

Apoptosis and mitotic slippage following drug intervention in leukaemia cells

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Apoptosis and Mitotic Slippage Following Drug Intervention in Leukaemia Cells

This Thesis is Submitted in Fulfilment of the Requirements for The Degree of Master of Philosophy (MPhil)

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Faculty of Medicine

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Abstract.

The response of leukaemia cells to therapeutic agents includes cell cycle arrest and apoptosis. The former response is useful in retarding disease progression, but induction of the latter is essential for disease eradication. Cell death is often related to toxicity so reducing drug-concentration or sensitising target cells to apoptosis is desirable. The relationship between the cell cycle and cell death has been at the centre of recent investigation focusing on mechanisms of cell death that are not driven directly by apoptotic responses. These mechanisms included mitotic catastrophe and mitotic slippage.

The K562 myeloid leukaemia cell line exhibits a combination of p53 negativity and carries the Bcr-Abl t (9:22) Philadelphia chromosome. Bcr-Abl is a powerful anti-apoptotic translocation and is the hallmark of chronic myeloid leukaemia (CML). The absence of p53-mediated apoptosis and the anti-apoptotic effects of Bcr-abl delays drug-induced cell death, leaving a window of opportunity to investigate the effects of different agents on leukaemia cells.

My investigations show that when DNA-targeting agents are used against myeloid leukaemia cells, G2 cell cycle arrest and apoptosis do not occur together i.e. cell cycle arrest precludes cell death; cells may escape G2 arrest as a result of mitotic slippage. In contrast, when anti-mitotic agents are used, it is necessary to induce mitotic arrest to subsequently induce apoptosis; thus lower concentrations are more effective in inducing apoptosis than higher drug concentrations. Evidence is provided suggesting reduced concentrations of both genotoxic agents and anti-mitotic agents may share a common pathway in inducing cell death that is related to events at mitosis and I suggest that this pathway has potential for exploitation by new agents currently in clinical trials, such as UCN-01, Purvanol, Roscovitine and agents that target the passenger proteins, in reducing the concentration of more conventional agents required to kill the Bcr-Abl positive leukaemias.

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Chapter 1

Introduction

1.1 Cell cycle:

Somatic cells divide into two daughter cells by means of an orderly sequence of events called the cell cycle. Resting cells, in the presence of a mitogen, enter the cell cycle; they pass through interphase, which includes phases G1, G2, and S phase, and enter mitosis where they divide into two daughter cells. The process requires a range of factors including work load, growth factors, cytokines and hormones (Murray et al, 1992).

Non-cycling cells at G0 phase first enter the cell cycle at G1-phase where the protein synthesis needed for the cell growth increases. The cytoplasm expands and the cell organelles are reproduced. Normally, G1 phase is followed by S phase where DNA replication occurs, and the cell leaves this phase with doubled chromosomes to enter G2 phase which is a gap between DNA synthesis and mitosis (Cortz et al, 1997). During G2 phase regulatory molecules such as kinases are synthesised. These are enzymes needed for protein phosphorylation during mitosis. At the same time, the cell continues to grow and to produce proteins needed for the next step which is mitosis or M phase. Here, protein synthesis stops and the cell divides into two similar daughter cells (Clarke et al, 2006). The duration of mitosis is shorter than interphase. These events are summarised in (Figure 1.1). After completion of the cell cycle the cell may return to the G0-phase where the cell leaves the cycle, and stops dividing or re-enter the cell cycle and continue to proliferate (Voorhees et al, 1976).

1.1.1 Key regulatory molecules:

1.1.1.1 Cyclins:

Progression throughout the cell cycle depends largely on the activity of a group of kinases, which control the activity of various regulatory proteins by phosphorylation and dephosphorylation. These activities are coordinated by associations between cyclins and their partner enzymes, cyclin dependent kinases. After binding with kinases cyclin activity is altered by phosphorylation, dephosphorylation and degradation.

The various cyclins are synthesised and degraded rapidly at different and specific times of the cell cycle (Figure 1.1) In addition to cell cycle progression, cyclin gene expression also controls DNA synthesis (Obaya et al, 2002).

1.1.1.2 Cyclin Dependent Kinases

There are large numbers of kinases which have different roles and actions during different phases of the cell cycle. Each kinase binds with a partner cyclin to form complexes which alter the activity of each other. The changing presence of these various complexes implies specific functions at each phase in the cycle. Also, intracellular levels of the various complexes fluctuate during the cell cycle, thus they appear to function at different stages of the cell cycle. (Morgan et al, 1995).

1.1.1.3 P53 gene:

DNA damage activates the ATM (Ataxia Telengectasia Mutated gene) which slows the rate of cell growth to allow damaged DNA to be repaired. Simultaneously, ATM activates DNA-PK. Both of these proteins stimulate transcription of p53, a key protein which prevents a mutated cell from progressing through the cell cycle (Hofmann et al, 2001). Thus p53 acts as a tumour suppressor gene (Casenghi et al, 1999).

Stress associated signals received by the cell also activate the p53 gene, and cause stabilization of p53 protein. Both mechanisms increase the cellular level of p53 which inhibits cell growth. Other signals received from the p53 gene can also cause cell cycle arrest at G1 phase thus p53 helps the cell to repair damage after genotoxic insults, and this repair enables the cell to pass through a normal cell cycle. P53 also acts as a transcription factor, and can control the transcription of p21, Bax and ∞ 14-3-3 (Keeshan et al, 2002). Dysfunction of these genes frequently results from a p53 gene stop or point mutation or from a defect in gene transcription, causing loss of p53 function. The regulation of p53 is complex due to its multiple interconnections and the wide range of its functional modifications which include both phosphorylation and acetylation (Vousden et al, 2002). P53 transcription is promoted directly by p300 or indirectly by ∞ 14-3-3. P53 transcription is inhibited by PRAP, RPA and Mdm2. Mdm2 binds to the N-terminal of p53 by phosphorylation which is carried out by Chk1/2 at ser-18 or ser-20 to form a stable complex. The negative feedback loop of Mdm2 –p53 regulation results in cleavage of Mdm2 by caspase 3 (Villunger et al, 2003).

p53 is transported from the nucleus to the cytoplasm under the control of import and export signals. In the cytoplasm, p53 is degraded by its control enzyme E3 ubiquitin ligase an APC/C protein.

The importance of p53, the 'guardian of the genome', in monitoring DNA damage and control of the cell cycle makes it a crucial tumour suppressor gene and p53 mutations are an important event in carcinogenesis for many tumours(Clifford et al, 2003).

Cell cycle regulation

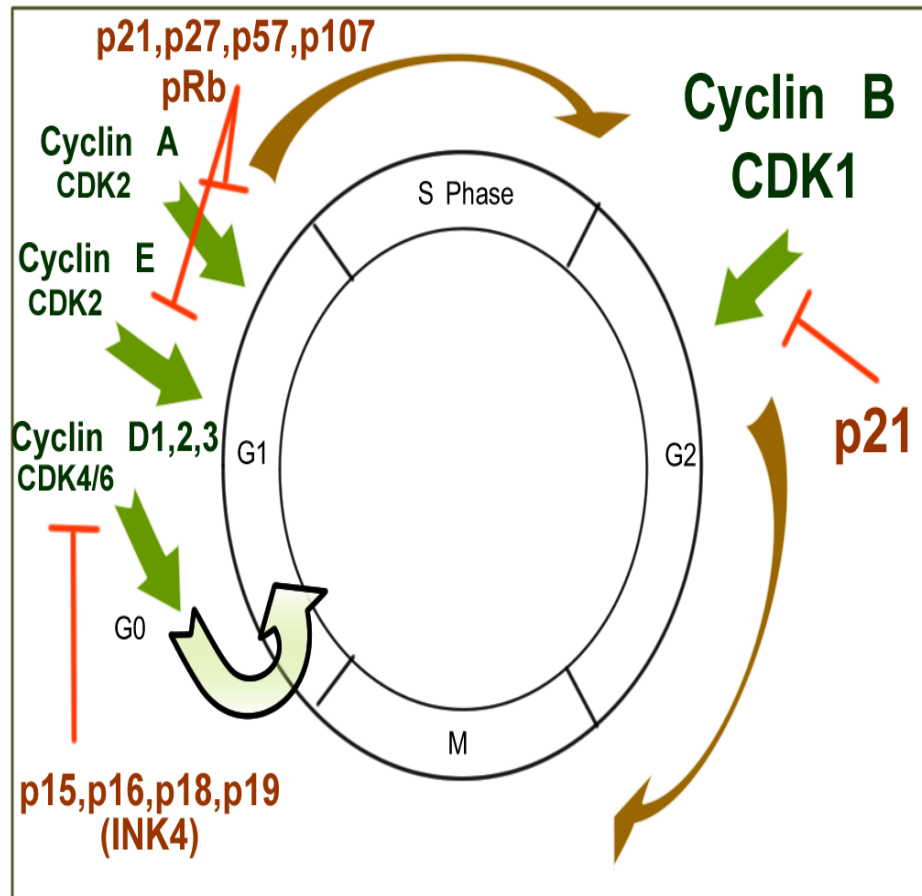


Figure 1.1:

The main cyclins and cyclin dependent kinases and their inhibitors involved in the regulation of cell cycle in each phase of the cell cycle (Modified from Golias et al, 2004).

1.2 Control of the cell cycle :

1.2.1 Entry into the cell cycle G0 to G1-phase.

Cells will enter the cell cycle in the presence of a mitogen. Cyclin D is the dominant cyclin in this phase. Cyclin D synthesis is induced as the cell leaves quiescence to enter the first gap phase of the cell cycle. It forms an activated complex by binding to CDK4 or 6. Formation of these complexes not only stimulates the cell to enter the cell cycle, but the action of these complexes must be sustained for progression through the cell cycle. (McGowan et al, 2003).

1.2.2 G1 phase:

Transition through G1 requires continued cyclin D/CDK4/6 activity. Towards the end of G1, cyclin E is synthesised and this binds to CDK2 to form an active complex that takes cells through to the end of G1 (Voorhees et al, 1976).

1.2.3 G1-S transition:

Cyclin E/CDK2 complex interacts with E2F family of cell cycle transcription factors to activate transcription of proteins needed at G1-S phase. This is important for full activation of the S-phase transcription programme. Cyclin A synthesis begins at the G1-S boundary and cyclin E/CDK2 activity reaches its peak at mid S-phase. Cyclin A gradually down regulates cyclin E by phosphorylation, which allows its recognition by the proteasome. This destroys cyclin E which helps to initiate DNA replication and prepares the cell for S phase (Cortez et al, 1997).

1.2.4 S Phase:

In parallel with the degradation of cyclin E|CDK2 complexes, cyclin A is expressed simultaneously and forms cyclin A/CDK2 complexes. Cyclin A/CDK2 complexes are important for the completion of DNA replication (Obay et al, 2002). The activity of these complexes is down regulated by dephosphorylation after binding to E2F. This effect completes S phase and allows progression to the next phase, mitosis (Boye et al, 2008).

1.2.5 G2 phase:

Throughout interphase cyclin B/Cdk1 remains inactive by inhibitory phosphorylation of the cyclinB|Cdk1 complex due to phosphorylation of Cdk1 at threonine-14 by Myt-1 and at tyrosine-15 by Wee1. Activation of the complex by threonine-161 phosphorylation also occurs but the complex remains inactive until the final activation step. At the end of G2 this drives the cell to mitosis by dephosphorylation of threonine 14 and tyrosine 15 by Cdc25 phosphatase which is regulated by cyclinB-Cdk1 itself and by Plk1 during mitosis (Hutchins et al, 2004). (Figure 1.2)(Figure 1.3)

Activation of cyclin B/Cdk1 complex

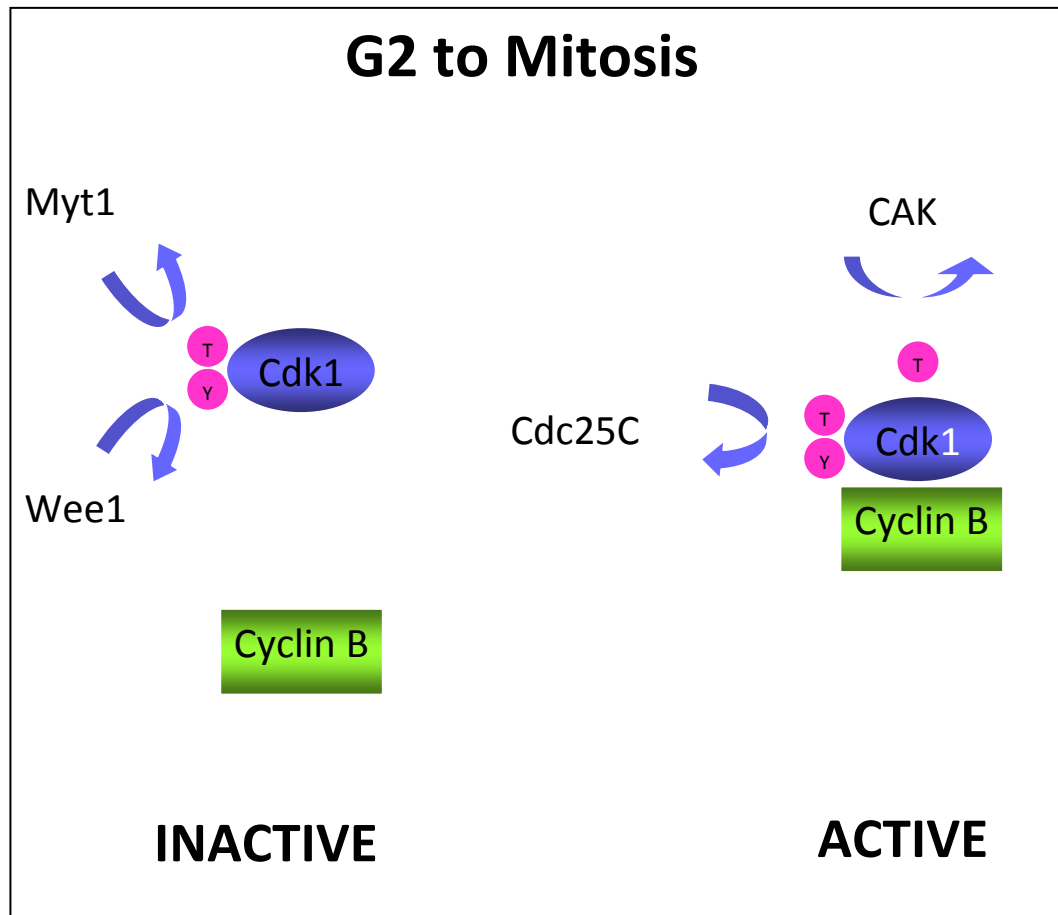


Figure 1.2:

An inactive cyclin B/Cdk1 complex is formed by inhibitory phosphorylation of CDK1 at threonine-14 and tyrosine-15 during interphase. The complex is activated by phosphorylation of Cdk1 threonine-161, then for the final activation step it requires dephosphorylation of threonine 14 and tyrosine 15 by Cdc25 phosphatase in mitosis (Hutchins et al, 2003).

1.2.6 Mitosis:

Conventionally, mitosis is divided into prophase, prometaphase, metaphase then anaphase, telophase and cytokinesis. In prophase, nuclear chromatin becomes tightly coiled and condensed and DNA coiling allows chromosomes to become prominent. The nucleolus is lost early in mitosis. In the cytoplasm the centrioles of the mitotic spindle move to the cell poles and microtubules extend from each centriole (Oegema et al, 2006).

In prometaphase, fragmentation of the nuclear membrane is followed by growth and migration of microtubules from the centrioles. Microtubules extend towards the centre of the cell, where they attach to chromosome centromeres on the sister chromatids via the kinetochores (Zachariae et al, 1999). Kinetochores are highly complex protein structures that congregate on the spindle side of centromeres of each chromosome. The kinetochores of each sister chromatid must interact with the microtubules that originate from opposite spindle poles, to ensure equal separation of the genetic material. When the cell begins to divide tension created by contraction of the attached microtubules will cause the chromosomes to start moving apart (Alberghina et al, 1975).

In metaphase, the metaphase plate forms in the centre of the dividing cell. The chromosomes are aligned in relation to the metaphase plate by the spindle fibres, which are now attached to the kinatochores of the centromeres. Each sister chromatid is attached to spindle fibres originating from the opposite pole. At this stage the spindle apparatus is fully formed with each centriole now placed on opposite poles of the cell (McGowan et al, 2003).

In anaphase, the paired chromosomes are pulled to opposite sides of the cell. This is brought about by formation of the central spindle mid-zone. Shortening of the spindle microtubules pulls on the kinetochores and the chromosomes move to the opposite ends of the cell (Harper et al, 2002).

Telophase is the last phase (Figure 1.4). The chromosomes uncoil and become less condensed, and the spindle apparatus disappears. Two daughter nuclei start to develop nuclear membranes. Mid-bodies form, connecting the two daughter cells, while each is surrounded by newly synthesised cytoplasmic membranes (Altieri et al, 2003).

G2M Checkpoint activation

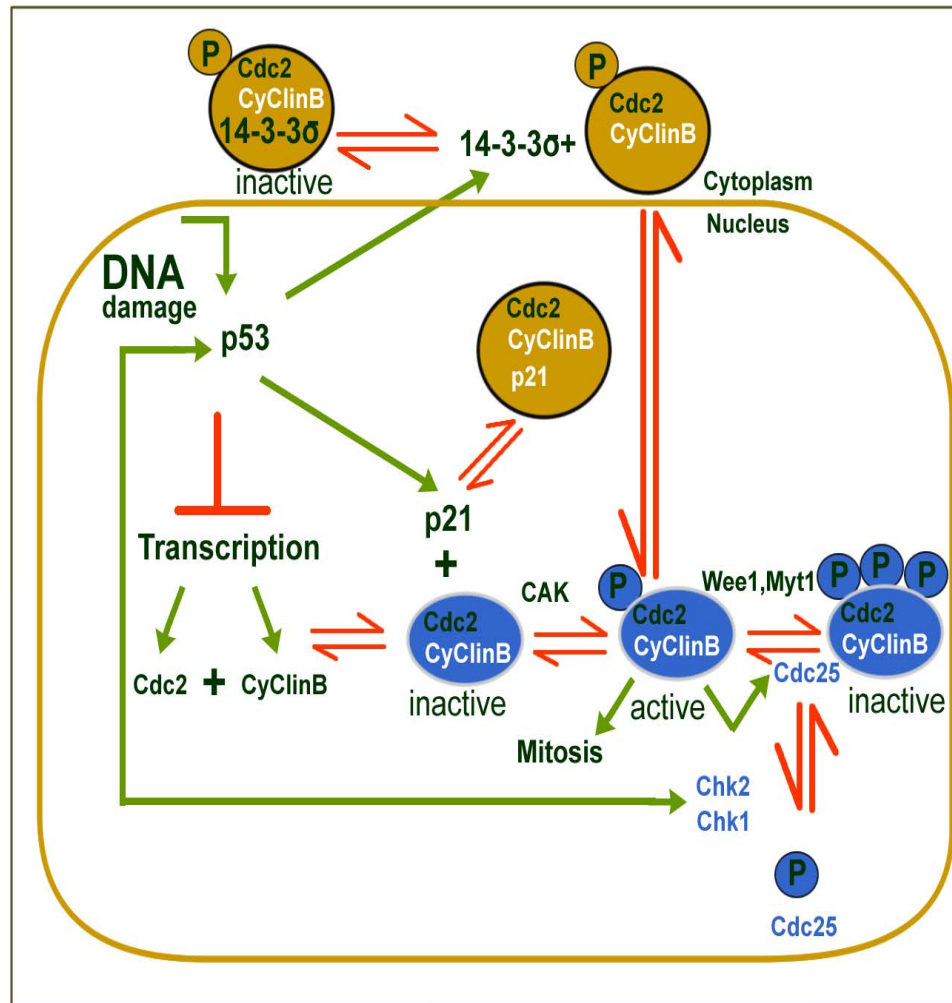


Figure 1.3:

In response to DNA damage p53 is responsible for increases in the synthesis of the Cdk1 (cdc2) inhibitor p21 and 14-3-3 proteins. The result is a complex interaction of proteins to bring about cell cycle arrest at the transition from G2 to mitosis (O'Connor et al, 2000)

1.2.7 Cytokinesis:

After mitosis cytokinesis is synchronized with nuclear partition. Cytoplasmic division starts at the completion of chromosome segregation. First, in the outer cytoplasmic membrane a cleavage furrow forms a ring at the level of the old metaphase plate. The ring contains actin and myosin filaments and their contraction initiates the splitting into two daughter cells (Altieri et al, 2001).

Cytokinesis.

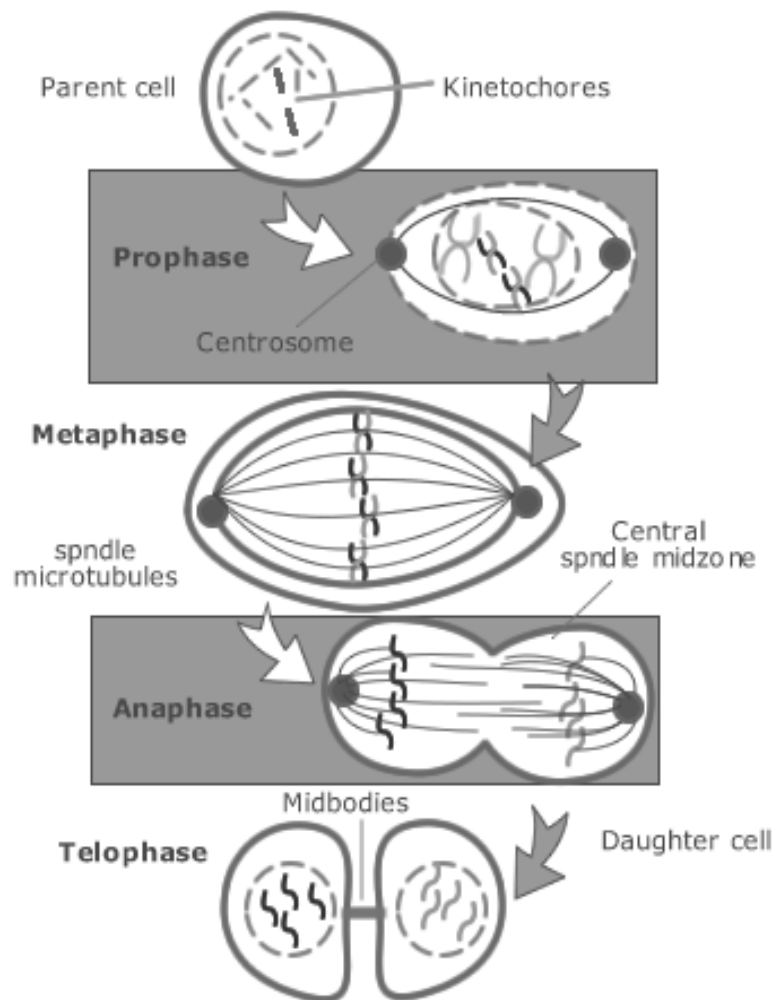


Figure 1.4:

Stages of mitosis showing the components required: kinetochores, centrosomes, spindle microtubules, central spindle mid-zone and mid-bodies (Altieri et al, 2001).

1.3 Cell cycle checkpoints:

These constitute a control system which monitors the orderly progression through the phases of the cell cycle. Cell cycle checkpoints have internal and external controls to direct the timing and order of critical events by a system of checkpoint signalling. A problem in any of the cell cycle pathways can produce stop signals which inhibit the operation of all other pathways and retard cell cycle advancement. Sometimes, in the presence of genomic instability, checkpoint signalling fails to respond to cellular defects. This is one of the mechanisms that predisposes to cancer (Kastan et al, 2004).

1.3.1 G1/S-phase or the DNA damage checkpoint:

The first checkpoint is at the G1/S-phase border. Of the various control systems, the most important is p53 and its antagonist Mdm-2. Phosphorylation of Mdm-2 activates and stabilizes p53 which allows p21 gene transcription. In response to DNA damage, p21 inhibits cyclin A/CDK 2, cyclin E/CDK2 complexes and therefore arrests the cell at G1 phase (McGowan et al, 2003).

1.3.2 G2 DNA damage checkpoint:

Detection of DNA damage stimulates two responses: one is the repair of the damaged DNA itself, and the other is creation of enough time for DNA repair by delaying cell cycle progression. This delay is achieved by down regulation of cyclin dependent kinase activity (Figure 1.2). Inhibition of kinases prevents the cell cycle progressing to mitosis by activation of Chk1 which indirectly inhibits Cdk1 (Damelin et al, 2007).

1.3.4 G2M checkpoint:

This is controlled mainly by phosphorylation of the dominant kinase which forms cyclin B/Cdk 1 complex. Cyclin B/Cdk1 complex is known as the maturation promoting factor (MPF). The Myt1 and Wee1 kinase phosphorylates threonine 14 and tyrosine15 on Cdk1 throughout interphase. The Cdk1/cyclin B complex is then activated by Cdc-25 which dephosphorylates tyrosine 15 and threonine 14 at the end of G2 and through mitosis (Nurse 1990)(Cummings et al, 2002). p53-mediated transduction of p21 may also target this checkpoint by directly inhibiting the cyclin B/Cdk1 complex (Figure 1.3)

However, following DNA damage cyclin B/Cdk1 complex can be inhibited by the sequestration of Cdc25 which is bound to 14-3-3 which in turn is activated by Chk-1&2 via ATM/ATR(Morgan et al, 1995).

Un-replicated or damaged DNA keeps the cyclin B/Cdk1 complex inactive. This is a mechanism that leads to cell cycle arrest at G2 phase (Smits et al, 2001).

1.3.5 Spindle assembly checkpoint:

After DNA duplication the chromosomes consist of pairs of sister chromatids joined by complexes of proteins known as cohesin. During mitosis the pairs must be pulled apart by the mitotic spindle fibres which shorten from their kinetochore end (Taylor et al, 2007).

The spindle assembly check point is regarded as bipartite: one arm monitors kinetochore occupancy by microtubules and the other arm monitors the spindle tension applied to the kinetochores (Adams et al, 2003).

The main protein components of the spindle assembly checkpoint are Bub1, BubR1, Mad1 and Mad2. In early mitosis BubR1, Mad-1 and Mad-2 are all located on the kinetochores, while BubR1 is essential to the spindle assembly checkpoint (Vogt et al, 2008). Activation of these proteins in response to spindle damage arrests mitosis in metaphase. They activate APC/C by inhibition of the action of Mad-2 after binding with Mad-1 where they form an inhibitory complex with Cdc-20 (Vogel et al, 2004).

Cdc-20 is the key activator of APC/C by Mad2-Cdc20. Mad2-Cdc20 complex generates an anaphase stop signal to allow correct arrangement of the chromosomes via their kinetochores and the microtubule spindle apparatus.

BubR1 is the connection between the kinetochore microtubule interaction and the APC/C. The activity of BubR1 is regulated by CENP-E which binds to it and targets it to its position in the kinetochores (Morrow et al, 2005).

1.4 Anaphase Promoting Complex/Cyclosome (APC/C):

The anaphase promoting complex (APC/C) is essential for the progression of the cell through the early phases of mitosis and its exit from anaphase into telophase. The APC/C is a multi-protein complex consisting of eleven subunits: Apc1, Apc2, Apc3/Cdc27, Apc4, Apc5, Apc6/Cdc16, Apc7, Apc8/Cdc23, Apc10/Doc, Apc11 and Apc13 (Kumar et al, 2005).

APC/C uses two co-activators: the first, Cdc20, operates in early mitosis and then Cdh1/Hct1 acts in late mitosis to help progression to anaphase. The activity of the APC/C complex is regulated by phosphorylation. The phosphorylation is controlled by Polo like kinase-1 (PLK1) which works in harmony with the mitosis promoting factor cyclin B/Cdk1 complex.

The cyclin B/Cdk1 complex also activates the APC/C complex. Since cell cycle progression also requires controlled degradation of regulatory proteins, protein kinase A (PKA) modifies and inhibits the complex (Plyte et al, 2007).

1.4.1 APC/C at mitosis:

The catalytic activity of the APC/C complex is controlled by a protein called the early mitotic inhibitor 1 (Emi1). Emi1 is phosphorylated by PLK1 during prophase, causing its inactivation or destruction throughout pro-metaphase. This allows dissociated Cdc-20 to conjugate with APC/C. Inhibition of this process is achieved by Rassf1A. At the end of pro-metaphase, APC/C, associated with its cofactor Cdc20 becomes active in degrading proteins such as cyclin-A which will later disappear in metaphase. Further progression of the cell to anaphase is induced by the degradation of cyclin B by APC/C –Cdc20, and destruction of securin, PLK1 and Aurora B by APC/C –Cdh1 during late mitosis (Gladfelter et al, 2006).

1.4.2 APC/C at spindle assembly checkpoint:

The spindle assembly checkpoint is turned on by unattached kinetochores which stimulate the spindle assembly attached proteins including Bub1, BubR1, Bub3, Mad1, and Mad2. These proteins produce the anaphase stop signal that inhibits APC/C-Cdc20 (Morrow et al, 2005). This creates time for the unattached kinetochores to take their place on the centromeres. When the repair is finished, the properly placed kinetochores-associated proteins will revert to their original places. Inhibitory signals to APC/C-Cdc20 complex will stop and thus its activity is retained. This helps the cell to progress through, and out of, anaphase (Baker et al, 2007) (Figure 1.5).

Regulation of APC/C

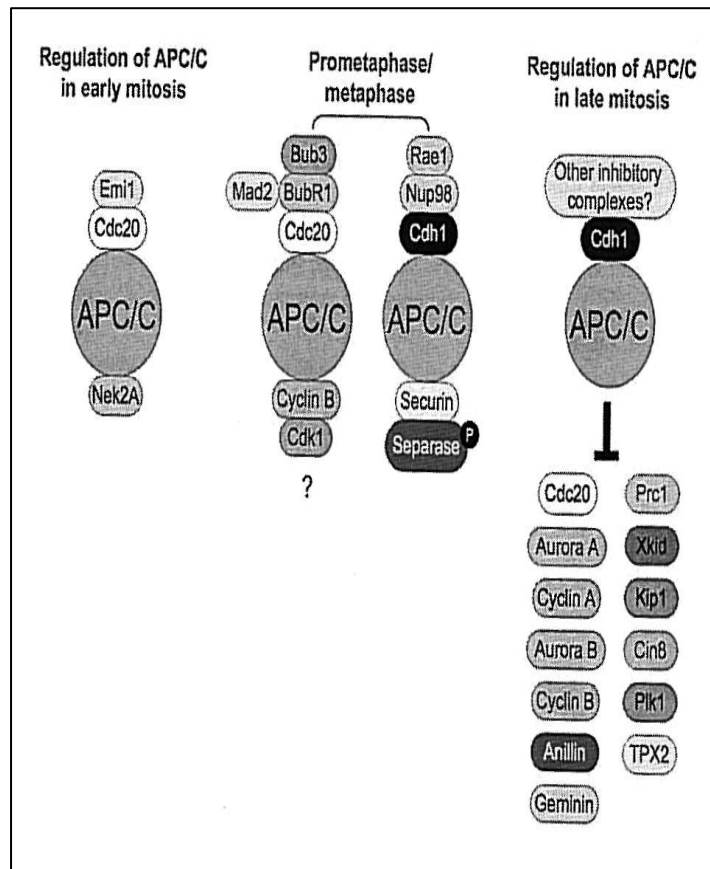


Figure 1.5:

Regulation of APC/C at the mitotic checkpoint in early mitosis, prometaphase /metaphase and regulation of mitosis stimulating and inhibitor factors (Baker et al, 2007).

1.5 Cell death:

Cell death occurs in different ways: these include apoptosis, mitotic catastrophe and necrosis. Each form of cell death has different morphological and biochemical manifestations.

Apoptosis is known as programmed cell death. It is associated with specific enzymatic reactions before the cell dies. Electrophoresis of DNA extracted from apoptotic cells shows a characteristic ladder formation on gels indicating fragmentation due to the action of endonucleases (Taylor et al, 2000).

Mitotic catastrophe is due to complete failure of mitosis. It is characterised by multipolar mitotic figures showing many uncondensed chromosomes contained within large cells. Mitotic catastrophe is then often followed by apoptosis (Roninson et al, 2001).

Necrosis follows direct damage to the cell. Often contiguous cells or sheets of cells are involved. Such necrosis is often followed by a characteristic inflammatory response after cell membrane and nuclear destruction (Castedo et al, 2004).

1.5.1 Apoptosis:

Apoptosis, also has specific morphological features. These include loss of attachment of the cell membrane, cell shrinkage, nuclear and chromosomal fragmentation, chromatin condensation, cellular blobbing and budding and fragmentation. Eventually cell fragments give rise to characteristic apoptotic bodies which will eventually be phagocytosed by macrophages or adjacent cells without damage to the surrounding cells or an inflammatory reaction (Taylor et al, 2000).

1.5.2 Caspase activation:

Factors involved in apoptosis include cascades of caspase enzymes which are cysteine proteases. Proteolytic cleavage causes zymogen dimerization. Normal precursors contain an N-terminal prodomain with small and large destruction sub units which are activated by cleavage at aspartate residues. Activation of the caspase cascade via the initiator caspases 8 and 9, via death receptors, via formation of an apoptosome or other modes of action, activate effector caspases such as caspase 3, 6 and 7 (Shi. et al, 2002). The effector caspases must be cleaved by the initiator caspases 8-9. (Boatright et al, 2003).

There are two pathways of apoptosis activation: intrinsic and extrinsic. Intrinsic or stress pathway activation begins by the formation of the apoptosome. This is a complex of Apaf-1, pro-caspase 9, ATP and cytochrome C; the latter being released after mitochondrial membrane responses to Bax, Bak and Bcl-2 interactions. Bcl-2 is an antiapoptotic protein released from the endoplasmic reticulum (Thomadaki et al, 2006). This controls Bax and/or Bak activation. Bax and Bak are pro-apoptotic proteins which participate in the release of cytochrome C (Garrido et al, 2006) (Figure 1.6).

The extrinsic pathway operates mainly through the 'death receptors' in the cytoplasmic membrane via the adaptor protein FADD (fas –associated death domain) CD95 or tumour necrosis factor receptors 1 and 2 (TNFR1&2) via the adaptor protein TRADD (Figure 1.7). Other receptor mechanisms include TRAIL. These receptors stimulate aggregation and activation of caspase 8 and the downstream caspase cascade, especially caspase 3, will then be stimulated (Spierings et al, 2005). This pathway can lead to apoptosis without involvement of mitochondria (Elmore et al, 2007).

In some cell types such as hepatocytes the death receptor associated caspase-8 is insufficient to activate downstream caspases, so in this case, caspase-8 acts by cleaving Bid (Bcl-2 homology-3 containing protein) to form tBid, which stimulates mitochondrial release of cytochrome-C and hence the activation of downstream pathway (Fulda et al, 2006).

1.5.3 The Role of Mitochondria:

Mitochondria are essential for cellular metabolism. The archetypal function of mitochondria is the production of adenosine triphosphate (ATP) which is accomplished by oxidative phosphorylation and the respiratory chain. Mitochondria are crucial for metabolism of carbohydrates, fatty acids and amino acids. However mitochondria also regulate cell death and play a significant role in apoptosis. Mitochondria contain several active proteins such as cytochrome C, apoptosis inducing factor (AIF), endonuclease G, and Smac /Diablo (Madash et al, 2002). The release of these proteins activates the caspase cascades, while translocation of AIF to the nucleus also stimulates apoptosis via a caspase independent pathway (Garrido et al, 2006).

The Bcl-2 family of proteins increase the permeability of the mitochondrial membranes. Bax or Bak are essential for the permeability process as both can oligomerise to form pores in the mitochondrial outer membrane. Uptake of water causes swelling and rupture of mitochondrial membranes enhancing the release of mitochondrial proteins essential for apoptosis. Anti-apoptotic proteins e.g. Bcl-2, Bcl-XL can negate the effects of Bax and Bak by heterodimerisation thus preventing mitochondrial-dependent apoptosis. De-repression of anti-apoptotic proteins by other Bcl-2 family proteins such as Puma and Noxa, however, can negate anti-apoptotic proteins thus bringing another layer of control of cytochrome C release. (Caroppi et al, 2009).

Mitochondrial apoptosome

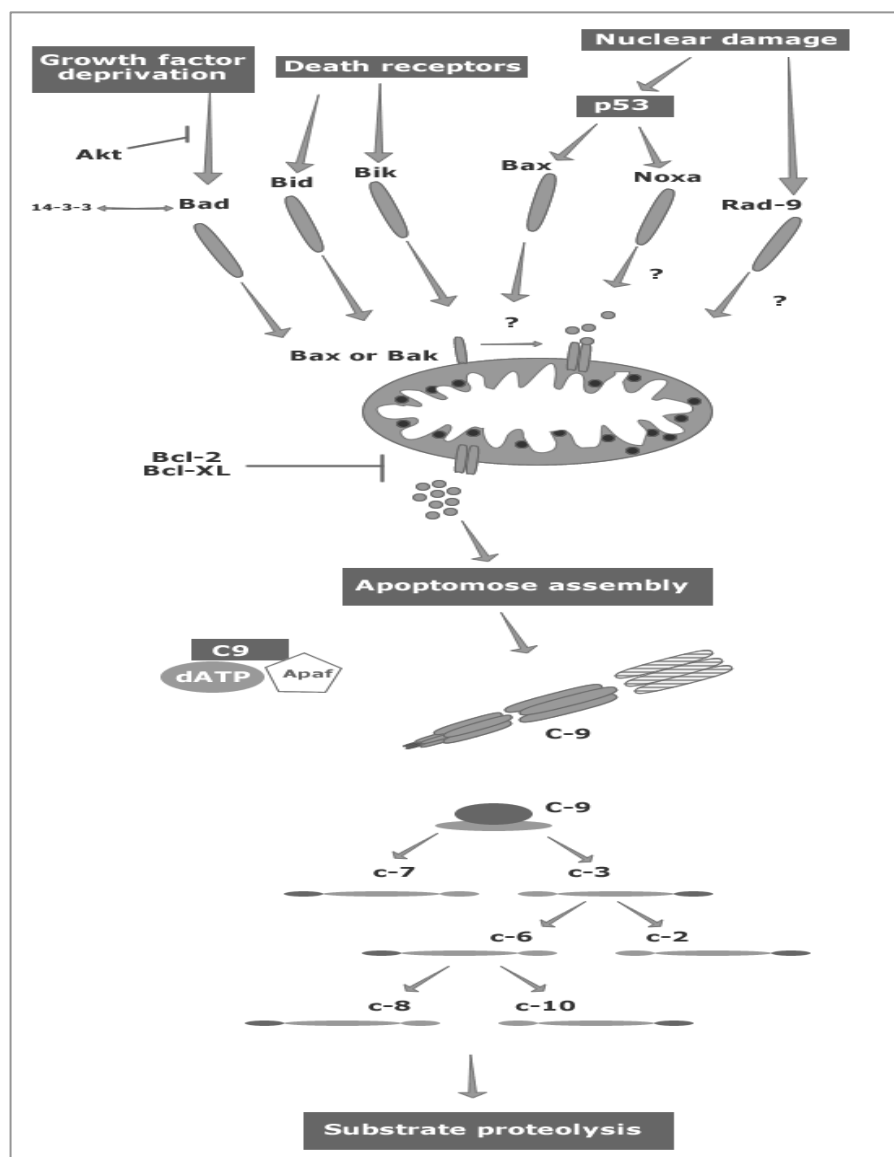


Figure 1.6:

The death promoting members of Bcl-2 (Bid, Bax, Bad, Bak, and Bik) are induced in response to stress and take their places within mitochondrial membrane. This triggers the release of cytochrome c which can be blocked by death inhibitory members of the Bcl-2 family (Bcl-2, Bcl-XL). Binding of cytochrome c to Apaf-1 promotes binding with caspase 9 to form a complex resulting in the activation of caspase 9 which then directly activates caspase 3, 6 and 7 resulting in cellular destruction. (Modified from the mitochondrial apoptosome (Martines, et al 2001)).

1.5.4 Mitotic catastrophe:

This type of cell death is caused by mitotic failure due to failure of chromatin condensation or damage to the spindle formation (Vakifahmetoglu et al, 2008). The morphological features include chromatin condensation and micro nucleation of the cell. Many factors predispose to mitotic catastrophe: these include p53 gene which regulates the G1 checkpoint, cyclin-B/Cdk1, aurora kinase and survivin (Castedo et al, 2004).

In p53 deficient cells caused by mutation, mitotic catastrophe can follow failed mitosis with no apparent apoptosis. p53 mutated cells are resistant to cell death following genotoxic insults caused by chemotherapeutic drugs. Microtubule depolymerising agents and DNA damaging agents induce premature mitosis without checkpoint activation and this predisposes to mitotic catastrophe (Smith et al, 2001).

Activation of CyclinB/Cdk1 complex requires many control steps and is important in the induction of mitosis and chromatin condensation. DNA damage stimulates checkpoints which in turn activate kinases such as Wee1/Myt1 and Chk1, and Chk2 to stop progression to mitosis by inhibiting Cdk1. Similarly, inhibition of APC stops degradation of cyclin-B at the spindle assembly checkpoint. Therefore any aberrant entry to mitosis in the presence of incomplete DNA replication causes mitotic catastrophe (Hoffmann et al, 2001).

1.6 Mitotic slippage:

Mitotic slippage, also known as checkpoint adaptation, is an escape from mitosis in spite of normal levels of factors controlling the spindle assembly checkpoint (Musacchio et al, 2007). In human cells slippage is usually associated with cyclin B destruction (Taylor et al, 2007).

When the signals from spindle fibers are abnormal or absent, kinetochores release Mad2 which binds to Cdc20 and dissociates it from APC/C; this deactivates APC/C and prevents destruction of securin which accumulates and prevents the action of separase. This creates more time for the cell to correct its situation. APC/C activation achieved by association with Cdc20 inhibits cyclin-B/Cdk1 complex activity at mitosis by proteolytic cleavage of cyclin-B. This allows a cell to escape mitosis with an incomplete spindle assembly checkpoint.

The spindle assembly checkpoint delays the exit of a cell with inadequately attached kinetochores from mitosis. There is destruction of cyclin B by APC/C proteolysis. This inhibits cyclin B/Cdk1 activity and allows the cell to escape mitosis in the presence of an incompletely active spindle assembly checkpoint (Herzog et al, 2009). Other opinions suggest that depletion or lack of accumulation of active chromosomal passenger complex in centromeres at mitosis might lead to premature mitotic exit accompanied by the loss of Mad2 and Bub1 from unattached kinetochores (Stoepel et al, 2005).

1.7 Chromosomal passenger complex:

The chromosomal passenger complex refers to a number of proteins that change their location throughout mitosis. So far, six chromosomal passenger proteins have been isolated from human cells. These consist of INCENP (inner centromere protein), Aurora B, Borealine /Darsa B, survivin, CSC-1 and TD-60 (Vagnarelli et al, 2004).

The chromosomal passenger complex is usually localized to inner centromeres. However they can change their position as the cell passes through metaphase to anaphase. They move to the mid-zone of the spindle apparatus during anaphase, and finally they localize to the mid-body during telophase. Diminution of any one of the chromosomal passenger constituents by any means triggers the elimination and mislocalization of the others (Carvalho et al, 2003).

1.7.1 Function of chromosomal passenger complex:

The chromosomal passenger complex contributes to chromosome organization, tension sensing, BubR1 and Mad2 targeting and microtubule dynamics. It is necessary to sustain spindle checkpoint activity (Terada et al, 2001). The role of Aurora B at the spindle assembly checkpoint, as part of the chromosomal passenger complex, is to arrest the cell cycle temporarily to prevent the tension between the kinetochores. Aurora B can detect and stabilize faulty attachments of microtubules to the kinetochore. It regenerates unattached kinetochores that are detected by the spindle assembly checkpoint, particularly when cells are under stress such as caused by drugs that affect the kinetochores (Lens SM et al 2006). The chromosomal passenger complex indirectly inhibits APC/C. However, they allow the cell to transmit lack of tension back to the attached microtubules (Gassmann et al, 2004).

In prophase the chromosomal passenger complex helps in kinase activation. During anaphase, the chromosomal passenger complex acts on spindle structure and stability. It targets MKLP1, and contributes to cytokinesis (Lens et al, 2003).

1.7.2 Survivin:

Survivin is a highly conserved intracellular protein of 70 amino acids and a molecular weight of 16.5 kDa. (Yigong Shi et al 2000). There are three splice variants known as survivin, survivin- 2B and survivin- Dex3 (Li et al, 2003). Survivin is produced mainly at G2M phase of the cell cycle (Kasof et al, 2001). Survivin has a long C-terminal helix motif zinc-coordinating Cys/His at the terminal half of the protein that interacts with polymerized microtubules and homodimerizes in solutions (Altieri et al, 2006).

It is a known member of the inhibitor of apoptosis (IAP) gene family whose members include: NAIP, cIAP1, cIAP2, XIAP, S-XIAP, ML-IAP, Apollon and survivin. Not all of the IAP family members are cell death inhibitors. The IAP proteins are inhibited by the mitochondrial protein Smac (Wei et al, 2008) (Figure 1.7)

Survivin contains only one BIR domain which is consistent with a potential role in inhibiting the apoptotic pathway at the level of caspase 3 and 7 but not 8 (Kaufmann et al, 2001). Survivin acts downstream of the Bax signaling pathway to inhibit apoptosis. Survivin is highly expressed in foetal tissue and in the majority of tumours, but not in normal adult tissue (Altieri et al, 1999). Its presence in high levels in tumours is associated with a poor patient prognosis, due to advanced cancer progression, high recurrence rate, drug resistance and short survival rate (Reed et al, 2001). This function of survivin is separate to its function as a passenger protein.

The IAPs

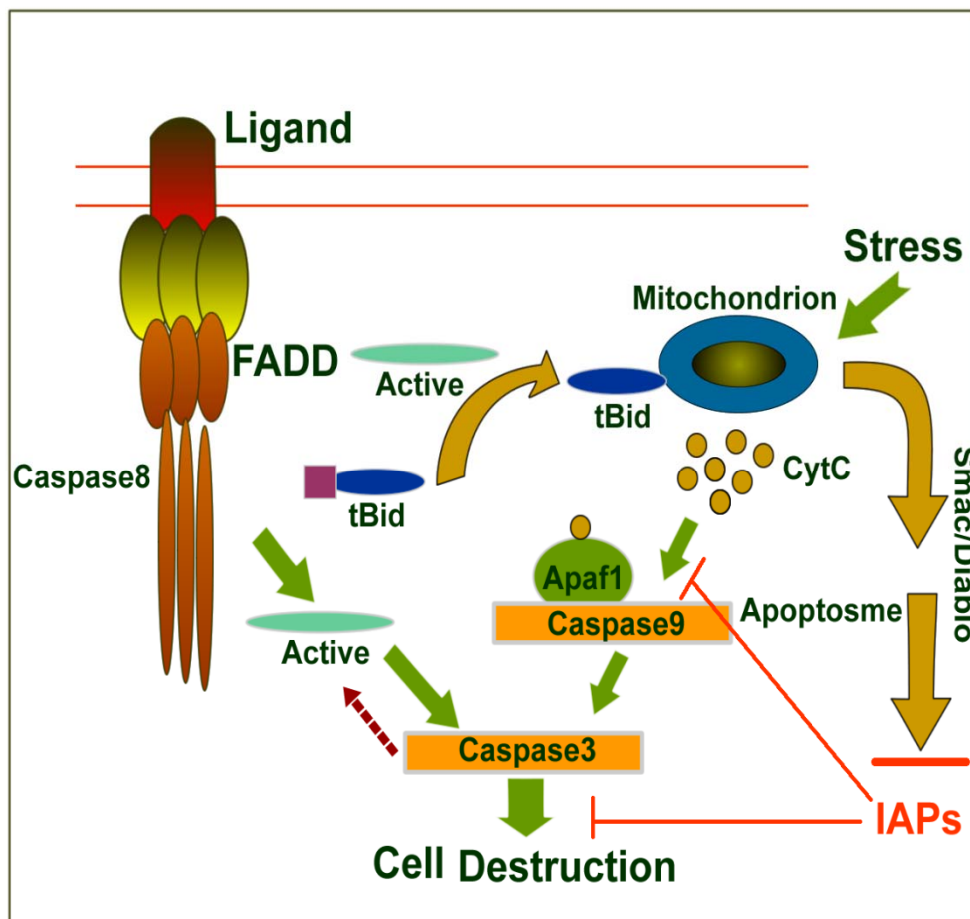


Figure 1.7:

The IAPs including survivin are able to inhibit caspases 9 and 3. This ability can be stopped by release of Smac a mitochondrial protein. This allows the completion of apoptosis. (Modified from Altieri et al, 2001).

1.7.5 Localization of survivin as a passenger protein

During mitosis survivin is associated with the microtubules of the mitotic spindle (Alteiri et al, 2000). This forms the chromosomal passenger complex with INCENP (inner centromere-binding protein) and Aurora B kinase (Sessa et al, 2005). Survivin was detected in MCF-7E breast cancer cells at G1/S where it blocks vitamin D3, allowing G1 arrest and resulting in accumulation of cells in S and G2M phases. (Li et al, 2006).

1.7.6 Function:

In dividing cells survivin regulates mitosis (Lens et al, 2006Dec). It also inhibits apoptosis. Survivin begins its anti apoptotic role by being activated by phosphorylation at threonine 34 by CDK1-cyclin B complex which is a mitotic kinase, this process is associated with sequestration of caspase 9

Survivin also plays a role in mitosis and microtubule function (Altieri et al, 2004). Survivin and the inner centromere protein INCENP show similar cell cycle localization and gene knockout phenotypes. From late prophase until metaphase they are localised at centromeres while at anaphase they localize to the mitotic spindle mid-zone. Later in anaphase and in telophase, survivin is localised with the microtubules (Wheatley et al, 2005).

1.7.3 Aurora B kinase:

Aurora B kinase is one of three serine/threonine protein kinases A, B and C that regulate pathways of cell division. Aurora B kinase is another chromosomal passenger protein that is involved in a number of processes during mitosis (Vader et al, 2006). In proliferating tissues Aurora B expression and activity is cell cycle dependent. Its concentration peaks at G2M transition and the kinase activity is highest at mitosis. Aurora B is phosphorylated by CDK activity and localizes at centromeres throughout pro-metaphase. When activated it is responsible for the phosphorylation of histone H3 serine 10 during mitosis (Murnion et al, 2001).

Aurora-B is necessary for chromosome condensation. It localizes on kinetochores at the cytokinesis phase of the cycle. When spindle tension is disturbed, a spindle assembly checkpoint occurs (Andrews et al, 2005). In the inner centromere Aurora B promotes the release of bound microtubules and therefore promotes the formation of monotelic attachments where chromosomes become attached to both spindle poles (Taylor et al 2004). In p53 deficient cells over expression of Aurora kinase leads to failure of cytokinesis and progression of the cell cycle then produces aneuploidy which characterises aggressive tumours (Adams et al, 2001).

1.7.4 Inner centromere protein (INCENP):

INCENP derives its name from its localization in the inner centromere of chromosomes. It consists of 60-80 amino acids near the C terminus of a protein known as the IN-box and is consequently phosphorylated here. This phosphorylation improves the activity of Aurora B kinase. INCENP is important for chromosomal segregation and cytokinesis (Carmena et al, 2003).

1.7.5 Borealine/Darsa B:

Borealine is a 31kda protein which has two related polypeptides called Borealine2/DarsaA. In mitotic cells Borealine is associated with survivin and also binds to INCENP. Borealine B is required for the chromosomal passenger complex to take its place at the centromere (Gassmann et al, 2004).

It is similar to chromosome segregation and cytokinesis defective-1(CSC-1): CSC-1 is a 27-kda protein which has similar function to Borealine. Both have short domains at both ends. Although CSC-1 requires zinc to bind to BIR-1 which is the binding site of survivin and INCENP, Borealine B does not need it for its binding to survivin. CSC-1 is required to target kinases (Vagnarelli et al, 2004).

1.7.6 Telophase disk -60 (TD-60):

Telophase disk -60 is a 60-kDa protein found at the spindle mid-zone and equatorial cortex. Borealine-B which is essential for localization of TD-60 at mitosis acts as a specific exchange factor for Rac1. TD-60 is essential for the localization of the chromosomal passenger complex and for progression of mitosis (Vagnarelli et al, 2004).

A complete understanding of the cell cycle and particularly of crucial checkpoints, gives opportunities to design drugs and other strategies for the treatment of human tumours.

1.8 Chronic Myeloid Leukaemia (CML):

Leukaemia is a disorder resulting from malignant transformation of the primitive haemopoietic stem cell. It can involve both lymphoid and myeloid lineages. The WHO classification shows CML as one of seven entities in the category of chronic myeloproliferative disease (Vardiman et al, 2002).

1.8.1 Incidence:

CML is a disease of all age groups but more common in a middle age (45-55 years) with male to female ratio of 2:1. It accounts for 15- 20% of adult leukaemia. It is more common in Caucasians. The incidence is about 1-2 per 100,000 population per year (D'Antonio 2005).

1.8.2 Clinical presentation:

Clinically, CML patients have fatigue, weight loss, abdominal fullness, bleeding, purpura splenomegaly, leukocytosis, thrombocytosis, and anaemia. The disease progresses from a chronic phase, through an accelerated phase, then a terminal blast crisis or acute leukemic phase (D'Antonio , 2005).

1.8.3 Karyotype:

CML is characterised by a karyotypic abnormality. Invariably there is a chromosomal translocation of chromosomes 9 and 22 (t (9; 22)) which creates the “Philadelphia chromosome”. This translocation forms the abnormal BCR/ABL fusion gene which is required for the development of 95% all cases of CML (Sattler et al, 1997) (Figure 1.8)

1.8.4 BCR/ABL:

Expression of the BCR-ABL gene is found in haematopoietic stem cells (HSCs). The t(9: 22) translocation which forms the Philadelphia chromosome results in a molecular rearrangement between the c-ABL proto-oncogene on chromosome 9 and the BCR (break point cluster region) on chromosome 22. This forms BCR/ABL fusion gene which codes for a p210-KDa protein. The N-terminal breakpoint cluster region (BCR) fuses to the ABL tyrosine kinase (TK) gene causing enhancement and activation of the tyrosine kinase (Pear et al, 1998).

The BCR/ABL chimeric protein stimulates a range of intra-cellular signalling and inhibits apoptosis; these effects can stimulate cell proliferation in the absence of growth factors which facilitates tumour progression and metastasis (Pasternak et al, 1998).

1.8.5 Action of BCR/ABL:

The oncogenic activity of BCR/ABL is responsible for the tetramerization of BCR/ABL which augments tyrosine kinase activity (Sattler et al, 1997). BCR/ABL induces interleukin-4 and 7 production (IL4, IL7), as well as granulocyte/macrophage – colony stimulating factor (GM-CSF) (Wetzler et al, 1993). As ABL can phosphorylate tyrosine residues the BCR-ABL gene product is also a tyrosine kinase. This is a continuously active gene which can stimulate and speed up the cell cycle. However there is associated DNA damage as it inhibits DNA repair and therefore causes genomic instability (O'Brien et al, 2005).

BCR/ABL activity results in activation of several downstream pathways: these include the phosphatidylinositol-3 kinase/AKT (PI-3K/AKT) pathway, the single transducer and activator of transcription (STAT) pathway and Ras/mitogen-activated protein

kinase (Ras/MAPK) (Cortez et al, 1997). Ras activation leads to phosphorylation and activation of MAPK protein signalling pathway which includes extra-cellular signal-regulated kinase (ERK) and c-jun N terminal kinase (JNK). The MAPK activation signals in BCR/ABL lead to enhancement of proliferation and survival signalling (Woessmann et al, 2001). The outcome is therefore that myeloid leukemic cells have a longer life than normal leukocytes.

1.8.6 Management:

Management of CML varies. Marrow ablation by chemotherapy and allogenic stem cell transplant is the only curative therapy. However there are side effects and a significant mortality. The degree of histocompatibility between donor and recipient influences a marrow transplant performed in the chronic phase of the disease. There is a 6-8% chance of recurrent leukaemia at 5 years; however, if performed at the accelerated phase the 5 year survival increases to 50%.

Interferon alpha ($INF\alpha$) is a glycoprotein with an anti-proliferative action. It induces a therapeutic response in 35-55% of patients. It was an effective therapy for CML and was commonly used until the arrival of the Imatinib. Imatinib mesylate is a synthetic ATP inhibitor. It competitively inhibits ATP binding to BCR-ABL residues by tyrosine phosphorylation and helps its entry to the nucleus so that it loses its antiapoptotic functions. This drug has changed the management of CML. It was recently approved as a first line treatment of CML at all stages of the disease and a complete cytogenetic remission and 5 year survival rates of almost 90% have been achieved (Martinelli et al, 2005)

WHO Classification of malignant haematological diseases

Classification	FAB	WHO	Comments
Myeloid malignancies	<ul style="list-style-type: none"> Chronic myeloproliferative diseases Myelodysplastic syndromes Acute myeloid leukemia 	<ul style="list-style-type: none"> Chronic myeloproliferative diseases Myelodysplastic/myeloproliferative diseases Myelodysplastic syndromes Acute myeloid leukemia's 	The WHO classification system puts a few diseases that show characteristics of both myeloproliferative and Myelodysplastic conditions into a new, separate group (myeloproliferative/Myelodysplastic diseases).
Chronic myeloproliferative diseases (MPD)	<ul style="list-style-type: none"> Chronic myelogenous leukemia (CML) Agnogenic myeloid metaplasia with myelofibrosis (MF) (Idiopathic myelofibrosis) Polycythemia Vera (EV) Essential thrombocytemia (ET) 	<ul style="list-style-type: none"> CML Ph+: t(9;22)(qq34;q11), BCR/ABL Chronic neutrophilic leukemia Chronic eosinophilic leukemia/hypereosinophilic syndrome Chronic idiopathic myelofibrosis Polycythemia Vera Essential thrombocytemia 	<p>CML</p> <p>The most important change is that only the Ph+ cases are called CML by the WHO. The Ph- cases (which show Myelodysplastic signs, and are known to have significantly worse prognosis) are called a CML (atypical CML), and belong to the newly created Myelodysplastic/myeloproliferative group. The aCML term is somewhat misleading, because it is not CML at all, but it was kept, having no better alternative.</p>

Myelodysplastic/ myeloproliferative diseases	<ul style="list-style-type: none"> • CMML belonged to the MDS in the FAB classification. About one half of the cases show proliferative, the other dysplastic signs, but it looks like these are just different forms of the same disease. 	<ul style="list-style-type: none"> • Atypical myelogenous leukemia (aCML) • Chronic myelomonocytic leukemia (CMML) • Juvenile myelomonocytic leukemia (JMML) 	
Acute myeloid leukemia's (AML)	<ul style="list-style-type: none"> • M0: minimally differentiated • M1: myeloblastic leukemia without maturation • M2: myeloblastic leukemia with maturation • M3: hypergranular promyelocytic leukemia • M4: myelomonocytic leukemia • M4Eo: variant, increase in marrow eosinophils • M5: monocytic leukemia • M6: erythroleukemia (DiGuglielmo's disease) • M7: megakaryoblastic leukemia 	<ul style="list-style-type: none"> • AML with recurrent cytogenetic translocations • AML with t(8;21)(q22;q22) AML1/CBFalpha/ETO • Acute promyelocytic leukemia: AML with t(15;17)(q22;q12) and variants PML/RARalpha • AML with abnormal bone marrow eosinophils inv(16)(p13;q22) vary t(16;16)(p13;q22) CBFbeta/MYH1 • AML with 11q23 MLL abnormalities • AML with multilineage dysplasia • With prior MDS • Without prior MDS • AML with Myelodysplastic syndrome, therapy related <ul style="list-style-type: none"> • Alkylating agent related • Epipodophyllotoxin related • Other types • AML not otherwise categorized • AML minimally differentiated • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monocytic leukemia • Acute erythroid leukemia • Acute megakaryocytic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis 	The changes are easily summarized: the leukemia's with consistent cytogenetic abnormalities and those that are MDS related were taken into separate groups; the rest of the old FAB classification was put under the "AML not otherwise categorized" entry.

Philadelphia (Ph) chromosome

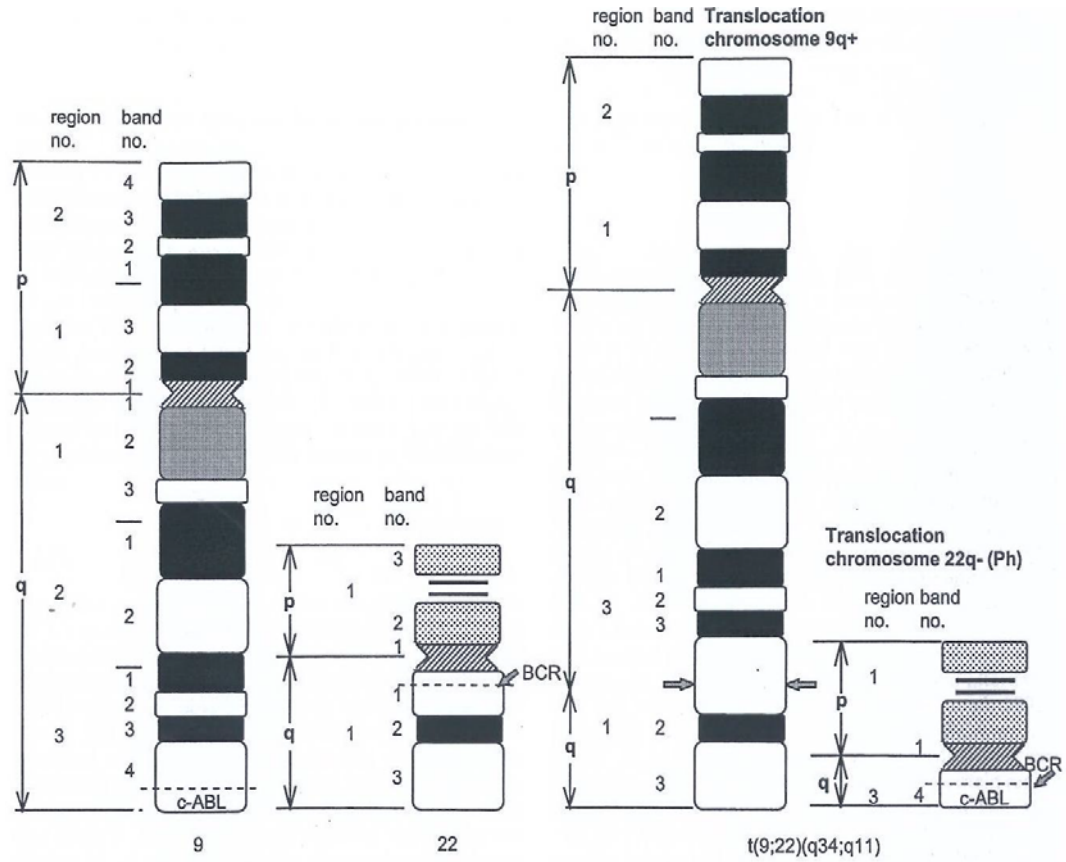


Figure 1.8:

A diagram showing the Philadelphia (Ph) chromosome, chromosome 9 and 22 and the translocation 9;22 (q34;q11) (Gunter et al, 1998).

1.9 Chemotherapeutic drugs:

1.9.1 Cytarabine:

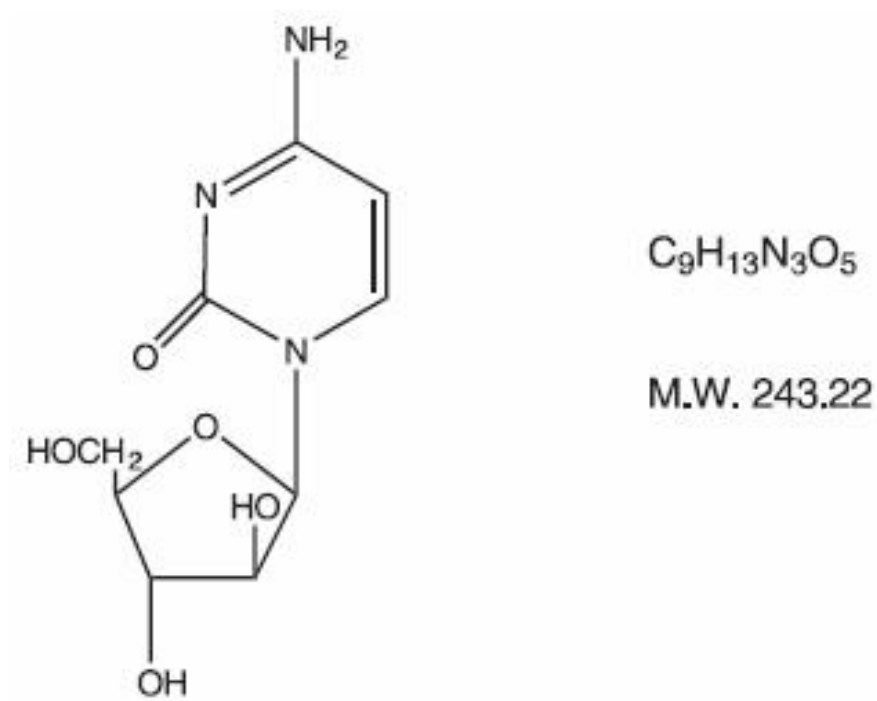


Figure 1.9:

Cytarabine (cytosine arabinoside) is an antimetabolic agent which specifically is a pyrimidine antagonist. Its chemical name is 1 β - arabinofuranosylcytosine. Its chemical formula is $C_9H_{13}N_3O_5$ and its molecular mass is 243.217 g/mol. In vivo, 13% is protein bound and there is 20% bioavailability when taken orally. The half life is biphasic: the initial is 10 minutes and the secondary is 1-3 hours and longer with continuous intravenous infusion. It is metabolized in the liver and 70-80% is excreted in urine. The common side effect is cerebellar toxicity.

Cytarabine acts only when it's rapidly converted into cytosine arabinoside which competitively inhibits DNA polymerase. This results in DNA damage and causes cell cycle retardation at S-phase during the synthesis of DNA. DNA incorporation leads to chain elongation and causes defective DNA fragmentation at S phase of rapidly dividing cells. Cytosine arabinoside inhibits both the DNA and RNA polymerases and nucleotide reductase enzymes needed for DNA synthesis. Cytarabine is metabolised by rapid conversion into the inactive uracil derivative (Nowrousian et al, 1985) (Figure 1.9).

1.9.2 Etoposide:

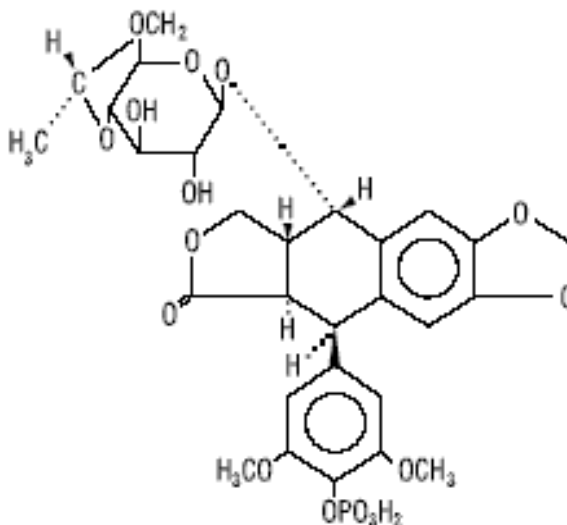


Figure 1.10:

Etoposide is a chemotherapeutic drug used to treat certain types of human cancer. It is a topoisomerase 2 inhibitor and is derived from podophyllotoxin of plant origin. Its systematic name is 4-demethyl-epipodophyllotoxin 9-{4, 6-O-(R)-ethylidene-beta-d-glucopyranoside} 4-di-hydrogen phosphate. Its chemical formula is C₂₉H₃₂O₁₃ and it has molecular mass of 588.557g/mol.

It has 25-75% bioavailability and 97% protein binding and a half-life of 6 hours when taken orally; this extends up to 12 hours if given intravenously. It is metabolized in the liver and excreted in urine and faeces. The common side effects are low blood pressure, hair loss, metallic taste and bone marrow suppression. Topoisomerases are essential enzymes which exist in all pro-and eukaryotic cells. They play a crucial role in the maintenance and replication of DNA during cell proliferation. These ubiquitous enzymes remove any defect formed by transient double-strand breaks (Baldwin EL et al 2005). Topoisomerase 2 inhibition results in stabilization of DNA double strand breakage. Thus accumulation of cleavage

complexes in treated cells forms permanent DNA strand breaks which cause mutagenesis and chromosomal translocation. Etoposide acts via the known topoisomerase 2 enzymes which are multi subunit proteins which use ATP to adjust DNA topology by passing an unbroken helix through a transient double – strand break in DNA. After strand passage the DNA backbone is held and DNA structure restored. Etoposide changes topoisomerase 2 into a poison that introduces a high level of transient protein-associated breaks in the genome of treated cells (Kenneth et al 2006). Resistance to etoposide in multi-drug resistance (MDR) phenotype cells occurs in many different ways; mutations at ser-1106 in the topoisomerase 2 molecule inhibits enzyme phosphorylation and reduces its response to etoposide(Baldwin et al, 2005)Figure 1.10

1.9.3 Nocodazole:

This anti-neoplastic agent is an abenzimidazole derivative. Its chemical formula is $C_{14}H_{11}N_3O_3S$ and it has a molecular weight of 301.32 kDa. It is used in chemotherapy because of its ability to rapidly depolymerise microtubules. Microtubules are linear protein polymers whose action is to regulate movement of vesicles, organelles such as Golgi apparatus, endoplasmic reticulum and most importantly, the movement of chromosomes during mitosis (Grigoriev et al, 1999). Microtubules are found in transition between one of two states: either elongation or rapid shortening. Nocadazole acts by depolymerising microtubules and preventing the formation of the metaphase spindle. This arrests mitosis, as the drug prevents the attachment of microtubules to the kinetochores. This detachment prolongs mitotic arrest and stimulates the spindle assembly checkpoint leading the cell to apoptosis. High doses of nocodazole prevent progression of cells into mitosis because of rapid depolymerization of microtubules. This causes mitotic apoptosis. A low concentration alters the microtubule dynamics arresting the cells at mitosis (Vasquez et al, 1997).

1.9.4 Vinca alkaloids:

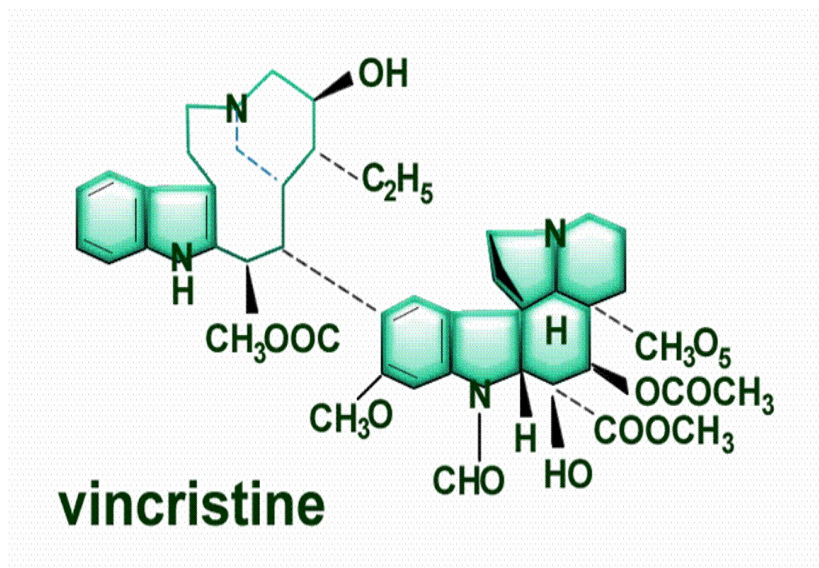


Figure 1.11:

Vincristine is an alkaloid obtained from *vinca rosea*, a periwinkle plant. Its chemical formula is C₄₆H₅₆N₄O₁₀ and it has a molecular mass of 923.958g/mol. It has 75% protein binding and a half-life in vitro of 19-155 hours after intravenous infusion. It is metabolized in the liver where 90% is excreted in the bile and 10% excreted in the urine. The common side effect is peripheral neuropathy, hyponatremia, constipation and hair loss. This is one of several vinca alkaloids and

is a powerful anti-tumour drug. It targets tubulin, a member of a protein family where the most common members are α tubulin and β tubulin. Normally they bind to microtubules with tubulin GTP dimer and cause microtubule assembly. The mechanism of action of vincristine involves binding to tubulin and inhibiting the assembly of microtubules. In addition, vincristine causes depolymerization of microtubules. Spindle damage interferes with chromosomal segregation and leads to mitotic arrest at metaphase (Gigant et al, 2005).

A low concentration of vincristine binds to microtubule tips and suppresses the dynamic instability of plus ends. A higher dose leads to microtubule depolymerization, causing proto-filament spirals and curls (Jordan et al, 1993). Vincristine (Figure 1.11) can be used as a tool to control the dynamic behaviour of microtubules in living cells (Gigant et al, 2005).

1.10 Aims:

Many drugs used in cancer chemotherapy have been used for decades with varying degrees of success. Enhanced efficacy of these agents could come about by the design of new “small molecules” that would act as adjuvants to conventional regimens. Cells that are relatively resistant to chemotherapy provide a “time lapse” or “window of opportunity” to analyse the response of cells to chemical insult.

As the BCR-ABL Philadelphia chromosome imparts a degree of drug resistance to cells, the aim of this study is to review and investigate the responses of K562, a BCR-ABL positive chronic myeloid leukaemia cell line, to etoposide and vincristine, drugs with differing modes of action

I aim to:

Determine the effect of each drug on proliferation

Determine the effect of each drug on cell death.

Determine the effect of drug concentration on these parameters

Determine the effect of time on these parameters

To investigate and discuss any common pathways that may lead to synergy for these agents

To investigate and discuss potential targets for new alternative therapeutic targets that may enhance the effectiveness of these more traditional agents in managing leukaemia

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell lines & culture conditions:

K562 cells were obtained from the European Collection of Animal Cell Cultures (ECACC) Proton Down, UK. These cells are derived from the K562 erythromyeloid leukemia cell line. They remain p53 negative in long term culture. The cells were propagated in RPMI 1640 medium containing 25mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid (HEPES), 10% v/v heat inactivated fetal bovine serum and 2mM L-glutamine.

The cells were cultured in either 25 cm³ or 75 cm³ flasks and kept in a humidified incubator containing 5% CO₂ at 37°C. Aseptic techniques and a laminar flow cabinet was employed to maintain the sterility of the cultures. Cells were grown in suspension at a density of 0.2-1.0×10⁶ cells/ml for exponential cell growth and subculture every two-three days.

2.1.2 Cryopreservation and replenishment of leukemic cell line:

Cells in exponential phase of growth (2-3 days of culture) were frozen in Cryopreservation medium. The cells were washed in RPMI 1640 medium, resuspended at a concentration of 1×10^7 /ml in the above medium and aliquot was stored in 1 ml ampoules. These were placed in a 750 cm³ Nalgene™ cryo 1°C freezing container filled with 250 ml of isopropanol this achieves a cooling rate of -1°C/min. The Nalgene™ cryo 1°C freezing container was placed at -70°C for 12 hours. The ampoules of cells were then transferred to vats containing liquid nitrogen for long term storage. Cells were removed from liquid nitrogen every six months, washed three times in Hank's balanced salt solution (HBSS) and resuspended in supplemented RPMI 1640 medium. At least three passages of the retrieved cells were allowed to occur before the fresh cells were used in any experiments.

2.1.3 Cell viability testing:

Cell viability was assessed by trypan blue dye exclusion. Since dead cells lose the integrity of their plasma membrane they are unable to exclude macromolecules such as trypan blue dye and viewed with a phase contrast microscope. 50 µl of 0.1% w/v trypan blue in HBSS and 50 µl of cells in suspension were mixed together and placed in a 96-microwell plate and incubated for 10 minutes at 37°C. All cells were counted using a haemocytometer. Dead cells showed blue staining of the cytoplasm and nucleus. Cell viability was calculated by: Percentage cell viability = $100 \times \frac{\text{total number of viable cells (unstained cells)}}{\text{total number of cells (stained and unstained)}}$. Optimal culture conditions were designated as greater than 95% cell

viability and experiments were only performed if the background level of cell death was less than 5%.

2.1.4 Testing the effect of chemotherapeutic and chemical agents:

K562 cells were incubated with, 0 µg/ml, 1 µg/ml and 20 µg/ml of cytarabine and etoposide for 4, 8, 24, 36 and 48 hours. A titration of vincristine dose was performed, a vincristine concentrations of 0.0, 0.1, 40 µg/ml were tested for at 2, 4, 6, 24, 48, 72 and 96 hours. For all experiments cells were seeded at a concentration of 0.25×10^6 cells/ml. Nocodazole was used at a concentration of 1 µg/ml all experiments.

2.2 Cell cycle analysis:

2.2.1 Apoptosis and G2M arrest by flow cytometry:

Exponentially growing K562 cells, were harvested, washed in medium and resuspended in supplemented RPMI 1640 medium at concentration of 0.25×10^6 cells/ml. 2ml aliquots of this suspension were incubated in 24 well plates in the presence and absence of etoposide, nocodazole and vincristine.

Aliquots of 0.25×10^6 cells were removed washed twice in phosphate buffered saline (PBS) and then fixed and permeabilised in 500 μ l 70% ice-cold ethanol for 30 minutes at 4°C. The cells were then washed three times in PBS, resuspended and incubated at 37°C for 60 minutes in 1 ml of PBS containing 200 μ g/ml RNase and 50 μ g/ml propidium iodide. The fluorescence of propidium iodide stained nuclei was measured on a flow cytometry using either a Coulter Epics XL4-MCL or a Becton Dickinson FACS Vantage flow cytometer with pulse processing for aggregate discrimination, a band pass filter on PMT3 at a wavelength of 620nm and System II Lyses 3 software or Cell Quest software respectively.

Analysis of 10,000 cells yielded a histogram of DNA content distribution. Apoptotic cells were defined as the sub G0 peak the lower content of DNA was therefore a feature used to detect apoptotic cells compared to the controls which are untreated cells.

2.2.2 Detection of apoptosis with annexin V immunostaining:

K562 cells, were harvested, washed in medium and resuspended in supplemented RPMI 1640 medium at 0.25×10^6 cells/ml in 2ml volumes in 24 well plates in the presence and absence of etoposide, and vincristine . Aliquots of 0.5×10^6 cells were then removed and washed with cold (PBS) and then cells were resuspended in 100 μ l of 1x binding buffer in a test tube to which was added 5 μ l of fluoresceinated annexin V antibodies.

The cells were mixed gently and incubated in 400 μ l of binding buffer at 20c° for 15mints in the dark and then analyzed by flowcytometry within 1 hour. In each experiment, controls were untreated cells from the same culture stained by similar protocols.

2.3 Whole cell protein extraction:

2×10^7 cells were washed twice in HBSS and centrifuged at 1,400 rpm for 5 minutes to pellet the cells. The cells were then homogenized in 1 ml radioimmunoprecipitation (RIPA) buffer Cells were sheared on ice by repeated passage through a 19 gauge microlance needle and a 1 ml syringe. The sample was left on ice for 60 minutes and the cells were sheared every 15 minutes for twenty strokes each time. Insoluble cellular debris was removed by centrifugation for 10 minutes at 4,000 rpm. The supernatant was removed, transferred to Eppendorf tubes and stored at -70 °C.

2.3.1 Protein quantification using the direct Lowry method:

The Lowry reaction works by peptide bonds forming a blue colour when complexes with an alkaline cupric tartrate reagent. Protein standards using different concentration of bovine serum albumin were prepared in a volume 1 ml of 10 µl of protein lysate samples were diluted with 990 µl of deionised water. 1 ml of Lowry reagent solution was added to the standard and sample tubes and left at room temperature for 20 minutes. With rapid and immediate mixing, 500 µl of Folin & Ciocalteu's Phenol Reagent Working Solution was added to each tube and the color allowed developing for 30 minutes. The samples were then transferred in triplicate to a 96-microwell enzyme-linked immunosorbant assay (ELISA) plate and read at a wavelength of 750nm on an ELISA plate reader. The readings were completed within 30 minutes. The sample concentrations were calculated from the prepared protein standards by linear regression analysis. The protein concentration of the samples were determined using the equation $Y = a + bX$ where Y is the optical density value and X is the protein concentration.

2.4 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein preparation:

Precisely 50 µg of cellular lysate as determined by the direct Lowry method were placed in 20 µl of RIPA buffer and 10 µl SDS-PAGE sample loading dye. The samples were heated to 100°C for 5 minutes in a dry block heater, allowed to cool and then centrifuged at 1,300 rpm for 1 minute in a minicentrifuge.

2.4.1 Relative molecular weight determined by SDS-PAGE electrophoresis:

Pre stained standard protein molecular weight markers ranging from 14.3 kDa to 220 kDa were prepared by mixing 10 μ l of the pre stained Rainbow™ marker with 10 μ l of RIPA buffer and 10 μ l of SDS-PAGE sample loading dye. The mixture of molecular weight markers was heated to 100°C for 5 minutes in a dry block heater, allowed to cool and then centrifuged at 1,300 rpm for 1 minute in a minicentrifuge. The pre stained standard protein molecular weight markers were run alongside the samples to determine the relative size of the protein of interest.

2.5 Western Blotting:

2.5.1 Preparation of SDS-PAGE mini gel

The two glass plates of the Bio-Rad Mini Protein II™ western blotting electrophoresis tank were cleaned in detergent, washed in de-ionized water and wiped with 70% methanol and allowed to air dry. The plates were assembled in a clamp holder using 0.5mm spacers and transferred onto a casting platform. An SDS-PAGE solution was prepared. Different percentage gels were prepared according to the molecular weight of the protein to be analyzed 3.5 ml of the gel solution was poured between the glass plates mounted in the assembled clamp holder, covered with 0.5 ml water and allowed to polymerise for approximately 1 hour. When the gel had set the water was washed off. The gel was then overlaid with a stack gel A 10 well comb was inserted into the stack gel solution which was also allowed to polymerize for 1 hour.

2.5.2 Protein electrophoresis:

The comb was removed from the polymerised SDS-Polyacrylamide gel (PAG), which was transferred in the clamp holder to an electrode assembly where it was clipped into position to form the inner reservoir chamber. 500 ml of electrode buffer was prepared and used to fill the inner reservoir chamber. The apparatus was transferred to the gel tank containing the remaining electrode buffer. Air bubbles were allowed to disperse from the wells before the samples were loaded. The Rainbow™ coloured protein molecular weight marker was loaded in the first lane of each gel, followed by precisely 50 µg per lane of cellular protein lysate. Electrophoresis was performed at a constant voltage of 100V for 90 minutes.

2.5.3 Electroblotting onto Immobilon-P polyvinylidene difluoride (PVDF) membranes:

After the completion of electrophoresis, the gels were removed from the glass plates and the stack gel discarded. The SDS-PAGE along with two blotting pads and a sheet of PVDF membrane were presoaked in separate containers containing transfer buffer for 30 minutes. One of the presoaked blotting pads, on which the gel was positioned, was placed in a clamp holder, the PVDF membrane was then layered on top of the gel and air bubbles removed by rolling a pipette over the membrane. The final blotting pad was placed on top of the membrane and the clamp holder closed and clipped shut. The clamped assembly was then positioned in the immersion electro blotter transfer tank with the gel facing the cathode and the membrane facing the anode. The tank was then filled with transfer buffer. A cooling system was

inserted into the transfer tank and the gels were electro blotted at a constant voltage of 30V for 14 hours. After the transfer was complete the membranes were allowed to air dry for 5 minutes before being stored at 4°C between two dry blotting pads, covered in Saran wrap.[®]

2.5.4 Immune-staining of blots:

Determination of expression of CDK1, Bax, cyclin a, cyclin B, survivin, Aurora B, cyclin B tyrosine 15 phosphorylation. Membranes were immunostained with an anti- β -actin antibody to ensure equal loading of the samples per lane. The PVDF membrane was rehydrated for one hour in Tris (hydroxymethyl amino methane buffered saline (TBS) (Appendix 9.1.13) containing either 0.05% v/v or 0.1% v/v Tween-20[®] (TBST) (Appendix 9.1.13) for monoclonal and polyclonal antibodies respectively. Whilst the membrane was hydrating it was cut into strips to separate the lanes containing protein which were visible during the rehydration process. The hydrated membrane strips were positioned inside 50 ml Falcon tubes, placed on a rolling mixer platform and non specific protein binding was blocked at 4°C by incubation in 5% w/v dried milk powder in TBST solution for one hours. The membrane was then briefly washed in TBST for 5 minutes before being incubated in a 50 ml Falcon tube containing a solution of primary antibody (at predetermined optimized concentrations, Appendix 9.1.13) and 1% w/v dried milk powder in TBS for 1 hour at 4°C on a rolling mixer platform. The membrane was then washed six times, for 10 minutes each in TBST before being incubated in a 50 ml Falcon tube

containing the horse radish peroxidase (HRP) conjugated secondary antibody (either goat anti-mouse antibody or goat anti-rabbit antibody as appropriate (Appendix 9.1.13) in 1% w/v dried milk powder in TBS for 1 hour at 4°C on a rolling platform mixer. Excess secondary antibody was removed by a further four washes of 10 minutes each in TBST followed by two washes for 10 minutes in TBS.

2.5.5 Anti-phospho-tyrosine antibody immunostaining:

The PVDF membranes were re-hydrated in 0.1% v/v TBST for one hour. Whilst the membrane was hydrating it was cut into strips to separate the visible lanes containing the protein. The hydrated membrane strips were placed in a 50 ml Falcon tube on a rolling mixer platform. Non specific protein binding was blocked in 5% w/v BSA in TBST (Appendix 9.1.14) at 4°C for one hour. The membrane was briefly washed in TBST before being incubated in a 50 ml falcon tube, containing the HRP conjugated anti-phospho-tyrosine antibody (Appendix 9.1.14) in TBST, at 4°C for one hour on a rolling platform mixer. Excess antibody was removed by four a further for 10 minutes washes TBST followed by two washes for each in TBS.

2.5.6 Visualization of protein bands using enhanced chemiluminescences:

Enhanced chemiluminescence's (ECL) (Appendix 9.1.15), utilizes the highest emitted reaction when hydrogen peroxidase catalyses the oxidation of luminal in alkaline conditions. Following oxidation. Luminal is in an excited state which then decays to ground state via a light emitting pathway. Since Tween-20[®] greatly diminishes the light output, Tween-20[®] was omitted from the wash solution for the last two washes. Membranes were incubated with ECL for 2 minutes before the

excess was blotted away. The membranes were then placed in a Rexel plastic wallet holder and exposed to blue light sensitive autoradiography film, 'Hyperfilm' (Appendix 9.1.15). The Hyperfilm was exposed to the membrane for between 5 seconds and 1 hour depending upon the strength of the bands achieved after post development. The Hyperfilm was developed in Xograph Imaging Systems Compact ×4 automatic developing machine.

2.5.7 Immunoprecipitation of survivin and Cdk1 by western blotting:

The phosphorylation status of the tyrosine residue 15 in CDK1 is indicative of its function. To assess differences in CDK1 phosphorylation status between cultures, CDK1 was immunoprecipitated using an anti-CDK1 antibody.

250 µl of appropriate anti-class specific coated Dynabeads (Appendix 9.1.16) were added to an Eppendorf tube and placed on a magnetic particle concentrator for 2 minutes. The supernatant (preservation buffer containing sodium azide) was carefully removed so as not to disturb the collected beads. The Eppendorf tube containing the beads was then removed from the magnetic particle concentrator and 1.5 ml of 0.1% w/v BSA in PBS (PBS/BSA) was mixed with the beads. The Eppendorf tube containing the beads was then placed on the magnetic particle concentrator for 2 minutes. Once again the supernatant was removed and the process of washing the beads in PBS/BSA to remove any traces of sodium azide (which inhibits antibody binding) was repeated three times. These washed beads were then incubated with 15 µg of primary antibody in 1.5 ml of PBS/BSA for 30 minutes at room temperature and placed on a rolling mixer platform. After five washes in PBS/BSA to remove the excess primary antibody, 75 µl of coated beads

were resuspended in 250 μ l of PBS/BSA and added to 500 μ g of cell lysate which was made up to a final volume of 1.5 ml with PBS/BSA. This was incubated at 4°C for 1 hour on a rolling mixer platform. The magnetically selected beads containing target Protein complexes were once again washed five times in PBS/BSA to remove any cell lysate containing non-bound protein.

The washed beads with bound target-protein complexes were then eluted directly into 20 μ l of SDS-PAGE loading dye. The samples were then heated to 100°C for 5 minutes in a dry block heater to denature the protein and to destroy the antibody-bead, antibody-antibody, and antibody-protein bonds. The samples were left to cool then centrifuged at 1,300 rpm for 1 minute in a micro centrifuge. The beads were separated from the sample by using the magnetic particle concentrator and the collected proteins were loaded onto an SDS-PAGE gel as previously described (2.4.2). The gel was then transferred onto PVDF membranes by Western blotting (2.4.3) and the membranes were then stained with an anti-phospho-tyrosine antibody (2.4.5). Survivin Immunoprecipitation was done with same technique.

Result chapter 3

Effects of cytarabine and etoposide on the cell cycle of K562 and HL60 cells

3.1 Introduction:

Cytarabine is an anti metabolic agent which damages DNA. It is rapidly converted to the active form cytosine arabinoside, which by competing with pyrimidine, inhibits DNA and RNA polymerase and nucleotide reductase enzymes. This inhibition of DNA synthesis arrests the cell cycle during DNA replication (S-phase).

Etoposide, on the other hand, inhibits topoisomerase 2, one of the important DNA repair enzymes. Inhibition of topoisomerase 2 causes persistence of DNA strand breakages. In the absence of repair by topoisomerase 2, these DNA breakages lead to permanent mutations and even chromosomal translocations. Persistence of DNA strand breaks caused by topoisomerase 2 inhibition also induces other repair or apoptosis mechanisms mediated by p53 gene (Baldwin et al, 2005).

The following experiments show the effects of these two drugs, cytarabine and etoposide on K562 cells and HL60 cells both of which are p53 deficient. The use of apoptosis resistant K562 cells allows the accumulation of DNA damaged cells which facilitates the study of the mechanism of cell death and resistance to genotoxic drugs.

Flow cytometry measurement of DNA and immunostaining using an anti-phospho histone H3 ser10 antibody were used to detect cells in mitosis.

3.2 Aims:

-To study the effect of the DNA damaging agents cytarabine and etoposide on the induction of apoptosis and cell cycle distribution of K562 and HL60 myeloid leukemia cells line.

-To determine whether these effects are time and concentration dependent.

3.3 Results:

3.3.1 Effect of cytarabine on K562 cells:

K562 cells were incubated with different concentrations of cytarabine (0-80 μ g/ml). The harvested cells were stained with propidium iodide. The degree of apoptosis and cell cycle distribution was determined via flow cytometry.

Induction of apoptosis in K562 cells with 80 μ g/ml cytarabine was measured as 7.7% \pm 1.9, 9.3% \pm 2.3, 17.0% \pm 4.2, 15.3% \pm 3.3, 17.1% \pm 4.2 of the total population at 4, 8, 24, 36 and 48hrs respectively. This shows an induction of apoptosis significantly greater than control cultures at time points of 24hrs and longer. Other concentrations of cytarabine did not induce appreciable amounts of apoptosis when compared to control cultures although small standard errors in measurement resulted in statistical significance in some instances. Thus, the percentage apoptosis induced by 20 μ g/ml was 7.2% \pm 1.9, 10.3% \pm 3.1, 11.2% \pm 3.0, 10.2% \pm 2.1, 10.0% \pm 2.1 at 4, 8, 24, 36 and 48hrs respectively; a dose of 5 μ g/ml induced apoptosis of 7.7% \pm 1.9, 10.3% \pm 2.3, 11.2% \pm 3.0, 10.2% \pm 2.1, 10.0% \pm 2.1 at 4, 8, 24, 36 and 48hrs respectively and a dose of 1 μ g/ml cause apoptosis of about 8.3% \pm 1.6, 10.9% \pm 2.0, 7.4% \pm 1.5, 10.1% \pm 0.6, 8.4% \pm 0.9 at 4, 8, 24, 36 and 48hrs respectively (figure 3.1).

The percentage of cells accumulating in G2M after incubation with cytarabine was also measured. As with the induction of apoptosis, significant changes in G2M were observed only in cells cultured with drug at 80 μ g/ml for 24hrs or longer when compared to control. G2M arrest in K562 cells induced with 80 μ g/ml was 6.0% \pm 1.2,

6.3% \pm 1.3, 6.4% \pm 0.7, 6.5% \pm 1.1, 6.7% \pm 1.0 at 4, 8, 24, 36 and 48hrs respectively. The timepoints of 24hr or longer showed a reduction in G2M arrest when compared to controls. Other concentrations of cytarabine did not significantly alter G2M accumulation although some G2M arrest was seen at 24hrs with lower concentrations of cytarabine. Thus, a dose of 20 μ g/ml caused a percentage G2M arrest of 6.8% \pm 1.7, 6.3% \pm 1.2, 12.4% \pm 1.3, 12.5% \pm 0.9, 11.6% \pm 1.5 at 4, 8, 24, 36 and 48hrs respectively; a dose of 5 μ g/ml induce G2M arrest of 7.9% \pm 1.7, 6.5% \pm 0.9, 19.6% \pm 2.6, 12.8% \pm 1.6, 11.7% \pm 2.4 at 4, 8, 24, 36 and 48hrs respectively and a dose of 1 μ g/ml induced G2M arrest of 7.57% \pm 1.7, 6.3% \pm 0.7, 17.7% \pm 1.5, 11.8% \pm 1.8, 11% \pm 2.3 at 4, 8, 24, 36 and 48hrs respectively (figure 3.4).

There was also a concomitant change in the percentage of cells accumulating in the G1 and S-phases of the cell cycle. The percentage of K562 cells in the G1 phase after incubation with 80 μ g/ml cytarabine was 55.3% \pm 1.6, 55% \pm 1.8, 46.5% \pm 2.5, 43.9% \pm 2.9, 41.6% \pm 4.1 at 4, 8, 24, 36 and 48hrs respectively. Dose of 20 μ g/ml induced G1 phase percentages of 54.5% \pm 1.1, 52% \pm 2.1, 31.8% \pm 1.6, 30.9% \pm 5.6, 34.2% \pm 5.7 at 4, 8, 24, 36 and 48hrs respectively. Dose of 5 μ g/ml induce G1 cells at 52.2% \pm 1.1, 50.4% \pm 1.8, 35.2% \pm 4.6, 43.4% \pm 2.4, 46.4% \pm 4.3 at 4, 8, 24, 36 and 48hrs respectively. Dose of 1 μ g/ml induce G1 cells at 50.6% \pm 0.4, 48.3% \pm 1.4, 44.4% \pm 2, 48.8% \pm 1.3, 50.9% \pm 2.5 at 4, 8, 24, 36 and 48hrs respectively (figure 3.2).

The percentage accumulation of K562 cells in S phase after incubation with 80 μ g/ml cytarabine was 28.7% \pm 2.5, 29.3% \pm 3.4, 26.4% \pm 3.1, 29.7% \pm 1.8, 29.5%

+/_1.7 at 4, 8, 24, 36 and 48hrs respectively. Dose of 20µg/ml induce G1 cells at 29.6% +/_2.2, 30.0% +/_1.9, 40.5% +/_3.5, 42.5% +/_5.6, 40.2% +/_5.7 at 4, 8, 24, 36 and 48hrs respectively. Dose of 5µg/ml induce G1 cells at 30.2% +/_2.7, 32.0% +/_1.7, 32.2% +/_3.6, 31.0% +/_4.4, 29.6% +/_4.7 at 4, 8, 24, 36 and 48hrs respectively. Dose of 1µg/ml induce G1 cells at 31.1% +/_2.1, 32.7% +/_1.8, 26.25 +/_1.4, 27.5% +/_2.6, 27.5% +/_2.6 at 4, 8, 24, 36 and 48hrs respectively (figure 3.3).

This data shows that cytarabine has little effect on the induction of apoptosis over an 8hr period. However, after 24hrs the highest concentration induced a significant increase in apoptosis but there was no subsequent increase over a further 24 hr period. Lower concentrations did not induce apoptosis suggesting this effect is not concentration dependent. The induction of apoptosis by 80µg/ml cytarabine was mirrored by a significant fall in the percentage of cells detected in G2M.

3.3.2 Effect of etoposide on K562 cells:

K562 cells were incubated with different concentrations of etoposide (0-80 μ g/ml). The harvested cells were stained with propidium iodide and cell DNA content was measured by flow cytometry to study apoptosis and the distribution of cell in the stages of cell cycle.

The percentage apoptosis induced in K562 cells with 80 μ g/ml was 13.1% \pm 3.7, 14.4% \pm 3.2, 29.8% \pm 7.9, 27.0% \pm 5.3, 41.8% \pm 3.3 at 4, 8, 24, 36 and 48hrs respectively. The percentage of apoptotic cells induced by 20 μ g/ml was 10.4% \pm 3.0, 10.4% \pm 2.6, 19.1% \pm 4.8 and 31.0% \pm 5.8 at 4, 8, 24, 36 and 48hrs respectively. The percentage apoptosis induced by 5 μ g/ml was 9.7% \pm 2.3, 9.3% \pm 2.6, 10.5% \pm 2.8 and 16.3% \pm 3.8 at 4, 8, 24, 36 and 48hrs respectively and 1 μ g/ml induce apoptosis of 9.2% \pm 2.1, 9.7% \pm 2.8, 7.8% \pm 2.3, 13.1% \pm 1.9 at 4, 8, 24, 36 and 48hrs respectively. These data show in contrast to cytarabine a time and concentration dependent induction of apoptosis (figure 3.5).

The percentage of cells accumulating in G2M after incubation with etoposide was also measured. The percentage G2M arrest of K562 cells induced with 80 μ g/ml etoposide was 7.8% \pm 2.6, 6.9% \pm 1.7, 5.5% \pm 0.4, 6.9% \pm 1.4, 5.3% \pm 1.1 at 4, 8, 24, 36 and 48hrs respectively. Dose of 20 μ g/ml induce G2M arrest at 7.8% \pm 1.2, 10.0% \pm 2.3, 12.2% \pm 1.8, 13.0% \pm 2.2, 13.8% \pm 4.0 at 4, 8, 24, 36 and 48hrs respectively. Dose of 5 μ g/ml induce G2M arrest at 8.0% \pm 0.9, 9.7% \pm 2.1, 22.2% \pm 3.9, 20.4% \pm 4.3, 29.1% \pm 7.8 at 4, 8, 24, 36 and 48hrs respectively and a dose of 1 μ g/ml induce G2M arrest at 8.9% \pm 1.8, 10.6% \pm 2.6, 26.3% \pm 3.8, 21.9% \pm 4.8,

23.9% \pm 5.9 at 4, 8, 24, 36 and 48hrs respectively. Again, a concentration-dependent effect is seen when measuring this parameter. Low concentrations of etoposide induced cell cycle arrest at G2M whereas higher concentrations abolished cell cycle arrest and therefore the G2 and mitotic cell cycle checkpoints (figure 3.8).

There was also a change in cell accumulation in G1 and S-phase. The percentage K562 cells accumulated at G1 phase at a dose of 80 μ g/ml was 43.7% \pm 1.2, 45.1% \pm 1.0, 34.6% \pm 4.0, 39% \pm 1.6, 27.5% \pm 1.3 at 4, 8, 24, 36 and 48hrs respectively. Dose of 20 μ g/ml induce cell accumulation at G1 phase of 45.4% \pm 0.9, 43.6% \pm 0.7, 25.7% \pm 3.3, 26.6% \pm 2.6, 19% \pm 3.1 at 4, 8, 24, 36 and 48hrs respectively. Dose of 5 μ g/ml induced cells in G1 phase arrest of 43.2% \pm 1.6, 43.4% \pm 0.9, 40.6% \pm 5.7, 40.5% \pm 4.4, 29.6% \pm 7.8 at 4, 8, 24, 36 and 48hrs respectively and a dose of 1 μ g/ml induced G1 phase arrest of 43.4% \pm 2.1, 44.9% \pm 1.8, 33.8% \pm 4.2, 36.1% \pm 5.8, 35.1% \pm 5.9 at 4, 8, 24, 36 and 48hrs respectively (figure 3.6).

The percentage accumulation of K562 cells in S-phase after incubation with 80 μ g/ml was 33.3% \pm 2.7, 33% \pm 3.2, 25.1% \pm 2.8, 25.5% \pm 3, 27.5% \pm 1.3 at 4, 8, 24, 36 and 48hrs respectively. A dose of 20 μ g/ml induced S phase arrest of 34.1% \pm 2.8, 35.9% \pm 2.5, 38.9% \pm 5.2, 39.9% \pm 4.7, 34% \pm 5.1 at 4, 8, 24, 36 and 48hrs respectively. A dose of 5 μ g/ml induced S phase arrest of 37.6% \pm 3.3, 36.1% \pm 3.1, 40.6% \pm 5.7, 40.5% \pm 4.4, 29.6% \pm 2.6 at 4, 8, 24, 36 and 48hrs respectively and a dose of 1 μ g/ml induce accumulation of cells at S phase of 35.2% \pm 2.7, 27.5% \pm 7.5, 29% \pm 2.2, 29.7% \pm 2.7, 24.9% \pm 1.6 at 4, 8, 24, 36 and 48hrs respectively (figure 3.7).

This data show that the effects of etoposide, in contrast to the effects of cytarabine, are both time and concentration dependent. However, the inverse relationship between the induction of apoptosis and G2M cell cycle arrest was true for etoposide as well as cytarabine.

3.3.3 Effect of etoposide on HL60 cells:

A similar series of experiments with etoposide were performed on the HL-60 cell line. A similar effect was observed to that seen in K562 cells although time courses and drug concentrations were shorter and smaller respectively, indicative of a cell line far more sensitive to the effects of etoposide. The relationship between G2M arrest and lack of apoptosis was again demonstrated in this cell line. HL60 cells were incubated with etoposide at concentrations of 0 and 0.1 µg/ml. After 24 hours the harvested cells were stained with propidium iodide and the DNA concentrations analyzed by flow cytometry to assess their distribution to the phases of cell cycle. Treated HL60 cells showed high levels of G2M arrest of >60% compared to 25% in control cells ($p < 0.05$). Significant G2M arrest was seen at 24 hours (Figure 3.10).

DNA content analysis cannot distinguish between G2 and mitosis as the DNA content is the same. Therefore cells were stained for a mitotic marker and analysed by flow cytometry. HL-60 cells were incubated with etoposide to induce G2M cell cycle arrest. Nocodazole was used as a positive control to induce mitotic cell cycle arrest. Very few cells incubated with etoposide were positive for the mitotic marker, phosphorylated histone H3 ser10 (figure 3.11). More specifically, when the cells in G2M were gated, less than 5% of etoposide treated cells were positive for the mitotic marker, compared to 35% of control cells ($p < 0.05$). These findings confirm that etoposide causes G2 phase arrest and these cells do not enter mitosis.

Effect of cytarabine on the rate of apoptosis in K562 cells

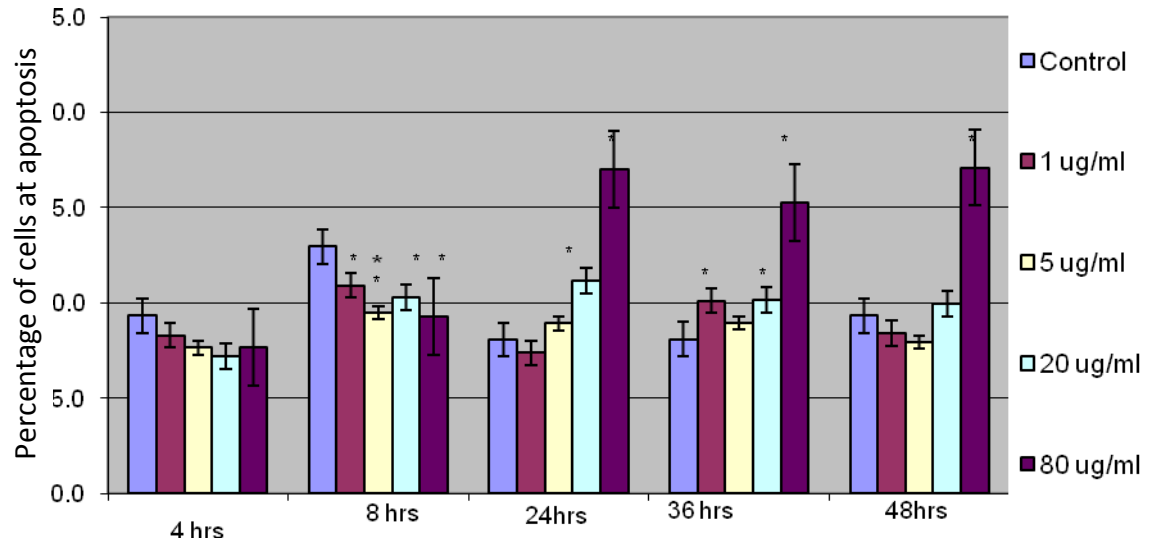


Figure 3.1:

Flow cytometric analysis of K562 cells showing the percentage of apoptotic cells. All test cultures were compared to control cultures by unpaired student t-test (* $P < 0.05$).

Effect of cytarabine on G1-phase in K562 cells

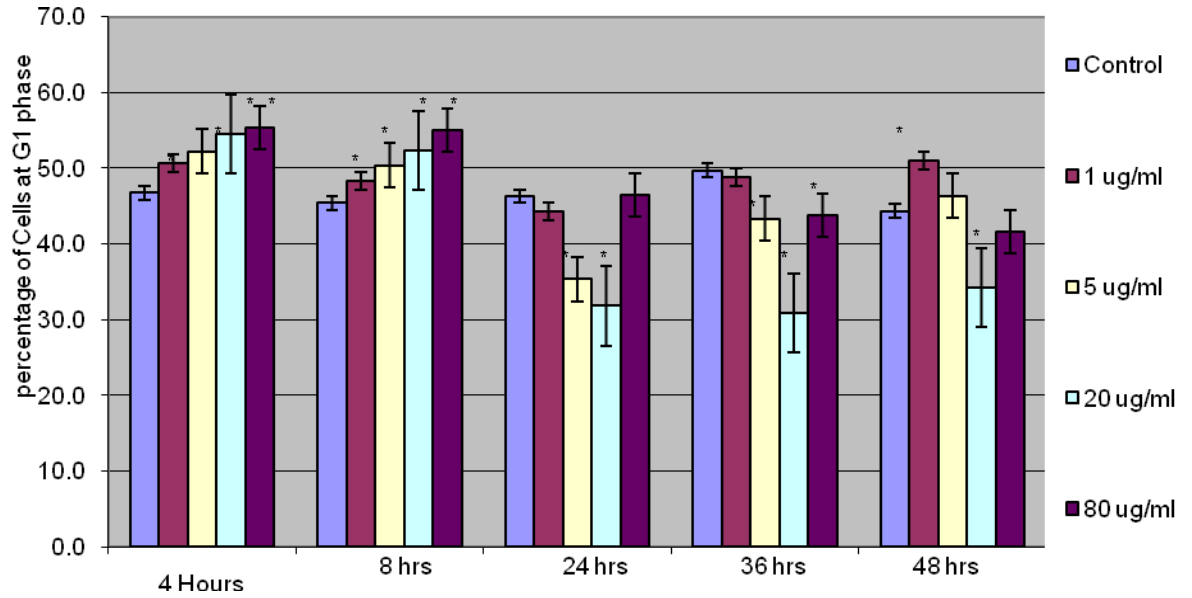


Figure 3.2:

Flow cytometric analysis of K562 cells showing the percentage of cells at G1 phase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of cytarabine on S - phase in K562 cells.

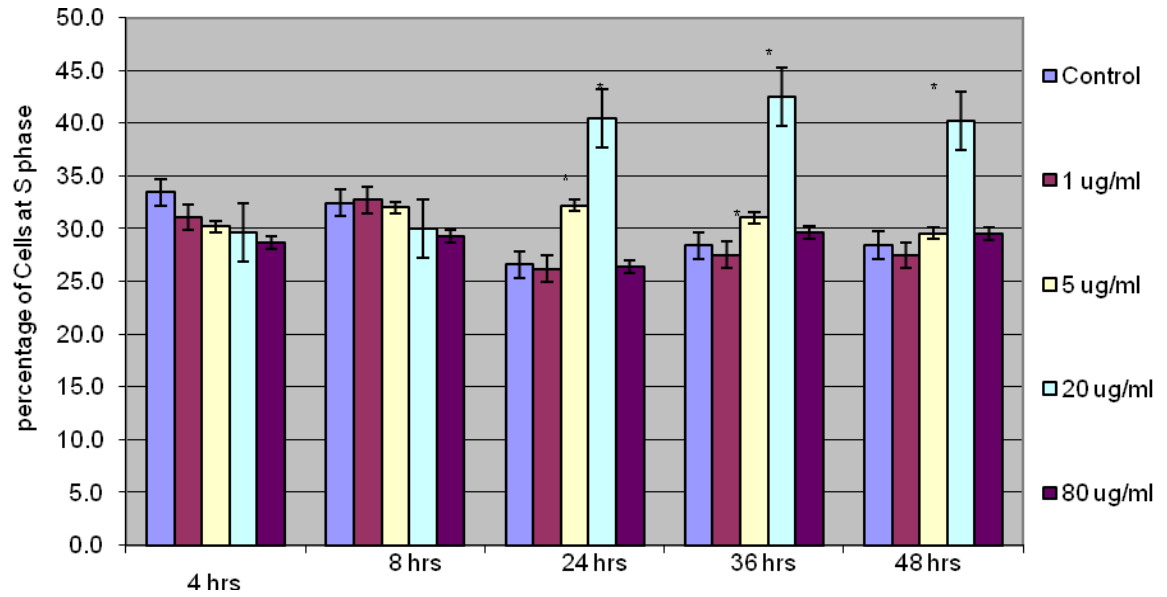


Figure 3.3:

Flow cytometric analysis of K562 cells showing the percentage of cells at S- phase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of cytarabine on G2M phase in K562 cells.

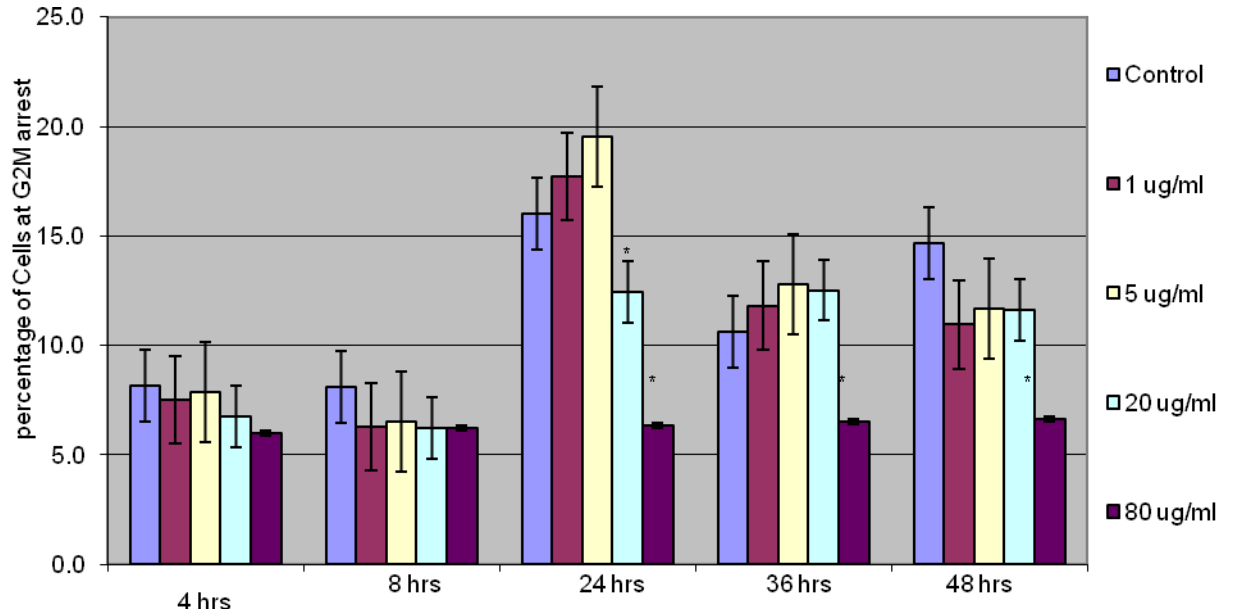


Figure 3.4:

Flow cytometric analysis of K562 cells showing the percentage of cells in G2M phase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of etoposide on apoptosis in K562 cells.

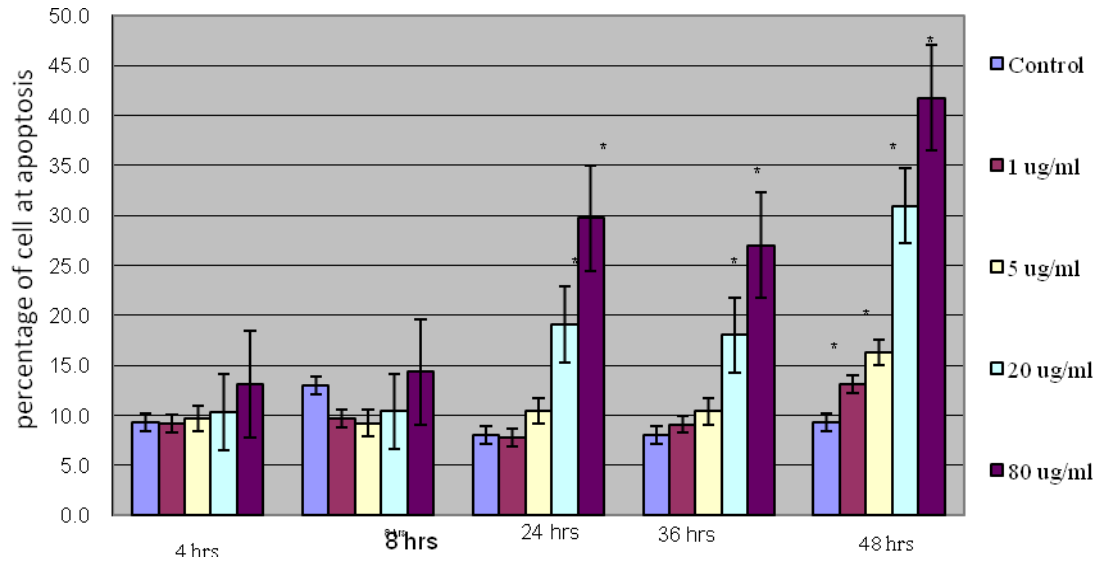


Figure 3.5:

Flow cytometric analysis of K562 cells showing the percentage of cells at G2Mphase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of etoposide on G1-phase in K562 cells.

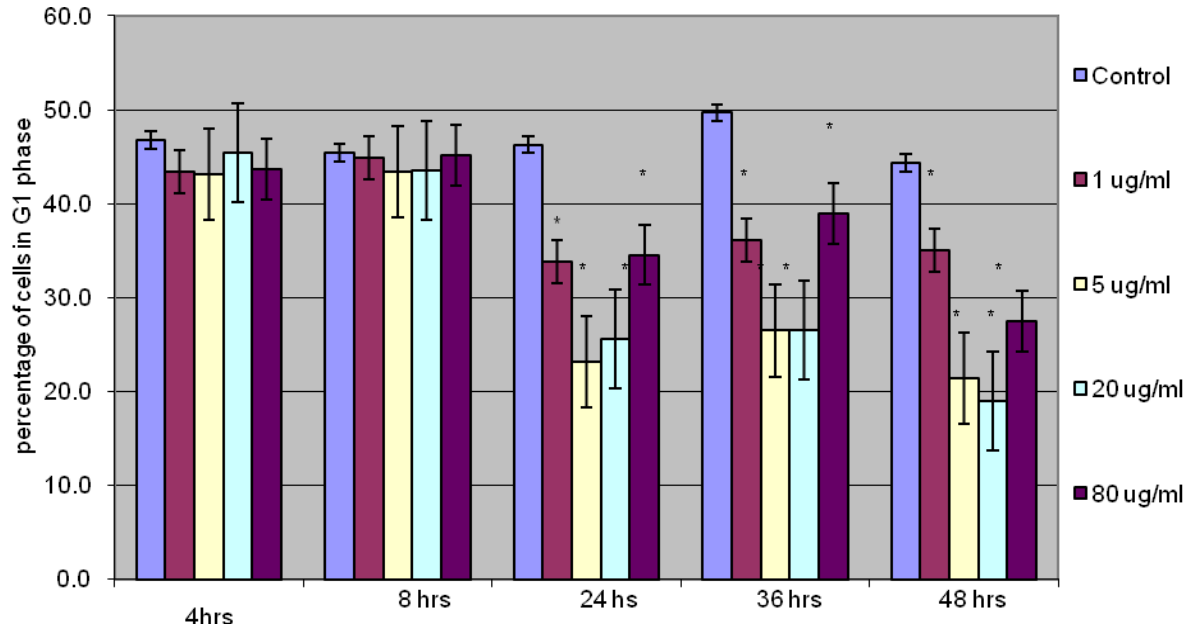


Figure 3.6:

Flow cytometric analysis of K562 cells show the percentage of cells at G1 phase. All test cultures were compared to control cultures by unpaired student t-test (* $P < 0.05$).

Effect of etoposide on S-phase in K562 cells.

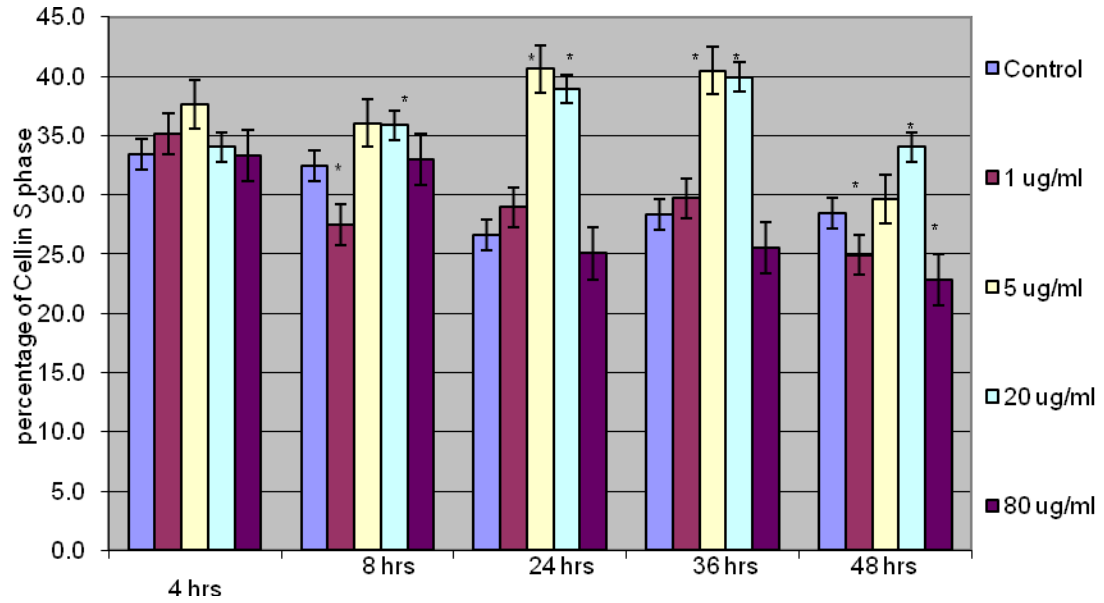


Figure 3.7:

Flow cytometry analysis of K562 cells showing the percentage of cells at S-phase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of etoposide on G2M phase in K562 cells.

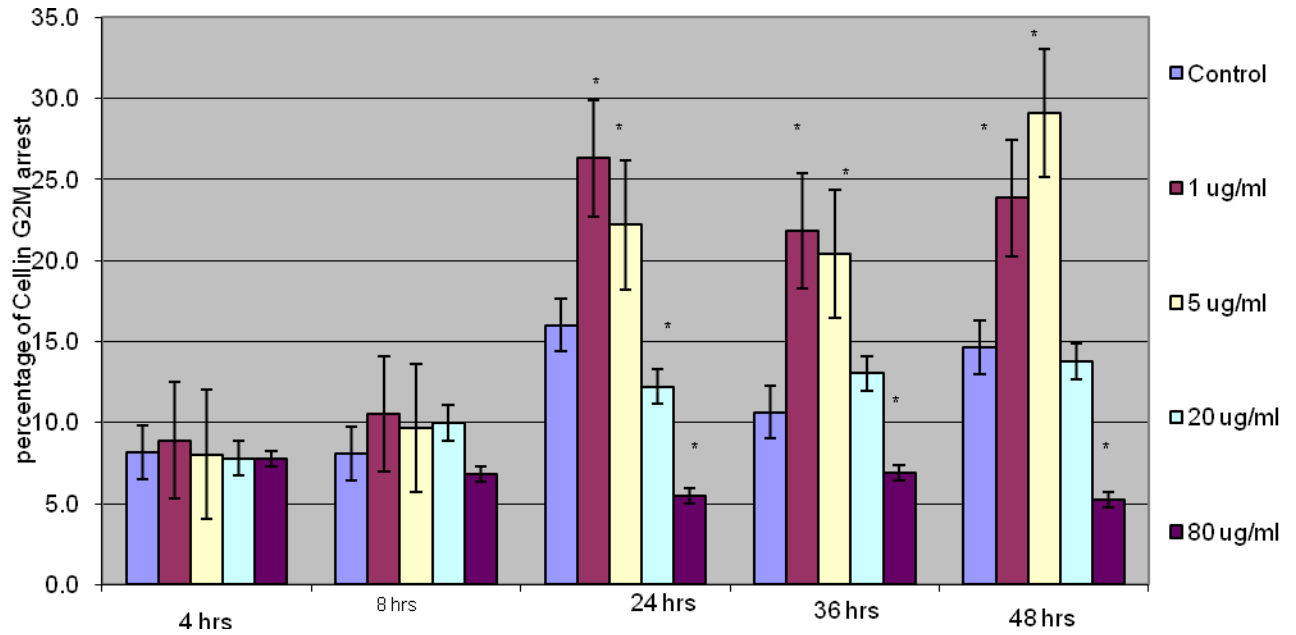


Figure 3.8:

Flow cytometric analysis of K562 cells showing the percentage of cells at G2M-phase. All test cultures were compared to control cultures by unpaired student t-test (*P<0.05).

Effect of etoposide on G2M phase of HL60

cell line.

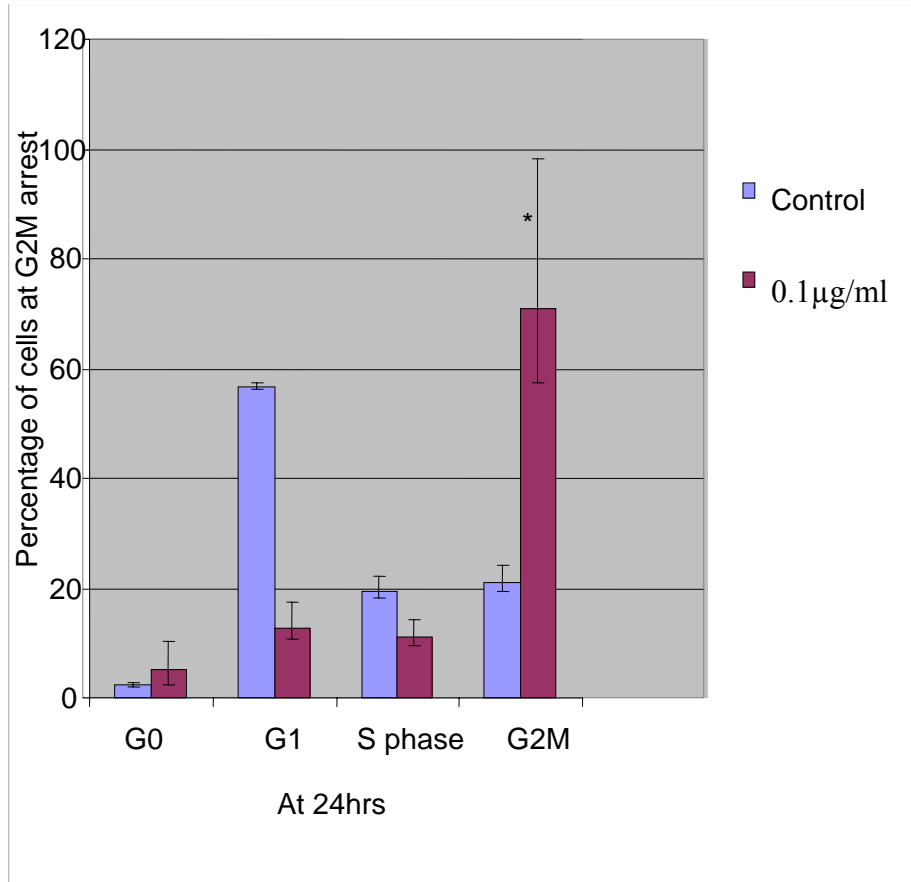


Figure 3.9:

Flow cytometric analysis of HL60 cell line showing the percentage of cells at G2M phase treated with 0.1 µg/ml etoposide after 24hrs. All test cultures were compared to control cultures by student t-test (*P<0.05).

Detection of histone H3 serine 10 phosphorylation as a marker of mitosis

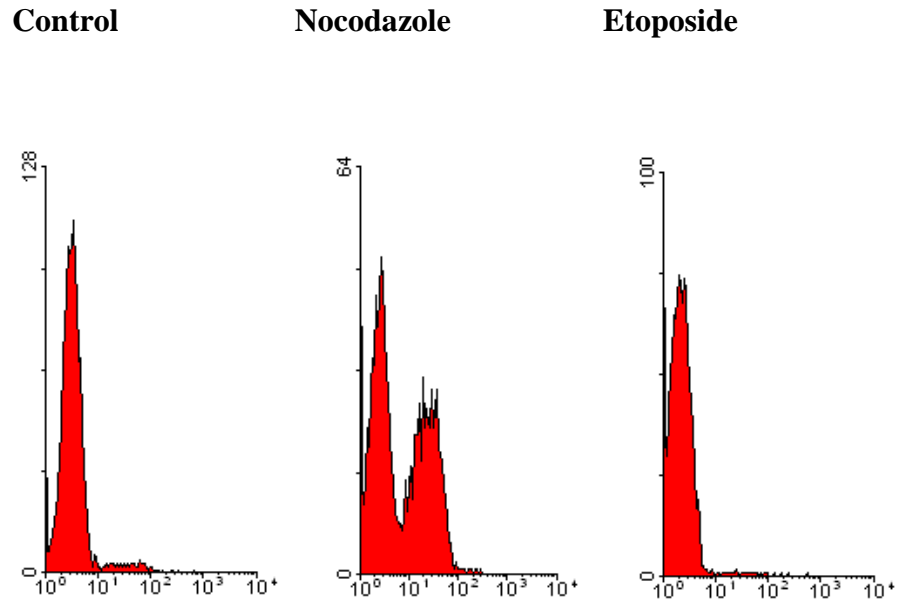


Figure 3.10:

Representative flow cytometry histograms of low-percentage staining of cells in etoposide-induced cell cycle arrest indicative of G2 phase. Nocodazole was used as a positive control to induce mitotic cell cycle arrest. (From Davies D and Allen P (Supervisor). DNA Analysis by Flow Cytometry pp165-179. In Flow Cytometry, Principles and Applications. Ed Marion G Macey. Humana Press 2007).

Effect of etoposide on the immunostaining of HL60 cells stained with H3Ser10 fluorescent antibody.

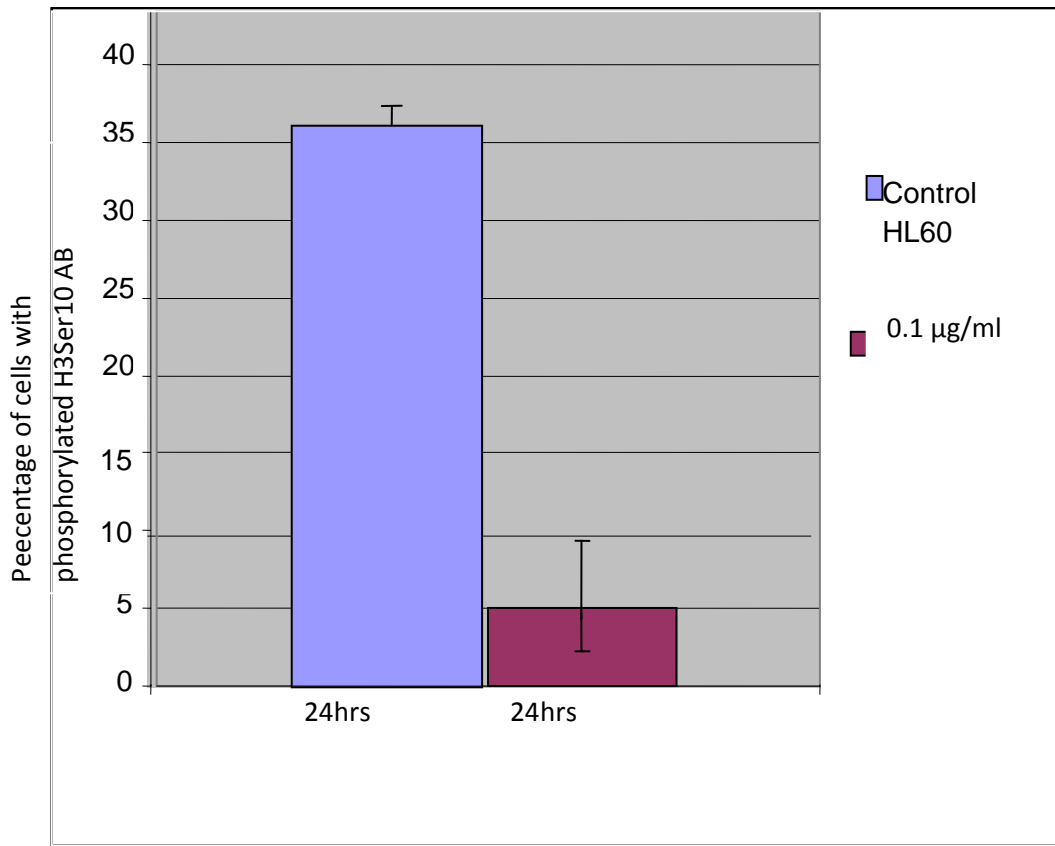


Figure 3.11:

Flow cytometric analysis of HL60 cells showing the percentage of cells at G2M phase after treatment with 0.1µg/ml for 24hrs of etoposide, and then stained with anti phospho-H3Ser10 antibody, a mitosis specific marker. All test cultures were compared to control cultures by unpaired student t-test ($P < 0.05$).

3.4 Discussion:

My experiments show the effect of etoposide and cytarabine on a K562 CML cell line which is resistant to apoptosis. The effect of etoposide and cytarabine was analysed by flow cytometry. This technique showed that etoposide treatment induced G2 arrest; however the highest doses also caused apoptosis. These findings confirm experiments by other workers in different models (Martins et al, 1997). More than one mechanism may be involved: the first having a lower threshold and causing G2M arrest while the second could have a higher threshold that causes apoptosis.

In contrast, the effects of cytarabine were not dose related. This may be because cytarabine requires an enzyme to generate the active form and this is a rate limiting step. So even though the dose of cytarabine was increased there was no additional increased effect on the cells, presumably because the rate of conversion to the active form remains steady.

This contrasts with etoposide which acts directly and does not require enzyme conversion to an active form. The higher the dose of etoposide, the more apoptosis is induced. Low doses cause cell cycle arrest; high doses cause apoptosis. Both of these observations have important implications for the treatment of leukaemia patients by chemotherapy.

In eukaryotic cells the chromatin contains four core histone proteins known as H2A, H2B, H3 and H4. Phosphorylation of Ser10 of histone H3 is associated with chromosomal condensation during mitosis (Hendzel et al, 1997). Phosphorylation of

Ser 10 on H3 is therefore an indicator for mitosis. In this experiment immunostaining cells with anti-phospho H3 ser10 antibody confirms that the percentage of cells in mitosis was very low, suggesting that the arrested cells are restricted to the G2 phase of the cell cycle. Therefore, HL60 cells treated with etoposide showed high G2M arrest by 24hours compared to controls which was subsequently identified as significant G2 arrest. Similar results were seen with etoposide in K562 cells (data not shown).

Mechanistically, this is due to DNA strands breaks not being repaired during topoisomerase 2 inhibition. DNA damage therefore stimulates Chk1 which phosphorylates Ser-216 of Cdc25C and results in deactivation of cyclin B/Cdk1 as a key part of the G2M checkpoint (Williams et al, 2001).

Escape of cells from mitosis may allow the cells to enter G1 phase at the border of the G1 checkpoint. This accumulation of abnormal cells in G1 phase may induce apoptosis. This can be caused by p21 activation and mediated by Bax proapoptotic protein. Activation of apoptosis in this model prevents damaged cells from re-entering the cell cycle. This situation would fit in with the cellular adaptation theory. My data show that cells that die are not blocked at either G2 or M. This could be seen as a collapse of both the G2 and mitotic checkpoints i.e. cell cycle arrest and apoptosis are mutually exclusive. Thus another possibility is that entry into mitosis is the trigger for mitotic apoptosis. That is to say cells die immediately on entering mitosis and therefore cannot accumulate. An alternative form of cell death such as mitotic catastrophe may also take place .

3.5 Conclusions:

- G2 cell cycle arrest and apoptosis do not occur together in either K562 or HL60 cells.
- When there is G2 cell cycle arrest, apoptosis does not occur.
- When apoptosis occurs there is diminished cell cycle arrest.
- Cell death induced by etoposide post G2 arrest is due mainly to apoptosis.

Results chapter 4

Effect of vincristine on the cell cycle of K562 cells.

4.1 Introduction:

K562 cells are a p53 negative cell line. They are resistant to cytotoxic drugs because their lack of p53 gene expression prevents p53-mediated apoptosis and leads to G2M cell arrest. Vincristine damages the mitotic spindle by depolymerising its microtubules. This arrests the cell at mitosis as a result of spindle assembly checkpoint activation. Under normal circumstances, the spindle assembly checkpoint is activated as a result of chromosome delay in attachment to the spindle. It recruits its active components Mad1, Mad2, Bub1, and BubR1 which then attach to kinetochores, accompanied by the chromosomal passenger protein survivin and Aurora B kinase.

Activation of the spindle assembly checkpoint followed by mitotic slippage initiates apoptosis, by activating Bax which is a member of the Bcl-2 family of proteins. The Bcl-2 proteins alter mitochondrial membrane potential and this leads to release of cytochrome C and AIF. These proteins form a complex with procaspase 9, and by activation of caspase 9 they initiate apoptosis.

4.2 Aims:

- To study the effect of various concentrations of vincristine on K562 cells.
- To determine any relationship between cell cycle arrest and apoptosis
- To determine evidence of mitotic slippage being a requirement for vincristine induced cell death.

4.3 Results:

4.3.1 Effect of vincristine on K562 cells:

Cells cultured with vincristine were stained with propidium iodide and analysed by flow cytometry to detect apoptosis and establish the distribution of cells in the various phases of the cell cycle.

Apoptosis in K562 cells after incubation with a dose of 40µg/ml of vincristine was observed at 3.8% +/-0.6, 6.4% +/-1.2, 8.9% +/-0.7, 12.9% +/-1.3 and 23% +/-4.2 at 4, 24, 48, 72 and 96hrs respectively; 20µg/ml of vincristine increased apoptosis to 4.5% +/- 1.1, 6.0% +/-1.0, 8.2% +/-0.4, 10.3% +/-1.4 and 15.3% +/-2.3 at 4, 24, 48, 72 and 96hrs respectively; 10µg/ml of vincristine increased apoptosis to 3% +/-0.3, 5.1% +/-0.1, 6.6% +/-0.8, 8.4% +/- 1.0, 9.5% +/-0.5 at 4, 24, 48, 72 and 96 hrs respectively; 5µg/ml of vincristine increased apoptosis to 3.3% +/-0.3, 5.2% +/-0.3, 6.1% +/-0.3, 9.3% +/-0.5, 10.2% +/-1.0 at 4, 24, 48, 72 and 96hrs respectively when compared to control values of 2.6% +/- 0.3, 2.2% +/- 0.4, 1.6% +/- 0.1, 2.2% +/- 0.7, 2.7% +/- 0.7 at the same time point (figure 4.1).

The percentage of K562 cells at G2M arrest increased significantly after incubation with 40µg/ml of vincristine to 39.1% +/-1.9, at 72 hrs ; 20µg/ml of vincristine increased G2M arrest to 49.5% +/-2.6, at 72hrs; 10µg/ml of vincristine induced G2M arrest of , 52.5% +/-6.0, 55.8% +/-4.2, 51.7% +/-1.3, 44.8% +/-4.7 at 24, 48, 72 and 96hrs respectively; 5µg/ml of vincristine increased G2M arrest to 57.3% +/-5.9, 57.2% +/-5.4, 51.6% +/-0.9, 41.4% +/-3.6 at 24, 48, 72 and 96hrs respectively

when compared to control values of 26.5% \pm 1.9, 32.9% \pm 0.8, 27.2% \pm 1.1, 30.3% \pm 1.2, 25.8% \pm 2.8 at same time points (figure 4.2).

There were also compensatory changes in G1 and S-phases of the cell cycle. G1 phase percentage of K562 cells when incubated with 40 μ g/ml of vincristine was significantly decreased to 30.6% \pm 2.4, 32.6% \pm 3.0 at 72 and 96hrs respectively; 20 μ g/ml of vincristine caused a G1 phase percentage decreased to 31.6% \pm 3.8, 24.6% \pm 1.9, 32.3% \pm 4.2 at 24, 48, 72 and 96hrs respectively; 10 μ g/ml of vincristine reduce G1 phase percentage to 38.7% \pm 4.5, 20.2% \pm 2.4, 20.4% \pm 1.6, 23.6% \pm 2.0 at 24, 48, 72 and 96hrs respectively; 5 μ g/ml of vincristine reduced G1 phase percentage to 22.8% \pm 3.9, 20.7% \pm 4.0 at 24, 48hrs respectively when compared to control values of 42.5% \pm 1.7, 44.3% \pm 0.5, 47.8% \pm 1.1, 45.3% \pm 0.6, 49.9% \pm 2.2 at the same time points (figure 4.3).

The S phase rate of K562 cells treated with 40 μ g/ml of vincristine was significantly reduced to 20.2% \pm 0.6 at 48hrs ; 20 μ g/ml of vincristine reduced S phase percentage to 18.4% \pm 1.5 at 48hrs ; 10 μ g/ml of vincristine reduce S phase percentage to 4.3% \pm 1.9, 17.3% \pm 2.2, at 24 and 48hrs respectively; 5 μ g/ml of vincristine increased S phase percentage of 28.6% \pm 2.3 at 4hrs and then reduced to 17.1% \pm 2.4, 19.2% \pm 1.5, 26.2% \pm 3.8 at 48, 72 and 96hrs respectively. When compared to control values of 26.5% \pm 2.4, 20.4% \pm 0.9, 23.7% \pm 1.2, 22.5% \pm 1.3, 21.3% \pm 2.4 respectively at the same time points (figure 4.4).

Effect of vincristine on apoptosis in K562 cells

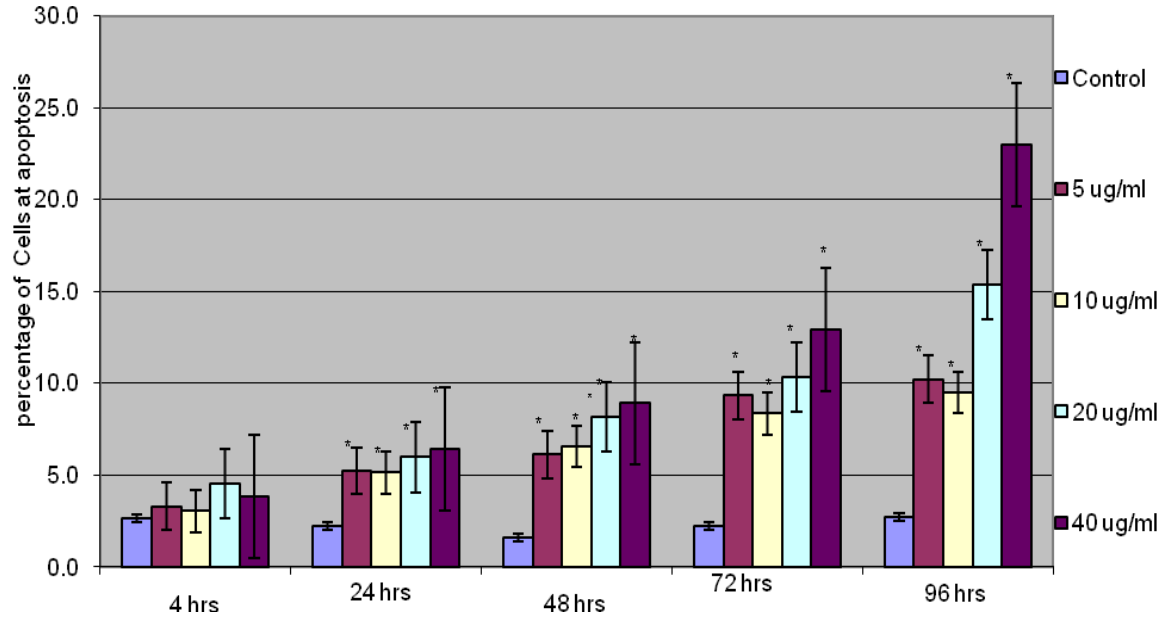


Figure 4.1:

Flow cytometric analysis of K562 cells showing the percentage of apoptotic cells. All test cultures were compared to control cultures by unpaired students t-test (*P<0.005).

Effect of vincristine on G2M in K562 cells

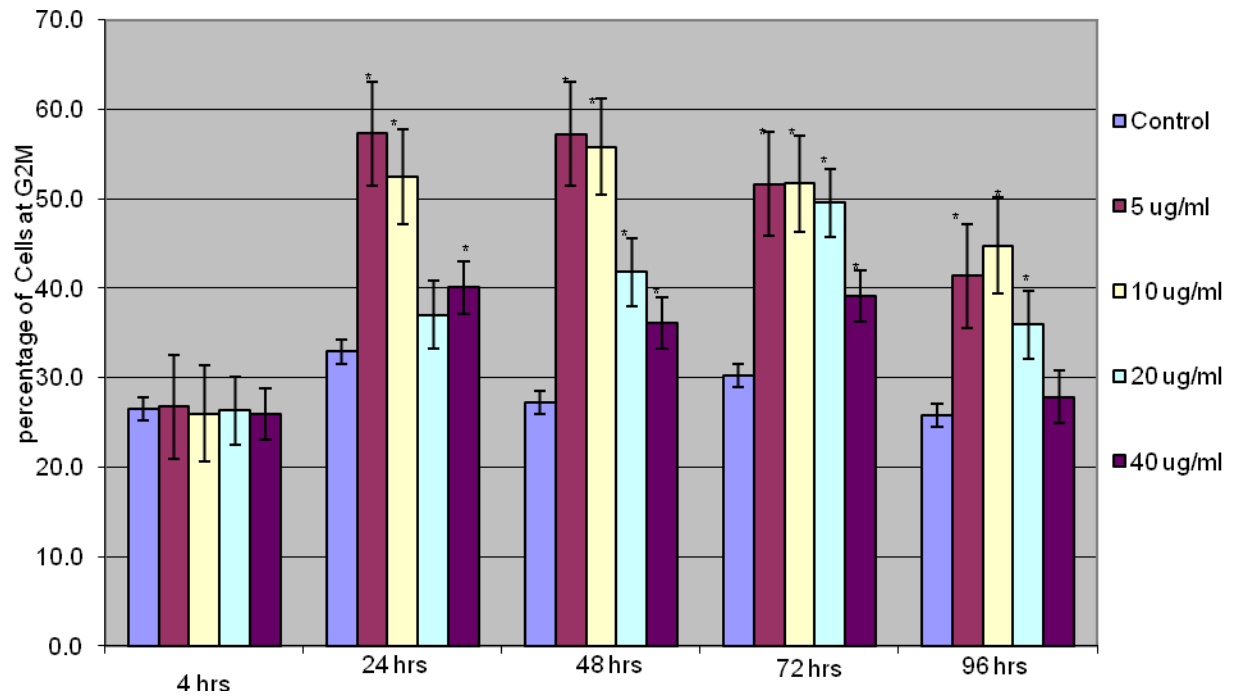


Figure 4.2:

Flow cytometric analysis of K562 cell line showing the percentage of G2M cells. All test cultures were compared to control cultures by unpaired students t-test (*P<0.005).

Effect of vincristine on G1 phase in K562 cells

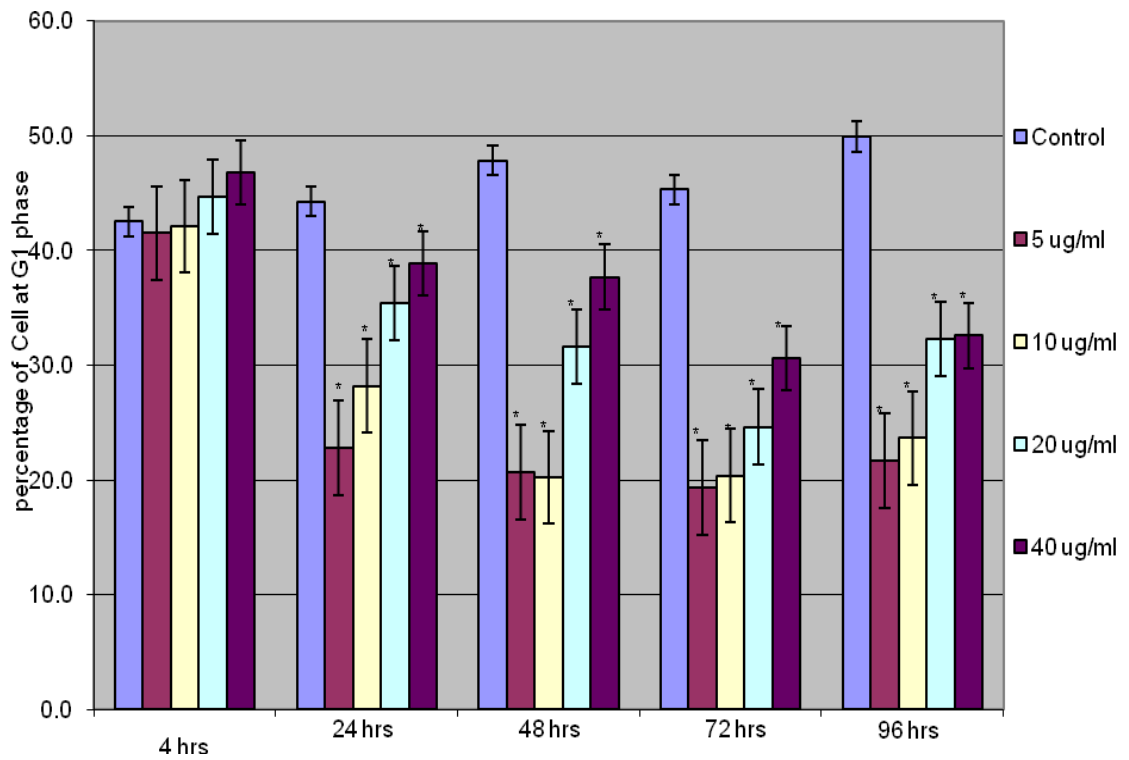


Figure 4.3:

Flow cytometry analysis of K562 cell line showing the cells at G1 phase. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

Effect of vincristine on S phase in K562 cells

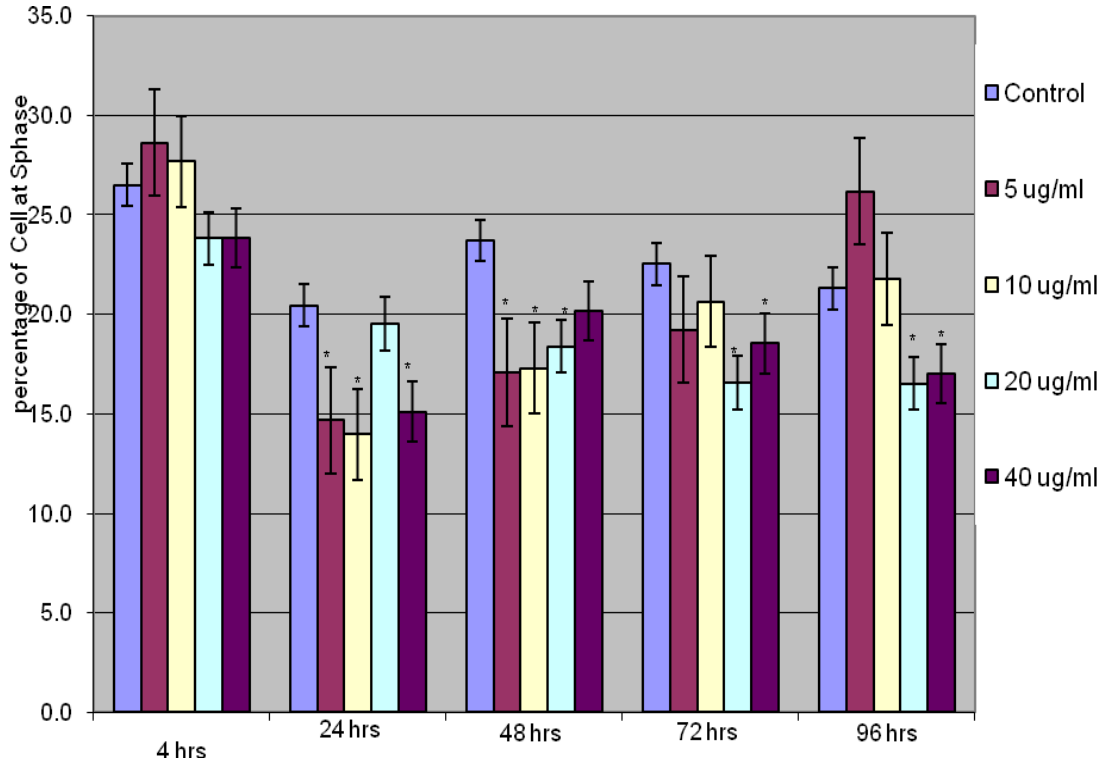


Figure 4.4:

Flow cytometry analysis of K562 cell line showing the cells at S-phase. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

4.3.1 Effect of low doses vincristine on K562 cells:

It was noted from figure 4.1 that the apoptosis induced by vincristine did not change with increasing drug concentrations in the lower range. There was very little difference in the apoptosis induced by 5, 10 or 20 μ g/ml up to 72hrs.

Therefore a closer investigation of lower dose vincristine was carried out. Figure 4.5 shows that over the course of the experiments the induction of apoptosis was actually inversely related to the concentration of vincristine at drug concentration below 20 μ g/ml. This was particularly evident at 48 and 72hrs where 0.02 μ g/ml was a more potent inducer of apoptosis than either of 0.10, 0.50, 2.50, 5.00 or 10 μ g/ml. Indeed at 72hrs, 0.02 μ g/ml was a better inducer of apoptosis than a 500 fold increase in drug concentration of 10 μ g/ml.

Figure 4.6 shows that over the course of the experiments the induction of G2M arrest also decreased as the drug concentration of vincristine was increased up to 20 μ g/ml. This was particularly evident at 24 and 72hrs where 0.02 μ g/ml was a more potent inducer of G2M arrest than either of 0.10, 0.50, 2.50, 5.00 or 10 μ g/ml. Indeed at 72hrs, 0.10 μ g/ml was a better inducer of G2M arrest than a drug concentration of 40 μ g/ml. So 0.02 μ g/ml vincristine was a potent inducer of apoptosis as well as G2M arrest. Increasing the concentration of vincristine up to 10 μ g/ml resulted in less apoptosis at 48hrs and 72hrs (figure 4.5) as well as less G2M arrest at 4,6 and 24hrs (figure 4.6)

To verify this counter-intuitive finding, low-dose vincristine was compared directly with a high concentration of vincristine. Therefore, K562 cells were incubated with either 0.1 μ g/ml or 40 μ g/ml of vincristine to take a closer look at cell response to this drug. The percentage of apoptosis induced by 0.1 μ g/ml vincristine was 4.39%, 7.85%, 10.14% and 18.64% at 24, 48 and 72hrs and 96hrs respectively. At 48 and 72hrs this was a higher percentage than that induced by 40 μ g/ml (figure 4.7). The greater effectiveness of low-dose vincristine to induce apoptosis was mirrored by a greater ability to induce G2M cell cycle arrest (figure 4.8). This is in contrast to the response of cells to etoposide where G2M arrest precluded induction of apoptosis.

Effect of low doses vincristine on K562 cells at apoptosis

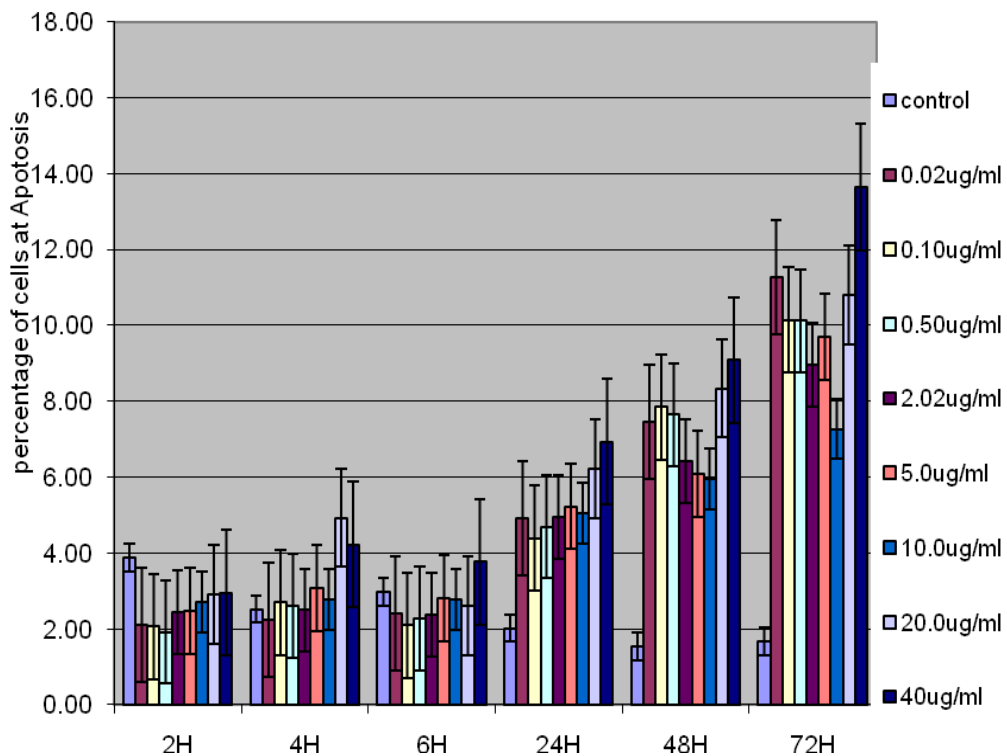


Figure 4.5:

Flow cytometry analysis of K562 cell line treated with low doses of vincristine ug/ul showing the cells at apoptosis. All test cultures were compared to control cultures by unpaired students t-test (result at 24, 48, 72hrs are $P < 0.05$).

Effect of low dose of vincristine on K562 cells at G2M

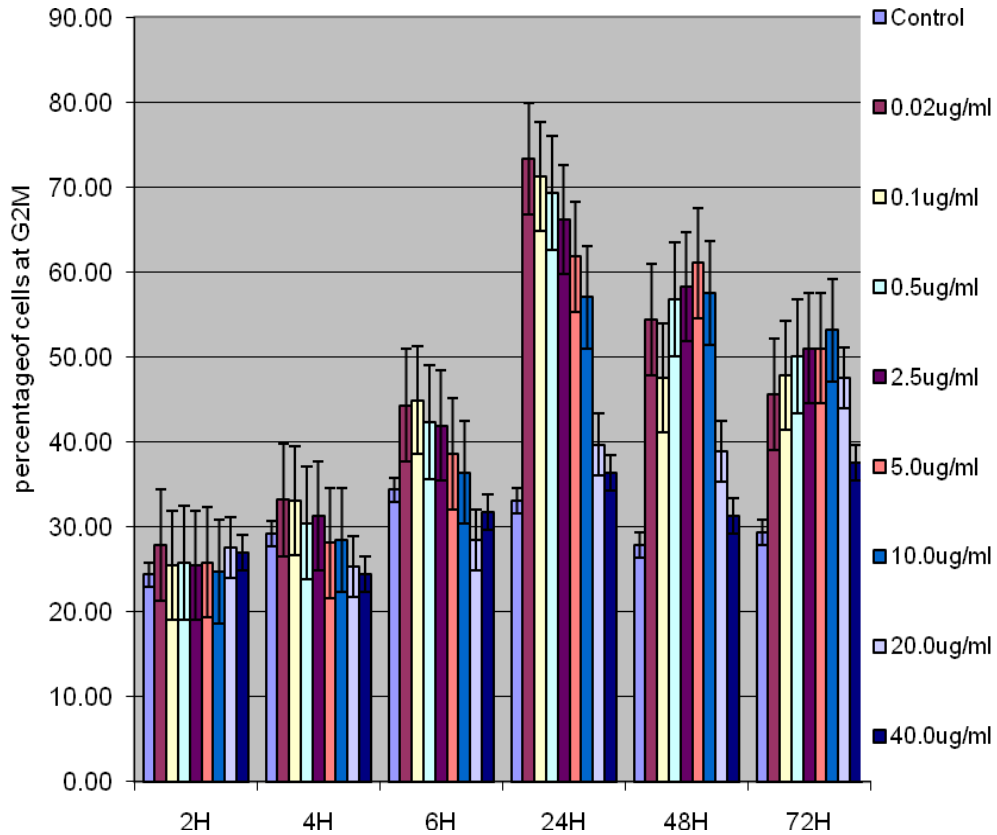


Figure 4.6:

Flow cytometry analysis of K562 cell line treated with low doses of vincristine ug/ul showing the cells at G2M phase. All test cultures were compared to control cultures by unpaired students t-test (result at 24, 48, 72hrs are $P < 0.05$).

Effect of vincristine on apoptosis in the K562 cells

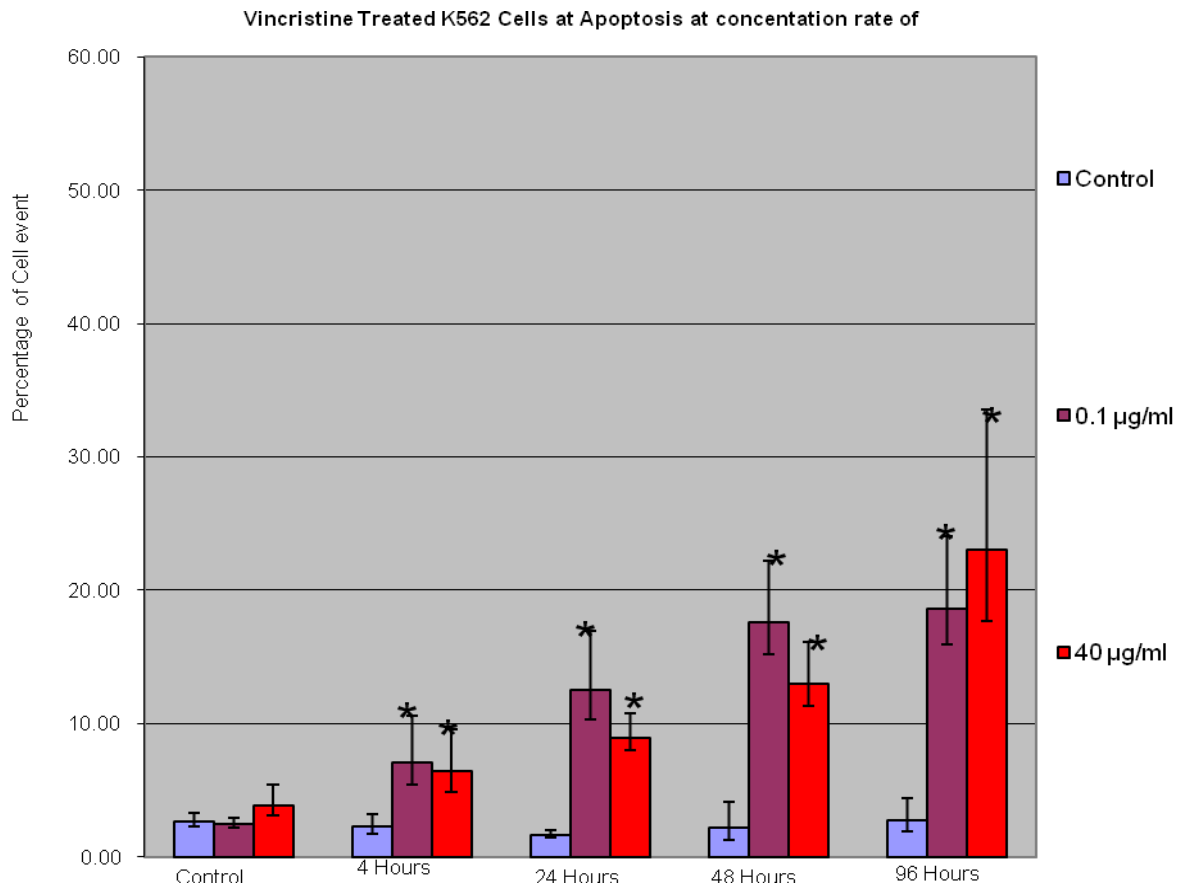


Figure 4.7:

Flow cytometry analysis of K562 cells showing the percentage of apoptotic cells stained with propidium iodide only. All test cultures were compared to control cultures by unpaired students t-test (* $P < 0.05$).

Effect of vincristine on induction of G2M arrest in K562 cells.

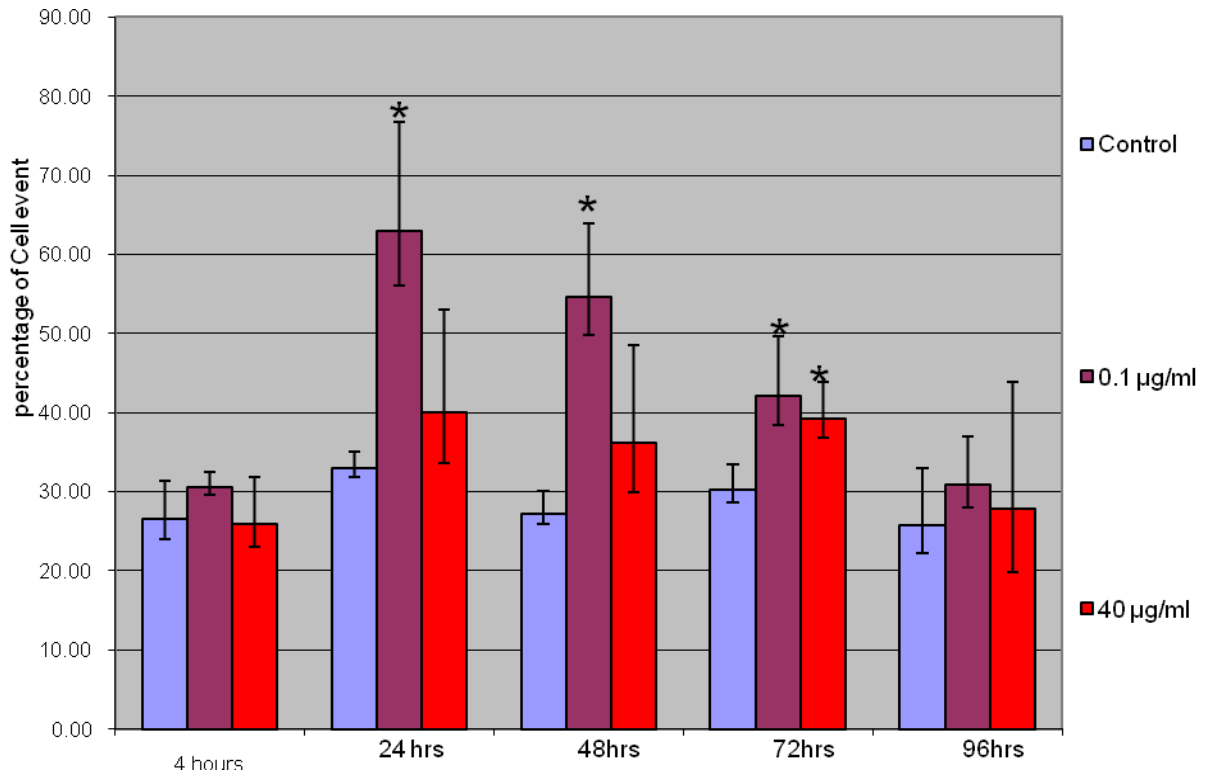


Figure 4.8:

Flow cytometry analysis of K562 cell line showing the percentage of cells at G2M stained with propidium iodide. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

4.3.2 Effect of vincristine on apoptosis in K562 cells by Annexin V immunostaining:

Annexin V is a cellular protein which has high affinity to phosphatidylserine (PS). PS is expressed on the surface of apoptotic cells. (Koopmann et al 1994). K562 cells were incubated with vincristine at different concentrations of (0, 0.1, 5, 10, 20, 40 μ g/ml) for specific time points (24, 48, 72, 96 hours).

Harvested cells were stained with Annexin V and propidium iodide and then analysed by flow cytometry for detection of apoptosis. Cells binding Annexin V are considered apoptotic and exclude propidium iodide. The data in (figure 4.9) of cells positive for both parameters does not reflect data generated using the DNA technique to determine vincristine-induced apoptosis in figure 27. However, it does support the notion that over a 48hr period there is no large disparity between 0.1 μ g/ml and 40 μ g/ml, as would be predicted.

A closer look reveals that there is very little difference in Annexin V binding due to vincristine used at either 0.1 or 40 μ g/ml and this observation is extended to 72hrs for this parameter (figure 4.10). However, much of the cell death induced by 40 μ g/ml vincristine can't be attributed to necrosis since the uptake of propidium iodide is greater for this concentration than that of 0.1 μ g/ml vincristine (figure 4.11). The apparent loss of propidium iodide uptake in cells at 96hrs (figure 4.11) is accounted for, because at this time point necrotic and apoptotic cells are positive for both parameters as seen in figure 29. This highlights the limitations of this technique for distinguishing between apoptosis and necrosis.

Nocodazole, another anti-mitotic agent confirmed this type of drug causes G2M arrest at low concentrations (figure 4.12) and histone H3 serine 10 phosphorylation studies verified that this cell cycle arrest was indeed mitotic (figure 4.13).

Effect of vincristine on the rate of apoptosis detected by annexin V staining of K562 cells.

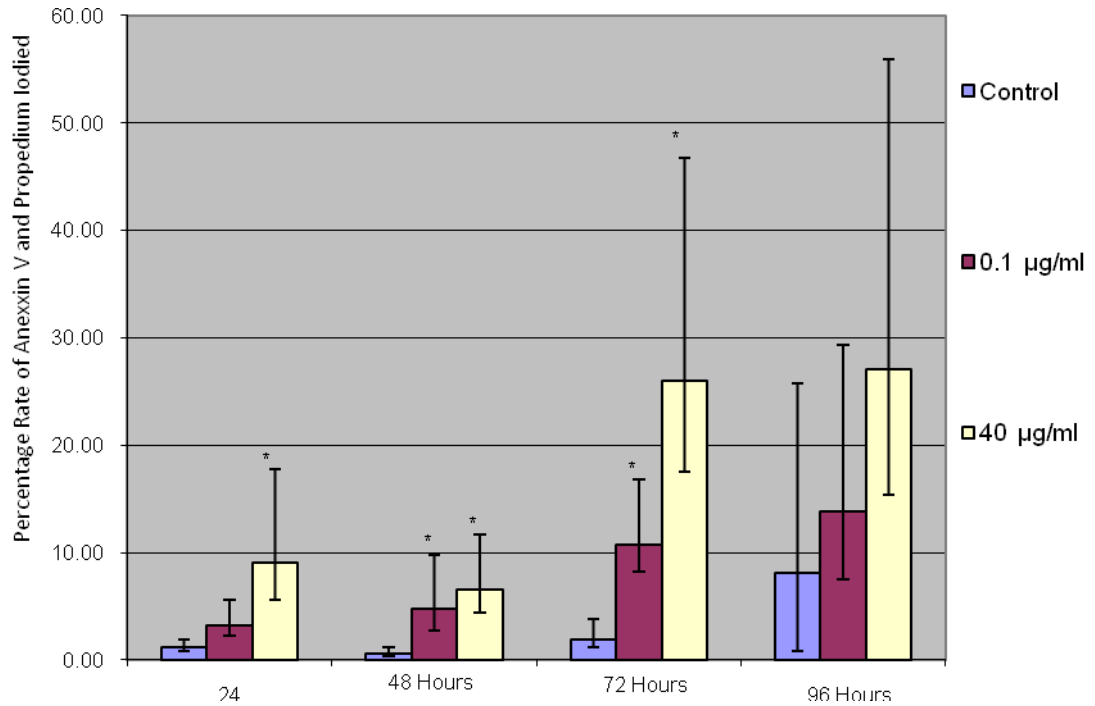


Figure 4.9:

Flow cytometry analysis of K562 cells treated with vincristine and stained with Annexin V and propidium iodide to study the percentage of apoptotic cells and post apoptotic cells positive of both annexin V and propidium iodide. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

Effect of vincristine on the rate of apoptosis detected by annexin V staining of K562 cells.

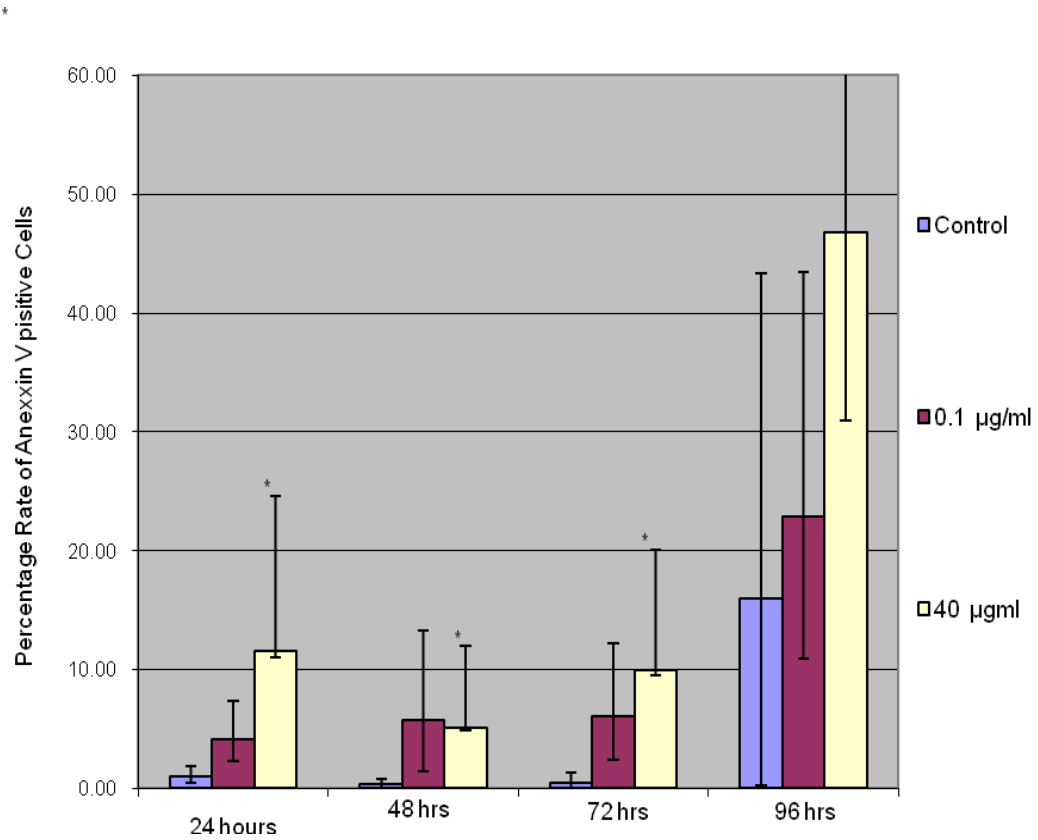


Figure 4.10:

Flow cytometry analysis K562 cells treated with vincristine and stained with Annexin V only to study the percentage of apoptotic cells and post apoptotic cells that are annexin V positive. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

Effect of vincristine on the rate of apoptosis detected by propidium iodide staining in K562 cells

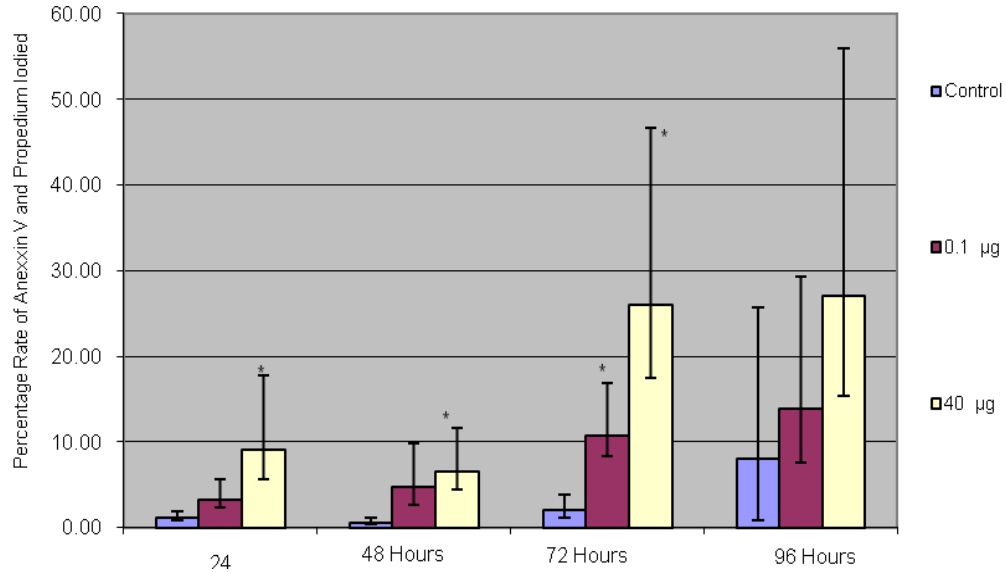


Figure 4.11:

Flow cytometry analysis of K562 cells treated with vincristine and stained with propidium iodide only to study the percentage of apoptotic cells and post apoptotic cells that are propidium iodide positive. All test cultures were compared to control cultures by unpaired students t-test (*P<0.05).

4.4 Discussion:

This chapter examined the effects of the microtubule inhibitor vincristine on K562 cells. The initial studies suggested that overall perturbation of the cell cycle and the induction of apoptosis followed a similar pattern to that observed for the effects of etoposide on myeloid leukemia cell lines. i.e. that the induction of cell death was time and drug-concentration dependent. This view was supported by the initial data. Over a vincristine drug-concentration range of 5 to 40 ug/ml the induction of apoptosis was drug concentration dependent and that the lower concentrations were more effective at inducing cell cycle arrest. Thus, like etoposide, cell cycle arrest precluded apoptosis. However, the relationship between cell cycle arrest and cell death was not so clear as that seen for topoisomerase II inhibitor. In fact, when low-concentrations of vincristine were studied, which are probably more pharmacologically relevant, it was found that over a range of 0.02 to 10ug/ml vincristine, the lower concentrations were more effective at inducing apoptosis than the higher concentrations. These concentrations were also potent at inducing mitotic cell cycle arrest. A direct comparison of 0.01ug/ml with a 400 fold greater concentration of 40ug/ml vincristine showed little difference in the induction of apoptosis when measure by DNA fragmentation on the flow cytometer. However, the lower concentration was much more effective in inducing mitotic cell cycle arrest over a 48hr period. An alternative Annexin V binding assay for the detection of apoptosis suggested that different mechanisms were at work. The lower concentration was as effect at inducing apoptosis as the higher as shown by Annexin V binding. However, the higher concentration took up propidium iodide far quicker

than the lower, indicative of necrotic cell death. The ability to distinguish between apoptosis and necrosis using Annexin V binding at high cell death levels is not therefore possible as necrotic cells will also bind Annexin V as the necrotic cell permeabilises, a limitation of this technique.

This observation has connotations. Why is a far lower concentration of vincristine as effective as or even better, at inducing apoptosis than a higher drug concentration. Also, what is the role of mitotic cell cycle arrest in this process as lower concentrations induce mitotic arrest as well as apoptosis when compared to control cell cultures. This contrasts with the role of the cell cycle checkpoint at G2 (just prior to mitosis) induced by etoposide which was seen as a preventer of apoptosis. This has clinical relevance since much lower concentrations of vincristine may be used thus reducing side effects for patients requiring this treatment with this drug.

Conclusions:

High concentrations of vincristine will induce cell death in target cells.

Lower concentrations of vincristine can be as effective, if not better, than high concentrations in inducing apoptosis in myeloid leukaemia cells.

The induction of apoptosis is associated with a concomitant induction of cell cycle arrest at mitosis.

This suggests that the cell cycle arrest could be inherent to the induction of cell death by vincristine.

Chapter 5

Effects of vincristine, etoposide and nocodazole on Cdk1 phosphorylation and expression of survivin, Aurora B kinase and Bax at drug-induced mitosis, G2-arrest and apoptosis.

5.1 Introduction:

Work for this thesis indicates that events just prior to mitosis in G2 and in mitosis itself may be important to the mechanisms of cell death induced by etoposide and vincristine respectively. One key element of this is the cyclin B/Cdk1 complex, also known as the maturation promoting factor (MPF) which drives cells through G2 and into mitosis. In G2 phase of the cell cycle the cyclin B/Cdk1 complex is inactive due to an inhibitory phosphorylation of Cdk1 at threonine-14 and tyrosine-15 during interphase. The complex is activated by phosphorylation of Cdk1 at threonine-161, then for the final activation step it requires dephosphorylation of threonine 14 and tyrosine 15 by Cdc25 phosphatase mid G2-phase. Following DNA damage by genotoxins or mutations, the ATM/ATR protein kinases will be activated. This kinase activity activates Chk1 and Chk2 which in turn phosphorylates Cdc25C at S216. This phosphorylation promotes binding of Cdc25C to 14-3-3 protein which thereby sequesters Cdc25C to the cytoplasm and thus prevents it from activating nuclear Cdk1. Lack of activated Cdk1 causes cell cycle arrest at G2.

Activation of Bax, a proapoptotic protein, leads to the release of cytochrome C from mitochondria (Chipuk JE et al 2004). So long as Bcl-2 does not negate Bax, by forming a Bcl-2/Bax complex, Bax will stimulate the mitochondria to release cytochrome C. These events promote apoptosis via the caspase system.

Survivin, a chromosomal passenger protein also has an anti-apoptotic action. It inhibits the apoptotic pathway by inhibiting caspases and is known as an inhibitor of apoptosis or IAP protein. Survivin is expressed mainly at G2M phase to protect cells from mitotic death during cell division. However, it is constitutively raised in many

cancers. An alternative role for survivin is as a passenger protein, where it localizes to the spindle microtubules and, together with the other passenger proteins, ensures the normal ordered events of mitosis occur. (Kasof et al 2001).

The spindle assembly checkpoint (SAC) delays the exit of a cell from the anaphase stage of mitosis when chromosomal kinetochore attachment to the spindle is inadequate. Correct chromosomal alignment via kinetochores to the mitotic spindle leads to destruction of cyclin B by APC proteolysis and that inhibits cyclin B/Cdk1 activity. The result is an exit of anaphase and eventually an exit from mitosis. Prevention of anaphase means cells are held in metaphase if chromosomes are incorrectly aligned. However, arrest in metaphase is not permanent and the cell can escape mitosis despite an activated spindle assembly checkpoint (Daniela et al 2006). This process is known as mitotic slippage.

Vinca alkaloids are powerful antitumor drugs which inhibit cell division at mitosis by their action on microtubules. Vincristine damages tubulin. They prevent the action of microtubules by inhibiting polymerization of tubulin and causing depolymerisation of already formed tubules (Jordan et al 1986, 1991).

A low dose of nocodazole, a non-clinically used anti-mitotic agent, also alters the microtubule dynamics, arresting the cells in mitosis. A high dose of nocodazole also prevents progression of the cells into full mitosis because of rapid depolymerisation of microtubules. This can cause apoptosis. (Vasquez et al 1997).

Etoposide on the other hand is a topoisomerase 2 inhibitor. Inhibition of this enzyme allows damaged DNA to persist and arrest the cell at G2 phase even in low doses.

5.2 Aims:

-To study the relation between the chromosomal passenger protein survivin and the spindle assembly checkpoint.

-To study the relationship between the apoptotic protein Bax and the spindle assembly checkpoint.

-To study the relation between Cdk1 phosphorylation status and the spindle assembly check point.

5.3 Results:

5.3.1 Effect of vincristine on Cdk1 expression and survivin expression in K562 cells:

In the last chapter it was established that 0.1ug/ml vincristine induced mitotic cell cycle arrest followed by apoptosis whereas the higher concentration induced a combination of apoptosis and necrotic markers without cell cycle arrest in the K562 cell line. To shed some light on the mechanisms of cell cycle arrest the expression of the pivotal G2/mitotic kinase was investigated at both low and high concentrations of vincristine. The expression of Cdk1 remained constant over a 72 hr period for both drug concentrations when compared to the control no matter whether the cells had been incubated with 0.1 or 40ug/ml vincristine. However, 0.1ug/ml vincristine induced expression of survivin when compared to the control which was not seen with the higher drug concentration (figure 5.1).

One of the regulatory elements of Cdk1 activity is its phosphorylation status at tyrosine 15. Using an anti phosphotyrosine antibody specific to Cdk1-Y15, it was established that there was an apparent tyrosine phosphorylation of Cdk1 with both drug concentrations over a 72 hr period. However, there was a small but gradual reduction of this phosphorylation over time at the lower drug concentration (figure 5.2). This slight reduction in phosphorylation status was also seen when cells were incubated with 40µg/ml vincristine. Pro-apoptotic Bax was also induced by vincristine at both concentrations when compared to control (figure 5.3)

Effect of vincristine on expression of Cdk1 and survivin at G2M arrest and apoptosis in K562 cells.

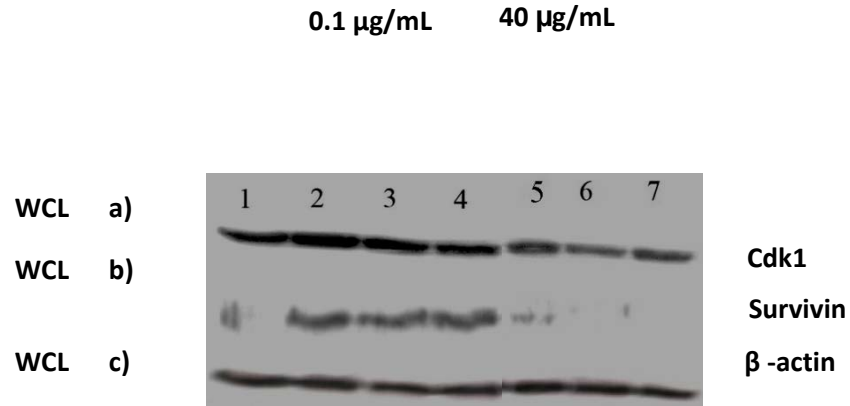


Figure 5.1:

a) Western blot of whole cell lysates of K562 cells treated with vincristine at different doses and different length of time, have been immunostained for Cdk1, survivin and β-actin as control.

Lane 1 control cell.

Lane 2 vincristine of 0.1 µg/ml for 24 hours.

Lane 3 vincristine of 0.1 µg/ml for 48 hours.

Lane 4 vincristine of 0.1 µg/ml for 72 hours.

Lane 5 vincristine of 40 µg/ml for 24 hours.

Lane 6 vincristine of 40 µg/ml for 48 hours.

Lane 7 vincristine of 40 µg/ml for 72 hours.

b) The same membrane stained for survivin.

c) The same membrane stained for β-actin.

Effect of vincristine on G2M arrest and apoptosis phosphorylation of tyrosine 15 on Cdk1 in K562 cells

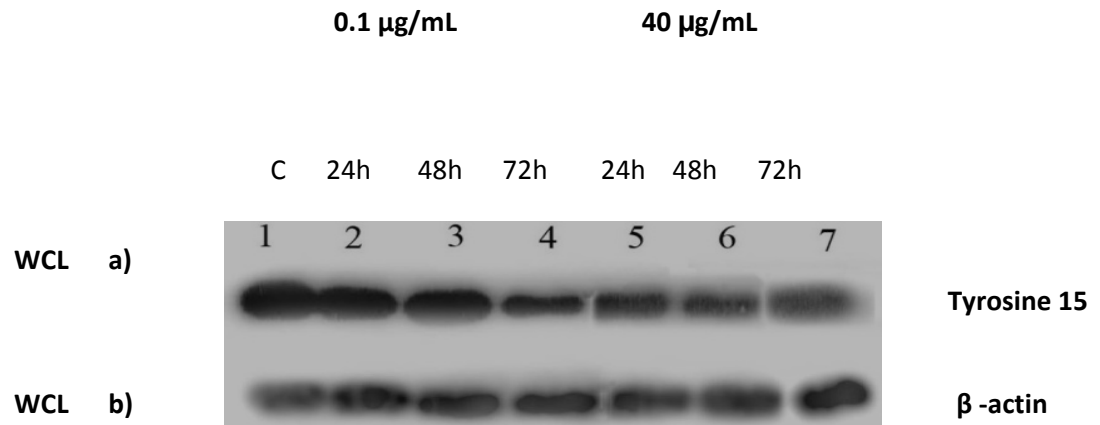


Figure 5.2:

a) Western blot of whole cell lysates of K562 cells treated with vincristine at different doses and different times then immunostained for Cdk1 phosphorylated at tyrosine 15.

Lane 1 control cell.

Lane 2 vincristine 0.1 µg/ml at 24 hour.

Lane 3 vincristine 0.1 µg/ml for 48 hours.

Lane 4 vincristine 0.1 µg/ml for 72 hours.

Lane 5 vincristine 40 µg/ml for 24 hours.

Lane 6 vincristine 40 µg/ml for 48 hours.

Lane 7 vincristine 40 µg/ml for 72 hours.

b) Same membrane stained for β-actin.

**Effect of vincristine on G2M arrest and apoptosis for expression of Bax in
K562
Cells.**

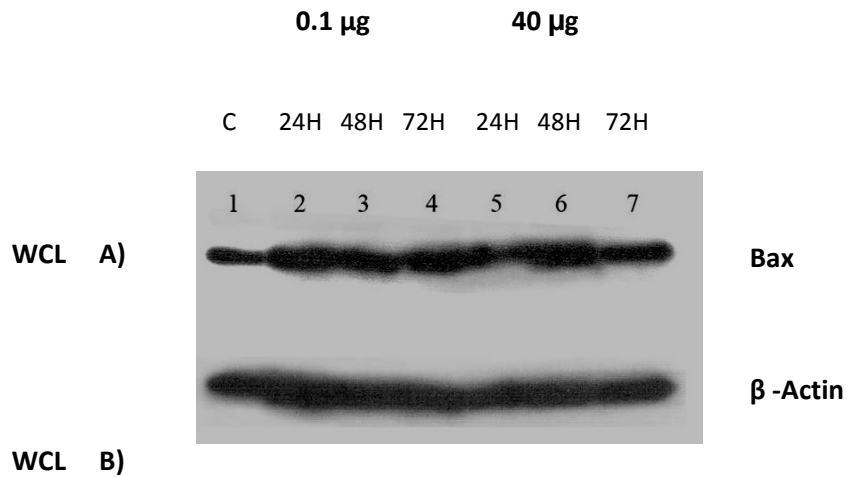


Figure 5.3:

A) Western blot of whole cell lysate of K562 treated with vincristine at different doses and different time points, stained for Bax and β-actin.

Lane 1 control cells

Lane 2 vincristine 0.1µg/ml for 24 hour,

Lane 3 vincristine 0.1µg/ml for 48 hours,

Lane 4 vincristine 0.1µg/ml for 72 hours,

Lane 5 vincristine 40 µg/ml for 24 hours,

Lane 6 vincristine 40 µg/ml for 48 hours,

Lane 7 vincristine 40 µg/ml for 72 hours stained for Bax.

B) Same membrane immunostained for β-actin.

5.3.2 Further analysis of the effects of vincristine on Cdk1 phosphorylation status in K562 cells:

To analyse further the phosphorylation status of Cdk1 during incubation of cells with vincristine it was decided to look at Cdk1 expression and its phosphorylation status by immunoprecipitation. K562 cells were treated with vincristine of 0.1 and 40µg/ml at 24, 48, 72 hours as before. Protein extraction was done after flow cytometry analysis at each dose and at the time points indicated.

Cdk1 was immunoprecipitated and the immunoprecipitated protein lysate was analysed by gel electrophoresis, then transferred onto PVDF membranes which were immunostained with anti-phosphotyrosine antibody, then stripped and restained with an anti-phospho-Y15 Cdk1 antibody (Figure 5.4).

Cdk1 tyrosine phosphorylation was readily detected in control cells and cells incubated with 0.1µg/ml vincristine for 24 and 48hrs. By 72 hrs, when apoptosis was detectable, there was a reduction in phosphotyrosine residue expression. The high-concentration vincristine produced a similar loss of tyrosine phosphorylation but this was seen earlier at 48hrs using this drug concentration as well as at 72hrs.

However these two longer cultures for 48 and 72 hours show higher rates of apoptosis than that observed at 24 hours. Thus a loss of Cdk1 tyrosine phosphorylation was detected at time points or drug concentrations that induce apoptosis. The data suggests that the changes in tyrosine phosphorylation can be attributed to tyrosine 15 as the total tyrosine phosphorylation has the same pattern of expression as that for tyrosine 15 phosphorylation (figure 5.4).

**Effect of vincristine on tyrosine phosphorylation status of Cdk1 at G2M arrest
and apoptosis in K562 cells**

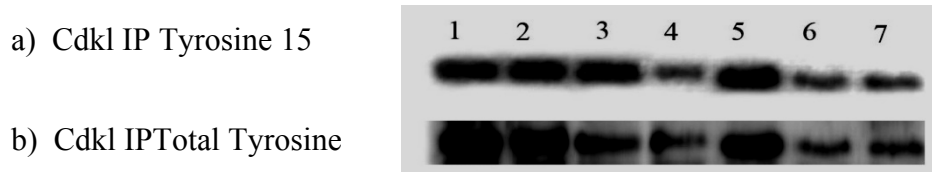


Figure 5.4:

a) Immunoelectrophoresis of immunoprecipitated Cdk1 from whole cell lysates of K562 cells treated with vincristine of different doses for different time points immunostained for total tyrosine phosphorylation

Lane 1 control cells.

Lane 2 vincristine 0.1 µg/ml for 24 hours.

Lane 3 vincristine 0.1 µg/ml for 48 hours.

Lane 4 vincristine 0.1 µg/ml for 72 hours.

Lane 5 vincristine 40 µg/ml for 24 hours.

Lane 6 vincristine 40 µg/ml for 48 hours.

Lane 7 vincristine 40 µg/ml for 72 hours.

b) Same membrane re-stained for Tyrosine 15 phosphorylation.

**Effect of vincristine on phosphorylation status of Cdk1 bound to cyclin A at
G2M arrest and apoptosis in K562 cells**

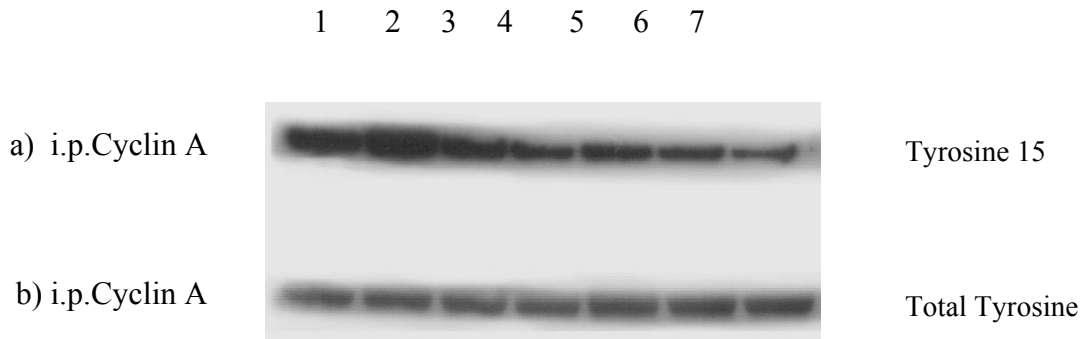


Figure 5.5:

a) Immunoelectrophoresis of Cdk1 that immunoprecipitated with cyclin A from whole cell lysate of K562 cells treated with vincristine of different doses at different time points then immunostained for total tyrosine phosphorylation of Cdk1 .

Lane 1 Control.

Lane 2 vincristine 0.1µg/ml for 24 hours.

Lane 3 vincristine 0.1µg/ml for 48 hours.

Lane 4 vincristine 0.1µg/ml for 72 hours.

Lane 5 vincristine 40µg/ml for 24 hours.

Lane 6 vincristine 40µg/ml for 48 hours.

Lane 7 vincristine 40µg/ml for 72 hours.

b) Same membrane stained for tyrosine 15 phosphorylation

The majority of Cdk1 is bound to cyclin B. However, a small proportion also binds to cyclin A, the cyclin associated with transition through S-phase and the initial entry into G2. My group has published data associating changes in tyrosine phosphorylation with etoposide-induced cell death that was not related to tyrosine 15. This was achieved by examining Cdk1 in cyclin B depleted lysates of etoposide treated cells and looking at the phosphorylation status of Cdk1 that co-precipitates with cyclin A.(Higginbottom et al 2007)

To determine whether this phosphorylation was apparent in vincristine-induced cell death, K562 cells were incubated with 0.1 and 40 μ g/ml of vincristine for 72 hrs and the phosphorylation status of Cdk1 that co-precipitated with cyclin A was examined (figure 5.5)

The data contrasted to that presented in figure 36, where the reduction of overall phosphorylation reflected the reduction of tyrosine 15 phosphorylation. In this experiment the reduction of tyrosine phosphorylation associated with prolonged incubation of cells with 0.1 μ g/ml vincristine (lanes 3 and 4, figure 5.5) or all time periods with 40 μ g/ml (lanes 5-7) was not reflected by a loss of overall tyrosine phosphorylation on the Cdk1 associated with cyclin A (figure 5.5). The degree of tyrosine 15 phosphorylation detected after 24hrs of 0.1 μ g/ml vincristine (lane 2) was the same as the control. However, after 72hrs a clear reduction of tyrosine 15 phosphorylation could be seen (lane 4). Also, 40 μ g/ml caused a reduction in tyrosine 15 at all time points (figure 5.5). However, there was no change in overall tyrosine phosphorylation relative to control. This indicates the loss of phosphorylation seen on cyclin A-associated Cdk1 is associated with tyrosine 15 in

cells primed to die via vincristine. The fact that this is not reflected in the overall tyrosine phosphorylation of cyclin A associated Cdk1 opens the possibility that there is an additional compensatory tyrosine phosphorylation of cyclin A-associated Cdk1 in dying cells as reported by my group for etoposide. Thus a different tyrosine phosphorylation pattern is seen on Cdk1 bound to cyclin A to that seen on Cdk1 bound to cyclin B when cells are incubated with vincristine to cause cell death.

This is not an antibody phenomenon since the same antibodies were used to follow tyrosine phosphorylation shown in figure 36 where the two antibodies showed concordance

5.3.3. Effect of etoposide and nocodazole on survivin expression in K562 cells.

The investigation into the effects of vincristine on K562 cells at G2M suggested that a drug concentration that induced cell cycle arrest was associated with induction of the cell survival protein survivin, which was not induced at high drug concentrations when compared to controls. To determine if this was also true for the DNA-damaging agent etoposide, the induction of survivin was examined in K562 cells following incubation with low and high-dose etoposide. Nocodazole, a non-clinical anti-mitotic agent, was used to compare events at G2 (low dose etoposide) with mitotic events (low dose nocodazole).

PAGE and Western blotting were performed on lysate of K562 cells to determine the effect of etoposide and nocodazole on survivin expression at G2M arrest and apoptosis, and the relationship of Cdk1 with survivin at the same stages of the cell cycle.

The results show similar expression of survivin by cells treated with etoposide at doses which cause G2 arrest and by doses that cause apoptosis at 24 hours. Exposure of the cells to the same doses for 48 hours caused very little change in expression of survivin (figure 38). Nocodazole, which is a mitotic inhibitor like vincristine, also had little effect on survivin expression at concentrations that cause mitotic arrest.

The relationship of survivin with Cdk1 was then examined. Figure 33 had shown loss of survivin in lysates from cells induced to die by vincristine but not in those induced to undergo mitotic arrest. Mitotic cell cycle arrest was induced in K562

cells by nocodazole and compared to the effects of etoposide at a concentration that also induces cell cycle arrest. The data shows that expression of survivin is maintained in K562 cells in both G2 arrest (etoposide) and in mitotic arrest (nocodazole). Moreover, it immune-precipitates with Cdk1 in both situations. However, survivin no longer immuno-precipitates with Cdk1 when etoposide is increased to a apoptosis-inducing concentration. Interestingly, Aurora B kinase, a protein that interacts with survivin when survivin functions as a mitotic passenger protein does not co-precipitate with Cdk1 at either low or high concentrations of etoposide (figure 5.7). However, survivin and Aurora B were shown to co-precipitate with each other, in the absence of Cdk1 when cells were incubated with etoposide or low-dose nocodazole. This was shown by an immuno-precipitation of survivin followed by gel electrophoresis and staining the resulting western blot for Aurora B kinase (figure 5.8).

Effect of etoposide and nocodazole on the expression of survivin in K562 cells.

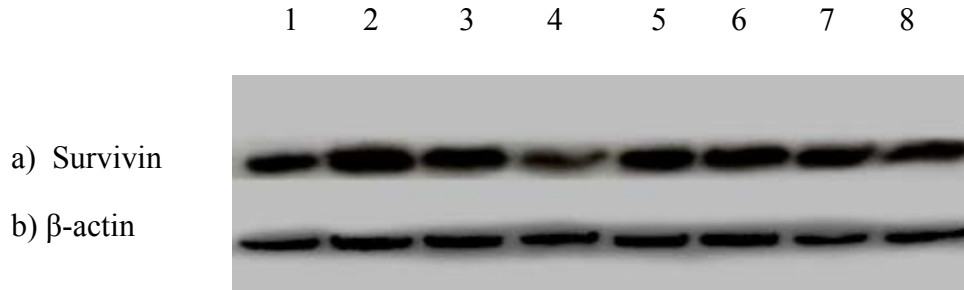


Figure 5.6:

a) immunoelectrophoresis of whole cell lysates of K562 cells treated with etoposide and nocodazole, and immunostained for survivin. I

Lane 1 control cell.

Lane 2 nocodazole 0.1 μg/ml for 24hours.

Lane 3 etoposide 0.1 μg/ml for 24 hours.

Lane 4 etoposide 40 μg/ml for 24 hours.

Lane 5 control cells.

Lane 6 nocodazole 0.1 μg/ml for 48 hours.

Lane 7 etoposide 0.1 μg/ml for 48 hours.

Lane 8 etoposide 40 μg/ml for 48 hours.

b) Same membrane immunostained for β-actin

Effect of etoposide on apoptosis and expression of chromosomal passenger proteins in relation to Cdk1 in

K562 cells at G2M arrest.

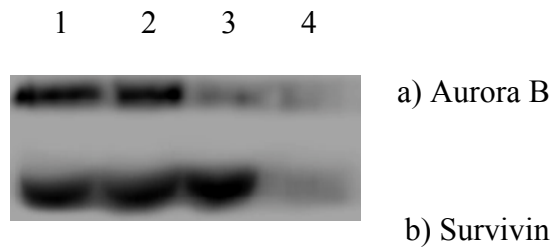


Figure 5.7:

a) Immunoelectrophoresis of whole cell lysates of K562 cells, and treated with etoposide and nocodazole at apoptosis and G2M arrest, immunoprecipitated for Cdk1 and stained for Aurora B.

Lane 1 control cell.

Lane 2 nocodazole 0.1µg/ml for 24 hours.

Lane 3 etoposide 0.1 µg/ml for 24 hours.

Lane 4 etoposide 40 µg/ml for 24hours.

b) Same membrane immunostained for Survivin.

Effect of etoposide and nocodazole on the co-precipitation of Aurora B kinase with survivin in K562 cells.

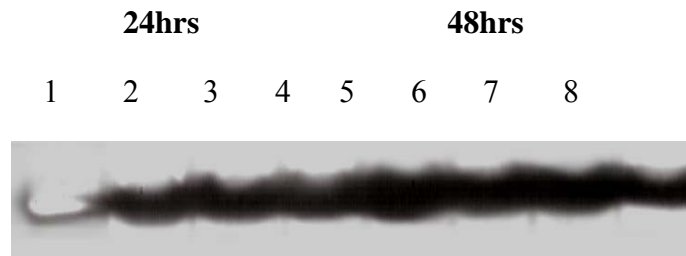


Figure 5.8:

Lane 1 control cell.

Lane 2 nocodazole 0.1 $\mu\text{g/ml}$ for 24hours.

Lane 3 etoposide 0.1 $\mu\text{g/ml}$ for 24 hours.

Lane 4 etoposide 40 $\mu\text{g/ml}$ for 24 hours.

Lane 5 control cells.

Lane 6 nocodazole 0.1 $\mu\text{g/ml}$ for 48 hours.

Lane 7 etoposide 0.1 $\mu\text{g/ml}$ for 48 hours.

Lane 8 etoposide 40 $\mu\text{g/ml}$ for 48 hours.

5.4 Discussion:

Work in previous chapters had shown some fundamental differences in the response of cells to a DNA damaging agent (etoposide) and an anti-mitotic agent (vincristine) in the relationship between cell cycle arrest and cell death.

Data presented in this chapter shows that vincristine will induce the anti-mitotic protein survivin, at concentrations that induce mitotic cell cycle arrest followed by apoptosis, but not at concentrations that induce cell death directly. It has no effect on Cdk1 expression although tyrosine phosphorylation of Cdk1 is diminished under conditions that induce cell death. Interestingly, vincristine induced Bax as though priming the cell for cell death at all concentrations. A closer look at Cdk1 tyrosine phosphorylation indicated the loss of phosphorylation was on the regulatory tyrosine 15 residue. However, the proportion of Cdk1 that binds cyclin A, rather than cyclin B may have other phosphorylation responses as overall tyrosine phosphorylation was not affected by vincristine, although tyrosine 15 phosphorylation was again reduced on induction of death in target cells.

Etoposide on the other hand caused a slight increase in survivin expression at all drug concentrations and time points. Cdk1 co-precipitated with survivin in control cells and with a drug concentration that induced G2 cell cycle arrest (0.1 µg/ml) but not with a higher drug concentration that induced apoptosis (40 µg/ml). This suggests that Cdk1 can target survivin for phosphorylation under non-fatal conditions. Interestingly, another passenger protein Aurora B did not co-precipitate with Cdk1 under conditions of either cell cycle arrest or cell death which may

suggest survivin is acting as an IAP in these circumstances and not as a passenger protein.

5.5 Conclusions

Vincristine does not alter the expression of Cdk1

Low-concentration vincristine (0.1 μ g/ml) induces survivin expression but a higher concentration (40 μ g/ml) does not induce it.

A slight reduction in Cdk1 tyrosine 15 phosphorylation was detected in whole cell lysates in association with vincristine-induced cell death i.e. cells incubated with 0.1 μ g/ml for 48 to 72 hrs and 40 μ g/ml at all time points.

Closer inspection of Cdk1 immuno-precipitates confirmed this observation on Cdk1.

The overall tyrosine phosphorylation of Cdk1 bound to cyclin A did not diminish under the conditions cited above although some loss of tyrosine 15 phosphorylation was detected. It may be a possibility that there is some further phosphorylation of Cdk1 bound to cyclin A in cells induced to die.

Vincristine may prime cells for apoptosis by augmenting Bax expression..

Unlike vincristine, etoposide had little effect on survivin expression.

Survivin co-precipitates with Cdk1 in cells in G2-arrest but not in cells induced to die. Aurora B, another passenger protein, does not co-precipitate with Cdk1 in either etoposide-arrested cells nor in cells induced to die.

Survivin and Aurora B are found to co-precipitate in cells incubated with low (G2 arrest) and high-concentration (apoptosis) etoposide.

Chapter 6

Discussion

6.1 Introduction:

Cancer chemotherapy damages cells in various ways and some of this damage activates the cell cycle checkpoints. Checkpoint activation causes cell cycle arrest and triggers repair mechanisms. When cell repair cannot be achieved, cell death usually occurs. However, within intact animals and tissues, cells are heterogeneous. Therefore, one way of studying the detailed responses of cells to damage is to isolate them in culture (Clarke et al, 2006).

The cultured K562 and HL60 cells used in these experiments are human myeloid leukemia cell lines. Both cell lines are p53 negative but the former also contains the Bcr-Abl fusion gene created by the chromosomal translocation that forms the Philadelphia chromosome. A combination of p53 negative and Bcr-abl positive contributes towards a delay in apoptotic responses in this cell line allowing a window of opportunity to investigate cell death responses to cellular insult. Multiple genetic events are required to establish immortal cell lines. This means that results of studies of such cell lines should be interpreted with caution. However, in the present work similar results were obtained from these two similar cell lines strengthening the validity of the experiments and enabled one of them (K562 cells) to then be used for subsequent experiments (Martin et al, 1997).

DNA can be directly damaged by anticancer treatment; alternatively, DNA repair enzymes such as topoisomerase 2 can be inhibited by drugs such as etoposide. Inhibition of DNA repair enzymes enables faults in DNA replication to persist. Detection of this DNA damage in turn stimulates the DNA damage checkpoints,

resulting in stimulation of p53 gene expression. p53 is a transcription factor and is the most important tumor suppressor gene. p53, 'the guardian of the genome' helps to repair DNA damage by arresting cells at G1 phase while repair mechanisms are activated. However, because K562 and HL60 are p53 gene deficient, the lack of p53 expression causes an activation of the Chk1 kinase pathway that arrests the cells at G2 phase (Levesque AA et al 2008). The other class of chemotherapeutic drugs used in these experiments, vincristine and nocodazole, are microtubule damaging agents which stimulate the spindle assembly checkpoint, an important antitumor target. Thus, knowledge of the specific in vitro responses of cancer cells to therapeutic agents will reflect the fate of target cells in treated patients. Events at the G2 checkpoint and in mitosis have now been shown to determine cell survival or cell death particularly in p53 compromised cells(Gomes et al, 2010).

Between these two cellular outcomes is cell cycle arrest. Depending on the response, cells may die either by apoptosis or by mitotic catastrophe. Alternatively, the cell may revert into the cell cycle, perhaps with abnormal chromatin containing uncorrected DNA damage or other abnormalities (Sun et al, 2009).

In my experiments in p53 deficient cells which are resistant to apoptosis, the cells responded in different ways to damage. Levels of apoptosis and cell cycle arrest varied with both treatment doses and treatment duration as described below.

6.2 Effects of cytarabine and etoposide on the cell cycle of K562 cells:

In my experiments the effects of cytarabine were not dose related probably because cytarabine is converted by an enzyme to an active form and this rate of conversion constitutes a rate limiting step. Thus, increasing doses of cytarabine had no additional effect on the cells probably because the rate of conversion to the active form remained steady, in spite of increasing doses of the precursor. An alternative possibility is that one of the mechanisms which leads to cell death is a rate limiting factor. This is less likely since other drugs such as etoposide which also induce apoptosis have dose related effects. The effect of cytarabine contrasts with the dose related action of etoposide which acts directly and does not require enzyme conversion to an active form (Loughlin et al, 1996).

The effects of etoposide on K562 cells were analysed by flow cytometry. The technique showed that etoposide treatment induced prolonged G2 arrest. However, the highest doses of etoposide also caused apoptosis. These findings confirm other experiments . There is more than one possible explanation: first, the cells resist the effects of low doses by compensatory mechanisms which may be overcome with higher doses. Alternatively, the cells could have a lower threshold for G2M arrest, while higher doses activate different mechanisms possibly with a higher threshold and cause apoptosis.

Low doses cause cell cycle arrest; high doses cause apoptosis, and the higher the dose of etoposide, the more apoptosis is induced. Both of these observations have

important implications for the treatment of leukaemia patients by chemotherapy.

Etoposide caused similar changes in HL60 cells.

6.3 Effect of etoposide and nocodazole studied by immunostaining H3Ser10:

Studies of the four core histone proteins (H1-H4) have shown that phosphorylation of Ser10 on histone H3 is associated with chromosomal condensation during mitosis (Hendzel et al, 1997). Therefore phosphorylation of Ser 10 on H3 is an indicator for mitosis. Immunostaining cells with H3Ser10 antibody confirmed that the percentage of cells in mitosis was very low, confirming that the arrested cells were restricted to G2 phase arrest. Therefore HL60 cells treated with etoposide showed high levels of G2 arrest which occurred by 24hours. The H3Ser10 data show that etoposide induces G2 arrest and not mitotic arrest. DNA strand breaks are not repaired during topoisomerase 2 inhibition. DNA damage therefore will stimulate Chk1 which phosphorylates Ser216 on Cdc25C. This deactivates cyclin B/Cdk1 as part of the involvement of the G2M checkpoint (Williams et al, 2001).

DNA damage increases Cdk1 phosphorylation and decreases the activity of cyclin B/Cdk1 complex (Zhang et al, 2009). This may causes either G2M arrest or an abnormal mitosis. Escape from mitosis may allow the cells to enter G1 phase at the border of the G1 checkpoint. This accumulation of cells with abnormal DNA in G1 phase may induce apoptosis. This is caused by p21 activation and mediated by Bax, a proapoptotic protein (Maddika et al, 2007). We confirm here that this activation of apoptosis prevents damaged cells from entering the cell cycle.

Etoposide induces G2M arrest precisely at G2 phase. This was confirmed by lack of H3Ser10 antibody staining which showed that the cells had not entered mitosis. Thus any lethal effect of etoposide does not occur during mitosis. The cells

accumulate at the border of G1-phase and the lethal effect is probably due to mitotic slippage which stimulates apoptosis.

The conclusions from this work are that G2 cell cycle arrest and apoptosis do not occur together in either K562 or HL60 cells. When there is G2 cell cycle arrest, apoptosis does not occur. The results suggest that cells can escape G2-arrest and mitotic arrest, the latter probably by mitotic slippage. When apoptosis occurs there is no cell cycle arrest. Thus cell death induced by etoposide post G2 arrest is due predominantly to apoptosis. Evidence that entry into, and out of, mitosis may be responsible for the induction of apoptosis will be discussed in a following section.

6.4 Effects of antimicrotubule agents on K562 cells:

Vinca alkaloids such as vincristine are used in human cancer treatment. They inhibit microtubule assembly by depolymerising microtubules. This prevents formation of the metaphase spindle. The abnormal metaphase spindle prevents attachment of the microtubules to the kinetochores on the chromosomal centromeres. This failure of attachment arrests the cell at mitosis. Prolonged mitotic arrest causes cells to accumulate in prometaphase as a consequence of mitotic checkpoint signalling from unattached kinetochores. Continued treatment with vincristine leads to cell death(Lentini et al, 2008).

Short term drug treatment can cause sustained mitotic arrest until the drug is cleared. This mechanism of action is cytostatic, with renewed cell cycling after drug removal. The second outcome is adaptation in which cells exit long term mitotic arrest while still exposed to the drug. In this case cytokinesis fails but the cells still enter G1. Adapting cells could escape to G1 then continue dividing despite continued mitotic checkpoint signalling. The inappropriately elevated levels of checkpoint inhibitors then provoke apoptotic death in interphase (Moore et al, 2009). A final outcome is activation of a death pathway from mitosis. Cell death by apoptosis following such long term arrest is mediated by caspase dependent pathways accompanied by activation of Bax (Beth et al, 2005)

My data show that the effects of vincristine are both concentration and time dependent. Low-doses of vincristine induced G2M arrest that was subsequently associated with apoptotic cell death. Interestingly, high doses appear to induce less

G2M arrest which was associated with necrotic death rather than apoptosis. This is in contrast to the etoposide data which show that low doses cause G2 arrest and high dose induce cell death. Thus, disparity between the disproportionately high levels of G2M arrest and low levels of apoptosis induced with high dose vincristine may be explained by low doses causing enough microtubule damage to cause cells to undergo G2M arrest. Some of these cells will subsequently undergo apoptosis. Higher doses which cause more microtubule damage will also cause G2M arrest, but the microtubules may be too damaged to allow cells to undergo either mitotic slippage or apoptosis even though there is activation of proapoptotic mechanisms as shown by raised annexin V immunostaining by flow cytometry and high expression of Bax by western blotting.

The spindle assembly checkpoint delays the exit of a cell with inadequately attached kinetochores, or lack of spindle tension, from mitosis. Destruction of cyclin B by APC proteolysis inhibits cyclin B/cdk1 activity (Perera et al, 2007). This will allow the cell to escape mitosis in the presence of an uncompleted spindle assembly checkpoint (Daniela et al, 2006). In other opinions depletion or lack of accumulation of active chromosomal passenger complex in centromeres at mitosis leads to premature exit from mitosis accompanied by the loss of Mad2 and Bub1 from unattached kinetochores (Stoepel et al, 2005). Reduction of any one of the chromosomal passenger constituents by any means triggers the concurrent elimination and mis-localization of the others. (Carvalho et al, 2003). However, another possibility is that activation of the spindle assembly checkpoint may itself, be responsible for the induction of apoptosis at mitosis under these circumstances

6.5 Effect of microtubule and DNA damage on Cdk1 phosphorylation status and associated chromosomal passenger proteins at G2M, mitosis and apoptosis.

An attempt was made to understand the underlying mechanisms for the observations made. The work for this thesis has shown that low-concentration vincristine caused mitotic arrest which was then followed by apoptosis. This introduces the concept of mitotic slippage and the possibility that mitotic arrest, or activation of the spindle assembly checkpoint, is required for the apoptotic response.

The data in chapter 5 indicated that low-concentration vincristine induced survivin expression whereas high concentrations did not induce survivin expression. This suggests a survival signal is induced at these lower concentrations. Also all concentrations of vincristine induced enhanced expression of pro-apoptotic Bax, suggesting that the cells become primed to die and will do so in the absence of a survival signal such as survivin. The reduction in tyrosine 15 phosphorylation seen in vincristine-treated cell is in agreement with the concept of Cdk1 activation during entry into mitosis. However, active Cdk1 also supports the notion that survivin may be acting as a substrate for Cdk1. This is important because Cdk1 phosphorylation of survivin at Thr34 enables survivin to sequester caspases and hence block caspase-mediated apoptosis (Temme et al,2003). This is supported by the observation that Cdk1 co-precipitated with survivin when leukaemia cells were incubated with another mitotic spindle inhibitor, nocodazole (figure 5.7), Thus the initial response of K562 leukaemia cells to low-concentration vincristine is to establish a pro-survival signal through induction of survivin expression; de-phosphorylated (and hence active) form of Cdk1 is observed raising the possibility of caspase 9

sequestration at the time of the mitotic cell cycle arrest seen in chapter 4 (Allan et al, 2009). Pro-apoptotic Bax is present throughout these events but my data cannot distinguish whether this is the active form or the inactive form of Bax. The literature states that in other cell systems, Bax becomes activated after activation of mitotic spindle assembly checkpoint (Wong et al, 2008). Hence the death or survival of a leukaemia target cell in response to vincristine depends upon a balance between survival signaling as a result of an induction of survivin expression, its phosphorylation by an active mitotic Cdk1/cyclin B complex and sequestration of caspases on one hand and the induction of activated pro-apoptotic Bax by the spindle assembly checkpoint on the other hand (Thomadaki et al, 2009).

These data relating to low-dose vincristine are more relevant to the physiological situation, as the combination of apoptosis and cell death seen with high-concentration vincristine would not be tolerable in vivo.

Some of this data for vincristine contrasts with that observed for etoposide. As etoposide targets DNA, it activates the G2 DNA damage checkpoint brought about by a predicted inhibition of Cdk1 activity via tyrosine/threonine phosphorylation or cytosolic sequestration of the Cdk1/cyclin B complex. Cells did not enter mitosis (unlike mitotic cell cycle arrest induced by vincristine where Cdk1 is active). Low-dose etoposide-induced Cdk1 tyrosine phosphorylation has been shown by other workers in my group (Higginbottom et al, 2002). However, in common with the mitotic cell cycle arrest induced by the microtubule inhibitor nocodazole, survivin co-precipitated with Cdk1 during an etoposide-induced G2 arrest indicative of a survivin-mediated sequestration of caspase 9 thus promoting a status of cell survival

during cell cycle arrest. An interesting contrast between mitotic cell cycle arrest and G2 cell cycle arrest was the additional co-precipitation of Aurora B kinase with Cdk1 and survivin with the former agent (figure 39). Also of interest was that when the concentration of etoposide was increased to a cell death-inducing concentration, neither survivin nor Aurora B co-precipitated with Cdk1.

6.6 Is there a common pathway between DNA damaging agents and microtubule assembly inhibitors in killing p53 negative leukemia target cells.

The data in this thesis has utilised two agents used to treat neoplasia, one targeting DNA and the other targeting mitotic tubule assembly. Both drugs induce cell cycle arrest at relatively low drug concentrations. Etoposide induces a cell cycle arrest at G2 and vincristine induces a mitotic cell cycle arrest. However, the cell cycle arrest induced by etoposide protects cells from apoptosis whilst the mitotic arrest induced by vincristine appears to be required for a later induction of apoptosis. In both scenarios, survivin provides the initial survival signal during cell cycle arrest.

How vinca alkaloids such as vincristine induce apoptosis is still not known. However, activation of the spindle checkpoint along with a transient delay in cell cycle progression is required (Thomadaki et al, 2009). Cdk1/cyclin B is active until late anaphase (Hershko et al, 1999). My data show induction of survivin expression and a potential role for Aurora B and survivin, possibly as mitotic passenger proteins, in maintaining the temporary checkpoint until Bax activation. Several examples of cell death after mitotic adaptation implicate Bax in the pro-apoptotic response.

Some of these events may facilitate etoposide-induced apoptosis in p53 compromised cells. Data from chapter 4 indicates that cell death is associate with a collapse of the post S-phase checkpoints (G2 and mitotic checkpoints) on induction of apoptosis. To induce Bax activation in p53 negative cells following drug-induced DNA damage, Vogel et al have suggested that a balance occurs between the mitotic checkpoint protein Mad2 on the one hand, and survivin and Aurora B kinase on the

other. They used an agent called UCN-01 which inhibits Chk1 and thus prevents accumulation of cells at the G2 checkpoint in the presence of DNA damage. Chk1 induces the G2 checkpoint in the presence of DNA damage by inactivating Cdc25 (Vogel et al, 2007). Cdk1/cyclin B remains phosphorylated and thus inactive in this situation causing cell cycle arrest. Inhibition of Chk1 has the opposite effect; Cdc25 remains active in the presence of DNA damage, thus Cdk1/cyclin B remains active and cells enter mitosis. The authors showed that the ensuing apoptosis, seen as a result of cells with drug-induced DNA damage entering mitosis, was dependent on the spindle checkpoint protein Mad2. Mad2-induced apoptosis is in turn dependent on activation of the spindle checkpoint that also requires the passenger protein Aurora B kinase and the passenger protein function of survivin. However, survivin can act as an anti-apoptotic protein when phosphorylated on thr 34 by sequestering caspase 9. Vogel et al were also able to show that cell death induced by DNA damage in cells that entered mitosis as a result of treatment with UCN-01 could be sensitized to enhanced apoptosis in this model by shRNA interference of survivin, or by chemical inhibition of Aurora B kinase. The proposed mechanism being that survivin is responsible for a stabilization of Bcl-2 through phosphorylation of Bcl-2 and a subsequent prevention of Bax activation. Presumably active Aurora B kinase stabilises survivin; it is also known that Cdk1 activity stabilizes survivin so inhibition of Cdk1 and/or Aurora B kinase contributes to the pro-apoptotic signal (Vogel et al,2007) .

Thus it is feasible that both vincristine and etoposide could share a common and potentially synergistic activation of Bax in mitosis.

My data has also highlighted a novel aspect to these events. Figure 5.5 shows that the total tyrosine phosphorylation of Cdk1 bound to cyclin A, rather than cyclin B, does not reflect that of tyrosine 15 when cells were incubated with vincristine. This suggests that cyclin A-associated Cdk1, under conditions that induce apoptosis after mitotic arrest (0.1 μ g/ml for 72hrs), and that induced by high-concentration vincristine, is phosphorylated on residues other than tyrosine 15. This has the potential to change Cdk1/cyclin A activity or substrate. These data substantiate that published by our group for etoposide- induced apoptosis (Higginbottom et al, 2002).

6.7 Concluding Remarks and Future Directions:

The data in this thesis and the accompanying review of the literature have attempted to highlight the importance of the G2 and M phases of the cell cycle in leukaemia cell chemotherapy of p53 negative cells. In the absence of the “guardian of the genome” the G2 and mitotic phases of the cell cycle and their inherent checkpoints should be major targets for exploitation by therapeutic agents.

It is desirable to reduce the toxicity of common anti-cancer agents such as etoposide and vincristine, by reducing their active concentrations. It is also desirable to enhance the efficiency in which drugs kill target cells. Therefore, it will be important to understand events at G2M in this respect. A systematic analysis of the interaction of mitotic checkpoint proteins with pro and anti-apoptotic molecules using shRNA technology for example, would assist in this aim

Any future work would investigate a possible adjuvant effect of UCN-01 with etoposide as my data show that drug resistant cells such as K562 will not die from G2. Abolition of this checkpoint induce by low-dose etoposide, by UCN-01 may enhance its efficacy. The question then arises as to what effect addition of low-dose vincristine would have on UCN-01 manipulated cells treated with etoposide. Would etoposide and vincristine then synergise in activating Bax from mitosis? Would mitotic checkpoint proteins play a role in this synergy and if so, which ones would be required in driving apoptosis. Where would the resistance to apoptosis come from in this situation; survivin would be a prime target for investigation. If survivin did play a role in resistance to mitotic driven apoptosis then factors such as its

phosphorylation status, its ability to sequester caspase 9, its role in Bcl-2 phosphorylation and Bcl-2 stabilisation could be investigated (Gascoigne et al, 2009)

Other possibilities include the prior manipulation of cells with Aurora B kinase inhibitors to de-stabilise survivin; Cdk1 inhibitors such as Roscovitine or Purvanol could be investigated in their role in preventing Cdk1 phosphorylation of survivin, which also promotes survivin de-stabilisation, as possible sensitizing agents for low-dose vincristine and/or etoposide induction of mitotic apoptosis. Roscovitine, purvanol and Aurora B inhibitors are currently in clinical trials (Kaestner et al, 2009).

New agents will also come on line which disrupt events at mitosis. I would also predict that any agent mimicking cyclin A-bound-Cdk1, phosphorylated on tyrosine residues not including tyrosine 15 would facilitate mitotic apoptosis.

Chapter 7

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Chapter 8

Abbreviations

Abbreviations

ADP	Adenosine 5'-diphosphate
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
Ann V	Annexin V
APC/C	Anaphase Promoting Complex/Cyclosome
APS	Ammonium Persulphate
Ara-C	Cytosine Arabinoside
ARF	Alternative Reading Frame
Asp	Aspartate
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphate synthase
ATR	Ataxia Telangiectasia Related
BH3	Bcl-2 homology domain 3
BIR	Baculovirus IAP repeat
BSA	Bovine Serum Albumin

°C	Degrees Celcius
Ca ²⁺	Calcium ions
CAD	Caspase-activated deoxyribonuclease
CAK	CDK Activating Kinase
CARD	Caspase recruitment domain
Caspase	cysteine aspartate protease
CDC (cdc)	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CML	Chronic Myeloid Leukaemia
CO ₂	Carbon Dioxide
cpm	Counts per Minute
C-terminal	Carboxy terminal
DNA	Deoxynucleic Acid
DR	Death Receptor
E1	Ubiquitin activating
E2	Ubiquitin conjugating
E3	Ubiquitin ligating

ECACC	European Collection of Animal Cell Cultures
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbant Assay
FADD	Fas-associated death domain
FITC	Fluorescein Isothiocyanate
G	Gram
G0	Gap 0
G1	Gap 1
G2	Gap 2
G2M	Gap2/Mitosis
GMCSF	Granulocyte/Macrophage-Colony Stimulating Factor
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HHT	Homoharringtonine
HRP	Horse Raddish Peroxidase
HTLV-1	Human T cell lymphotropic virus type 1

IAP	Inhibitor of apoptosis protein
IL	Interleukin
JAK	Janus Protein Kinase
kDa	KiloDalton
KOH	Potassium Chloride
L	Litre
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
M	Molar
M	Mitosis
MDR	Multidrug Resistance
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar

mm	Millimeter
MPF	Mitotic Phase Promoting Factor
mV	Millivolts
ng	Nanogram
nm	Nanometer
Noc	Nocodazole
N-terminal	Amino terminal
PAG	Polyacrylamide Gel
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
Ph	Philadelphia
PI	Propidium iodide
PMSF	PhenylMethaneSulphonyl Fluoride
PS	Phosphatidyl serine
PVDF	Polyvinylidenedifluoride
RB	Retinoblastoma
Rf	Refractive Front

RIPA	Radioimmunoprecipitation
RNA	Ribonucleic Acid
RNAse	Ribonuclease
Rpm	Revolutions per Minute
RPMI	Roswell Park Memorial Institute
S	Synthesis
SAC	Spindle activation complex
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SSC	Sodium Chloride Sodium Citrate
STAT	Signal Transducer and Activators of Transcription
T	Threonine
T14	Threonine residue 14
T161	Threonine residue 161
TBE	tris borate EDTA
TBS	Tris(hydroxymethyl)aminomethane buffered saline
TBST	TBS Tween-20 [®]
TEMED	N,N,N',N'-tetramethylethylenediamine

Thr	Threonine
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor
TRADD	TNF-R1-associated death domain
TRAF	TNF receptor associated factor
TRAIL	TNF α -related apoptosis-inducing ligand
tris/Trizma	Tris(hydroxymethyl)Aminomethane
TRITC	Tetramethylrhodamine Isothiocyanate
Tyr	Tyrosine
Ubq	Ubiquitin
V	Volts
v/v	Volume per Volume
w/v	Weight per Volume
Y	Tyrosine
Y15	Tyrosine residue 15
Y15-P	Tyrosine residue 15 phosphorylation
Y-P	Tyrosine phosphorylation
Yx	Tyrosine residue(s) other than Y15

Chapter 9

Appendix

9.0 APPENDICES:

9.1 APPENDIX A. REAGENTS, CHEMICALS AND BUFFER PREPARATION

9.1.1 Reagents and equipment used for cell culture

Supplemented RPMI 1640 Medium

RPMI 1640 medium containing 25mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) was purchased from Sigma, UK. This was supplemented with 10% v/v heat inactivated foetal bovine serum (Sigma, UK), 2mM L-glutamine (Sigma, UK), 60 µg/ml sodium benzylpenicillin (Britannia Pharmaceuticals Ltd, UK) and 100 µg/ml streptomycin sulphate (Evans Medical Ltd, UK).

9.1.2 Reagents used for cryopreservation and replenishment of leukaemic cell lines:

Cryopreservation Medium

RPMI 1640 was supplemented with 50% v/v heat inactivated foetal bovine serum (Sigma, UK) and 10% v/v dimethylsulphoxide (DMSO) (BDH, UK).

Chemicals and Equipment for Freezing Cells

Isopropanol (BDH, UK) was used to fill the Nalgene™ cyro 1°C freezing container.

9.1.3 Reagents used for cell viability testing:

Trypan Blue Dye Solution

1 mg of Trypan blue dye (Sigma, UK) was dissolved in 1 ml of HBBS (Sigma, UK) and sterilised by passing through a 0.22 µm Millipore filter. This working solution of 0.1% w/v trypan blue/HBBS was stored at 4°C.

9.1.4 Chemotherapeutic and chemical reagents used in experiments:

Cytarabine

Etoposide (VP-16) (Vespid) at a concentration of 20, 40 µg/ml was obtained from Bristol Myers, UK.

Nocodazol (Sigma, UK) 10 mg of was dissolved in 1 ml of DMSO (BDH, UK) to make a 10 mg/ml stock solution.

Vincristine 0.02 -80µg/ml

9.1.5 Buffers and reagents used for the analysis of apoptosis by flow cytometry:

Phosphate Buffered Saline (PBS) pH 7.2

PBS tablets were purchased from Oxoid, UK. 1 PBS tablets was dissolved in 100 ml of deionised water.

Chemicals Used for Fixing and Staining Cells

Ethanol (Absolute Alcohol) was purchased from Hayman Ltd, UK

1 mg RNase (Sigma, UK) and 250 µg of Propidium Iodide (Sigma, UK) were dissolved in 5 ml PBS (Oxoid, UK)

Annexin V V-FITC (BD Biosciences pharmingen) 5µl per test used.

9.1.6 Reagents for mini-agarose gel electrophoresis:

10× Tris Borate Ethylenediamine tetraacetic acid (TBE) Buffer

108 g Tris(hydroxymethyl)aminomethane (Tris) (Sigma, UK)

55 g of boric acid (Sigma, UK)

40 ml of 0.5M Ethylenediamine tetraacetic acid (EDTA) pH8.0 (Sigma, UK).

1 L deionised water.

1.8% w/v Agarose TBE Gel

0.63 g of Agarose (Seakem, FMC Bioproducts, Flowgen, UK) was added to 3.5 ml of 10× TBE buffer which was made to a final volume of 35 ml using deionised water this solution was boiled for 1 minute before being cast and allowed to cool.

9.1.7 Reagents for whole cellular lysis:

RIPA Buffer

PBS (Oxoid, UK) containing:

1% v/v Nonidet-P40 (NP40) (Sigma, UK)

0.5% w/v Sodium Deoxycholate (Sigma, UK)

0.1% w/v Sodium Dodecyl Sulphate (SDS) (BDH, UK)

4% v/v aprotinin (Trasylol 10,000 Kallikrein Inactivator Units (KIU)/mL Bayer UK)

1% v/v Sodium Orthovanadate (0.1M Stock Solution) (Sigma, UK)

0.5% v/v PhenylMethaneSulphonyl Fluoride (PMSF) (0.1M Stock) (Sigma, UK).

9.1.8 Reagents used in the direct Lowery protein assay method:

Sigma Protein Assay Kit

2 mg of freeze dried bovine serum albumin (Sigma, UK) was reconstituted with 5 ml of deionised water to make a stock concentration of 400 µg/ml. Protein standards of 0 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml and 200 µg/ml in a final volume of 1 ml were prepared. Modified Lowery reagent (Sigma, UK) (Lowery reagent and sodiumdodecylsulfate) was reconstituted with 40 ml deionised water,

Folin and Ciocalteu's Phenol Reagent (Sigma, UK) was reconstituted with 90 ml deionised water.

9.1.9 Reagents used in the preparation of protein for SDS-PAGE electrophoresis:

Sodium DodecylSulphate (SDS) Loading Dye

4 ml deionised water

1 ml 0.5M Tris-HCl pH 6.8 (See 9.1.13)

800 µl Glycerol (Sigma, UK)

1.6 ml 10% w/v SDS (Sigma, UK)

400 µl 2 β-Mercaptoethanol (Sigma, UK)

200 µl 0.2% w/v Bromophenol blue (Sigma, UK)

9.1.10 Reagents for the determination of relative molecular weight:

Reagents Used in Molecular Weight Determination

Rainbow™ coloured protein molecular weight markers (Amersham Pharmacia Biotech, UK) consist of a mixture of:

Myosin	220 kDa
Phosphorylase b	97 kDa
Bovine Serum Albumin	66 kDa
Ovalbumin	45 kDa
Carbonic Anhydrase	30 kDa
Trypsin Inhibitor	20.1 kDa
Lysozyme	14.3 kDa

9.1.11 Reagents for the preparation of SDS-PAGE mini gels:

Chemicals Used for SDS-PAGE Gels

Butanol (Sigma, UK) was mixed in equal volumes with deionised water and the solution was shaken to saturate the butanol. The solution was allowed to settle before the top layer of saturated butanol was used.

Buffers Used in the Preparation of SDS-PAGE Gels :

1.5M Tris-HCl pH8.8

18.17 g Trizma Base (Sigma, UK) was dissolved in 100 ml deionised water, this solution was adjusted to pH 8.8 with Hydrochloric acid (Sigma, UK).

0.5M Tris-HCl pH 6.8

6.06 g Trizma Base (Sigma, UK) was dissolved in 100 ml deionised water, this solution was adjusted to pH 6.8 with Hydrochloric acid (Sigma, UK).

10% SDS-PAGE gels(for detection of Cyclin A):

4.0 ml Deionised water

3.3 ml 30% Acrylamide/bis Acrylamide (Sigma, UK)

2.5 ml 1.5M Tris-HCl pH 8.8

100 µl 10% w/v SDS (Sigma, UK)

100 µl 10% w/v APS (Sigma, UK)

6 µl TEMED (Sigma, UK)

12% SDS-PAGE gels (for β -Actin, Bax, Survivin, Aurora B, Tyrosine total, Tyrosin 15 detection):

3.3 ml Deionised water

4.0 ml 30% Acrylamide/bis Acrylamide (Sigma, UK)

2.5 ml 1.5M Tris-HCl pH 8.8

100 µl 10% w/v SDS (Sigma, UK)

100 µl 10% w/v APS (Sigma, UK)

6 µl TEMED (Sigma, UK)

STACK gel:

2.7 ml Deionised water

0.67 ml 30% Acrylamide/bis Acrylamide (Sigma, UK)

0.5 ml 0.5M Tris-HCl pH 6.8

40 μ l 10% w/v SDS (Sigma, UK)

40 μ l 10% w/v APS (Sigma, UK)

4 μ l TEMED (Sigma, UK).

9.1.12 Buffer for the electrophoresis of protein:

5 \times Electrode (Running) Buffer

7.5 g Tris Base (Sigma, UK)

36 g Glycine (Sigma, UK)

2.5 g SDS (Sigma, UK)

500 ml deionised water

1 \times Electrode buffer was prepared by diluting 100 ml of 5 \times Electrode buffer with 400 ml deionised water.

9.1.13 Buffer for the electroblotting of protein on to PVDF membranes:

5× Transfer Buffer

7.5 g Tris Base (Sigma, UK)

36 g Glycine (Sigma, UK)

500 ml deionised water

1× Transfer buffer was prepared by diluting 100 ml of 5× Transfer buffer with 50 ml Methanol (BDH, UK) and 350 ml deionised water.

9.1.14 Reagents used for the antibody staining of western blots:

Tris Buffer Saline pH 8.0

100 mM Trizma Base (Sigma, UK)

150 mM Sodium Chloride (Sigma, UK)

This was prepared by dissolving 3.63 g Trizma base and 26.3 g sodium chloride in 3 L deionised water this was adjusted to pH 8.0 with hydrochloric acid (Sigma, UK) .

Buffer for Monoclonal Antibodies

0.05% v/v Tween-20[®] (Sigma, UK) in TBS (TBST).

Buffer for Polyclonal Antibodies

0.1% v/v Tween-20[®] (Sigma, UK) in TBS (TBST).

Blocking Solution

5% w/v dried milk powder (Marvel) in TBST was used to block endogenous proteins.

Optimised Primary Antibody Concentrations:

β-Actin, mouse monoclonal antibody (Sigma, UK) dilution of 1:40,000

Bax, rabbit polyclonal antibody (Santa Cruz, Insight Biotechnology, UK) dilution of 1:8,000

Cyclin A, mouse monoclonal antibody (Santa Cruz, Insight Biotechnology, UK) concentration of 2 µg/ml.

Phospho-tyrosine¹⁵ specific CDK1, rabbit polyclonal antibody (Calbiochem, Novabiochem, UK) dilution of 1:1,000.

Survivin monoclonal antibody (Santa Cruz, Insight Biotechnology, UK) dilution of 1:5,000.

Aurora B polyclonal antibody.

Primary (and Secondary) Antibody Incubation Solution

1% w/v dried milk powder (Marvel) in TBST .

Optimised Secondary Antibody Concentrations

Horse Radish Peroxidase (HRP) conjugated Goat anti Mouse (Santa Cruz, Insight Biotechnology, UK) dilution of 1:2,000. HRP conjugated Goat anti Rabbit (Santa Cruz, Insight Biotechnology, UK) dilution of 1:3,000.

9.1.15 Reagents for the phospho-tyrosine staining of western blots:

Blocking Solution

5% w/v BSA (Sigma, UK) in 0.1% v/v Tween-20[®] (Sigma, UK) in TBS (TBST).

Optimised Primary Antibody Concentration

Phospho-tyrosine (HRP conjugated) mouse monoclonal (Upstate Biotechnology, Insight Biotechnology, UK) concentration of 0.05 µg/ml.

Antibody Incubation Solution and Wash Buffer for Phospho-tyrosine Antibody

0.1% v/v Tween-20[®] (Sigma, UK) in TBS (TBST).

9.1.16 Reagents for the visualisation of protein bands:

ECL Reagent

Supersignal[™] CL-HRP Substrate System Kit (Pierce and Warriner, Perbio Science UK Ltd) solution A and solution B were mixed together in equal volumes immediately prior to use for detection.

Autoradiography Film

Hyperfilm[®] was purchased from Amersham Pharmacia Biotech, UK.

9.1.17 Reagents for the immunoprecipitation of CDK1 and c-ABL:

Antibody Class Specific Dyna Beads

1.5×10^8 Rat anti mouse IgG2a coated Dynabeads[®] (Dynal, UK) were coated with 15 μ g of CDK1 (cdc2 p34) mouse monoclonal IgG2a antibody. 1.5×10^8 Sheep anti mouse IgG1 Dynabeads[®] (Dynal, UK) were coated with 15 μ g of c-ABL mouse monoclonal IgG1 antibody.

Wash Buffer

0.1% w/v BSA (Sigma, UK) in PBS (Oxoid, UK).

9.1.18 Reagents and buffers for the functional assay of CDK1:

Kinase Assay Lysis Buffer (for the Immunoprecipitation of Functional Kinases)

50 mM Tris-HCl pH 7.4 (Sigma, UK)

250 mM Sodium Chloride (Sigma, UK)

1 mM EDTA (BDH, UK)

50 mM Sodium Fluoride (Sigma, UK)

1 mM DTT (Sigma, UK)

0.1% v/v Triton[®] X-100 (Sigma, UK)

4% v/v aprotinin (Trasylol 10,000 Kallikrein Inactivator Units (KIU)/mL Bayer UK)

1% v/v Sodium Orthovanadate (0.1M Stock Solution) (Sigma, UK)

0.5% v/v PMSF (0.1M Stock) (Sigma, UK)

Antibody Class Specific Dyna Beads:

1.5×10^8 Sheep anti mouse IgG1 Dynabeads[®] (Dynal, UK) were coated with 15 μ g of Cyclin B IgG1 mouse monoclonal antibody.

9.2 APPENDIX B. SUPPLIERS OF REAGENTS:

Reagents, chemicals and equipment used in this study were obtained from the following suppliers:

Alpha Laboratories. Parham Drive, Eastleigh, Hampshire.

Amersham Pharmacia Biotech Ltd. Amersham Place, Little Chalfont, Buckinghamshire.

Bayer Plc. Bayer House, Strawberry Hill, Newbury, Berkshire.

BDH Chemical Ltd. Poole, Dorset.

BD Biosciences pharmingen. Europe.

Bioquote Ltd. The Raylor Centre, York.

Boehringer Mannheim UK Ltd. Bell Lane, East Sussex.

Bristol Myers. Lancaster Place, South Marston Park, Swindon, Wiltshire.

Britannia Pharmaceuticals Ltd. Redhill, Surrey.

Calbiochem/Novabiochem (CN Biosciences). Padge Road, Beeston, Nottingham.

Digital Scientific Ltd. Sheraton House, Castle Park Cambridge.

Dynal Biotech UK. Croft Business Park, Bromborough, Wirral.

Evans Medical Ltd. Langhurst, Horsham, West Sussex.

Fisher Scientific UK Ltd. Bishop Meadow Road, Loughborough, Leicestershire.

Flowgen. Novara House, Excelsior Road, Ashby de la Zouch, Leicestershire.

Gibco Lifetechnologies Ltd. Fountain Drive, Inchinnan Business Park, Paisley.

Hayman Ltd. Eastway Industrial Park, Witham, Essex.

Insight Biotechnology Ltd. Wembley Middlesex.

Oxoid, Unipath Ltd. Bassingstoke, Hampshire.

Perbio Science UK Ltd. Upper Northgate Street, Chester.

Promega UK Ltd. Delta House, Chilworth Research Centre, Southampton.

Shandon Scientific Ltd UK. Chadwick Road, Astmoor Runcorn Cheshire.

Sigma-Aldrich Chemicals. Gillingham, Dorset.

Vector Laboratories Ltd. Accent Park, Bakewell Road, Orton Southgate,
Peterborough.