DNA damage, mediate apoptosis via this pathway **[54]**. These stimuli cause changes in the inner mitochondrial membrane permeabilization which leads to an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential ($\Delta\psi_m$) and release of two main groups of apoptotic promoting proteins normally sequestered in the intermembrane space into the cytosol. The first group consists of cytochrome c, the second mitochondria-derived activator of caspase/direct IAP-binding protein with low isoelectric point (Smac/Diablo), and the high temperature requiment protein HtrA2/Omi which is a serine protease **[55-56]**. All these apoptotic promoting proteins activate the

caspase dependent mitochondrial pathway. The second group consists of apoptosis-inducing factor (AIF), endonuclease G (EndoG) and caspase-activated DNase (CAD). These proteins are released from the mitochondria at the late phase of apoptosis after the cell has committed to die. They play role in apoptosis in either caspase independent or dependent manner.

Cytochrome c in the cytosol interacts with apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP, and procaspase-9 forming an apoptosome **[57]**. Apaf-1 contains a CARD which interacts with caspase-9, and a WD-40 repeat domain that may play role to maintain protein inactivity in the absence of cytochrome c. Interaction of cytochrome c and Apaf-1 in the presence of ATP/dATP, Apaf-1 undergoes a conformational change by self aggregation **[58]**. The CARD of Apaf-1 is exposed and induces the recruitment of procaspase-9 which is subsequent autoproteolytic cleaved to active caspase-9. Caspase-9 then directly activate effector caspases, caspases-3, -6 and -7 (Fig. 7), which results in the sequentially cell death by proteolytic clevage of numberous downstream targets. Smac/DIABLO and HtraA2/Omi in the cytosol can bind to the inhibitor of apoptosis proteins (IAPs) to disrupt their association with active caspase-9, thus allowing caspase-9 to activate caspase-3, resulting in apoptosis **[59]**,

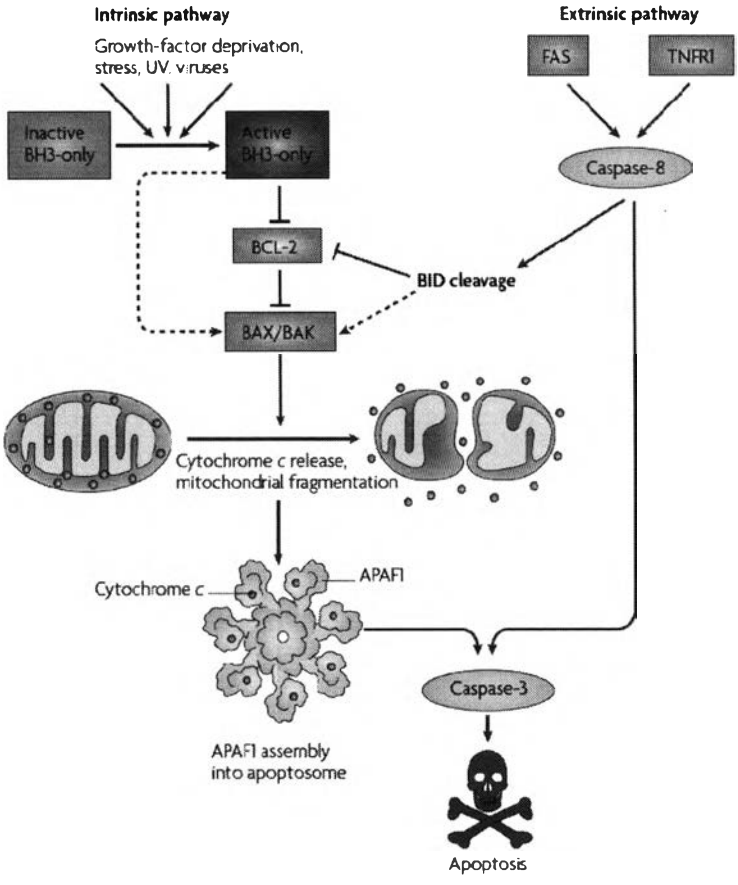

Figure 7: The intrinsic pathway of apoptosis **[60]**.

The group II apoptotic promoting proteins from mitochondria intermembrane space, including AIF and EndoG, can induce apoptosis in a caspase-independent manner. Once release from the mitochondria, AIF translocates into the nucleus and causes chromatin condensation and large-scale DNA fragmentation to fragments of approximately 50-300 kb pieces **[61]**. EndoG also translocates to the nucleus and cleaves nuclear chromatin to nuclear DNA fragments **[62]**.

The proteins in the Bcl-2 family

The mitochondrial membrane permeabilization (MMP) is regulated by a various Bcl-2 (B-cell lymphoma 2) family proteins. These proteins are classified based on their Bcl-2 homology domains (BH1–4 domains) into three groups, the anti-apoptotic BCL-2, the pro-apoptotic BCL-2 and the BH3-only proteins (Fig. 8) **[63-64]**.

The anti-apoptotic BCL-2 proteins, including BCL-2, BCL-w, BCL-XL, A1 and MCL-1, contain BH domains 1- 4. Most of them are trnsmembrane proteins containing

transmembrane domains (TM). They are integrated in the endoplasmic reticulum (ER), the nuclear envelope and the outer mitochondrial membrane. Their functions at the ER and the nuclear envelope are not clear. These proteins block the mitochondrial pathway of apoptosis. They prevent cytochrome c release from mitochondria via directly bind to and inhibit the pro-apoptotic BCL-2 proteins. This leads to preserve cell survival **[65]**.

The pro-apoptotic BCL-2 proteins, BAK and BAX, contain BH 1-3 domains. They are critical for inducing permeabilization of the outer mitochondrial membrane by forming a dimer or high-order oligomers to create the proteolipid pore. This pore is responsible for the release of cytochrome c and DIABLO/SMAC which leads to caspase activation **[66]**.

The BH3-only proteins, including BAD, BID, BIK, BIM, BMF, Harakiri (HRK), Noxa and PUMA (p53-upregulated modulator of apoptosis), contain a conserved BH3 domain that can bind and regulate anti-apoptotic BCL-2 proteins. They are pro-apoptotic proteins which play role as initial sensors of apoptotic signals. They act as pathway-specific sensors for various stimuli that provoke cell stress or damage. Activation of these proteins is crucial for overcoming the effect anti-apoptotic BCL-2 proteins **[60]** and promoting the assembly of BAK–BAX oligomers within mitochondrial outer membranes. These oligomers permit the efflux of intermembrane space proteins into the cytosol and result in apoptotic induction.

Expression of some Bcl-2 family proteins especially pro-apoptotic proteins are positively regulated by p53 which is a tumor suppressor gene product. P53 is a transcription factor that plays essential role in the expression of several pro-apoptotic proteins and cell cycle inhibitors. A numerous stimuli that induce DNA damage result in the p53 expression and activation and lead to cell cycle arrest or apoptosis of DNA damaged cells **[67]**. BAX, NOXA and PUMA genes contain a p53-responsive element in their regulatory sequences **[68-69]**. Apaf-1 has also been identified as a direct target of p53 activation **[70]**.

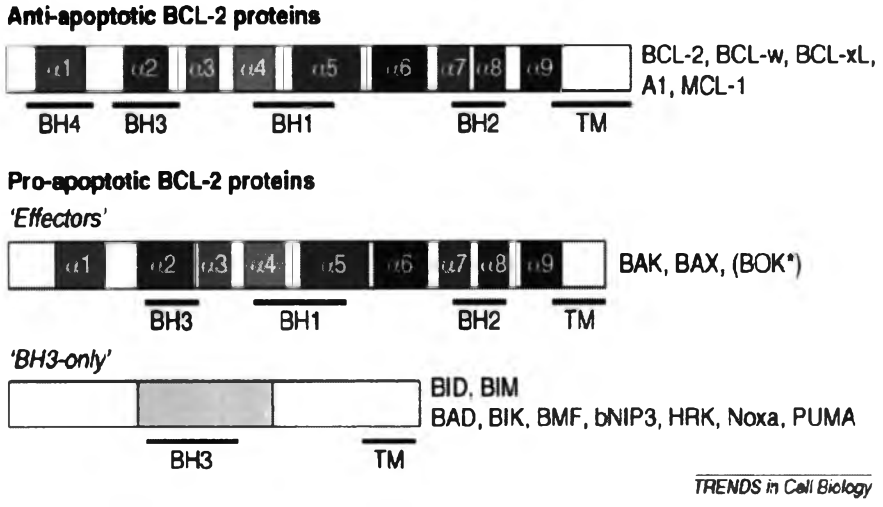

Figure 8: The Bcl-2 family of proteins **[65]**.

The granzyme B-mediated pathway of apoptosis

Granzyme B is a serine protease released from the secretory granules of activated cytotoxic lymphocytes and activated natural killer cells into target cells. It plays a pivotal role in natural killer (NK) cell and cytotoxic T lymphocyte (CTLs) functions to clear viral infected cell or tumor cells **[71]**. Within target cells, granzyme B cleaves its protein substrates after aspartatic acid residues and can promote caspase activation and apoptosis (Fig. 9). Granzyme B promotes apoptosis through two main pathways, BID-dependent mitochondrial permeabilization or direct caspase processing and activation. Granzyme B mediates proteolysis of BID into tBID which targets to mitochondria to induce oligomerization of BAX and/ or BAK in the mitochondrial outer membrane. This leads to cytochrome c release to initiate caspas activation. Granzyme B can directly activate effector procaspases 3 and -7 in to active caspases. It can also directly cleave the inhibitor of a DNase (CAD) and leads to internucleosomal DNA hydrolysis. It has been reported that granzyme B also directly cleave a variety of proteins implicated in the maintenance of nuclear integrity (Lamin B), as well as in protection against cell death (MCL-1), DNA repair (DNA-PKcs), microtubule dynamics (a-tubulin) and a host of autoantigens (NuMa, Mi-2) **[20]**

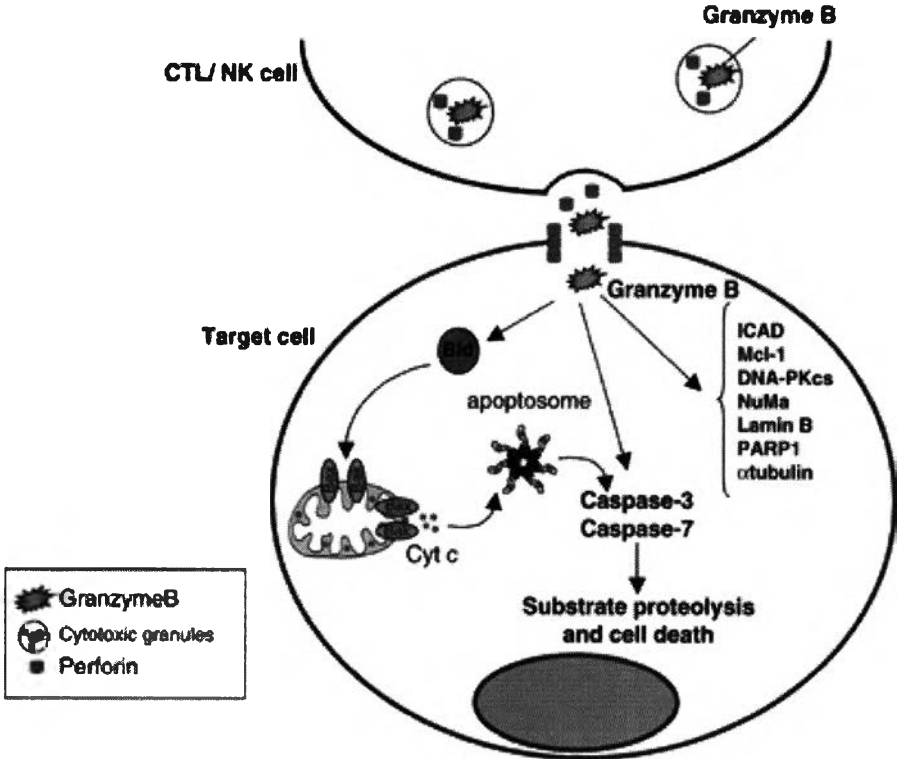

Figure 9: Granzyme B-mediated apoptosis pathway. [20]

The cell cycle

The cell cycle of eukaryotes can be divided into four phases (Fig. 10): M phase (mitosis), in which the nucleus and the cytoplasm divide; S phase (DNA synthesis) in which the DNA in the nucleus is replicated and all chromosomes are duplicated; G1 and G2 phases. G1 phase is a critical phase that responses to extracellular stimuli to either commitment to a further round of cell division or withdrawal from the cell cycle (G0) for differentiation **[72]**. The G1 phase is also involved in the control of DNA integrity before the onset of DNA replication. Between S and M phases is the G2 phase during which the cell checks the completion of DNA replication and the genomic integrity before go to mitosis.

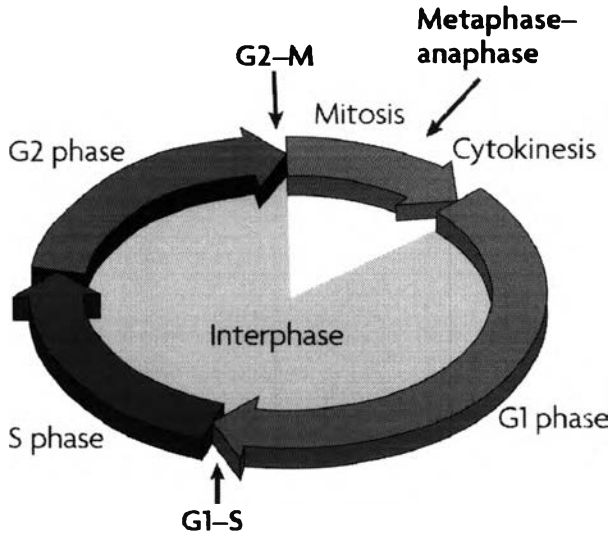

Figure 10: The stages of the cell cycle and checkpoints **[73]**.

Key element of the cell cycle regulation

The progression in orderly of a cell in the cell cycle is controlled by the following proteins; cyclin-dependent protein kinases (CDKs), cyclins and inhibitors of cyclins-dependent protein kinases (CKIs)

1. cyclin-dependent protein kinases (CDKs): These kinases are the key regulatory protein of the cell cycle. They are proteins of 34–40 kDa with Ser/Thr-specific protein kinase activities. There are 4 CDKs function during the cell cycle, CDK1 at G2 and M phases, CDK2 at G1 and S phase, and CDK4 and CDK6 at G1 phase. CDKs are active

at specific points in the cell cycle (Fig. 11). The CDKs have to associate with their corresponding cyclin to become heterodimer active CDKs **[74]**. Not only regulated by association with cyclin, the kinase activity of most CDKs is also regulated by phosphorylation and dephosphorylation in their activation segment and by inhibitors of cyclin dependent protein kinase (CKIs).

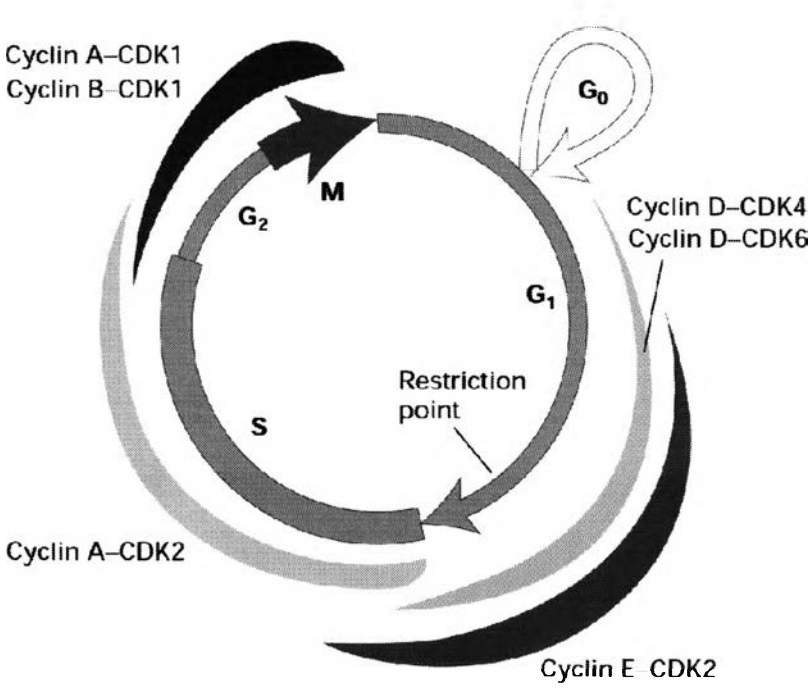

Figure 11: Activity of mammalian cyclin-CDK complexes through the cell cycle **[75]**.

2. Cyclins: They are activating subunits of CDKs. Each cyclin specifically associates with different CDKs. Different CDK-cyclins are required at different phases of the cell cycle (Fig. 11) as in the followings; D cyclin binds to CDK4 and CDK6 at G1 phase, E cyclin binds to CDK2 at G1 and early S phase, A cyclin binds to CDK2 at G2 phase and to CDK1 at M phase, and B cyclin binds to CDK1 at M phase. During the cell cycle, the CDK proteins remain stable, but cyclins are cyclically synthesized and destroyed at specific times in the cycle (Fig. 12).

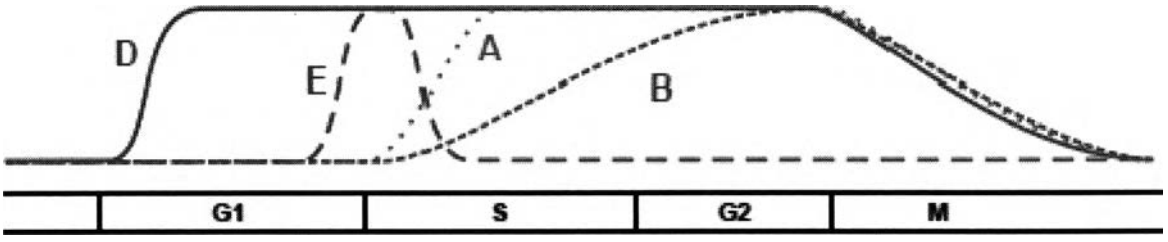

Figure 12: Concentration changes in cyclins during the cell cycle **[76]**

The amount of a distinct cyclin available for CDK activation is strictly controlled by the following mechanisms; regulation of cyclin expression, targeted degradation in the ubiquitin pathway, phosphorylation and subcellular distribution **[76]** (Fig. 13). The concentration of cyclin is regulated mainly at the transcriptional level. Growth factors relay signals via the MAPK pathway or other pathways to activate transcription factors (c-Jun, c-Myc) that up regulate cyclin transcription. Cyclins can be degraded by phosphorylation-dependent proteolysis **[77]**. Phosphorylation of cyclins promotes their ubiquitination by ubiquitin ligase such as Skp, Cullin, F-box containing complex (SCF complex) and anaphase-promoting complex (APC), and leads to destruction of these cyclin by the ubiquitin-proteasome pathway. Moreover, the concentration of cyclin can be changed by the subcellular distribution.

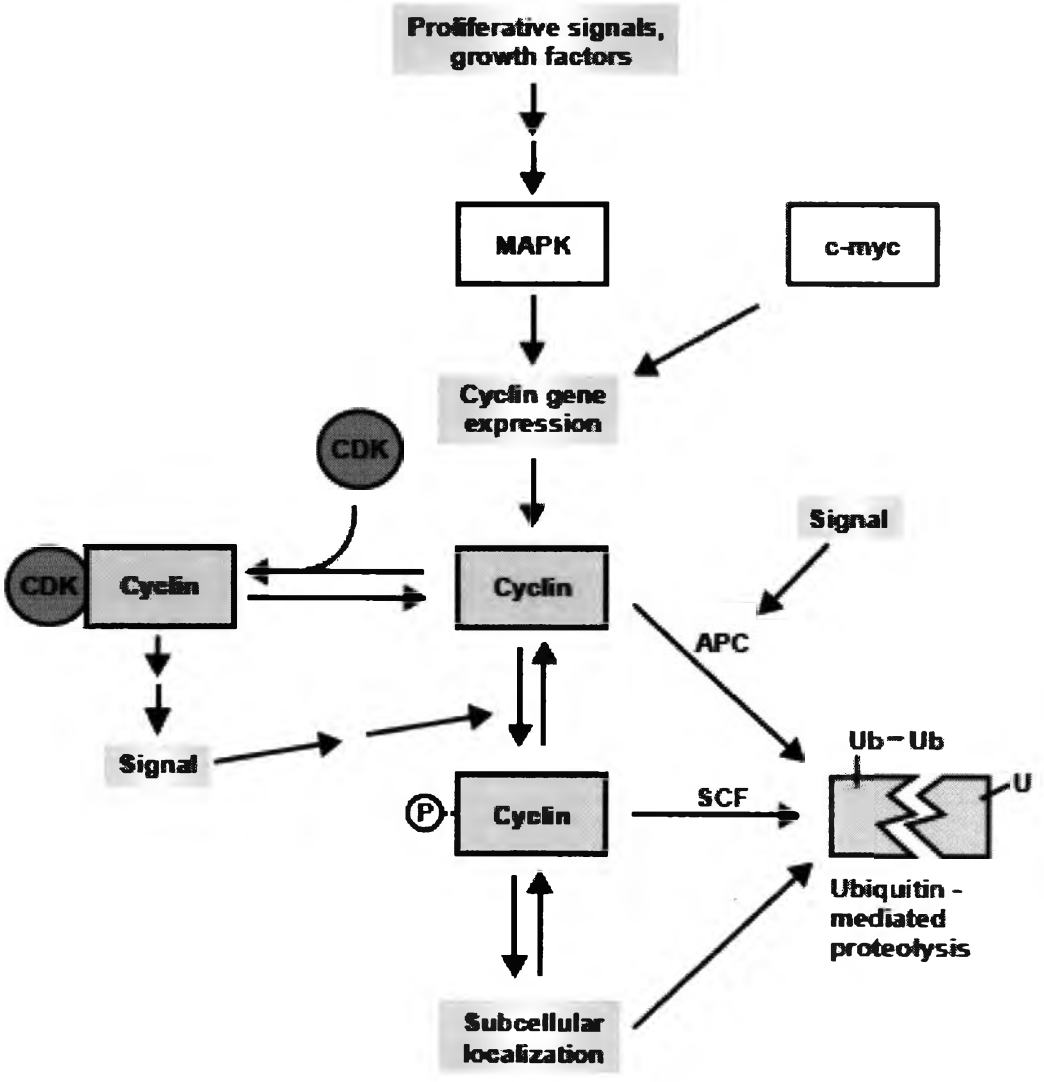

Figure 13: Processes influencing cyclin concentration. [76]

3. Inhibitors of cyclin-dependent protein kinases (CKIs): These inhibitors negatively control the CDK activity **[78]**. CKIs bind to CDK or to the CDK-cyclin complex and inhibit CDK activity **[79]**. They are encoded from two CKI gene families which are defined based on their evolutionary origins, structure, and CDK specificities; the inhibitors of CDK4 (INK4) gene family and the CDK inhibitory Protein/Kinase Inhibitor protein (Cip/Kip) gene family. CKIs in the INK4 family are $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Fig. 14). They specifically bind to CDK4 and CDK6 during the G1 phase and inhibit these CDK activities by interfering with their association with D cyclin. These CKIs prevent transition of a cell from G1 to the S phase. CKIs in the CIP/KIP family are $p21^{CIP1/Waf1}$, $p27^{KIP1}$and $p57^{KIP2}$ **[80]**. They bind to both cyclin and CDK subunits and activate the activities of cyclin D-, E-, A-, and B-CDK complexes.

CKIs are regulated by transcriptional, translational, proteolytic and localizational mechanism. The expression of $p21^{(WAF1/Cip1)}$ is up-regulated by a transcription factor p53 in response to DNA damage, and contributes to G1 cell cycle arrest. It has also been shown that p21 interacts with proliferating cell nuclear antigen (PCNA) to block DNA synthesis, although PCNA-dependent DNA repair is not affected. **[81]**.

Moreover, the transition from one phase of the cell cycle to the next is also controlled by cyclin–CDK complexes which ensure that all phases of the cell cycle are completed in the correct order. The complex regulatory and signaling pathways that regulate cell-cycle progression are highly conserved in eukaryotes. There are three checkpoints in the cell cycle (Fig. 10) including the G1/S checkpoint at the onset of S phase, the G2/M checkpoint at the entry of mitosis, and the metaphase/anaphase or spindle checkpoint at the exit of mitosis to control the order and timing of cell- cycle transitions (G1–S and G2–M).

The G1/S phase transition is made decision to enter S phase and derived cell to a new round of division, or to enter a resting state. When mitosis has been completed, the cell requires signals such as growth factors to direct towards a new round of division. The signals become effective in the early part of G1 phase. In this time window, the cell is programmed to begin a new cell cycle or to enter G0 phase. After a particular point, the restriction point R, no further signals are needed to continue the cell cycle.

The cell cycle apparatus is self-contained from this point onwards. S, G2, and M phase occur without external control.

The G2/M transition: At this point, if the DNA is not properly replicated or is damaged, the cell cycle can be arrested. It functions as an important checkpoint for progression through M phase. Entry into M phase is primarily determined by the activity of the cyclin B-CDK1 kinase complex, which is also called the mitosis-promoting factor, MPF.

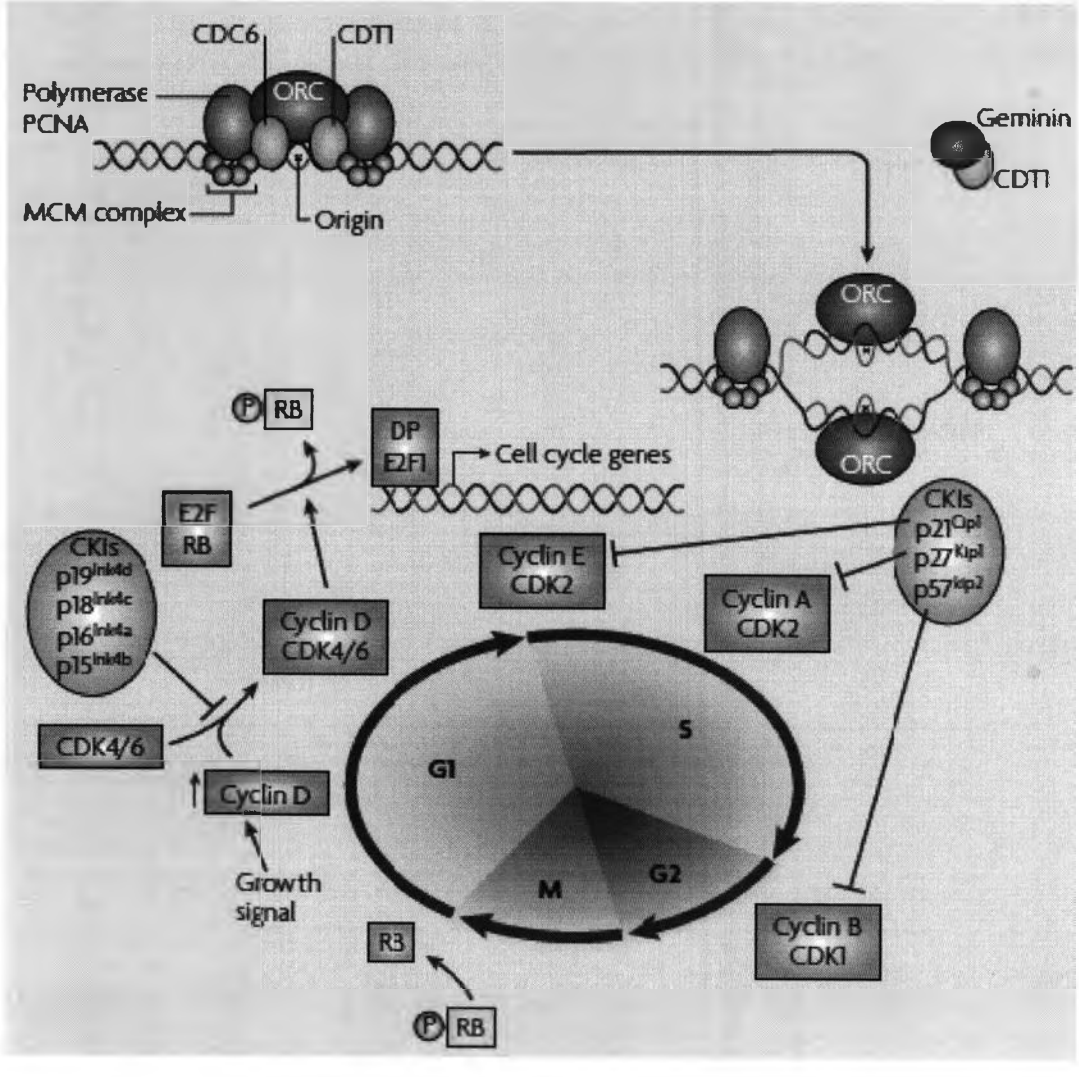

Figure 14: The cell cycle inhibitory proteins (CKI) **[82]**.

Cell cycle progression

The main events that control progression through the cell cycle are as follows **[76]**: G1 phase progression, following exit from mitosis and entry into G1 phase, cells can enter a quiescent state or they can continue in G1, which requires the presence of mitogenic signals in the form of growth hormones. Signaling by growth hormones increases the expression of D cyclins and leads to the formation of cyclin D-CDK4/6 complexes. The metabolism and growth of the cells are stimulated and the cells are able to reach the critical size required for crossing of the restriction point. Furthermore, the pRb protein becomes initially phosphorylated by the cyclin D-CDK4/6 complexes, and cells are thus prepared for crossing the restriction point. Activation of cyclin E/CDK2 and restriction point crossing, as a consequence of the increased formation of cyclin D-CDK4/6 complexes, the inhibitor $p27^{KIP1}$ is sequestered from complex formation with cyclin E-CDK2, and an initial amount of active cyclin E-CDK2 is available that continues phosphorylation of pRb and thereby initiates transcription of E2F-responsive genes, among which is the gene for cyclin E. Activation of cyclin E-CDK2 also requires active CDC25A phosphatase, which dephosphorylates the inhibitory Thr14- and Tyr15-phosphates. Now the requirements for restriction point crossing are fulfilled and the continued action of the E2F transcription factors provides for the enzymes that are necessary for entry into and progress through S phase. At this moment, cyclin E-CDK2 already plays an important role in driving the cell in G1 phase to enter the S phase **[81]**. S phase progression, among the target genes of the E2F transcription factors is the gene for cyclin A, which increases at the beginning of S phase. The cyclin A-CDK2 and the cyclin E-CDK2 complexes are thought to phosphorylate important components of initiation complexes of DNA replication and thereby induce the transition of pre-replication complexes to the post-replicative state. Shortly after entry into S phase, the cyclin E is targeted for degradation in the ubiquitin-proteasome pathway, and the activity of the cyclin E-CDK2 is shut off. Further progress through S phase requires the continued action of cyclin A-CDK2 complexes. G/M transition and progress through M Phase, during S phase and G2 phase, the cyclin B-CDC2 complex accumulates in an inhibited state and is activated by the action of the Cdc25B/C enzymes at the G2/M

transition. The active cyclin B-CDC2 complex phosphorylates numerous substrates and is inactivated by proteolysis only at the end of M phase and during G1 phase.

Normal cellular growth is dependent upon the tightly regulated activation and inactivation of cyclin-dependent protein kinases (CDKs) which enable the cell to pass through several regulatory transition points in the cell division cycle **[83]**. Failure to regulate CDK activity may accelerate cell cycle progression, resulting in unchecked cell proliferation and neoplasia **[84]**.

Micromelum hirsutum Oliver.

Micromelum hirsutum Oliv. is a plant in the genus *Micromelum* belongs to the sub-family Aurantioideae of the Rutaceae family **[85]**. The botanical features of *Micromelum hirsutum* Oliv. have been described as follows **[86]**; Shrub or small tree tomentose, leaves 6 to12 in. long; leaflets 9 to 25, lanceolate to oblong-lanceolate, base very oblique, edges obscurely serrate, tomentose beneath; nerves 5 to 10 pairs, 1.5 to 3.5 in. long, 0.8 to 1.5 in. wide; petioles up to 2 in. long, cymes very tomentose, lax, 4 to 6 in. across or less, flowers 25 in. across, white, calyx deeply 5-4 lobed, ovary very villous, berry orange. This plant is widely distributed in the Southeast Asia.

Chemical constituents from plants in the genus *Micromelum* and their pharmacological properties

Several studies have been revealed a number of chemical constituents isolated from plants in the genus *Micromelum* which are coumarins, flavonoids, quinolone alkaloids and carbazole alkaloids. It has been reported that the dichloromethane extract of the stem bark of *M. hirsutum* contains six carbazole alkaloids, lansine, 3-methylcarbazole, methyl carbazole-3-carboxylate, 3-formylcarbazole, 3-formyl-6-methoxycarbazole and micromeline, and one lactone derivative of oleic acid named micromelide **[87]**. Two coumarin, micromelin and magnolioside and three flavonoids; 5,7-dihydroxy-3,6,4'-trimethoxyflavone, 5,7-dihydroxy-3,6,8,4'-tetramethoxyflavone, and 7-hydroxy-3,5,6,8,4'-pentamethoxyflavone, have been identified from the leaves of this plant **[88]**. Reported pharmacological activities of chemical constituents from plants in the genus *Micromelum* are as follows;

Coumarins

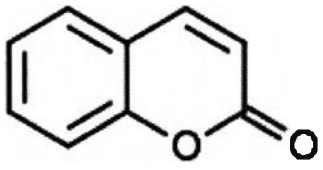

Figure 15: Chemical structure of coumarin

Several coumarins from plants in the genus *Micromelum* have been identified. They are micromelin and scopoletin from *M. integerrimum* **[89]**, 6-formyl-7-methoxycoumarin and micromelin from the leaves and stems of *M. zeylanicum* **[90]**, a numerous coumarins from *M. minutum* (micromelin, murralongin, murrangatin, dihydromicromelin A and B, acetyldihydromicromelin A, the *threo* diastereomer of murrangatin, and murrangatin were reports. **[91]** a typical coumarin (micromelin), a dihydrocinnamic acid derivative of micromelin (l,2-seco-dihydromicromelin), microminutinin, 6-methoxymicrominutinin **[92-93]**, In 2006, the chemical constituents in the acetone fraction from stems provided six new coumarins micromarin-A (1), -B (2), -C (3), -F (4), -G (5), and -H(6), along with six known ones **[94]**. 3",4"-dihydrocapnolactone 1, 2',3'-epoxyisocapnolactone 2, 8-hydroxyisocapnolactone-2',3'-diol 3, 8-hydroxy-3",4"-dihydrocapnolactone-2',3'-diol4 **[95]**, 8,4"-dihydroxy-3",4"-dihydrocapnolactone-2',3'-diol **[96]**, 6- and 8-prenylated coumarins **[85]**, and micromelin and magnolioside from the leaves of *M. hirsutum* **[88]**.

It has been demonstrated that naturally occurring or synthetic coumarin-related compounds from *Micromelum* show the suppressive effects on both lipopolysaccharide and interferon-γ induced nitric oxide (NO) generation in RAW 264.7 **[97]**. scopoletin isolated from *M. integerrium* exhibited anti-tumor activity on p-388 lymphocytic leukemia **[89]**.

Flavonoids (polyphenolic compound)

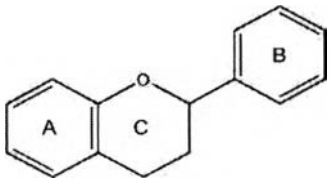

Figure 16: Chemical structure of flavonoid

Flavonoids are the most common polyphenolic compounds found ubiquitously in plants. Several flavonoids also identified from plants in the genus *Micromelum*. These include 5-hydroxy-3,3',4',7,8 pentamethoxyflavone from the leaves and stems of *M. zeylanicum* **[90]**, polymethooxylated flavonoids, 5,7-dihydroxy-3,4',8-trimethoxyflavone, 5-hydroxy-3,4',7,8-tetramethoxyflavone and 5-hydroxy-3,4',6,7,8-pentamethoxyflavone, 5,7-dihydroxy-3,6,8,4'-tetramethoxyflavone, dihydromicromelin A and B, acetyldihydromicromelin A, 7-methoxy-6-(3-methyl-1-oxo-2,3-epoxybutyl)-2*H*-1-benzopyran-2-one (hopeyhopin), and polyoxygenated flavonoids from the leaves of *M. minutum* **[98, 85, 99]**, 5,7-Dihydroxy-3,6,4'-trimethoxyflavone , 5,7-Dihydroxy-3,4',6,8-tetramethoxyflavone, 7-Hydroxy-3,5,6,8,4'-pentamethoxyflavone from the leaves of *M. hirsutum* **[88]**.

Several pharmacological properties of flavonoids have been evidenced. These include anti-allergic, anti-oxidant, anti-inflammatory, antimicrobial and anticancer **[100]**. However, there was no report of pharmacological activity of flavoniods from the *Micromelum*.

Quinolone alkaloid

Fews quinolone alkaloids have been identified from plants in the genus *Micromelum*. They are flindersine from the leaves of *M. pubescens* **[101]** and N-Methylflindersine N-methylswietenidine-B, 3-hydroxy-1-methyl-3-(2-oxopropyl)-quinoline-2,4(1H,3H)-dione and Methyl 2-(3-hydroxy-1-methyl-2,4-dioxo-1,2,3,4-tetrahydroquinolin-3-yl)acetate from the stem bark of *M. falcatum* **[102]**. There was no report of activities of these compounds.

Cabazole alkaloids

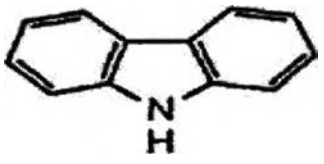

Figure 17: Chemical structure of carbazole alkaloid

Many carbazole alkaloids have been identified from plants in the genus *Micromelum*. These include oxazole, carbazole and bisindole alkaloids from *M. minutum* **[85]**, koenigine, p-sitosterol, and omethylhalfordinol from *M. zeylanicum* **[90]**, and lansine, 3-methylcarbazole, methyl carbazole-3-carboxylate, 3-formylcarbazole, 3-formyl-6-methoxycarbazole and micromeline from the stem bark of *M. hirsutum* **[5]**.

Mahanine is a well known carbazole alkaloid from *M. minutum* that exhibits anti-tumor activity in U937 and HL-60 leukemic cells, androgen-responsive, LNCaP and androgen-independent, PC3 cells **[103, 104, 105 and 106]**. It inhibited cell growth and induced apoptosis in U937 cells through a mitochondrial dependent pathway **[103]**. It reversed an epigenetically silenced gene, RASSF1A in prostate cancer cells by inhibiting DNMT activity that in turn down-regulates a key cell cycle regulator, cyclin D1 **[107]**. It also showed anti-mutagenicity against heterocyclic amines such as Trp-P-1 with an IC50 of 5.2 μM **[105]** and antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus* with MIC100 values of 6.25 and 12.5 μg/ml, respectively. **[105]**. The dichloromethane from the stem bark of *M. hirsutum* containing carbazole alkaloids and a lactone derivative showed in vitro anti-TB activity **[5]**. The dichloromethane extract of the branches and leaves of *M. hirsutum* showed the anti-herpes virus activity **[108]**.