

Alterations in Cell Cycle and Apoptosis in Drug Resistant Cells

A thesis submitted for the degree of Ph.D.

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

The effects the anthracycline Doxorubicin exerts on variants of the human lung carcinoma cell line DLKP was investigated in this thesis, in an attempt to understand the means by which the chemotherapeutic agent exerts its cytotoxic and cytostatic effects on human lung tumours. Sensitive DLKP-SQ and resistant variants DLKP-SQ/A250 10p#7, DLKP-A2B and DLKP-A5F were exposed to equitoxic doses of Doxorubicin, which resulted in approximately 50% cell kill after 72 hours. The subsequent effects on the cell cycle and apoptosis induction were studied in an attempt to identify any differences between sensitive and resistant cells, that may contribute to the resistant state.

Doxorubicin treatment induced a permanent G2/M arrest in sensitive cells by 24 hours, and a temporary arrest in resistant variants at an earlier time of 12-16 hours with a subsequent bypass of the G2/M arrest to re-emerge in and accumulate in G1. The transient G2/M arrest and subsequent progression into G1 indicated insufficient checkpoint monitoring of DNA damage induced by doxorubicin treatment in the resistant variants. The G2/M checkpoint controller complex cyclin B-cdk1, was investigated in an attempt to explain the G2/M over-ride seen in drug resistant cells. Doxorubicin treatment did not alter cyclin B or cdk1 protein levels, or the complex-forming ability of cdk1. The G2/M arrest seen in sensitive cells may be due to an increase in inhibitory phosphorylation on cdk1. In contrast Tyr15 phosphorylation did not change in resistant variants and cdk1 kinase activity seems to increase following drug treatment, unlike the sensitive cells which displayed little alterations in kinase activity upon drug treatment. Further investigation of the mechanism of G2/M override in the resistant variants involved examination of the cdk1 regulators wee1 kinase and cdc25 phosphatase. Initial studies on these regulators raised some mechanistic possibilities of G2/M override.

Apoptosis occurs in each of the four cell lines after Doxorubicin treatment, with kinetics of cell death dependent on the concentration of Doxorubicin and exposure time used. The mechanism of apoptosis induction in the cell lines was investigated. Analysis of procaspase expression revealed that caspase-3 is greatly downregulated in resistant cell lines, and caspase-8 is reduced to a lesser extent. Levels of procaspases did not decrease during apoptosis, indicating lack of caspase activation in general, and very low caspase-3 activation occurs during doxorubicin-induced apoptosis, raising doubts about caspase-3 involvement in the apoptotic pathway. The caspase-3 substrate PARP was not cleaved in any of the cell lines during apoptosis, although the cytoskeletal protein fodrin was cleaved in all four cell lines. Furthermore the caspase inhibitors zVAD-fmk, YVAD-fmk and DEVD-fmk which readily cross cell membranes, failed to protect the cells from doxorubicin-induced apoptosis, raising the possibility of caspase

redundancy or a caspase-independent mechanism in these cells. Involvement of the Fas-mediated pathway was also investigated, and resistant variants do not express altered levels of CD95 receptor compared to sensitive cells. When the cells were treated with inhibitors of the Fas signalling pathway, or with a combination of caspase inhibitors and CD95 inhibitors, they continued to die by apoptosis. The fact that cell permeable inhibitors of both caspases and CD95 signalling do not prevent doxorubicin-induced death lends further weight to the possible existence of a caspase-independent pathway.

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1.0 Introduction

1.0 Introduction

1.1 Chemotherapy in the treatment of cancer

Cancer is one of the major causes of death in the world today, with more than 100 cancer types occurring and 6 million new cases diagnosed every year. Approximately half of these cases results in death, and the remainder can be successfully treated by surgery, radiotherapy or chemotherapy.

The main aim of chemotherapy is to eliminate all cancer cells whilst inflicting as little damage as possible on the surrounding normal cells; maximum tumour cell kill accompanied with minimum normal cell kill. Since the commencement of chemotherapy in 1962, advances and breakthroughs in cancer research and drug design have led to the discovery of structurally and functionally diverse chemotherapeutic drugs such as anthracyclines, vinca alkaloids, epipodophyllotoxins, antimetabolites and covalent DNA binding drugs. These drugs can specifically target some of the alterations only seen in tumour cells, and can interfere with DNA, RNA and protein synthesis. However, the failure of tumours to respond to chemotherapy is a significant problem in the clinic; a cancer may respond well to initial treatment but after a time become resistant to further therapy.

1.1.1 Anthracyclines

Anthracyclines are popularly used in cancer chemotherapy especially doxorubicin (Adriamycin) and daunorubicin (Daunomycin). Their principal mode of action is through binding to DNA and intercalating between the paired bases in DNA. In doing this, they form a tight drug-binding complex which results in cytotoxicity. The anthracyclines bind to double stranded DNA, causing the DNA to uncoil at the site of binding and inhibit the action of topoisomerase II, an enzyme involved in the control of DNA supercoiling. Anthracyclines are also involved in the formation of reactive oxygen species which induce DNA strand breaks. Doxorubicin is cytotoxic to both exponentially growing and lag phase cells, although cells in the exponential phase are

more sensitive to adriamycin, probably due to increased DNA synthesis. Barlogie *et al.* (1976) revealed that cells in the S phase of the cell cycle are most sensitive to doxorubicin treatment perhaps due to the fact that the DNA is uncoiled at this phase for DNA replication.

Doxorubicin is used in chemotherapy of a broad range of cancers including breast, bladder, ovaries, lung, thyroid and stomach carcinoma, as well as bone and soft tissue sarcomas. Daunorubicin is principally used in the treatment of acute myelogenous leukaemia. Anthracyclines are administered intravenously, and exhibit two-phase pharmacokinetics, Doxorubicin has an initial half-life ($\alpha t_{1/2}$) of 10 minutes followed by a slow decline over 30 hours ($\beta t_{1/2}$). Daunorubicin has half-lives of 40 minutes ($\alpha t_{1/2}$) and 50 hours ($\beta t_{1/2}$). However anthracycline treatment is associated with serious side effects, the principle being cardiotoxic effects (Gaudin *et al.*, 1993; Hutchins *et al.*, 1993) and is believed to be due to the generation of reactive oxygen species (Pratt *et al.*, 1994). Anthracyclines are also mutagenic, carcinogenic and cause immunosuppression.

1.1.2 Epipodophyllotoxins

Podophyllotoxin is produced by the plant *podophyllum peltatum* (American mandrake) and is a mitotic inhibitor that acts by binding to tubulin, blocking polymerisation of tubulin thereby preventing the formation of microtubules (Long 1992). Semisynthetic derivatives of podophyllotoxin including etoposide (VP-16) and teniposide (VM-26) have been used in the treatment of human cancers such as testicular, lymphoma, leukaemia, and Kaposi's sarcoma (Van Maanen *et al.*, 1988a, 1988b).

Topoisomerase II is the major cellular target of these drugs in human cancers (Ross *et al.*, 1984). The drug acts by stabilising the complex between topoisomerase II and DNA, which causes an interference in the activity of topoisomerase II resulting in DNA double strand breaks and cytotoxicity. The main side effects of epipodophyllotoxins is bone marrow depression, which limits their dosage. VP-16

and VM-26 are usually administered intravenously and have a terminal half-life of approximately 4-8 hours, with 30-50% of VP-16 recovered as unchanged drug in urine (Pratt *et al.*, 1994).

1.1.3 Vinca Alkaloids

Vinca alkaloids are isolated from the periwinkle plant *catharanthus rosea*, and include vincristine and vinblastine. These drugs are commonly used in the treatment of acute leukaemia, Hodgkin's and non-Hodgkin's lymphoma, small-cell lung cancer, wilm's tumour, neuroblastoma, rhabdomyosarcoma and Ewings sarcoma (Pratt, 1994). Complete remission has been reported in 80-90% of acute leukaemias treated with vinca alkaloids (Pratt, 1994). Vinca alkaloids exhibit a relatively low toxicity to bone marrow cells, and are often used in combination with other chemotherapeutics.

The cytotoxic mechanism of action is through binding to microtubules, and disruption of the balance between polymerisation and depolymerisation of microtubules. These drugs are usually administered as bolus intravenous injection, and vinblastine has a terminal half life of approximately 24 hours, whereas vincristine has a terminal half-life of about 85 hours. The most common side effect of vinca alkaloid treatment is peripheral neuropathy, although vinblastine has less neurotoxic side effects than vincristine. Another relatively new, semi-synthetic vinca alkaloid is vinorelbine, which is commonly used as a single agent in the initial treatment of non small cell lung cancer (NSCLC). The incidence of neurotoxicity associated with vinorelbine is significantly lower than that observed with other vinca alkaloids (Hohneker, 1994).

1.1.4 Taxanes

Taxol was originally derived from the bark of the pacific yew tree (*Taxus brevifolia*), but semisynthetic derivatives had now been developed. This class of drugs is very effective in the treatment of metastatic breast cancer, refractory ovarian cancer, ovarian cancer, NSCLC and squamous cell carcinoma (Pratt, 1994). Neutropenia is the most common side effect of taxol treatment. Taxanes act by stabilising

microtubules, and induce formation of microtubule bundles, with minimal effects on the synthesis of DNA, RNA and protein.

1.1.5 Pyrimidine Analogues

Pyrimidine analogues are part of the antimetabolite family of drugs, which are structurally similar to existing compounds involved in cellular metabolic pathways. They induce cytotoxicity by interfering with production of nucleic acids by substituting themselves for purines or pyrimidines in the anabolic nucleotide pathways. For example 5-fluorouracil is a pyrimidine antagonist that is widely used in chemotherapy of the most commonly occurring types of solid tumours including colorectal, breast, head and neck, gastric and pancreatic cancers (Pratt, 1994). 5-fluorouracil is usually administered as a continuous infusion, and the most common side effects are gastrointestinal toxicity and bone marrow depression.

1.2 Chemotherapy Resistance

A recurrent problem in the treatment of cancer with antitumour drugs is the development of drug resistance. It is a major obstacle to successful chemotherapy. Over the past 25 years, extensive research has led to an understanding of some of the mechanisms responsible for drug resistance. Distinct biochemical and genetic alterations have been identified as key elements in the developments of drug resistance, and these resistance mechanisms can interplay with each other, further complicating the ability to overcome drug resistance. A cell may be intrinsically resistant (before drug treatment) or acquire resistance mechanisms after drug treatment. Many mechanisms of resistance have been identified such as development of drug efflux pumps (Pgp; MRP), alterations in expression of enzymes such as topoisomerases, and the drug detoxifying enzymes, glutathione-S-transferases (Bellamy, 1996).

1.2.1 P-glycoprotein

By far the best characterised mechanism of resistance is that involving p-glycoprotein. Pgp was originally identified as a 170 kDa protein overexpressed in drug resistant cells (Juliano and Ling, 1976). Pgp is a membrane-associated, energy-dependent efflux pump encoded by the MDR1 gene, which is present on chromosome 7q21 (Bosch and Croop, 1998). It is a member of the ATP-binding cassette (ABC) transporter family. ABC transporters widely occur in many organisms such as bacteria, yeast, plants, insects, animals and humans (Ames *et al.* 1992) and serve as transporters across membranes in diverse functions such as nutrient uptake, ion transport, cell signaling and extrusion of toxins. Pgp is normally found in adrenal glands, kidney, liver, colon and rectum tissue in humans indicating that it has roles other than drug efflux. There are two classes of Pgps, class I includes the drug-transporting p-glycoproteins and class II are those not involved in drug resistance.

The treatment of human cell lines with chemotherapeutic drug results in the upregulation of Pgp mRNA and protein (Mickley *et al.* 1989; Mazzanti *et al.* 1996), and Pgp is expressed in a wide variety of tumours that exhibit multiple drug resistance (MDR). An MDR cell has the ability to develop cross-resistance to a broad range of structurally and functionally-unrelated drugs. Overexpression of Pgp in tumour cells can confer cross resistance to such drugs as anthracyclines, epipodophyllotoxins, vinca alkaloids, Taxol and actinomycin D (Germann and Chambers, 1998). High levels of Pgp expression have also been detected in many intrinsically resistant tumours. Overexpression of Pgp is associated with decreased cellular uptake of a drug, and allow cells to survive in the presence of higher drug concentrations.

1.2.2 MRP

Multidrug resistance-associated protein (MRP) is another member of the ABC transporter superfamily and like Pgp is involved in conferring an MDR phenotype to tumour cells (Cole *et al.* 1998). Quite often non-Pgp mediated resistance is due to

the expression of MRP, and it has been identified in non-Pgp MDR from a variety of human cell types such as leukaemia, breast, non-small cell lung, small cell lung, cervix, prostate and bladder carcinomas. Cells can also co-express both Pgp and MRP, but the individual contributions from each protein is unknown. Several homologues of MRP exist in humans including cMOAT (multispecific organic anion transporter)/MRP2, hMRP3, hMRP4 and hMRP5 (Kool *et al.* 1997; Kool *et al.* 1999a, 1999b). These proteins are thought to play a role in the detoxification process, and MRP is thought to be a clinically relevant marker of drug resistance.

MDR can also develop as a result of an alternative resistance mechanism that prevents cytotoxic drugs from reaching their cellular targets, and one such mechanism involves lung resistance-related protein (LRP). LRP is a human major vault protein (Scheper *et al.*, 1993) and may play a role in MDR. Overexpression of LRP has been found in many drug resistant cells, and could serve as a clinically relevant marker of drug resistance *in vivo* (Izquierdo *et al.*, 1998).

1.3 Cellular effects of chemotherapy

1.3.1 Cell cycle effects

DNA damage is a consequence of chemotherapy in tumour cells, and this results in the cell shutting down its normal activities until DNA repair is completed. Many chemotherapy drugs affect the cell cycle by causing it to arrest either in G1/S or G2/M phases. Certain drugs target a specific phase in the cell cycle, for example etoposide induces a G2/M arrest by reducing the activity of cdk1 kinase, the main controller of mitotic entry (Lock and Ross, 1990a). Doxorubicin also induces a G2/M arrest, and at very high doses it can induce a G1 arrest (Barlogie *et al.*, 1976).

Understanding the mechanisms regulating cell cycle progression and checkpoint activation in response to drug treatment is important for elucidating pathways that regulate normal growth, and also to understand how genetic defects identified in tumour cells can deregulate the cell cycle. Furthermore, differences in the response of normal and cancer cells to DNA damaging agents could lead to improvements in chemotherapy or design of novel drugs specific for defects in cell cycle control mechanisms of tumour cells.

1.3.2 Apoptosis induction

The majority of chemotherapy drugs eliminate cells by interfering with normal cellular metabolism and triggering apoptosis (Reed, 1997; Toft and Arends, 1997). Depending on the intensity of insult to the cell, either apoptosis or necrosis can take place. Apoptosis usually occurs in response to low or moderate damage, but necrosis, an unregulated catastrophic mode of cell death, occurs when the cell is intensely damaged or stressed. The fact that apoptosis occurs in response to chemotherapeutic drug treatment indicates the presence of a genetically controlled cell death pathway in cells, that can be triggered, and allows an organism to eliminate damaged cells in an orderly fashion.

Resistance to undergo chemotherapy-induced apoptosis represents another problem for successful chemotherapy. Elucidation of the apoptotic pathway and signalling cascades induced by drugs will hopefully allow the resistance to be overcome, and result in design of drugs that can specifically target and activate the apoptotic pathway in tumour cells.

1.3.3 Role of p53 in cellular response to chemotherapy

p53 tumour suppressor is a potent inhibitor of cellular proliferation (Lane, 1992; 1993). The primary role of p53 is in maintaining genetic stability through elimination of cells with DNA damage from the viable cell population by cell cycle arrest or apoptosis. p53 is a critical mediator of a number of cellular responses to DNA damage including cell cycle arrest in G1 phase and induction of apoptosis.

In some cells, DNA damage can lead to an arrest in the G1 phase of the cell cycle (Pellegata *et al.* 1996). This phenomenon is largely co-ordinated through p53 accumulation and activation, which induces p21 transcription (El-Deiry *et al.* 1993; Dulic *et al.* 1994; Agarwal *et al.* 1995). p53 acts as a sensor of DNA damage, and can induce cell cycle arrest before entry to S phase where replication of damaged DNA would be lethal for the cell. p21 actively inhibits entry to S phase of the cell cycle, and is further discussed in section 1.4.6. The G2 checkpoint, however seems to be independent of p53; cells containing mutated or null p53 do not arrest in G1, but still arrest in G2 suggesting a separate checkpoint in G2 (Kastan *et al.* 1992; Kuerbitz *et al.* 1992; Han *et al.* 1995). p53 also plays a role in apoptosis induction in the presence of DNA damage induced by irradiation and anticancer drugs (Lowe *et al.* 1993; Allday *et al.* 1995). It acts as an activator of apoptosis when DNA is damaged, however cells lacking p53 can still undergo apoptosis following irradiation and chemotherapy (Han *et al.* 1995), so the G2 checkpoint is thought to control apoptosis induction. However, the Bcl-2 family also play a critical role in induction of apoptosis, and this is further discussed in section 1.5.9.

1.4 The Eukaryotic Cell cycle

1.4.1 Introduction

The animal cell cycle is a regulated process consisting of a round of DNA replication called S phase, followed by cell division or mitosis (M) in which the replicated chromosomes are segregated to form two identical daughter nuclei. Both these events are closely monitored to ensure that the S and M phases will occur in the correct sequence, so that M will not occur before S has completed and vice versa. Several control mechanisms have evolved to maintain the ordered sequence and prevent these events from occurring at inappropriate stages of the cell cycle. The cell cycle is outlined in figure 1.1. Gap phases termed G1 and G2 are present between M and S, and between S and M, respectively. These phases act as preparatory periods during which the cell can prepare for entry to S or M phases. There is also a resting phase termed G₀, in which cells can remain for an indefinite period of time, during which cells become quiescent, but can re-enter the cell cycle at any time when conditions become favourable for growth and division (Pardee, 1989).

The cell integrates signals from the environment into the cell cycle, and depending on whether conditions are sub-optimal or favourable for growth, the cell can either grow and divide or remain quiescent. Therefore the cell requires a stringent regulatory mechanism to control entry to the cell cycle and progression through the various phases. Today's understanding of the cell cycle comes from extensive studies in the budding yeast *S. cerevisiae*, fission yeast *S. pombe*, *Xenopus* eggs and more recently, mammalian cell systems.

The main controllers of the cell cycle are protein kinase complexes each consisting of a cyclin and a cyclin-dependent kinase (cdk). Nine cyclins have been identified to date, named in order of their discovery, cyclin-A, -B, -C, -D, -E, -F, -G, -H and I (Lew *et al.*, 1991; Xiong *et al.*, 1991; Koff *et al.*, 1991; Bai *et al.*, 1994; Bates *et al.*, 1996; Nakamura *et al.*, 1995) and these are the regulatory and activating subunits of the complex. Cyclins were first identified in *Xenopus* eggs as proteins whose levels oscillated through the cell cycle and dropped after every mitosis (Evans *et al.*, 1983),

suggesting that they might be proteins that control progress through the cell cycle and whose activity is regulated by the relative amount present. Cyclins share a 150 amino acid region of structural homology called the cyclin box (Hunt *et al.*, 1995), and this is required for binding to cdks.

Cyclins have been classified as G1 or G2 cyclins depending on which phase they are involved in. The G1 cyclins including cyclin-C, -D and -E, are short-lived proteins that are degraded soon after their activity has peaked. This degradation is facilitated by the presence of a PEST region in the C-terminus of these cyclins (Deshaies *et al.*, 1995). PEST is a sequence of four amino acids which confers instability to proteins by targeting it to the ubiquitination system of proteolysis. The G2 cyclins including cyclin-A and -B, are stable throughout interphase and are specifically degraded at mitosis. Degradation occurs due to the presence of a destruction box in the N-terminus of the protein; again, proteolysis occurs via ubiquitination (Glutzer *et al.*, 1991). Cyclin F is the largest of the cyclins; 100kDa and is also thought to be involved in cell cycle coordination throughout the cell cycle (Bai *et al.*, 1994). Cyclin-G1 and -G2 are unique in that they do not have a destruction box, or a destabilising PEST region, and expression increases after DNA damage, suggesting a role in negative regulation of the cell cycle (Bates *et al.*, 1996; Horne *et al.*, 1997). It has also been suggested that cyclin I may play a role in negatively regulating the cell cycle (Horne *et al.*, 1997) although no definitive evidence exists to date.

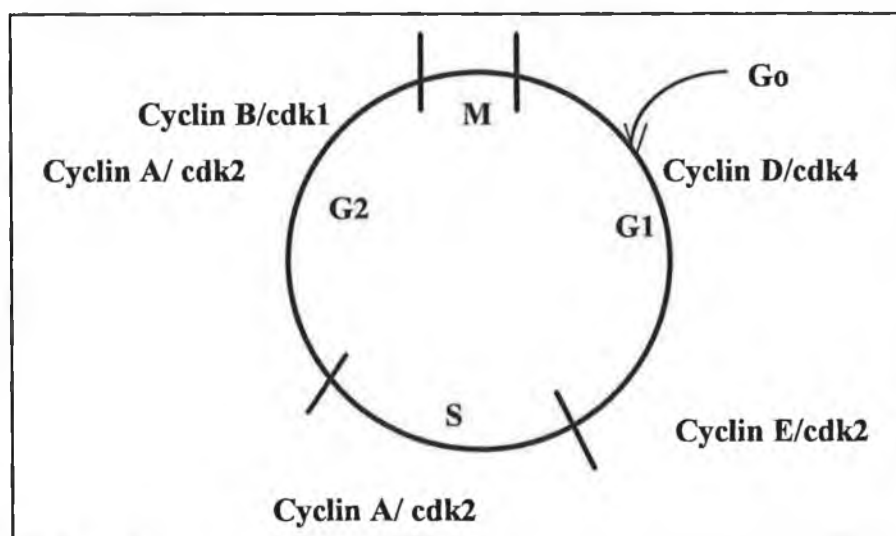


Figure 1.1 Mammalian Cell Cycle

Cdks are the catalytic subunit of the cyclin-cdk complex and require association with a cyclin to attain peak kinase activity. Cdks were originally identified in budding yeast and called cdc (cell division cycle) proteins (Hartwell, 1970; Hartwell, 1991) on the basis that when these proteins were mutated, yeast cell division was inhibited. Eight cdks have been identified in mammalian cells and all share structural similarities including a PSTAIR motif which is thought to bind to the cyclin box. Each cdk interacts with a specific subset of cyclins, and these interactions are listed in table 1.1.

CDK	CYCLIN PARTNER	ACTIVITY PHASE
cdk1 (cdc2)	cyclin A, -B	G2, G2/M
cdk2	cyclin A, -E	G1, S
cdk4	cyclin D	entry to G1
cdk5	cyclin D	entry to G1
cdk6	cyclin D	entry to G1
cdk7	cyclin H	activating cdks in all phases
cdk8	cyclin C	possible role in regulation of RNA polymerase

Table 1.1 cdks and their cyclin binding partners

1.4.2 Activation of cyclin-cdk complexes

Cyclin levels vary throughout the cell cycle, and the fluctuations in levels control the activation of inactive cdks which are present in constant levels in the cell. Once the cyclin-cdk complex has formed, it becomes a substrate for a number of kinases which either positively or negatively regulate its activity, as outlined in figure 1.2. Cdks are regulated by a series of complicated mechanisms (reviewed by Morgan, 1995, Lees, 1995). Activation of a cdk occurs when cyclin-cdk complex is phosphorylated on a conserved threonine residue, Thr161 on cdk1, Thr160 on cdk2 (De Bondt, 1993, Morgan and De Bondt, 1994, Russo *et al.*, 1996). This activating phosphorylation is carried out by cyclin activating kinase (CAK) which is composed of cyclin H and cdk7 (Fesquet *et al.*, 1993; Fisher and Morgan, 1994). Cyclin H and cdk7 also bind to a third protein to form a ternary complex, and this protein was identified as MAT1 (menage-à-trois 1) and is thought to act as an assembly factor for the cyclin and cdk (Devault *et al.*, 1995). The assembly factor in some way promotes binding of the cyclin and cdk and activates the cdk.

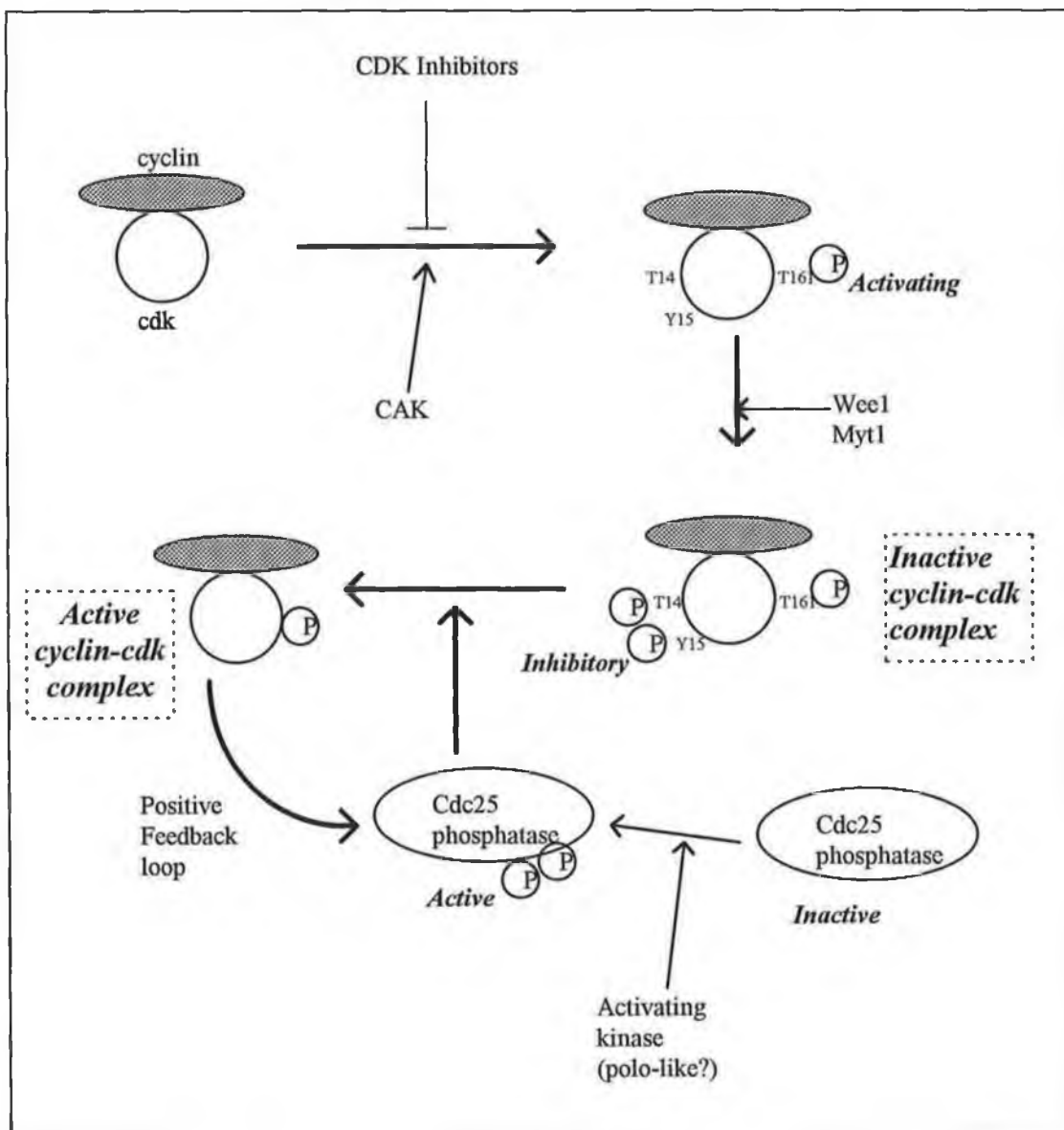


Figure 1.2 Regulation of cyclin-cdk complexes

1.4.3 Inhibition of cyclin-cdk complexes

Inhibitory phosphorylations occur on the cdk near the ATP binding site (see figure 1.2). Thr14 and Tyr15 are conserved phosphorylation sites in cdk1 and cdk2, and the presence of phosphates on these residues inhibits kinase activity, perhaps through interference with ATP binding. Wee1 HU (originally identified in *S. pombe*) is responsible for phosphorylation on Tyr15 (Parker *et al.*, 1992, McGowan and Russell, 1995, Watanabe *et al.*, 1995, Blasina *et al.*, 1997) and the human Myt1 can phosphorylate both Thr 14 and Tyr 15 (Mueller *et al.*, 1995, Liu *et al.*, 1997, Booher *et al.*, 1997), although Booher *et al.* (1997) revealed that Myt1 only phosphorylates cdk1-containing complexes. The wee1 kinase plays an important role in mitotic control which will be discussed in section 1.4.7. The inhibitory phosphates are removed by a dual specificity phosphatase cdc25 (Lee *et al.*, 1992) resulting in dephosphorylation and activation of the cyclin-cdk complex (see figure 1.2).

Three cdc25 homologues exist in humans, cdc25A, cdc25B and cdc25C. cdc25A is involved in activation of cyclin E-cdk2 (Hoffmann and Karsenti, 1994), cdc25C dephosphorylates and activates cyclin B-cdk1 (Hoffmann *et al.*, 1993), and cdc25B is thought to play a role in activating cyclinB-cdk1 also. All cdc25 phosphatases must be phosphorylated themselves in order to become active and a positive feedback mechanism had been identified, through which active cyclinB-cdk1 can activate further cdc25C. Cdks are also inhibited by the actions of a family of proteins called cell cycle inhibitors and will be discussed in section 1.4.6.

1.4.4 Entry to cell cycle and G1 progression

Cells in culture entering G1 require serum mitogens continuously for a period of time, after which they become serum independent. The point at which they switch from being serum-dependent to serum-independent is called the Restriction point (Pardee *et al.*, 1974), and at this point the cell commits itself to continue through the cell cycle.

The main cyclin-cdk complex involved in entry to G1 is cyclin D-cdk4. Cyclin D proteins are the rate limiting controllers of G1 phase progression, are induced by mitogens in G1 and synthesis throughout the cell cycle depends on persistent growth factor stimulation (Matsushime *et al.*, 1991a, 1991b, 1992). If growth factors are removed before S phase entry D-type cyclins are rapidly degraded and the cell fails to enter S phase. However, if growth factors are removed after the G1/S transition, cell division can still proceed. So cyclin D is most important in mid to late G1 when cells need mitogens to pass the restriction point. Cyclin D proteins therefore are mitogen-induced genes which act as growth factor sensors, linking the external environment to the cell cycle machinery. Cyclin D binds mainly to cdk4/cdk6 and kinase activity increases during mid G1 phase, peaking as the cells approach G1/S boundary (Matsushime *et al.*, 1994, Meyerson and Harlow, 1994). The retinoblastoma protein is one of the principle substrates of cyclin D-cdk4 (Kato *et al.*, 1993; Dowdy *et al.*, 1993).

The main role of the retinoblastoma protein (pRb) is to act as a signal transducer linking the cell cycle to the transcriptional controls in the cell. It is a key protein in the transition between cellular proliferation and growth inhibition, a molecular switch that can suppress expression of genes needed for the progression of the cell cycle (For review, see Weinberg, 1995). The phosphorylation state is the primary control mechanism of pRb, and phosphorylation levels vary during the cell cycle (De Caprio *et al.*, 1989,1992). When hypophosphorylated (mainly in G1), pRb acts as a growth suppressor and binds to and negatively regulates the activity of transcription factors such as E2F, p107, and p130. These transcription factors play a critical role in progression into S phase and have consensus binding sites in the promoter regions of

many genes required for S phase. When bound to E2F, pRb inhibits its function as a transactivator, thereby preventing induction of genes whose expression is required for cell cycle progression. However upon phosphorylation on specific sites, a conformational change occurs in pRb, and its growth suppressive effect on E2F is removed, releasing the transcription factor to activate genes required for G1/S transition and S phase, such as c-myc, dihydrofolate reductase, cyclin E, cyclin A and cdk1. The process of cyclin D-cdk4 mediated release of E2F is outlined in figure 1.3.

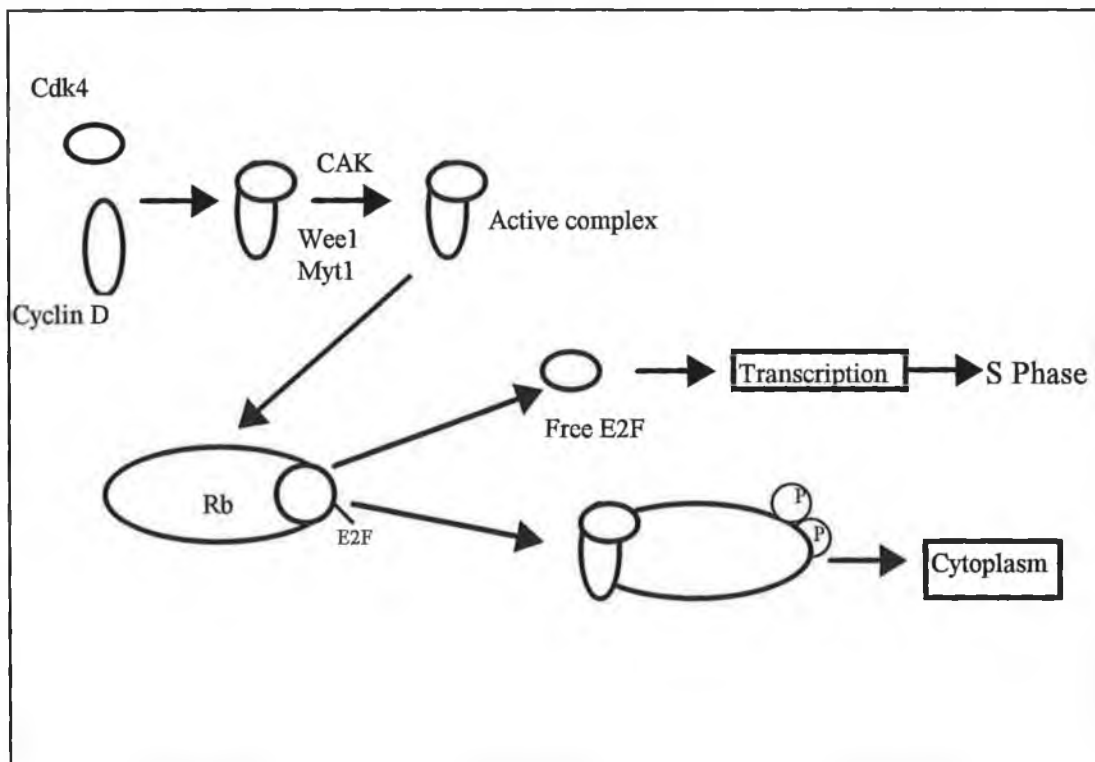


Figure 1.3 Effect of cyclin D-cdk4 on pRb

1.4.5 Entry to S phase

After induction of cyclin D, expression of cyclin E increases in late G1 and binds to cdk2, thereby resulting in an accumulation of cyclin E-cdk2 complex levels (Dulic *et al.*, 1992), and cyclin E-cdk2 activity peaks at the G1/S transition (Koff *et al.*, 1992, 1993). Cells that fail to form active cyclin E-cdk2 complexes cannot pass the G1/S transition confirming its role in control of entry to S phase (Koff *et al.*, 1993, Resnitzky and Reed, 1995). Once cells enter S phase, cyclin E is rapidly degraded resulting in the destruction of the cyclin E-cdk2 complex. However cdk2 can now form a complex with cyclin A, therefore cdk2 has two periods of oscillating activity, and it acts on different substrates depending on which cyclin it binds to.

Cyclin A-cdk2 activity is essential for initiation of S phase. Cyclin A levels begin to rise at the end of G1 phase, and cyclin A-cdk2 activity peaks at the onset of S phase. In early S-phase cyclin A-cdk2 binds directly to, and phosphorylates E2F, and in doing so inhibits its DNA binding ability, thereby inactivating E2F and turning off the G1/S phase genes that are no longer required once the DNA replication has begun. (Krek *et al.*, 1995, Xu *et al.*, 1994). Cyclin A also binds to cdk1 during G2 phase (Pagano *et al.*, 1992), and is required during the G2/M transition, so cyclin A can play a role in two cell cycle phases depending on which cdk it binds to.

Cardoso *et al.* (1993) demonstrated that cyclin A co-localises with sites of DNA replication during S phase, suggesting that it may directly participate in the assembly, activation or regulation of replication structures. In fact, accumulating evidence implicates cyclin A-cdk2 as a negative regulator of DNA replication. Before initiation of replication, a number of proteins such as origin recognition complex (ORC) proteins, cdc6 and mini-chromosome maintenance (MCM) proteins form preinitiation complexes at replication origins. Piatti *et al.* (1996) revealed that an absence of cdk2 kinase activity in G1 allows assembly of these proteins required for DNA replication. Low levels of cyclin A-cdk2 kinase activity during G1/S allows initiation of DNA replication, and once replication begins at the onset of S phase, the cdk2 kinase activity increases. High levels of cyclin A-cdk2 activity during S phase then negatively regulates DNA replication, by preventing association of MCM proteins

with chromatin (Hua *et al.*, 1997). This inhibitory activity serves to limit DNA replication to a single round per cell cycle. Hua and Newport (1998) also discovered that the cdc6 protein in the preinitiation complex is targeted by cdk2 to be degraded, and this is a key step in the initiation of DNA replication, because failure to remove cdc6 results in inhibition of DNA replication.

Recently the replication activity of DNA polymerase was investigated during the cell cycle in the presence of cyclin E-cdk2 and cyclin A-cdk2 (Voitleitner *et al.*, 1999). At peak activity in late G1, cyclin E-cdk2 phosphorylates DNA polymerase and stimulates its activity, thus initiating DNA replication and onset of S phase. However cyclin A-cdk2 phosphorylates DNA polymerase during S phase and reduces its ability to initiate DNA replication *in vitro*. This study confirms the roles of both cyclin E and cyclin A in the initiation and progression of S phase, showing that cyclin E-cdk2 is necessary for initiation of S phase, and once DNA replication has begun, cyclin A-cdk2 ensures that it cannot be initiated again thus preventing re-replication.

1.4.6 Cell Cycle inhibitors

In addition to inhibition by phosphorylation, cyclin-cdk complexes are inhibited and regulated by a group of proteins called cdk inhibitors (CKI). In animal cells two families of CKIs namely the Cip family and the INK4 family, have been identified based on protein sequence similarity, mechanism of inhibition and specificity. The Cip family (cdk interacting proteins) is comprised of p21/CIP1/WAF1 (Xiong *et al.*, 1993), p27/KIP1 (Toyoshima *et al.*, 1994) and p57/KIP2 (Matsuoka *et al.*, 1995), and they preferentially inhibit cdks of the G1/S phase (Harper *et al.*, 1995, Sherr, 1995). p21 is the most extensively studied of this family (Gartel *et al.*, 1996, review). It can bind cdk2 and cdk4 in complex with cyclins and inhibit the phosphorylation of pRb and prevent cell cycle progression (Harper *et al.*, 1993). It is under the control of p53, and after DNA damage, p53 can induce p21 transcription, resulting in a cell cycle arrest. p21 exists in a quaternary complex with a cyclin, cdk and proliferating cell nuclear antigen (PCNA), a DNA replication factor (Flores-Rozas *et al.*, 1994). By binding to PCNA p21 can inhibit DNA replication (Waga *et al.*, 1994). The stoichiometry of p21 molecules in a cyclin-cdk complex governs its ability to inhibit the cyclin-cdk activity, and multiple molecules are required for cdk inhibition. In fact, complexes containing one molecule of p21 are still active (Zhang *et al.*, 1994, Harper *et al.*, 1995).

p27 levels are elevated in terminally differentiated cells, and play a role in G1 checkpoint. It is responsible for blocking cells in G1 phase in response to TGF β treatment by potently inhibiting cyclin E-cdk2 (Polyak *et al.*, 1994). Even though p27 levels are high in quiescent cells, they decrease rapidly when the cells enter the cell cycle in the presence of mitogens. If mitogens are removed, p27 levels rise again to inhibit cell cycle progression in conditions that are unfavourable for growth. In cells lacking p27, serum deprivation does not prevent cells from entering G1 (Kato *et al.*, 1994).

The INK4 (inhibitor of cdk4) family consists of p15, p16, p18 and p19. As the name suggests these are selective for cdk4 inhibition, and can inhibit monomeric cdk4 as well as cdk4 complexed with a cyclin (Hannon and Beach, 1994, Guan *et al.*, 1994,

Hirai *et al.*, 1995). p15, p16, p18, p19, p21 and p27 have been shown to block phosphorylation of pRb by inhibiting cyclin D-cdk4 activity thus reducing E2F release and S phase entry (Xiong *et al.*, 1993, Hannon and Beach, 1994, Toyoshima *et al.*, 1994, Hirai *et al.*, 1995, Lukas *et al.*, 1995). Therefore the activity of these inhibitors is critical in the timing and progression of S phase and proliferation, and any defects may lead to deregulated cell cycle control and unrestrained proliferation. Some of these defects will be discussed later.

1.4.7 Entry to Mitosis

An understanding of the G2-M transition derived from genetic studies in a number of organisms such as fission yeast (*S. pombe*) and *Xenopus* eggs (Nurse, 1990, Norbury *et al.*, 1991). The key component of the mitotic pathway in cells is a mitotic regulator comprised of cyclin B (Pines and Hunter, 1989) and the kinase catalytic subunit cdc2/cdk1 (Draetta and Beach, 1988) which form mitosis-promoting factor (MPF) (Matthews and Huebner, 1985, Lee and Nurse, 1988).

MPF, once active can exert its effect on many cellular substrates to effectively and rapidly induce cytoskeletal rearrangement and cytokinesis. Indeed, the active kinase is intimately involved with reorganisation of the structural features of the cell during mitosis. Cyclin B-cdk1 causes dramatic changes in the microtubule network, the actin microfilaments and the nuclear lamina. The nuclear lamina is a structure that underlies the nuclear membrane during interphase, and is composed of lamin subunits. Lamins are hyperphosphorylated at mitosis, promoting their disassembly (Nigg *et al.*, 1992). Proteins in the intermediate filament network such as vimentin and desmin are also phosphorylated by cdk1, promoting their dismantling at mitosis (Chou *et al.*, 1990). Another significant substrate is caldesmon, which is a component of cytoplasmic filaments and binds actin and calmodulin (Yamashiro *et al.*, 1991). Histone H1 when phosphorylated by cyclin B-cdk1, possibly plays a role in chromosome condensation during mitosis (Langan *et al.*, 1989).

MPF is associated with a kinase activity that varies through the cell cycle, peaking at M phase and rapidly declining at the end of mitosis. Cyclin B levels accumulate gradually from G1, and upon reaching a threshold level it binds to cdk1 (Pines and Hunter, 1989). However the cyclin B-cdk1 complex is not active until modified by phosphorylations resulting in an abrupt increase in cdk1 kinase activity, promoting onset of mitosis. The switch that controls the transition between inactive cyclin B-cdk1 and active cyclin B-cdk1 is controlled by a number of phosphorylations as earlier described. Cdk1 must be phosphorylated on Thr161 by CAK for activity, and inhibitory phosphates occur on Thr14 by Myt1 and on Tyr15 through the action of the mitotic inhibitor weel (Parker and Piwnica-Worms, 1992). Wee1 was identified

as a protein kinase that is required for delay in entry to mitosis (Russell and Nurse, 1987, Featherstone and Russell, 1991). Wee1 is itself negatively regulated through phosphorylation by nim1 kinase. Cdk activity occurs through dephosphorylation of Thr14 and Tyr15 by the cdc25C phosphatase (Strausfeld *et al.*, 1991). These inhibitory phosphates play a critical role in timing of entry to mitosis, and cells that have mutated cdk1 which cannot be phosphorylated on Thr14 and Tyr15 fail to pause before entry to mitosis (Krek and Nigg, 1991, Norbury *et al.*, 1991).

1.4.8 Regulation of Mitotic cdk controllers

Regulation of wee1 kinase and cdc25 phosphatase activities is one of the key determining factors in the timing of mitotic progression. Cdc25 is regulated through several complex pathways. Both cdc25B and cdc25C are involved in entry to mitosis, and show different patterns of activation during the cell cycle indicating that they have different roles to play in the activation of cyclin B-cdk1. Cdc25B activity appears in late S phase and peaks in G2 (Lammer *et al.*, 1998). A rise in cdc25C activity was demonstrated by Izumi *et al.* (1992) as cells entered mitosis, and decreased upon exit from mitosis. The rise in cdc25C activity during mitosis is due to phosphorylation (Doree *et al.*, 1989, Hunt, 1989, Solomon *et al.*, 1990) and active cyclin B-cdk1 is capable of phosphorylating and directly activating cdc25C (Hoffmann *et al.*, 1993). This suggests that there is a positive feedback mechanism in which a small amount of already active cyclin B-cdk1 can activate cdc25C, and this in turn dephosphorylates cdk1 resulting in a rapid activation of cyclin B-cdk1. If phosphorylation sites in cdc25C are mutated, its mitosis promoting activity is reduced (Izumi and Maller, 1993) thus supporting the idea of a role for cdc25C activation in progression through mitosis. However, the question remains as to what the initial activator of cyclin B-cdk1 is, in order for the feedback loop to commence. Cdc25B is a good candidate for this, during G2 it is translocated into the cytoplasm, and cytoplasmic cyclin B-cdk1 is an excellent substrate for cdc25B during late G2. Cdc25B could then dephosphorylate cyclin B-cdk1 in the cytoplasm, promoting translocation of the active cyclin-cdk complex to the nucleus where it could phosphorylate and activate cdc25C thus initiating the feedback loop. However

dephosphorylation of cdk1 by cdc25B does not result in maximal cdk activity, but perhaps sufficient to phosphorylate cdc25C. Lammer *et al.* (1998) found that depletion of cdc25B prevented entry to mitosis in HeLa cells thus confirming a role for cdc25B in mitotic progression.

Cdc25 proteins can also be controlled by another family of proteins called polo-like kinases (Kumagi and Dunphy, 1996). In *Xenopus*, the polo-like kinase Plx can phosphorylate and activate cdc25C, and depletion of Plx suppresses cdc25C phosphorylation and cyclin B-cdk1 activation (Abrieu *et al.*, 1998).

1.4.9 Localisation control in mitosis

Many of the controller and effector proteins involved in mitosis are further regulated by their subcellular compartmentalisation during interphase and mitosis. Cyclin B has perhaps the most extensive restrictions. Three forms of cyclin B exist in different areas of the cell during the cell cycle. Cyclin B1-cdk1 is located in the cytoplasm with microtubules during interphase, due to the presence of a cytoplasmic retention signal (CRS) in cyclin B1 (Pines and Hunter, 1991, Heald *et al.*, 1993, Jackman *et al.*, 1995) but is rapidly translocated to the nucleus at the onset of mitosis. Cyclin B2-cdk1 complex is found in the Golgi apparatus, apparently serving to phosphorylate and induce golgi fragmentation during mitosis (Lowe *et al.*, 1998). Cyclin B3-cdk1 is predominantly found in the nucleus throughout the cell cycle, and studies in *Drosophila* suggest that it may not be required for mitosis (Jacobs *et al.*, 1998).

Wee1 protein kinase has nuclear location until the onset of mitosis at which time it is transported to the cytoplasm, thus removing it from the nucleus where it has an inhibitory role, allowing cdk1 activity to initiate mitosis (Baldin and Ducommun, 1995, Heald *et al.*, 1993). Cdc25C is also regulated by subcellular localisation. Cdc25C is found in the nucleus during interphase and early mitosis (Miller *et al.*, 1991). Cyclin B-cdk1 is localised to the cytoplasm during interphase (Pines and Hunter, 1991, Gallant and Nigg, 1991) and only enters the nucleus at the onset of mitosis, so by spatial separation cdc25C cannot be activated and consequently cyclin

B-cdk1 remains inactive until the required time. Thus the spatial organisation and temporal activation or inhibition of the MPF regulators is very important for the G2-M transition, onset of mitosis and cytokinesis.

1.4.10 Chemotherapy Drugs and G2/M Arrest

Treatment of cells with agents that cause DNA damage often leads to a delay or arrest in G2 of the cell cycle. G2/M arrest has been reported in cells after treatment with different drugs such as doxorubicin (Barlogie *et al.*, 1976, Krishan *et al.*, 1976, Kimler *et al.*, 1978), rubidazole, etoposide and melphalan (Barlogie *et al.*, 1978), also mitomycin C, bleomycin, hydroxyurea and lucanthone (Kimler *et al.*, 1978). This delay is enforced by altered regulation of the G2/M controllers, mainly cyclin B-cdk1 kinase activity. Alterations in the controllers of this kinase results in diminished activity and prevention of entry to mitosis, for example failure to dephosphorylate the inhibitory phosphates from cdk1 results in an inactive cdk1 which enforce a G2 delay. A summary of the possible alterations occurring after drug treatment is outlined in figure 1.4.

During a G2/M arrest there is altered regulation of cyclin B and cdk1 (Tsao *et al.*, 1992). Cyclin B protein levels were also found to be decreased after an ionising irradiation-induced G2/M arrest (Muschel *et al.*, 1991, Datta *et al.*, 1992), however no changes in cdk1 or cdc25 protein levels were reported, so the regulation of the proteins must be other than at a translational level (Datta *et al.*, 1992). Preliminary studies by Lock and Ross (1990, 1991) demonstrated a decrease and inhibition of cdk1 kinase activity during a G2 arrest after etoposide treatment. They consequently demonstrated that the inhibition of kinase activity after etoposide treatment was due to a failure to dephosphorylate the activating tyrosine residue (Lock, 1992), even though cyclin B-cdk1 complex forming ability is not affected (Lock and Keeling, 1993). More recently Ling *et al.* (1996) studied the effect of Doxorubicin on P388 cells and found inhibition of cdk1 activity due to absence of dephosphorylation on Tyr15. Other chemotherapy drugs such as cisplatin (Nishio *et al.*, 1993), camptothecin (Tsao *et al.*, 1992), nitrogen mustard (O'Connor *et al.*, 1992), 5-

fluorouracil (Okamoto *et al.*, 1996) and nucleoside analogues (Halloran *et al.*, 1998) have been shown to induce a G2/M arrest, induced by a failure to dephosphorylate Tyr15 of cdk1 resulting in a reduced cdk1 kinase activity. In addition, Wee1 activity is reportedly disrupted during a G2 arrest (Leach *et al.*, 1998). Cdc25C activation has been shown to decrease after irradiation, and reduction of cdc25C phosphatase activity results in increased inhibitory phosphorylation of cdk1 in cells after treatment with DNA damaging agents. These findings are summarised in figure 1.4.

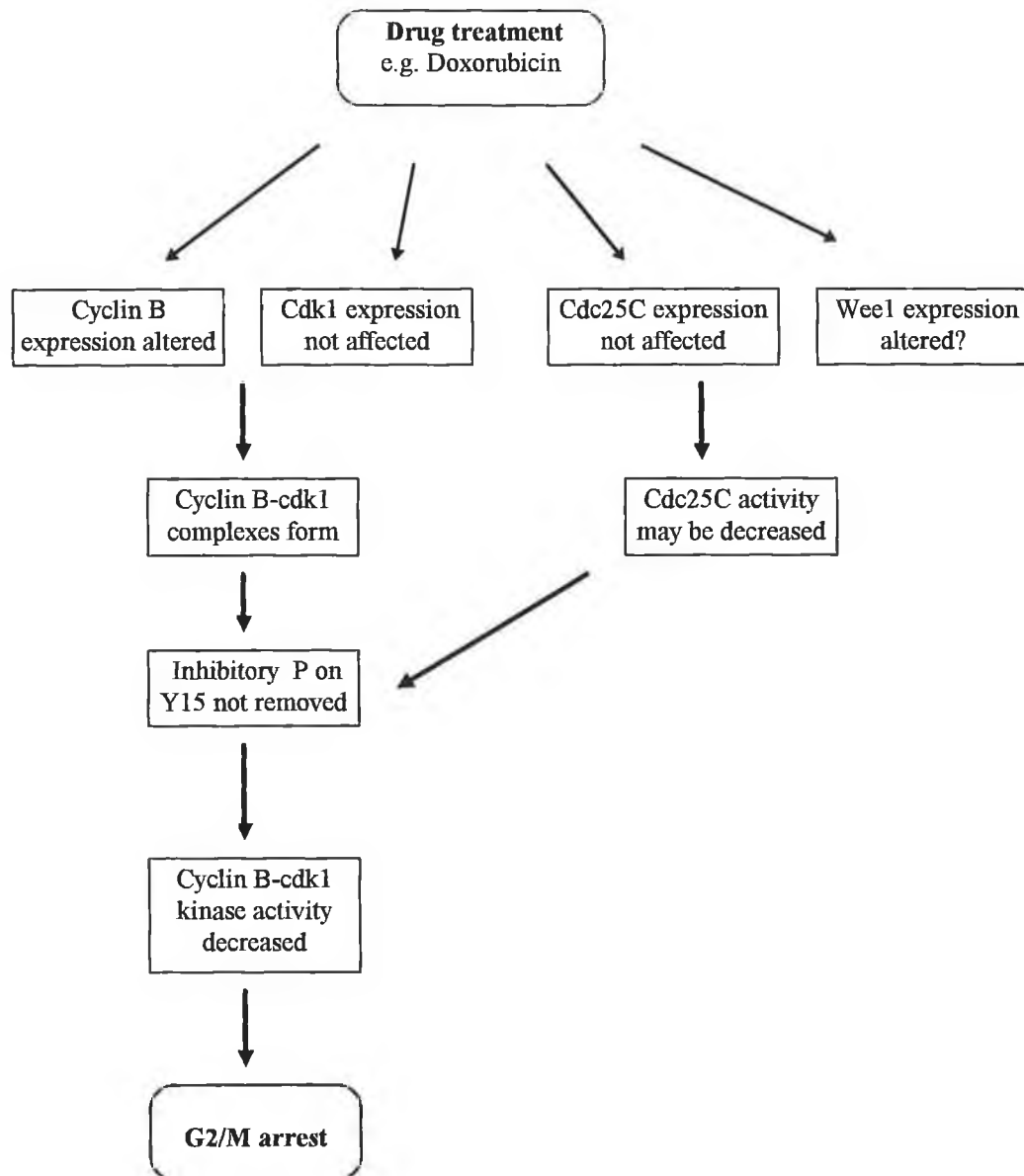


Figure 1.4 Summary of cellular effects of drug treatment resulting in G2/M arrest

1.4.11 DNA Damage Checkpoints

The fact that cells have the ability to arrest after experiencing damage to their DNA led to the discovery of cell cycle checkpoints. Checkpoints exist in the cell cycle to act as regulatory surveillance mechanisms to control the order and timing of the critical events of DNA replication in S phase and chromosome segregation at mitosis. Therefore the checkpoint response plays a significant part in maintaining genomic stability throughout the cell cycle, by monitoring the state of the DNA, and arresting cell cycle progression in response to DNA damage or inhibition of DNA replication (Elledge, 1996).

1.4.12 G2/M Checkpoint pathway in response to DNA damage

A family of DNA damage sensor proteins, called Rad proteins, exist in fission yeast (*S. pombe*) which are required for checkpoints to occur (Carr, 1997). Human homologues of these DNA damage sensor proteins have been identified demonstrating an evolutionary conserved checkpoint pathway (Review, Weinert, 1998). When DNA damage occurs, checkpoint proteins such as Rad24 bind to damaged DNA (Garvik *et al.*, 1995) which results in phosphorylation of Rad9 (Emili *et al.*, 1998, Sun *et al.*, 1998). Rad9 is essential for a checkpoint to occur, and is thought to phosphorylate another Rad family member, Rad53, also essential for cell cycle arrest in response to DNA damage (Walworth, 1998, Sun *et al.*, 1998). This process is illustrated in figure 1.5. Until recently the exact mechanism of interaction between checkpoint proteins and the cell cycle was unknown, but it has been suggested that in some way they could arrest the cell cycle through indirect inhibition of cdk1 kinase.

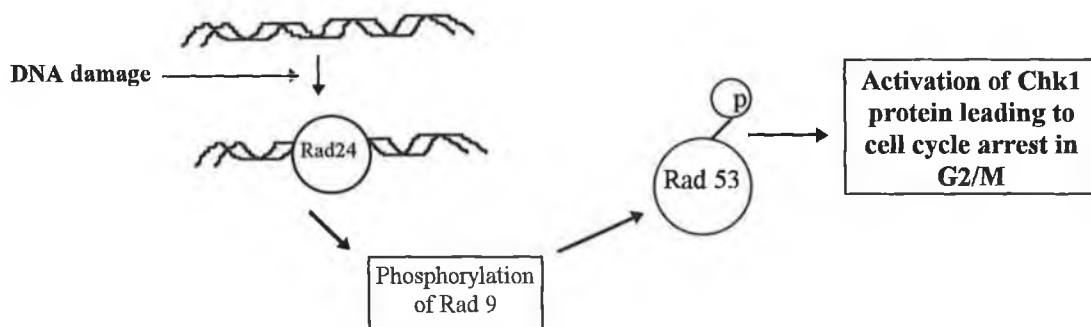


Figure 1.5 DNA damage-induced G2/M Checkpoint

The link between the DNA damage sensor proteins and the cell cycle emerged from the discovery that human cdc25C is phosphorylated on a specific residue in response to DNA damage (Peng *et al.*, 1997). Phosphorylation is carried out by Chk1 protein kinase, a homologue of the *S. pombe* Chk1 which is required for the DNA damage checkpoint. In response to DNA damage Chk1 is activated, presumably by the Rad 3 and other Rad proteins (Walworth *et al.*, 1996). When Chk1 is active, it phosphorylates Cdc25C on ser216, and induces a conformational change which creates a binding site for 14-3-3 proteins (Furnari *et al.*, 1997, Sanchez *et al.*, 1997) resulting in the inhibition of cdc25C's ability to dephosphorylate and activate cdk1. Therefore the key event in the pathway leading to G2/M arrest is the phosphorylation of cdc25C by Chk1 (see figure 1.6).

However the phosphorylation of cdc25C by Chk1, and binding of 14-3-3 protein to cdc25C does not reduce the enzymatic activity of cdc25C, so another regulatory mechanism must exist, perhaps involving limiting substrate availability. Cdc25C is cytoplasmic during interphase, and enters the nucleus at G2 to initiate M. Chk1 is nuclear, and when cdc25C is phosphorylated by Chk1 in the nucleus in response to DNA damage, a nuclear 14-3-3 protein binds (Lopez-Girona *et al.*, 1999). Rad24 is a 14-3-3 protein important in the DNA damage checkpoint pathway in fission yeast (*S. pombe*), and it is a sufficiently small protein that can passively diffuse in and out of the nucleus. It contains a hydrophobic region called a nuclear export signal (NES) which exports it from the nucleus. Lopez-Girona *et al.* (1999) recently demonstrated that Rad24 binds to phosphorylated cdc25C after DNA damage and escorts it from the nucleus, thus separating cdc25 from its substrate cyclin B-cdk1.

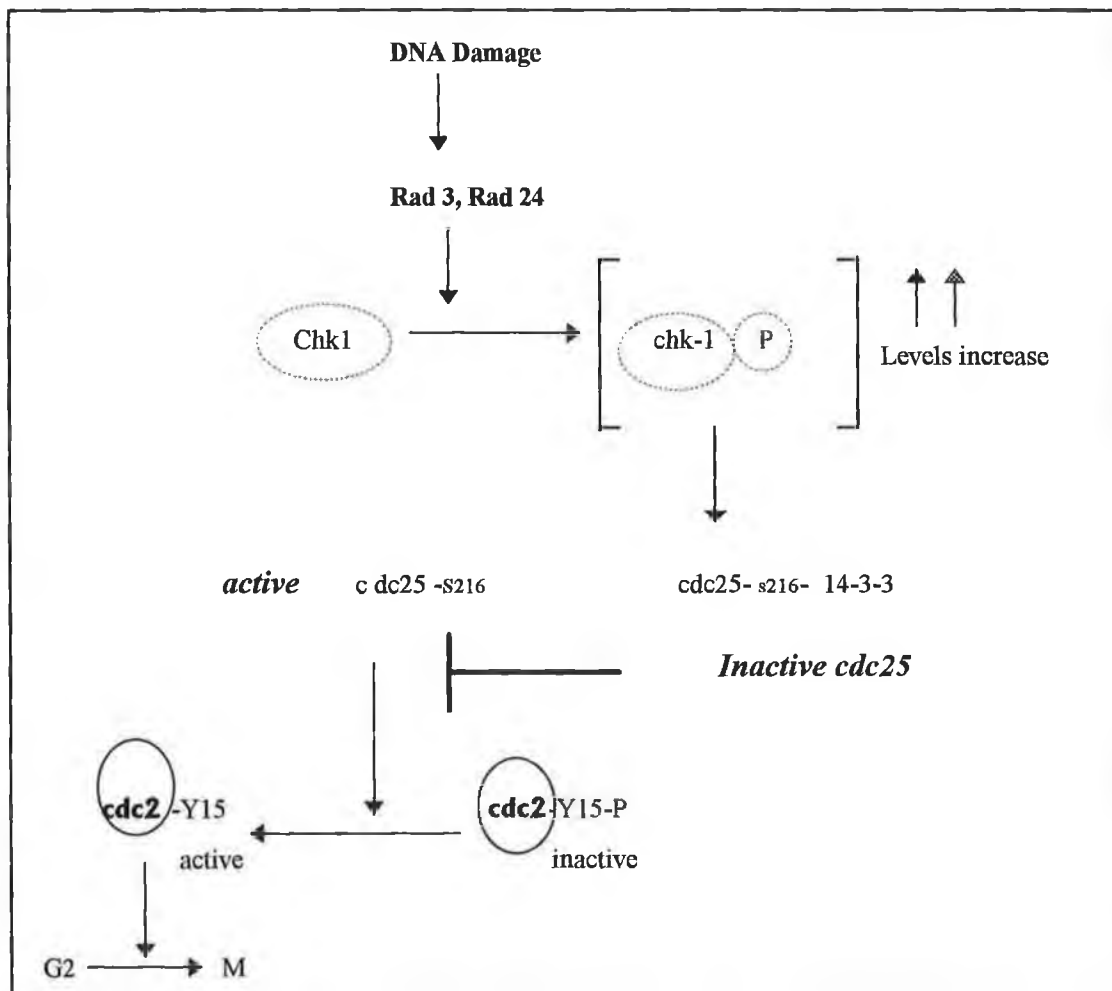


Figure 1.6 G2/M Checkpoint in response to DNA damage

Another mechanism regulating G2 arrest following DNA damage involves the cellular localisation of cyclin B. Smeets *et al.* (1994) demonstrated that cyclin B was cytoplasmic in cells arrested after DNA damage and the restriction of cyclin B to the cytoplasm contributed to the G2/M arrest (Jin *et al.*, 1996). Jin *et al.* (1998) reported that expression of cells with a mutant cyclin B which remains in the nucleus throughout the cell cycle results in cells that can override a G2/M arrest after DNA damage, thus confirming that cyclin B localisation assists the G2/M arrest after DNA damage. Consistent with these findings, Toyoshima *et al.* (1998) reported that cells expressing mutant nuclear cyclin B fail to exhibit a G2 delay following DNA damage. Recently Innocente *et al.* (1999) established that p53 arrests the cell cycle in G2 by

lowering intracellular levels of cyclin B and attenuating the activity of the cyclin B1 promoter. Taken together, these results suggest that maintenance of Thr14 and Tyr15 phosphorylation of cdk1 and nuclear export of cdk1-cyclin B are the two primary mechanisms through which eukaryotic cells achieve a G2 arrest following DNA damage.

1.4.13 Cell Cycle and Cancer

To maintain the stability of their genome, proliferating cells normally arrest in either G1/S or G2/M in response to cellular damage, and the presence of these checkpoints provides the cell with additional time for repair processes to remove any potentially lethal damage before DNA is replicated or segregated. A wealth of evidence supports the idea that loss of checkpoint control can lead to genomic instability, inappropriate survival of genetically damaged cells and the evolution of cells to a malignant state (Kaufmann *et al.*, 1995; Paules *et al.*, 1995).

Dysregulation of cell proliferation is a frequent event in tumorigenesis, and this unconstrained proliferation can be a result of gain or loss of function of the proteins that control the cell cycle. Many of the cell cycle proteins associated with G1- S transition in the cell cycle are altered in some way leading to a destabilisation of the normal growth control pathway and unrestrained proliferation. Moreover the majority of tumours have mutations in one or another of the genes involved in controlling progression throughout the G1 phase of the cell cycle. Malignant cells acquire independence from the mitogenic signals that are normally required for cell cycle progression. Cells lacking p53 fail to arrest in G1 or G2 after DNA damage, demonstrating that lack of p53 can result in defective checkpoint control and enhance the genomic instability and genomic rearrangements (Kastan *et al.*, 1991, Hartwell, 1992).

Cyclin D1 acts as a positive regulator of G1 by removing the growth suppressive function of Rb. Any defect in the action of cyclin D such as overexpression will lead to Rb being constantly hyperphosphorylated thus allowing free E2F to induce

transcription of S phase genes. Cyclin D1 has been mapped to the 11q13 locus close to the BCL1 region, which is frequently amplified in many tumours (see review, Motokura and Arnold, 1994). Overexpression of cyclin D1 is associated with poor prognosis of patients with breast cancer (Borg *et al.* 1991; Schuurin *et al.*, 1992; Michalides *et al.*, 1996, van Diest *et al.*, 1997) and human pancreatic carcinoma (Gansauge *et al.*, 1997). Antisense cyclin D1 can decrease cyclin D1 levels and prevent tumorigenicity in human colon carcinoma cell lines (Arber *et al.*, 1997).

p16 is frequently mutated, deleted or inactivated through methylation in many different types of human cancers, suggesting its role as a tumour suppressor. Deletions and mutations of chromosome 9p21, the location of p16, occurs very frequently in pancreatic, head and neck, non-small cell lung, bladder and ovarian carcinomas (Hall and Peters, 1996), and perhaps is the most common genetic alteration in human lymphoblastic leukemia (Kamb *et al.*, 1994, Hussussian *et al.*, 1994, Quesnel *et al.*, 1994). Furthermore, p16-null mice spontaneously develop aggressive tumours within 6 months (Serrano *et al.*, 1996). Cdk4 overexpression or mutation can be detected in various tumour types including glioblastoma and melanoma (He *et al.*, 1994, Bartkova *et al.*, 1996). There is also a possibility that cells may acquire resistance through altered expression of cell cycle regulatory genes (Hocchauser, 1997)

The G2 checkpoint is also implicated in tumorigenesis. Attenuation or inactivation of the G2 checkpoint can result in cells with increased genomic instability, and also promotes immortalisation in normal human fibroblasts (Kaufmann *et al.*, 1995). Paules *et al.* (1995) investigated the G2 checkpoint control in familial cancer syndromes and concluded that these cells have a defective G2 checkpoint which may have contributed to development of tumorigenesis.

1.5 Apoptosis

1.5.1 Introduction

Apoptosis is a genetically regulated biological process that is fundamental to the development of organisms and to homeostasis of tissues (Kerr *et al.*, 1972). Various stimuli can trigger diverse regulatory pathways which activate a conserved execution machinery. This results in dramatic structural changes such as cytoplasmic shrinkage and distortion, membrane blebbing and formation of membrane-bound cell fragments called apoptotic bodies. These changes are accompanied by chromatin condensation, a cytosolic increase in Ca^{2+} , decrease in cellular pH and DNA cleavage and fragmentation (Cobb *et al.*, 1996, Umansky (review), 1996).

All nucleated animal cells constitutively express the entire complement of proteins required to undergo programmed cell death (Tamaoki and Nakano, 1990). Apoptosis serves many functions in animal development such as sculpting structures; eliminating cells between developing digits, controlling cell numbers and deleting unwanted structures, such as female reproductive organs in males. Any abnormal, misplaced or harmful cells are also eliminated by apoptosis (Jacobson *et al.*, 1997).

The best characterised cell death pathway to date is in *Caenorhabditis elegans*. This roundworm is 1mm in length and during the course of its development, 131 out of 1090 cells are genetically programmed to die. Using genetics and loss-of-function/gain-of-function experiments, 13 genes involved in programmed cell death have been discovered which have helped to elucidate the apoptotic pathway in humans. These are summarised in table 1.2.

GENE	FUNCTION	HUMAN HOMOLOGUE
ces-1	prevents neuronal cell death	not known
ces-2	negatively regulates ces-1	HLF (hepatic leukemic factor)
egl-1	induces apoptosis	BH-3 domain proteins
ced-3	promotes apoptosis	Caspases
ced-4	in combination with ced-3, promotes apoptosis	Apaf-1
ced-9	prevents apoptosis	Bcl-2
ced-1	engulfment of cell corpses	ABC transporter (ced-7)
ced-6		
ced-7		
ced-2	engulfment of cell corpses and migration of distal-tip cells	DOCK180 (ced-5)
ced-5		
ced-10		
nuc-1	degradation of DNA	caspase-activated nuclease (CAD) (putative)

Table 1.2 Genes involved in the apoptotic pathway in *C. elegans*

Although this overall pathway is poorly understood, it is now accepted that caspases play a key role in the regulation of this biochemical process. Caspases comprise a family of cysteine proteases which have similar cleavage specificity in that they cleave their substrates after an aspartate residue. They are synthesised as a inactive precursor, which must be proteolytically cleaved at specific aspartate residues to become active. They can be cleaved themselves (autoprocessing) or by other enzymes.

1.5.2 Discovery of ICE

Initial studies on *Caenorhabditis elegans* revealed a ced-3 gene, which is essential for cell death to occur in the worm (along with ced-4). This ced-3 gene encoded a

protein, which has significant homology to the human interleukin-1 β -converting enzyme (ICE). ICE is a cysteine protease that cleaves pro-interleukin-1 β to generate the active form. ICE, synthesised as a 45 kDa proenzyme, is then cleaved into two subunits of 20kDa and 10kDa, the active enzyme consisting of two 20kDa and two 10kDa subunits.

Overexpression of genes encoding ICE induces apoptosis in mammalian fibroblasts (Miura *et al.* 1993) suggesting a role for ICE in the apoptotic pathway. However ICE- knockout mice seem to develop normally and appear healthy, thus questioning the role of ICE in apoptosis. In addition Kuida *et al.* (1995) found ICE-null mice to be resistant to killing by Fas-mediated apoptosis, while retaining their sensitivity to most other forms of apoptotic induction. Currently the only known substrate of ICE is pro-IL-1 β , this raised the possibility that additional proteases were involved in the apoptotic process.

Subsequent research led to the discovery of ICE-like proteases based on their structural homology to ICE and CED-3. Identification and studies of ICE-like proteases were carried out in a number of ways; gene-knockout studies to look at the effect of absence of a protease gene on apoptosis, transfection studies to look at the effect of overexpression of a protease gene on apoptosis and finally protease inhibitor studies to look at the effect of inhibiting a protease.

Since the discovery of the similarity between CED-3 and caspase-1 (ICE), a large family of proteases have been identified comprising of 14 (to date) caspases, and are listed in table 1.3. All identified caspases are structurally related, share amino acid homology and have an essential cysteine residue in their pentapeptide active site; QACxG.

CASPASE	OTHER NAMES	PROPOSED FUNCTION
caspase-1	ICE	n/a
caspase-2	Nedd-2 / Ich-1 _L	effector caspase
caspase-3	CPP32 / YAMA / apopain	effector caspase
caspase-4	ICE _{reIII} / Tx / Ich-2	development
caspase-5	ICE _{reIII}	inflammation
caspase-6	Mch2	effector caspase
caspase-7	Mch3 / ICE-LAP-3	effector caspase
caspase-8	MACH/FLICE/Mch5	initiator caspase
caspase-9	ICE-LAP6/Mch6	initiator caspase
caspase-10	Mch4	initiator caspase
caspase-11	Ich-3	inflammation
caspase-12	--	-----
caspase-13	ERICE	-----
caspase-14	MICE	-----

Table 1.3 Caspases involved in the apoptotic pathway

Caspases are expressed as inactive proenzymes and range in size from 20 to 50 kDa. The proenzyme is cleaved after a specific Asp residue to form two active subunits of 20 kDa and 10 kDa, although this can vary in size depending on the caspase in question. Caspases are composed of three domains, the prodomain at the NH₂-terminal, the large p20 subunit and the small p10 subunit. See Figure 1.7.

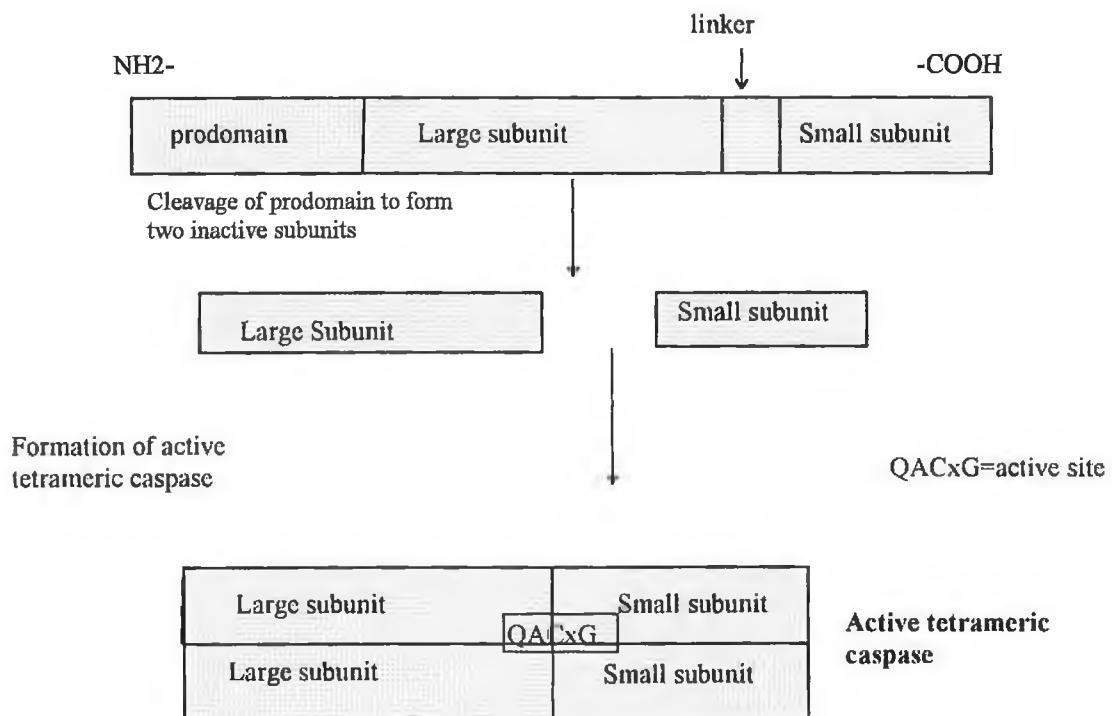


Figure 1.7 Activation of caspases

Activation of caspases involves proteolytic processing of the procaspase resulting in cleavage of the prodomain and two subunits. These subunits then heterodimerise and bind to another heterodimer of the same caspase to form a tetramer (see figure 1.7). A tetrameric formation is essential for catalytic activity of the complex and substrate binding (Walker *et al.*, 1994; Wilson *et al.*, 1994).

A linker domain sometimes separates these subunits. The function of the linker domain is as yet unknown but may play a role in caspase regulation. The prodomain is highly variable in length and sequence. Caspases with short prodomain include caspase-3, -6, and -7, whereas caspases with long prodomains include caspase-1, -2, -4, -5, -8, -9, and -10. It is thought that the main function of prodomains is in the regulation of the activation of procaspases. Van Creikinge *et al.* (1996) demonstrated

that the prodomain of caspase-1 (ICE) is absolutely required for dimerisation and autoactivation to occur, and that dimerisation occurs before activation.

1.5.3 Caspase Specificity

Caspases have a unique specificity in that they cleave directly after an aspartate residue. Caspases are among the most specific of proteases, with an absolute requirement for an aspartate residue in the P1 position of their substrate cleavage site. Therefore the sequence of amino acids at the N-terminal side of the cleavage site in the substrate is important, and four amino acids at the positions P1, P2, P3 and P4 directly influence the ability of a particular caspase to cleave a substrate. Sleath *et al.* (1990) originally established that ICE had a requirement for Asp in the P1 position, and all identified caspases share this phenomenon. The cleavage site in procaspases is located directly after the Asp residue, and from this evidence it was shown that caspases are involved in the cleavage of other caspases, and led to the emergence of the hierarchical theory. Based on the substrate specificity caspases can be grouped into three categories (Thornberry *et al.*, 1997; Talanian *et al.*, 1997). All caspases have stringent requirement for Asp in P1, the next most important residue is P4, and this is the determining factor for substrate specificity.

GROUP	SEQUENCE - P ₄ P ₃ P ₂ P ₁	MEMBERS
I	WEHD	Caspase-1, -4, -5
II	DExD	Caspase-2, -3, -7
III	L/V ExD	Caspase-6, -8, -9

Table 1.4 Sequence Specificity of caspases

Caspases have overlapping substrate specificities, and this may indicate a certain degree of redundancy. Group I caspases (caspase-1, -4, -5) can tolerate large aromatic

or hydrophobic amino acids in P4. Group II caspases (caspase-2, -3, -7) require an Asp residue in P4. Group III caspases (caspase-6, -8, -9) prefer amino acids with large aliphatic side chains but can tolerate many different amino acids in P4. P3 residue can be substituted generally without any major effects on enzymatic activity, except for caspase-9, which has an absolute requirement for His in this position. Glu is the preferred P2 residue for all caspases.

In addition to the above findings, Talanian *et al.* (1997) showed that caspase-2 requires an additional P5 residue in its substrates for maximum activity, but this can be one of a specific set of amino acids. The substrate specificity of caspase-6 is interesting because these preferred sequences are found in the activation site of caspase-3, indicating that caspase-6 may act upstream of caspase-3.

One other important finding by Thornberry *et al.* (1997) was that caspase-1, -2, -4, -5, -8 and -9 have long prodomains, and their substrate specificity sequences are very similar to their activating sequences, suggesting autocatalysis.

This finding of caspase substrate specificities led to an understanding of their biological functions. When the substrate specificity sequences of group III (-6, -8, -9) were compared to caspase amino acid sequences, it was noted that these specific sequences were found in the activation sites of both caspase-3, and -7, indicating that group III caspases were upstream components which could amplify an apoptotic signal in the caspase cascade. Caspase-3 and -7 must therefore be downstream effectors.

1.5.4 Caspase Inhibitors

The cowpox virus protein CrmA is a potent inhibitor of ICE. CrmA is a serpin (serine protease inhibitor) which contains a tetrapeptide sequence that ICE recognises as a cleavage site. It can therefore bind to ICE and prevent the active protease from its normal activity. CrmA expression can protect cells against apoptosis induced by many different agents (Gagliardini *et al.*, 1994, Tewari *et al.* 1995). Baculovirus p35

also inhibits ICE activity but has a much broader spectrum of activity (Bump *et al.*, 1995).

The fact that these proteins could suppress apoptosis by inhibiting ICE provided researchers with a very powerful tool with which they could further investigate the role of ICE-like proteases in apoptosis. This led to the development of synthetic inhibitors with specific target sequences to which the ICE-like proteases could bind and cleave.

As previously mentioned, there are three groups of caspases based on their substrate specificity. An important point to note is that the different families act on different substrates. Based on a specific substrate sequence, synthetic inhibitors can be designed to specifically inhibit a particular protease. All inhibitor peptides contain the aspartate residue, which is essential for cleavage. Caspases similar to ICE preferentially bind to YVAD, Y being hydrophobic, consequently ICE-specific inhibitor peptides contain a YVAD tetrapeptide. CPP32-like proteases must bind to a sequence with an anionic residue, usually another aspartate, DEVD, so CPP32-specific inhibitors utilise this sequence. This sequence is also seen in substrates such as PARP, U1-70kDa and DNA-dependent protein kinase. Depending on the group conjugated to the peptide, the inhibitor can be reversible or irreversible. Reversible inhibitors have nitriles, ketones or aldehydes conjugated to the peptide (e.g. Ac-VAD-CHO, Ac-YVAD-CHO), while irreversible inhibitors have thiomethylketones conjugated. Cell permeability can be achieved by addition of groups such as fluoromethylketones (e.g. DEVD.fmk).

Studies using such inhibitors have confirmed the involvement of various caspases in apoptosis. DEVD-CHO, the CPP32-specific inhibitor has been shown to inhibit apoptosis induced by Fas (Gamen *et al.*, 1996; Hasegawa *et al.*, 1996; Anel *et al.*, 1997) and by granzyme B (Anel *et al.*, 1997), therefore effectively inhibiting CPP32 activity and showing that caspase-3 has a dominant role in Fas- and granzyme B-mediated apoptosis. Similar studies have been carried out using the YVAD, the ICE-specific inhibitor (Shimizu *et al.* 1995, 1996) to investigate the possibility of the existence of an ICE cascade using ICE inhibitors YVAD and DEVD. When ICE is

specifically inhibited they found CPP32 to be also inactive, but when CPP32 was specifically inhibited, there was no effect on the activity of ICE. This suggests that ICE is active upstream of CPP32, and may participate in a protease cascade.

1.5.5 Proteolysis and caspase substrates

When apoptosis occurs in a cell it is accompanied by many ultrastructural changes, that are manifested as the prominent hallmarks of apoptosis - cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. The contribution of caspases to this process is not completely clear but there is a co-ordinated regulated hierarchical sequence of protein cleavage that leads to the eventual dismantling of the cell. Over 40 proteins have been identified which are cleaved during apoptosis, but so far only a small number of these have been shown to play a definite role in apoptosis. These proteins are modified in such a way as to alter their normal role and contribute to the induction of apoptosis. Caspases promote disassembly in a number of ways such as nuclear structural protein cleavage, cytoskeletal protein cleavage, cleavage of membrane transport proteins, and inactivation of protective proteins.

Lamin cleavage

Lamins are intermediate filament proteins responsible for the structural rigidity of the nuclear membrane. During apoptosis nuclear lamina collapse when lamins are cleaved allowing chromatin condensation to take place. Zhivotovsky *et al.* (1997) reported caspase involvement in the cleavage of lamins during apoptosis, but the caspase involved was not responsible for PARP cleavage. Caspase-6 and not caspase-3 is responsible for cleavage of lamin A and is the main laminase in the cell (Takahashi *et al.*, 1996; Orth *et al.*, 1996).

PARP cleavage

PARP is a 116kDa nuclear DNA repair enzyme, that is cleaved during apoptosis into a 98kDa and 25 kDa fragment, and is a widely used marker for onset of apoptosis. This cleavage may interfere with its role as a DNA repair enzyme. Tewari *et al.* (1995) and Nicholson *et al.* (1995) confirmed that PARP is specifically cleaved *in vitro* by

caspase-3, and this along with caspase-7 are the main caspases responsible for PARP cleavage (He *et al.*, 1998). The biological relevance of PARP cleavage is questionable however because PARP-deficient mice appear to develop normally (Wang *et al.*, 1995). PARP is now a readily accepted indicator of apoptosis, by monitoring the appearance of one or both of the cleavage products (for example see He *et al.*, 1998).

Fodrin Cleavage

Fodrin is an abundant membrane-associated cytoskeletal protein that is cleaved early during apoptosis, possibly allowing membrane blebbing and cell shrinkage to take place. Janicke *et al.* (1998) reported an absolute requirement for caspase-3 in fodrin cleavage during apoptosis in MCF-7 cells.

Gelsolin cleavage

Gelsolin is another important cytoskeletal protein cleaved during the onset of apoptosis. It is involved in Ca²⁺-dependent cleavage of actin filaments to form monomeric actin. However when gelsolin is cleaved during apoptosis, it cleaves the filamentous actin in a Ca²⁺-independent manner, and is thought to mediate morphological changes in apoptosis (Kothakota *et al.*, 1997).

Kinectin cleavage

An example of caspase-mediated inhibition of membrane transport during apoptosis is the cleavage of kinectin, which is a kinesin-binding integral membrane protein anchored in the endoplasmic reticulum. Kinesin is an ATP-driven motor protein involved in membrane trafficking pathways. During apoptosis kinectin is cleaved which in turn inhibits the kinesin activity (Machleidt *et al.*, 1998). Caspase-7 specifically cleaves kinectin, which is interesting given that the specificity profile for caspase-3 and -7 are almost indistinguishable.

Cleavage of I^{CAD}

DNA fragmentation is a critical event in the apoptotic process and is brought about through the activation of Dnases and nucleases which can cleave the DNA into fragments. The activation of Dnases has been found to be regulated by caspase activity. Caspases can cleave an inhibitor of Dnase, which then allows activation of Dnase. I^{CAD} (inhibitor of

CAD), also known as DNA fragmentation factor (DFF) is a subunit of CAD (Caspase Activating Dnase) and is cleaved during apoptosis in living cells. Intact I^{CAD} remains bound to CAD and inhibits Dnase activity. However, during apoptosis I^{CAD} is cleaved to form a 11kDa fragment, and cleavage results in the inhibitory actions being removed from CAD. Then CAD is free to activate Dnase and nucleases which bring about DNA fragmentation. Active caspase-3 is required for I^{CAD} cleavage and activation of the enzyme (Tang and Kidd, 1998). Liu *et al.* (1997) demonstrated that once caspase-3 cleaves I^{CAD} its activity is no longer required for DNA fragmentation to occur. This raises the possibility that caspase-3 mediated cleavage of I^{CAD} is the triggering factor for DNA fragmentation. CPAN (Caspase activated nuclease) is a homologue of I^{CAD} (Halenbeck *et al.*, 1998).

Altogether cleavage of these proteins results in the shutdown of many cellular activities like membrane trafficking, DNA repair, mRNA splicing and disruption of the cytoskeleton and nuclear membrane thus leading to the disassembly of the cell.

1.5.6 Apoptotic Execution pathway

Since all caspases cleave after an Asp residue, the notion that caspases could sequentially activate others arose, and was confirmed with evidence of the existence of a caspase cascade. Enari *et al.* (1996) demonstrated that Fas sequentially activates ICE-like and caspase-like proteases, and cpp32-like proteases act downstream together with a cytosolic component to induce apoptosis. Greidinger *et al.* (1996) also provided evidence of a sequence of caspase activation, which was accompanied by a sequence of substrate cleavage.

Initiator and Effector Caspases

When a pro-apoptotic stimulus "damages" the cell, the signal is transduced through the membrane-associated receptors and promotes activation of the initiator pro-caspases (such as caspase-8, -10, -9). These in turn activate the effector pro-caspases downstream, and the effector caspases in conjunction with certain cofactors implement the execution phase of apoptosis - namely the morphological and biochemical features of apoptosis. Caspase-8 has been shown to activate many of the other caspases indicating that it must be at the apex of the cascade. Recently procaspase-3 was confirmed as the major cellular target of caspase-8 activity (Stennicke *et al.* 1998). Different stimuli activate different sets of initiator caspases, but these distinct signals are thought to converge into one common pathway involving the effector caspases. An outline of this proposed system can be seen in figure 1.8 and will be discussed in the following sections.

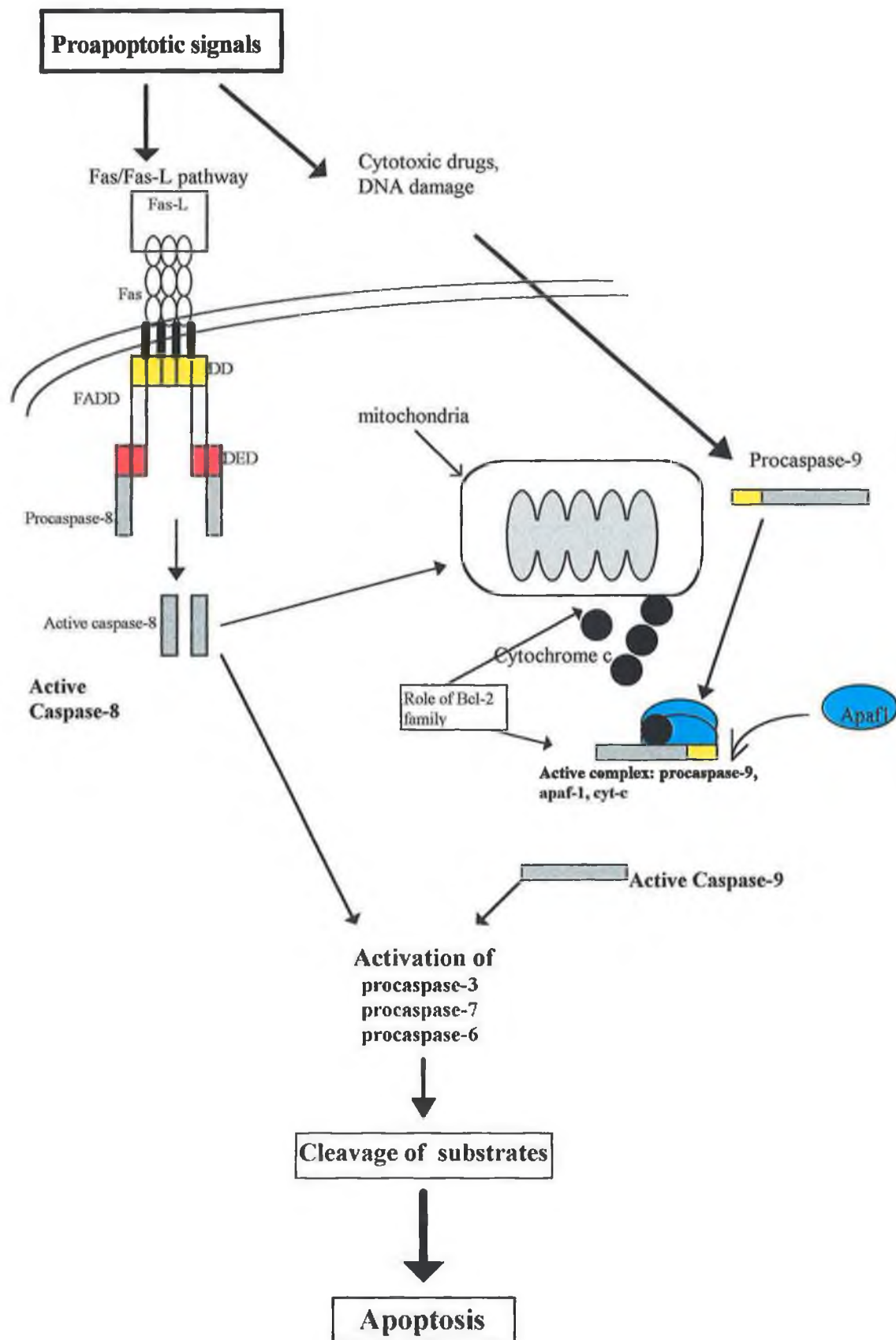


Figure 1.8 Outline of apoptotic pathways in the cell

1.5.7 CD95 Pathway

A growing number of cell surface receptors have been identified that play an important role in signal transduction in the apoptotic pathway. The best characterised receptors are CD95 (Fas/APO-1) and tumor necrosis factor receptor TNFR1 (p55/CD120a) (Nagata, 1997) which belong to the TNF superfamily of type I transmembrane receptors. Other receptors recently identified by their homology to the CD95 and TNF receptors are DR3 (Chinnaiyan *et al.*, 1996; Marsters *et al.*, 1996), DR4 (Pan *et al.*, 1997), DR5 (Wu *et al.*, 1997) and DR6 (Pan *et al.*, 1998).

These receptors share a homologous region in their intracellular domain called a death domain (DD) (Tartaglia *et al.*, 1993; Itoh *et al.*, 1993) that is capable of transmitting a cytotoxic signal to induce apoptosis. A family of ligands has also been identified that activate their corresponding receptor of the TNF superfamily, including CD95-L (Fas-L), TNF and TRAIL/Apo-2L (Pitti *et al.*, 1996). CD95-L binds to CD95 and TNF binds to TNFR1 (Beutler *et al.*, 1994). TRAIL can induce apoptosis by binding to DR4 and DR5 (Wiley *et al.*, 1995; Pitti *et al.*, 1996). DR6 ligand interactions remain elusive and the physiological function has not yet been identified. Yet another group of TNFR homologues exist which act as decoy receptors, and inhibit signal transduction, of which three have been so far identified; DcR1, DcR2 and DcR3, the first two being cell surface molecules and the latter a soluble secreted protein. The inhibitors DcR1 and DcR2 act by competing with the functional receptors DR4 and DR5 for TRAIL binding, thus acting as decoys that prevent TRAIL from inducing apoptosis (Ashkenazi and Dixit, 1998).

CD95 and its ligand play a critical role in various pathological and physiological forms of cell death (Nagata *et al.*, 1997) such as autoimmune disease due to dysregulation of the CD95 system. Overexpression of CD95 and hypersensitivity to apoptosis has been reported in HIV-1 infected T-lymphocytes (Debatin *et al.*, 1994). A role for CD95-induced apoptosis has been found in autoimmune diabetes (Chervonsky *et al.*, 1997) and in alcohol-induced hepatitis (Galle *et al.*, 1995). Many diverse stimuli can induce apoptosis via activation of the CD95 pathway, for example overexpression of c-myc can induce apoptosis dependent on the CD95/CD95L interaction (Hueber *et al.*, 1997). Chemotherapy drugs can also induce apoptosis mediated by the CD95 system (Friesen *et*

al., 1996; Muller *et al.*, 1997). However these findings are somewhat controversial given that other reports exist which demonstrate that chemotherapy-induced apoptosis can occur in a CD95-independent fashion (Eischen *et al.*, 1997; Gamen *et al.*, 1997; Villunger *et al.*, 1997).

The CD95 and CD95L interaction transduce an apoptotic signal by the binding of CD95 to adaptor molecules via a homologous region called a death domain (DD). The first of these adaptor molecules to be identified was FADD (Fas-associated death domain) by Chinnaian and co-workers (1995). Overexpression of FADD could induce apoptosis, thus confirming its role in apoptotic signaling. In particular the N-terminal alone of FADD was sufficient to induce apoptosis, and this domain was termed the death effector domain (DED). If just the c-terminal of FADD was overexpressed apoptosis did not occur, suggesting that DED is linked to the downstream signaling involved in apoptosis. FADD preferentially binds to CD95 through the homologous DD, whereas TRADD binds to TNFR. TRADD lacks a DED so must first bind to FADD in order to transduce signals downstream (see figure 1.9).

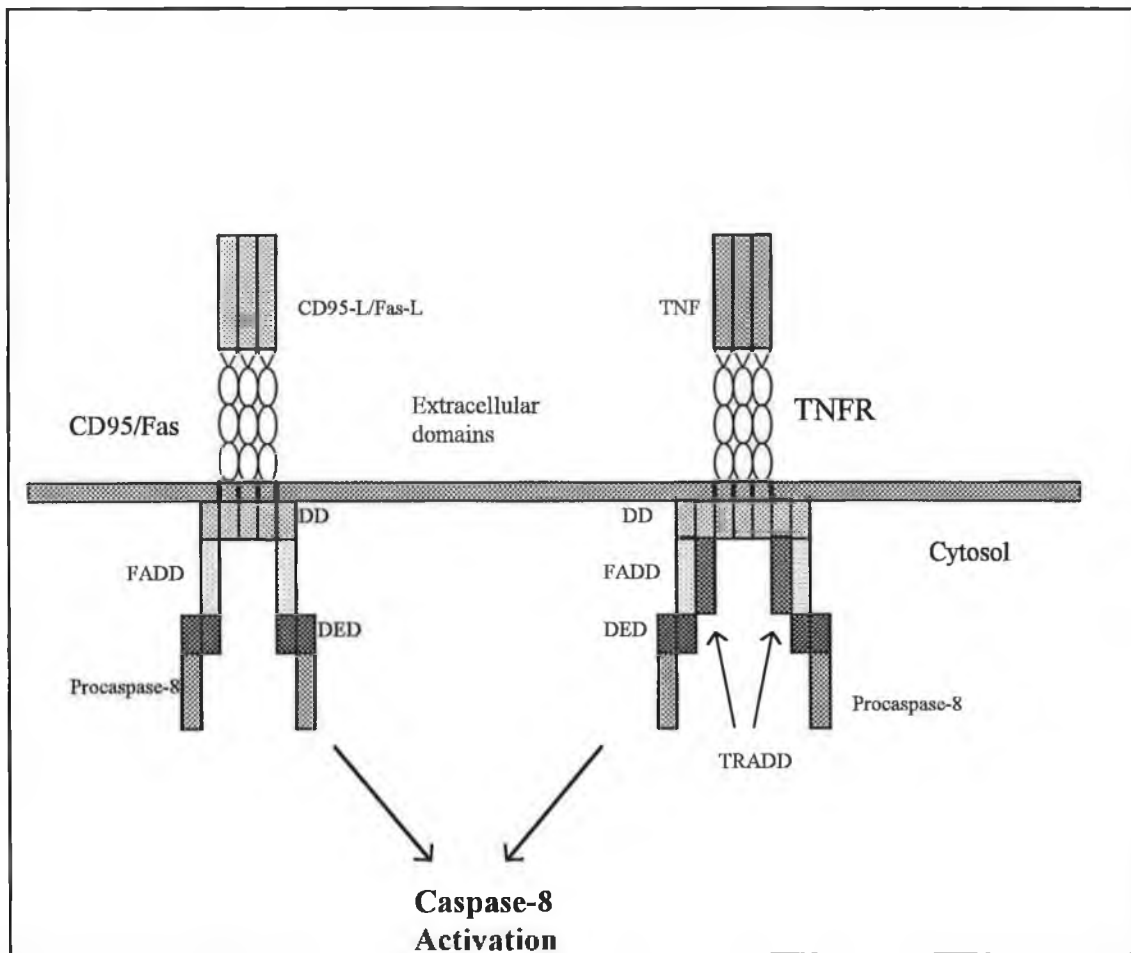


Figure 1.9 CD95/CD95L activation of procaspase-8

The initiator caspases, -8 and -10 differ from other caspases in one respect, that they possess two distinct sequences in their prodomains with homology to a region in FADD called the DED domain. The remainder of the proteins are similar to the other caspases. FADD plays a critical role as an double-adaptor protein in the CD95 pathway of apoptosis. When CD95-L binds to its transmembrane receptor CD95, the signal is transduced and FADD binds to Fas through homologous DD domains. Procaspase-8 can then bind to FADD through its DED domain, and consequently procaspase-8 is activated by autoprocessing. This is outlined in figure 1.9. It is thought that when caspase-8 binds to FADD some structural change may occur to facilitate its activation. Because caspase-8 is thought to be at the apex of the apoptotic pathway, its mode of activation remained elusive for some time, but recently the mechanism has been explained by an induced

proximity model. Muzio *et al.* (1998) confirmed that procaspase-8 contains an intrinsic caspase activity at a low level, however when procaspase-8 molecules come together and bind to FADD through homologous DEDs, the concentration of caspase activity increases to a sufficient level to allow proteolytic autoactivation (*i.e.* procaspase-8 molecules induce self-proteolysis to yield active caspase-8). Therefore FADD acts as a cofactor that brings molecules of procaspase-8 together for activation.

Once active, caspase-8 can process other downstream caspases such as caspase-3 in response to CD95-mediated apoptosis (Armstrong *et al.*, 1996; Schlegel *et al.*, 1996). Stennicke *et al.* (1998) confirmed that caspase-3 can be directly activated by caspase-8 and caspase-10, indicating that activation of caspase-3 is the central event in the further transmission of the apoptotic signal. Furthermore, Rohklin *et al.* (1998) demonstrated that caspase-3 and caspase-7 are activated during CD95-mediated apoptosis, presumably through the action of caspase-8.

Recently Juo *et al.* (1998) provided evidence for an absolute requirement for caspase-8 in the initiation of CD95-induced apoptotic cascade. Caspase-8-deficient cells were unable to activate downstream caspases such as caspase-2, -3, -7 and proteolytic cleavage was also blocked. Chemotherapeutic drugs however can induce apoptosis independently of caspase-8. In caspase-8 mutant cells, apoptosis still occurred when induced by adriamycin, etoposide, staurosporine or UV irradiation, although there was a 30-40% reduction in apoptosis (Juo *et al.*, 1998). This indicates that there must be another parallel pathway present in which another initiator caspase can transduce pro-apoptotic signals to the downstream caspases.

1.5.8 Activation of caspase-9

Liu *et al.* (1996) investigated the apoptotic pathway in a cell-free system and concluded that dATP and three cytosolic apoptotic protease factors (Apafs) are required for apoptosis to occur. They identified apaf-2 as cytochrome c, this protein was released to the cytosol upon apoptotic stimulation, and in combination with Apafs was responsible for the cleavage of procaspase-3 to its active form.

Cytochrome c is an essential component of the mitochondrial respiratory chain, and is normally located in the inner mitochondrial membrane and in the intermembrane space (Gonzales and Neupert, 1990). Numerous groups including Kluck *et al.* (1997) demonstrated cytochrome c release into the cytosol during the early stages of apoptosis thus supporting a central role for cytochrome c in the induction of apoptosis. Another essential protein for apoptosis to take place was identified as apaf-3 (Zou *et al.*, 1997), and Li *et al.* (1997) revealed this to be human caspase-9.

Further studies revealed apaf-1 to be a 130 kDa cytosolic protein with a region in its NH₂-terminus homologous to the prodomain of caspase-9. This region is called a caspase recruitment domain or CARD (Hofmann *et al.*, 1997). The presence of a CARD in apaf-1 implicates it as a recruitment protein for caspases. CARD regions have been found in other caspases with long prodomains, such as caspase-1, -2, and -4, but not in caspase-3 or -6, implying that caspases with a CARD may have roles as upstream initiators. Li *et al.* (1997) reinforced this by demonstrating that apaf-1 can interact with caspase-4, -8 and -9.

Apaf-1 also contains a WD40 repeat region, that is thought to be involved in the regulation of apaf-1 activity. Apaf-1 is capable of self-association, and this self-association can directly induce pro-caspase-9 binding and results in caspase-9 cleavage and activation (Srinivasula *et al.*, 1998; Hu *et al.*, 1998). However, deletion of the WD40 repeat region makes apaf-1 constitutively active and capable of processing procaspase-9 independent of cytochrome c and dATP, therefore the WD40 repeat region acts as a negative regulator of apaf-1 activity (Hu *et al.* 1998). Overexpression of apaf-1 in HL-60 cells increased apoptosis levels five-fold (Perkins *et al.*, 1998). Yoshida *et al.* (1998) demonstrated that apaf-1 is essential for apoptosis to occur in brain development.

Apaf-1 is thought to bring about caspase-9 proteolytic processing by first binding to cytochrome c to become active, then self-associating to form apaf-1 oligomers in the presence of dATP, thus a large multimeric apaf-1-cytochrome c complex is formed (Zou *et al.*, 1999). Caspase-9 precursor molecules are then induced to bind through

the homologous CARD domains (Li *et al.*, 1997; Pan *et al.*, 1998), which results in autocatalysis to form active caspase-9 (Srinivasula *et al.*, 1998). Upon activation, caspase-9 can then process procaspase-3, thus triggering the substrate cleavage events of apoptosis. An important finding by Li *et al.* (1997) was the requirement of caspase-9 for activation of caspase-3, and can also activation of caspase-7 (Srinivasula *et al.*, 1998). Caspase-9 can therefore be described as a central regulator of downstream caspases, and is positioned at the apex of the signaling cascade involving cytochrome c and apaf-1. A caspase-9 mutant which lacks the catalytic site yet retains the prodomain for binding to apaf-1, is a potent inhibitor of apoptosis, presumably by blocking the interaction between apaf-1 and normal caspase-9 (Seol and Billiar, 1999). Furthermore, an alternatively spliced endogenous form of caspase-9 can interact with apaf-1 through the CARD domain, and block caspase-9 activation, and subsequent caspase-3 activation (Srinivasula *et al.*, 1999). Taken together, these reports confirm that caspase-9 interaction with apaf-1 and cytochrome c is a critical pathway involved in apoptosis.

1.5.9 Regulation of apoptosis by Bcl-2 family

Bcl-2 plays an important role in apoptosis regulation, and overexpression of bcl-2 provides cells with a survival advantage. Bcl-2 is the mammalian homologue of ced-9, which was shown in *C. elegans* to prevent pro-apoptotic actions of ced-3 and ced-4. Bcl-2 blocks apoptosis after a wide variety of stimuli and in doing so, allows the cells that experience DNA damage to avoid death and enables the acquisition of genetic aberrations and the emergence of neoplasia. Bcl-2 is located in the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelope, however some Bcl-2 family members are largely cytosolic, such as Bax. Numerous Bcl-2 family members have been identified in mammalian cells (see table 1.5), and have been divided into pro-apoptotic members (e.g. Bcl-x_S, Bax, Bad) and pro-survival members (e.g. Bcl-2, Bcl-X_L).

MEMBER	ROLE	DOMAINS
Bcl-2	anti-apoptotic	BH-1, -2 and -3
Bcl-X _L	anti-apoptotic	BH-1, -2 and -3
Bcl-w	anti-apoptotic	BH-1, -2 and -3
Mcl-1	anti-apoptotic	BH-1, -2 and -3
NR-13	anti-apoptotic	BH-1, -2 and -3
CED-9	anti-apoptotic	BH-1 and -2
Bax	pro-apoptotic	BH-1, -2 and -3
Bad	pro-apoptotic	BH-1, -2 and -3
Bok	pro-apoptotic	BH-1, -2 and -3
Bcl-X _s	pro-apoptotic	BH-1, -2 and -3
Bid	pro-apoptotic	BH-3
Bad	pro-apoptotic	BH-3
Bik	pro-apoptotic	BH-3
Hrk	pro-apoptotic	BH-3
Bim	pro-apoptotic	BH-3
Blk	pro-apoptotic	BH-3

Table 1.5 Anti-apoptotic and Pro-apoptotic Bcl-2 members

All Bcl-2 family members contain at least one of four conserved motifs called Bcl-2 homology domains (BH domains). These domains participate in the formation of various dimerisations and in pore formation, the BH3 domain in particular being essential for pro-apoptotic function. Pro- and anti-apoptotic proteins can hetero or homodimerise, and depending on the ratio of pro- to anti-apoptotic proteins, the susceptibility of a cell to apoptosis can be determined. For example, overexpression of Bax enhances apoptosis, but if Bcl-2 is overexpressed it can heterodimerise with Bax and apoptosis is prevented. Overexpression of anti-apoptotic proteins such as Bcl-2 (Kamesaki *et al.*, 1993) or Bcl-X_L (Ibrado *et al.*, 1996; Schmitt *et al.*, 1998)

can inhibit caspase-3 activation (Ibrado *et al.*, 1996), indicating that the Bcl-2 family interfere with one of the central events in apoptosis, namely caspase activation.

1.5.10 Functions of Bcl-2 family - Regulation of Apoptosis

Bcl-2 proteins can regulate apoptosis in two ways, the first involving cytochrome c release from mitochondria and the second involving interaction with apaf-1. Pro-apoptotic proteins like Bax and Bid can directly induce cytochrome c release from mitochondria (Jurgensmeisier *et al.*, 1998; Luo *et al.*, 1998) perhaps via a pore forming ability. It has previously been shown that both anti-apoptotic and pro-apoptotic Bcl-2 family members can form ion channels *in vitro* (Minn *et al.*, 1997; Schendel *et al.*, 1998; Schlesinger *et al.*, 1997). Bcl-X_L and Bcl-2 can prevent cytochrome c release from mitochondria (Kharabanda *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997).

However the Bcl-2 family proteins can regulate apoptosis independently of dimerisation. Bax can antagonise Bcl-X_L without heterodimerising with Bcl-X_L (Simonian *et al.*, 1997). Luo *et al.* (1998) and Li *et al.* (1998) recently reported Bid to be a direct link between activated caspase-8 and the downstream caspases. Possessing only a BH3 domain, full length Bid usually exists as an inactive form in the cytosol of cells, but when caspase-8 becomes activated during the apoptotic signaling pathway, Bid is cleaved and activated by caspase-8. The truncated part translocates to the mitochondria, thus unleashing its pro-apoptotic activity and inducing cytochrome c release into the cytosol, which in turn initiates the downstream caspase activation and subsequent cell shrinkage and nuclear condensation. The countereffect of the anti-apoptotic protein Bcl-X_L inhibits all the apoptotic changes induced by the active Bid.

The second method of regulation is through binding to apaf-1, and consequently governing activation of procaspase-9. It is thought that Bcl-X_L can bind to apaf-1 and prevent caspase-9 from binding to it. If a pro-apoptotic member such as Bik is

present, heterodimers can form, allowing free apaf-1 to bind to procaspase-9. (see figure 1.10). Procaspase-9 is then activated through autocatalysis.

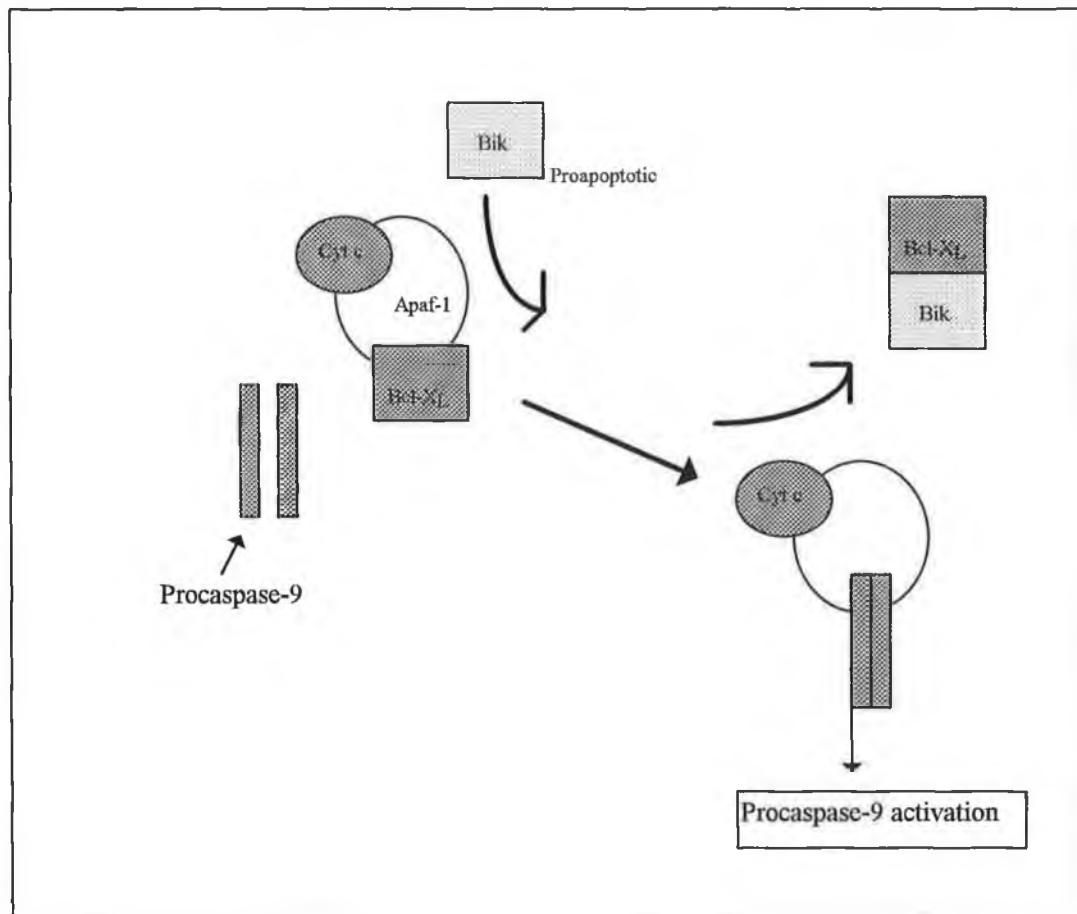


Figure 1.10 Apaf-1 regulation by bcl-2 family

Bcl-2 family members act downstream of caspase-8 activation. Srinivasan *et al.* (1998) demonstrated that Bcl-X_L can block apoptosis in cells with a catalytically active caspase-8. This may occur through the ability of Bcl-X_L to interact with apaf-1 and inhibit its association with caspase-9 (Hu *et al.*, 1998). Bcl-X_L shares a CED-4 domain with apaf-1. So through binding at this region Bcl-X_L may prevent caspase-9 from binding to the CARD region in the N-terminal of apaf-1. This finding indicates that Bcl-X_L can regulate apoptosis independently of its association with intracellular membranes.

Evidence that Bcl-X_L lies upstream of caspases was provided by Schmitt *et al.* (1998) who reported that Bcl-X_L delayed activation of effector caspases but it could not block caspase-3 activity once caspase-3 has been activated.

1.5.11 Regulation of Bcl-2 family

Playing such a critical role in apoptosis regulation suggests that Bcl-2 family members must be tightly regulated to maintain an environment which can switch on or prevent cell death at the introduction of a stimulus. Regulation occurs at different levels, both transcriptionally and post-translationally. In particular, the BH-3 domain-containing proteins seem to be regulated by being restricted to the cytosol of living cells, but in response to a death signal they can be activated as a result of increased transcription or translation or post-translational modification such as phosphorylation or cleavage, and then transported to the mitochondria. Bax is upregulated in response to DNA damage (Kozopas *et al.*, 1993; Akashi *et al.*, 1999) and translocates to the mitochondria (Wolter *et al.*, 1997). When survival signals exist in the cell, Bad is phosphorylated in response to the survival factor IL-3, binds to a 14-3-3 protein and becomes sequestered in the cytosol. The phosphorylation of Bad prevents it from heterodimerising with Bcl-X_L, thus allowing Bcl-X_L to promote cell survival. However, on removal of IL-3 Bad is dephosphorylated, and then can move to the mitochondria to induce apoptosis (Zha *et al.*, 1996). Bid is also activated in response to apoptotic stimuli, is cleaved and translocated to the mitochondria (Luo *et al.*, 1998; Li *et al.*, 1998).

Pro-survival members can also be modified through phosphorylation or cleavage. Depending on the phosphorylation sites occupied on Bcl-2 or Bcl-X_L, the anti-apoptotic activity can be increased or prevented (Haldar *et al.*, 1994; 1995; 1996; Maundrell *et al.*, 1997, Chang *et al.*, 1997, Ito *et al.*, 1997). Cheng *et al.* (1997) also demonstrated that Bcl-2 could be cleaved by caspases resulting in its conversion to a pro-apoptotic member, perhaps by activating the pro-apoptotic activity of its BH3 domain. Bcl-X_L can undergo a similar cleavage.

1.5.12 Apoptosis and DNA damaging drugs

Since there are a number of possible apoptotic signaling pathways present, each converging on the activation of effector caspases, the pathway resulting from chemotherapeutic drug treatment has very important implications for effective tumor cell kill and any resistance to undergo chemotherapeutic drug-induced apoptosis. Any defects in this pathway would result in defective apoptosis and ensuing resistance.

Numerous groups have verified the role of caspases in apoptosis induced by various DNA damaging agents with diverse chemical structures and mechanisms of action. DNA damaging agents such as Ara-C, cisplatin, etoposide, camptothecin (Datta *et al.*, 1996), CPT-II (Suzuki *et al.*, 1996) and MC540 (Pervaiz *et al.*, 1998) have been shown to activate caspase-3 activity. Faleiro *et al.* (1997) have reported that the major active caspases in cells induced to undergo apoptosis by etoposide are caspase-3 and caspase-6, and these caspases are present as multiple species which can vary in activity between cell type. Etoposide treatment of HL-60 cells also induces multiple species of caspase-3 and caspase-6, but procaspase-2 was not activated (Martins *et al.*, 1997b) indicating that the stimulus activated specific caspases. Simizu *et al.* (1998) recently demonstrated the requirement of caspase-3 for apoptosis (mediated by hydrogen peroxide production) induced by camptothecin, vinblastine, inostamycin and adriamycin. Keane *et al.* (1997) have also shown that caspase-3 activity is essential for apoptosis to occur after staurosporine treatment.

All of the reports confirm the role of caspase-3 in apoptosis induced by chemotherapeutic drugs, but caspase-3 is an effector caspase. So the question remains as to what are the initiator complexes involved in the chemotherapeutic drug signaling pathway? The main contenders are obviously CD95 or a caspase containing a CARD domain, namely caspase-9. A body of evidence exists for each of these initiators at present. Originally CD95 was thought to be the main signaling protein involved, but with the recent discovery of caspase-9, some very convincing reports prove the involvement of caspase-9 in cytotoxic induced apoptosis.

Fas-involvement

Fulda *et al.* (1997) showed that CD95 and CD95-L levels were upregulated on neuroblastoma cells after treatment with doxorubicin, VP-16 and cisplatin, and they proposed that this upregulation of CD95/Fas was responsible for apoptotic induction in the cells. Furthermore when CD95 and CD95-L production was inhibited by cyclosporin A, there was a significant (up to 50%) reduction in drug-induced apoptosis. They also demonstrated that CD95-resistant cells were resistant to doxorubicin and cisplatin suggesting a common signaling pathway. These findings indicate that the activation of caspases was most probably through the activation of the CD95/CD95-L pathway. These results are reinforced by a recent publication by Fulda *et al.* (1998) in which they report cleavage of the apical caspase-8, the downstream caspase-3 and PARP cleavage in doxorubicin treated neuroblastoma cells.

In contrast to these findings, a number of groups report that CD95-independent drug-induced apoptosis can also occur. Eischen *et al.* (1997) concluded that even though CD95- and chemotherapy-induced pathways converge on downstream apoptotic pathways, the mechanism of drug-induced apoptosis can occur in a CD95-independent manner. Using leukaemia cells which were resistant to CD95-induced apoptosis they demonstrated that these resistant cells were sensitive to apoptosis induced by a wide variety of chemotherapeutic agents such as etoposide, doxorubicin, cisplatin, staurosporine and methotrexate. Cells then treated with the ZB4 Fas-blocking antibody were still susceptible to apoptosis when cells were treated with etoposide or doxorubicin, suggesting a CD95-independent apoptotic pathway for these drugs.

Villunger *et al.* (1997) carried out similar experiments and found that drug treatment increased CD95-L expression, consistent with the findings of Fulda *et al.* (1997). In contrast to Fulda's notion that upregulation of CD95-L expression might render cells more sensitive to drug-induced apoptosis, Villunger *et al.* (1997) showed that apoptosis occurred independently of the CD95/CD95-L signaling pathway. In addition, Ferrari *et al.* (1998) reported that the two apoptotic pathways could be dissected by ATP depletion, and depletion of ATP resulted in inhibition of chemotherapy-induced apoptosis, but did not affect the CD95 system. Thus apoptosis

involving apaf-1 and caspase-9 was the pathway involved in chemotherapeutic-triggered apoptosis.

Caspase-9 Involvement

The finding that drug-induced apoptosis could occur independently of the CD95/CD95-L pathway led researchers to search for another upstream component of the apoptosis pathway which could act in an independent and parallel fashion, and yet converge on the similar pathway in which caspase-3 is activated and apoptotic events commence. The pathway involving caspase-9, apaf-1 and cytochrome c soon became apparent. When cytochrome c is released from mitochondria, apoptosis is induced. Apaf-1 is the human homologue of the *C. elegans* CED4 protein (Zou *et al.*, 1997), and this along with the cytochrome c (apaf-2) and caspase-9 have been shown to activate caspase-3 (Zou *et al.* 1997). Kuida *et al.* (1998) recently showed that caspase-9 deficient mice had reduced apoptosis, a result of inhibition of procaspase-3 activation. Caspase-9 is necessary *in vivo* for cytochrome c-mediated caspase-3 activation and apoptosis to occur. These findings indicate that caspase-9 is a key activator of the apoptotic cascade.

Hakem *et al.* (1998) investigated the physiological role of caspase-9, using caspase-9 deficient mice and their various cell types to look at the propensity of cells to undergo apoptosis when treated with various DNA damaging agents. Three different apoptotic pathways were proposed from results; 1) Solely dependent on caspase-9: caspase-9 deficient thymocytes are resistant to apoptosis induced by dexamethasone or γ -irradiation, but caspase-3 deficient thymocytes were sensitive to apoptosis induced by these stimuli. 2) caspase-3 dependent: caspase-9-deficient activated T-cells died after α -CD95 treatment, but caspase-3-deficient cells were resistant to apoptosis suggesting that caspase-8 (DED) or caspase-4 (CARD) may act upstream in this situation. 3) Pathway independent of both caspase-3 and caspase-9: thymocytes deficient to either caspase-3 or -9 were sensitive to apoptosis induced by UV-irradiation, indicating that thymocytes can die independently of either caspase. Taken together these results indicate that the requirement for caspase-9 is cell type-specific and stimulus specific.

Caspase-Independent Apoptosis

Chol-Ha *et al.* (1998) provided evidence of a caspase-independent apoptotic pathway in Bcl-2-overexpressing cells. Using polyamine analogues which resulted in a G2/M arrest and apoptosis, they found activation of caspase-3, DNA fragmentation, PARP cleavage and cytochrome c release. Yet when Bcl-2 was overexpressed all the apoptosis features were inhibited but the cells still underwent apoptosis suggesting the existence of a caspase-independent pathway.

Considering the numerous reports supporting both the CD95 and caspase-9-cytochrome c pathways, clearly the activation of either pathway is cell type-dependent and stimulus specific.

1.5.13 Drug Resistance and Apoptosis

The primary mechanism of action of chemotherapeutic drugs with different cellular effects is through induction of apoptosis in tumour cells. This pathway is highly regulated and influenced by many factors, and a defect in any of the steps in the pathway can alter or halt the pathway in such a way as to inhibit or interfere with apoptosis. Such defects may have important implications in drug resistance, and effective chemotherapy.

1.5.13.1 Contribution of Caspase Activation to drug resistance

A number of reports by Frankfurt *et al.* (1994a, 1994b) highlighted the fact that drug resistant cells were resistant to apoptosis following exposure to a variety of cytotoxic agents. It is now accepted that deregulation of the apoptotic pathway may be important in the development of drug resistance in tumours (Haq and Zanke, 1998). Gey *et al.* (1996) investigated this phenomenon and found that drug resistant T-cells were inherently resistant to apoptosis, using serum withdrawal as a stimulus in order to overcome any potential interference from P-glycoprotein. Cells with increased metastatic potential were also resistant to apoptosis induction (McConkey *et al.*,

1996). Antoku *et al.* (1997) demonstrated that down-modulation of caspases in Jurkat leukaemia cells by the naturally occurring caspase inhibitor CrmA, enhanced the resistance of the cells to chemotherapeutic drugs such as Ara-C, doxorubicin, etoposide and cisplatin. This suggests that decreased caspase activity may have a role in certain forms of drug resistance. Nagane *et al.* (1998) also reported suppression of caspase-3 activity in drug resistant human glioblastoma cells. Cisplatin resistant squamous carcinoma cells fail to activate caspase-3 when compared to parental cells (Kojima *et al.* 1998).

Droin *et al.* (1997) recently carried out a study of caspase gene levels in a panel of resistant leukaemia cells. In untreated cells, caspase gene expression is very heterogeneous, their expression seems to be independent of each other, and there is no clear correlation between the ability of cells to undergo apoptosis and the normal basal expression of caspase in mRNA. This may be due to the functional redundancy that exists between caspases or the fact that the caspases investigated (caspase-2,-3,-6) are very much downstream caspases and would be regulated at an earlier stage in the pathway. However, when the cells were etoposide-treated, an upregulation of caspase-2 and caspase-3 mRNA was noted in cells that were sensitive to apoptosis, with unaltered regulation in more resistant cells. These results suggest that the extent of up regulation of caspase genes after drug treatment in leukaemia cells could be predictive of their sensitivity to drug treatment and apoptosis induction.

1.5.13.2 Contribution of Bcl-2 to apoptosis resistance

Bcl-2 is a key regulator of caspase activation and therefore can influence greatly the caspase activation pathway. Depending on the presence of either pro- or anti-apoptotic Bcl-2 family members, caspase activation can be influenced accordingly. In the study by Kojima *et al.* (1998) of decreased caspase-3 activity and its links to multi-drug resistance, they found that overexpression of Bcl-X_L was responsible for the failure of cells to activate caspase-3. Bcl-X_L overexpression was induced as cells developed resistance to the selecting drugs vincristine and doxorubicin, and this overexpression had a direct effect on the processing of caspase-3.

Mitochondrial cytochrome c release was also inhibited upon overexpression of Bcl-X_L pointing to the role of the Bcl-2 family as pore forming regulators of cytochrome c release. Taken together these findings by Kojima *et al.* (1998) suggest that Bcl-X_L plays an important role in the development of multidrug resistance and by directly affecting the activity of the apoptotic machinery – caspase-3 and cytochrome c.

Overexpression of Bcl-X_L was shown by Nagata *et al.* (1998) to inhibit caspase-3 like protease activation in drug-resistant human glioblastoma cells. McConkey *et al.* (1996) originally found that elevated Bcl-2 levels correlated with increased metastatic potential of cells, presumably through modulation of caspase activities. Many other reports exist documenting the effect which Bcl-2 overexpression has on caspase activation and its subsequent contribution to drug resistance.

1.5.13.3 Contribution of CD95/CD95-L to Drug Resistance

Defective activation of the CD95/ CD95-L system in cells has been linked to drug resistance. Friesen *et al.* (1997) and Los *et al.* (1997) demonstrated that disruption of the CD95/CD95-L system leads to resistance of tumour cells to drug treatment. CD95 levels were found to be much lower in drug resistant cells, and CD95-L levels were not upregulated after drug treatment, unlike the case in parental cells. This highlights the importance of an intact CD95 signaling system for efficient apoptosis in anticancer therapy. Studies by Fulda *et al.* (1998a, 1998b) implicate deficient activation of the CD95 system in drug resistance, and found that *in vitro* chemosensitivity of solid tumour cells was directly related to a functional CD95 signaling pathway.

1.5.13.4 P-glycoprotein and Resistance to Apoptosis

Recent studies have implicated the drug-efflux pump P-glycoprotein (Pgp) in resistance to apoptosis. Smyth *et al.* (1998) and Johnstone *et al.* (1999)

demonstrated that P-glycoprotein additionally protected cells from apoptosis induced by a variety of stimuli including chemotherapy drugs and CD95. Resistance could be circumvented if the activity of Pgp was inhibited using a variety of blockers such as verapamil or antagonistic antibodies. Reduced caspase activation was seen as the major effect of Pgp activity, and the reduction in caspase-3 activity and apoptosis induction was not attributable to MDR effects.

1.6 Aims of the Thesis

The main aim of this thesis was to investigate the effects of doxorubicin on the cell cycle and apoptosis induction in variants of a human lung carcinoma cell line DLKP. This study was carried out to identify any alterations there may be in resistant cells compared to sensitive cells in their response to drug treatment. DLKP-SQ is a clonal population of DLKP which is sensitive to doxorubicin and other chemotherapeutic drugs, DLKP-A250 10p#7, DLKP-A5F and DLKP-A2B are variants with varying levels of resistance to doxorubicin and other chemotherapeutic drugs.

Analysis revealed differences in the response of resistant variants to doxorubicin treatment compared to the sensitive cells, particularly in the cell cycle response. The resistant variants exhibited an abrogation of the G2/M arrest, raising the possibility of a defective DNA damage-induced G2 checkpoint in resistant cells. This theory was further investigated by looking at the controllers of the G2/M transition in the sensitive and resistant DLKP cell lines.

The apoptotic pathway was also investigated in the DLKP cells following doxorubicin treatment. Apoptosis induction occurred in all four cell lines following drug treatment, but higher doxorubicin concentrations were used in the resistant cells. Numerous studies in the literature implicate defects in the key apoptotic executioners, caspases as a mechanism of resistance. This theory was investigated in the resistant cells, to determine if, in their resistance development, DLKP resistant variants acquire defects in their apoptotic pathways which could contribute to the resistant state.

2.0 Materials and Methods

2.1 Preparation for Cell Culture

2.1.1 Ultrapure Water

Ultrapure water, (UHP) was used for the preparation of all media and solutions. This water was purified to a standard of 12-18 M Ω / cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP). A conductivity meter in the system continuously monitored the quality of the UHP.

2.1.2 Glassware

The solutions utilised in the various stages of cell culture were stored in sterile glass bottles. These sterile bottles and other glassware required for cell culture related applications were prepared as follows: glassware and lids were soaked in a 2% solution of RBS-25 (AGB Scientific) for 1 hour. After this time they were cleaned and rinsed in tap water. The glassware was washed in an industrial dishwasher, using Neodisher detergent and rinsed twice with UHP. The materials were finally sterilised by autoclaving as described in Section 2.3.

2.1.3 Sterilisation Procedures

All thermostable solutions, water and glassware were sterilised by autoclaving at 121°C for 20 min at 15 p.s.i.. Thermolabile solutions were filtered through 0.22 μ m sterile filters (Millipore, Millex-GV SLGV025BS). Large volumes, (up to 10 litres) of thermolabile solutions were filter sterilised through a micro-culture bell filter (Gelman, 12158).

2.1.4 Preparation of cell culture media

The basal media used during cell culture were prepared as follows: 10X media were added to sterile UHP water, buffered with HEPES (N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)) and NaHCO_3 as required and adjusted to pH 7.5 using sterile 1.5 N NaOH and 1.5 N HCL. The media were then filtered through sterile 0.22 μm bell filters (Gelman, 12158) and stored in sterile 500mL bottles at 4°C. Sterility checks were performed on each bottle of media by inoculating samples of the media on to Colombia blood agar plates (Oxoid, CM217), Thioglycollate broths (Oxoid, CM173) and Sabauraud dextrose (Oxoid, CM217) and incubating the plates at 37°C and 25°C. These tests facilitated the detection of bacteria, fungi and yeast contamination.

Basal media were stored at 4°C for up to three months. The HEPES buffer was prepared by dissolving 23.8g HEPES in 80mL UHP water and this solution was then sterilised by autoclaving. Then 5mL sterile 5N NaOH was added to give a final volume of 100mL. NaHCO_3 was prepared by dissolving 7.5g in 100mL UHP water followed by autoclaving. Complete media was then prepared as follows: supplements of 2mM L-glutamine (Gibco, 11140-0350) and 5% foetal calf serum (Sigma, F-7524) were, in the majority of cases, added to volumes of 100mL basal media. 1mL 100X non-essential amino acids (Gibco, 11140-035) and 100mM sodium pyruvate (Gibco, 11360-035) were also added to MEM. Complete media were maintained at 4°C for up to a maximum of 1 week.

2.2 Cell Culture procedures

All cell culture work was carried out in a class II laminar air-flow cabinet (Nuair Biological Laminar Air-Flow Cabinet). All experiments involving cytotoxic compounds were conducted in a cytogard laminar air flow cabinet (Gelman Sciences, CG series). Before and after use the laminar air-flow cabinet was cleaned with 70% industrial methylated spirits (IMS). Any items brought in to the cabinet were also cleaned with IMS. At any time, only one cell line was used in the laminar air-flow cabinet and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes so as to eliminate any possibilities of cross contamination between the various cell lines. The cabinet was cleaned weekly with industrial detergents (Virkon or TEGO) and these detergents were alternated every month.

2.2.1 Cell lines

Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in table 2.2.1. All cells were grown in complete medium consisting of ATCC or RPMI with 1% L-Glutamine and 5% foetal calf serum. Cells were incubated at 37°C and where required, in an atmosphere of 5% CO₂. Cells were fed with fresh media or subcultured (see section 2.5.1) every 2-3 days in order to maintain active cell growth.

CELL LINE	BASAL MEDIUM*	CELL TYPE	SOURCE**
DLKP-SQ	ATCC	Clonal subpopulation of DLKP (Human Lung squamous carcinoma)	NCTCC
DLKP-A2B	ATCC	Clonal population of adriamycin-selected cell line DLKP-A	NCTCC
DLKP-A5F	ATCC	Clonal population of adriamycin-selected cell line DLKP-A	NCTCC
DLKPA25010p	ATCC	Adriamycin-selected variant of DLKPSQ	NCTCC
DLKP-A250 10p#7	ATCC	Clonal population of DLKP-SQ/A250 10p	NCTCC
HL-60	RPMI-1640	Human Leukaemia cell line	Dr. Melvin Center

* ATCC basal media consists of a 1:1 mixture of DMEM and Hams F12.

**NCTCC = National Cell and Tissue Culture Centre.

RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025)

Table 2.2.1 Source description and media requirements of cell lines used in experiments described in this thesis

2.2.2 Subculturing of adherent cell lines

The waste cell culture medium was removed from the tissue culture flask and discarded into a sterile bottle. The flask was then rinsed out with 1ml of trypsin/EDTA solution (0.25% trypsin (Gibco, 043-05090), 0.01% EDTA (Sigma, E9884) solution in PBS (Oxoid, BRI4a)) to ensure the removal of any residual media. 5mls of trypsin was then added to the flask, which was then incubated at 37°C, for approximately 5 minutes, until all of the cells detached from the inside surface of the flask. The trypsin was deactivated by adding an equal volume of complete media to the flask. (For subculturing of suspension cells, trypsin was not used). The cell suspension was removed from the flask and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 1000rpm for 5 minutes. The supernatant was then discarded from the universal and the pellet was suspended in complete medium. A cell count was performed and an aliquot of cells was used to reseed a flask at the required density.

2.2.3 Cell counting

Cells were trypsinised, pelleted and resuspended in media as described in section 2.2.2.. An aliquot of the cell suspension was then added to trypan blue (Gibco, 525) at a ratio of 5:1. The mixture was incubated for 3 minutes at room temperature. A 10µl aliquot of the mixture was then applied to the chamber of a glass coverslip enclosed haemocytometer. Cells in the 16 squares of the four grids of the chamber were counted. The average cell numbers per 16 squares were multiplied by a factor of 10^4 and the relevant dilution factor to determine the number of cells per ml in the original cell suspension. Non-viable cells stained blue, while viable cells with intact membranes excluded the trypan blue dye, and remained unstained. On this basis, percentage viability could be calculated.

2.2.4 Cell freezing

Cells for cryopreservation were harvested in the log phase of growth and counted as described in section 2.2.3. Cell pellets were resuspended in a suitable volume of serum. An equal volume of a 10 % DMSO/serum solution was added dropwise to the cell suspension. A total volume of 1ml of this suspension (which should contain approximately 7×10^6 cells) was then placed in cryovials (Greiner, 122278). These vials were then placed in the vapour phase of a liquid nitrogen container, which was equivalent to a temperature of -80°C . After a period of three-four hours, vials were removed from the vapour phase and transferred to the liquid phase (-196°C) for storage.

2.2.5 Cell Thawing

The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C . A volume of 9ml of fresh growth medium was added to a sterile universal. The cells were removed from the vials and transferred to the medium in the universal. The resulting cell suspension was centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in fresh culture medium. An assessment of cell viability on thawing was then carried out (Section 2.2.3). Thawed cells were then added to an appropriately sized tissue culture flask with a suitable volume of growth medium and allowed to attach overnight. Cells were fed with fresh medium the next day to ensure complete removal of any residual DMSO that may be present

2.2.6 Monitoring of sterility of cell culture solutions

Sterility testing was performed for all cell culture media and cell culture related solutions. Samples of prepared basal media were inoculated on to Colombia blood agar plates (Oxoid, CM331), Thioglycollate broths (Oxoid, CM173) and Sabouraud dextrose (Oxoid, CM217) and incubating the plates at 37°C and 25°C. These tests facilitated the detection of bacteria, fungus and yeast contamination. Complete cell culture media were sterility tested a least four days prior to use, using Columbia blood agar.

2.2.7 *Mycoplasma* analysis of cell lines

All cell lines were tested for possible *Mycoplasma* contamination at the National Cell and Tissue Culture Centre, Glasnevin, Dublin 9.

The protocol used is detailed in the following Sections 2.2.7.1 and 2.2.7.2.

2.2.7.1 Indirect staining procedure for *Mycoplasma* analysis

Mycoplasma negative NRK (Normal rat kidney fibroblast) cells were used as an indicator cells for this analysis. The cells were incubated with a sample volume of supernatant from the cell lines in question and then examined for *Mycoplasma* contamination. A fluorescent Hoechst stain was used in this analysis. The stain binds specifically to DNA and so stains the nucleus of the cell in addition to any *Mycoplasma* present. *Mycoplasma* infection was indicated by fluorescent bodies in the cytoplasm of the NRK cells.

2.2.7.2 Direct staining procedure for *Mycoplasma* analysis

Direct staining for *Mycoplasma* analysis involved inoculating samples on to a *Mycoplasma* culture broth (Oxoid, CM403). This was supplemented with 16% serum, 0.002% DNA (BDH, 42026), 2µg/mL fungizone (Gibco, 042 05920), 2×10^3

units penicillin (Sigma, Pen-3) and 10mL of a 25% yeast extract solution. Incubation was carried out at 37°C for a period of 48 hours. Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had been supplemented as described above. The plates were incubated for three weeks at 37°C while exposed to 5% CO₂. The plates were examined microscopically every 7 days. The appearance of small oval shaped colonies indicated the presence of *Mycoplasma* infection.

2.2.8 Safe handling of cytotoxic drugs

Cytotoxic drugs were handled with extreme care at all times to minimise any risks of exposure. Risks include carcinogenic and teratogenic effects as well as severe cytotoxicity. All work involving cytotoxic drugs was carried out in Gelman “Cytoguard” laminar air flow cabinets (CG series) and nitrile gloves or double latex gloves were worn when dealing with concentrated stocks and drug wastes. All drugs were stored in designated areas at room temperature, 4°C or -20°C. Table 2.2.8.1 details storage and disposal of cytotoxic drugs used in this thesis.

CYTOTOXIC AGENT	STORAGE OF CONC. STOCK	DISPOSAL
Doxorubicin	4°C in darkness	Inactivate with 1% hyperchlorite, and neutralise with sodium thiosulphate
Taxol	Room temperature in darkness	Incineration
5-Fluorouracil	Room temperature in darkness	Incineration

Table 2.2.8.1 Storage and Disposal of cytotoxic drugs

2.3 Establishment of clonal populations from the DLKP-SQ/A250 10p cell line

The DLKP-A250 10p cell line was established by pulse exposure of doxorubicin on the DLKP-SQ cell line (Nicamhlaoibh, 1997). Clonal populations were isolated from this cell line to facilitate flow cytometrical analysis of the cells.

To propagate a clonal population from the DLKP-A250 10p, individual cells were plated into wells of a 96-well plate and allowed to grow as an individual clonal population. A single cell suspension of DLKP-A250 10p was prepared at a density of 1 cell per 300µl of media. The cell suspension was then added to a 96 well plate, at 100µl per well, so that only one in three wells should have one cell. The plates were incubated at 37°C and 5% CO₂ and monitored for cell growth. Any wells identified with just one cell after 3 days were marked, and monitored for growth. This resulted in the development of a population derived from just one cell, i.e. a clonal population. When a clonal population had reached 80% confluency in a well, it was trypsinised as described in 2.2.2. and transferred to a 24-well plate (Greiner; 662160), and transferred again to a 25cm² flask once 80% confluency had been reached in the 24-well plate. Clonal populations were then frozen to maintain stocks, as described in 2.2.4.

2.4 Cytotoxic treatment of cells

2.4.1 Preparation of cells for drug treatment and sampling

Cells were set up at a density of 2×10^5 cells/ 25cm² flask 2 days before treatment. The media was removed and 5ml of 1x concentration of drug was then added for 2 hours. After this time the drug was removed and the flask was washed twice with 37°C sterile PBS and refed with fresh media.

Cells were analysed at various timepoints for effects of chemotherapeutic drugs on the cell cycle and apoptosis as follows: At the selected timepoints the media was removed and placed in sterile labelled universals to retain any floating or detached cells. The adherent cells were then trypsinised as described in section 2.2.2., and trypsinised cells were pooled with the corresponding medium. Cells were spun at 1000 rpm for 5 minutes. Pellets were washed twice with sterile ice-cold PBS and used for further analysis as required. Drugs and other reagents used throughout the course of this thesis are listed in table 2.4.1.

REAGENT	CONCENTRATION USED	SUPPLIER
Doxorubicin (Adriamycin)	0.6µg/ml - 100µg/ml	Farmitalia
Taxol	40ng/ml - 80ng/ml	Bristol Myers-Squibb
5-Fluorouracil	0 - 50µg/ml	David Bull Laboratories, Ltd.
zVAD.fmk	100mM	Calbiochem
zDEVD.fmk	100mM	Calbiochem
YVAD	100mM	Calbiochem
CD95 (CH-11)	100ng/ml	Pharmingen
ZB4	500ng/ml 250ng/ml	Pharmingen
NOK-2	2µg/ml 1µg/ml	Pharmingen

Table 2.4.1 Drugs and inhibitors used in experiments

2.4.2 Inhibitor treatments

Cells required for experiments investigating the role of caspase inhibitors and CD95 inhibitors were set up as described in 2.4.1, the appropriate concentration of inhibitor was added for 1 hour before doxorubicin treatment. Inhibitor was then removed, and drug added as a 2x concentration with 2x concentration of inhibitor. Cells were incubated either for 2 hours for 72 hours with this drug-inhibitor combination, after which time cell viability was determined using either crystal violet or acid phosphatase assays.

2.4.3 Serum Starvation

Cells were seeded at a density of 2×10^4 /ml one day prior to treatment. Cells were carefully washed with sterile PBS three times and then incubated in serum-free medium (ATCC with 1% L-Glutamine and no foetal calf serum) for 72 hours after which time cell viability was determined.

2.5 *In vitro* toxicity assays

2.5.1 *In vitro* toxicity assay -96 well plate

Cell suspensions containing 1×10^4 cells/ml were prepared in cell culture medium. Volumes of 100 μ l of these cell suspensions were added in to 96 well plates (Costar, 3599) using a multichannel pipette. The first column of the plate was not seeded with cell suspension and was used as a control containing only medium. Plates were agitated gently in order to ensure even dispersion of cells over the wells. Cells were then incubated overnight at 37°C in an atmosphere containing 5% CO₂. Cytotoxic drug dilutions were prepared at 2X their final concentration in cell culture medium, 100 μ l of the drug dilutions were then added to each well using a multichannel pipette in replicas of eight. Plates were then mixed gently as above. Cells were incubated for 6 days at 37°C and 5% CO₂ or until control wells had reached approximately 80-90% confluency. Assessment of cell survival in the presence of drug was determined by acid phosphatase assay (section 2.5.2). The concentration of drug which caused 50% cell kill (IC₅₀ of the drug) was determined from a plot of the % survival (relative to the control cells) versus cytotoxic drug concentration.

2.5.2 Assessment of cell viability - Acid Phosphatase assay

Following the incubation period of 6 days, media was removed from the plates. Each well on the plate was washed with 100 μ l PBS. This was then removed and 100 μ l of freshly prepared phosphatase substrate (10mM *p*-nitrophenol phosphate (Sigma 104-0) in 0.1M sodium acetate (Sigma, S8625), 0.1% triton X-100 (BDH, 30632), pH 5.5) was added to each well. The plates were then incubated in the dark at 37°C for 2 hours. The enzymatic reaction was stopped by the addition of 50 μ l of 1N NaOH. The plate was read in a dual beam plate reader at 405 nm with a reference wavelength of 620 nm.

2.5.3 Toxicity Assay - 25cm² flask

25cm² flasks were set up as described in section 2.4.1. After drug treatment cells were re-fed with complete medium and incubated for 72 hours. At this time media was removed from the flasks which were then washed twice with PBS and viability was analysed using crystal violet elution assay in 2.5.4.

2.5.4 Crystal Violet Elution assay

Crystal violet toxicity assays were carried out on cells growing in 25cm² flasks. The medium was removed and cells washed twice with PBS, fixed for 10 minutes in 10% formalin and allowed to air-dry. The cells were then stained with crystal violet for 10 minutes, excess dye removed by washing and allowed to air-dry. The dye was eluted by addition of 1ml of 33% glacial acetic acid, and the absorbance measured at 570nm with a reference of 620nm.

2.6 Protein Analysis

2.6.1 Protein Extraction - Total cell extract

At the selected timepoints, the cells were trypsinised and pooled with any floating cells and spun to obtain pellets. The resulting pellet was washed twice with ice-cold PBS. All procedures from this point forward were performed on ice. The pellet was resuspended in 200-400 μ l NP-40 lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5% NP-40 (Sigma, N6507), 50mM NaF) to which was added immediately before use, 1x stock of the following: 100mM Na₃VO₄ (Sigma, 6508) (in UHP stored at 4°C), 100 mM DTT (Sigma, D9779) (in UHP stored at -20°C), 100 mM PMSF (Sigma, P7626) (in 100% ethanol stored at 4°C), 25 x protease inhibitors (Boehringer Mannheim, 1 697 498) (in UHP stored at -20 °C). Samples were left on ice for 20 minutes and checked microscopically for lysis. If cells were not lysed at this stage they were then sonicated with 8 pulses lasting 0.7 sec, and then checked for lysis. The sample was centrifuged at 13,000rpm for 30 min at 4°C in a benchtop microfuge. The supernatant was removed, aliquotted and stored at -80 °C. Protein concentration was quantified as detailed in sections 2.6.3 (BioRad).

2.6.2 Protein extraction - Cytosolic extracts

Cytosolic extracts were used for fluorometric caspase assays which are detailed in section 2.9. Cells were set up as described in section 2.4.1. The pellet was then resuspended in buffer C (25mM HEPES, pH 7.5 at 4°C, 5mM MgCl₂ (Sigma, M-8266), 1mM EGTA (Sigma; E4378) with 1mM PMSF (Sigma, P7626), 25x protease inhibitors (Boehringer Mannheim, 1 697 498) immediately before use, and kept on ice. The cells were then stored at -80 °C or lysed directly by sonication as before. The samples were centrifuged at 13,000rpm for 15 min at 4°C in a benchtop microfuge. The supernatant was carefully removed, supplemented with 5mM EDTA and centrifuged at 40,000 rpm for 1hour at 4°C in a 70.1T rotor (Beckman). The supernatant was removed using a 32-gauge needle

and supplemented with 2mM DTT (Sigma, D9779). The samples were frozen at -80 °C. Protein concentration was quantified as detailed in section 2.6.4.

2.6.3 Protein Quantification - BioRad Method

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad, 500-0006) as follows. A 2mg/ml bovine serum albumin (BSA) solution (Sigma, A9543) was prepared in NP-40 lysis buffer, and frozen in aliquots at -20°C. A protein standard curve (0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml) was prepared from the BSA stock with dilutions made in UHP. The Bio-Rad reagent was diluted 1:5 in UHP water and filtered through Whatman paper before use. A 10µl volume of protein standard dilution or sample (diluted 1:5 in UHP) was added to 490µl of diluted dye reagent and the mixture vortexed. After 5 minutes incubation, absorbance was assessed at 570nm although samples could be read within 1 hour of preparation. The concentration of the protein samples was determined from the plot of the absorbance at 570nm versus concentration of the protein standard.

2.6.4 Protein Quantification - BCA Method

Components in buffer C for cytosolic extracts interfered with the BioRad reagent, so an alternative protein assay was used for determination of protein concentrations of cytosolic extracts. Protein levels were determined using the BCA protein assay kit (Pierce, 23220) as follows. A 2 mg/ml bovine serum albumin (BSA) solution (supplied with kit) was used to create a standard curve, with dilutions made in UHP. A working reagent was prepared by combining 50:1 BCA reagents. 10µl of each standard or sample (diluted 1:50 or 1:100 in UHP) was added to a 96-well plate, and 200µl working reagent was added to each well. Blanks were set up using 10µl UHP with 200µl of working reagent. The plate was mixed for 30 seconds and then incubated for 30 minutes at 37°C. Absorbance was measured at 562 nm, and protein

concentrations determined from plotting a graph of absorbance at 562nm against protein concentration.

2.6.5 Immunoprecipitation

Immunoprecipitations were carried out using 100µg extracted protein (detailed in section 2.6.1) as estimated using the BioRad assay in section 2.6.3. All steps were carried out on ice. 100µg protein was centrifuged at 13,000rpm at 4°C for 15 minutes in a benchtop microfuge. The supernatant was transferred to an eppendorf and precleared by addition of 1µg of normal mouse IgG (Sigma, I5381) and shaking for 1 hour at 4°C. 20µl protein A agarose (Santa Cruz, sc2001) was added and samples shaken for a further 30 minutes at 4°C, after which time they were spun at 5000rpm for 5 minutes at 4°C in a benchtop microfuge. The supernatant was carefully transferred to eppendorfs, taking care not to carry over any beads (high source of background). Primary antibody was added (10µl cyclinB; 2µl weel; 4µl cdc25C) to the supernatant, and eppendorfs were shaken gently for at least 2 hours or overnight at 4°C. 12µl protein A agarose was added to each sample, and shaken for 2-4 hours (30mins for weel) at 4°C. Samples were spun at 5000rpm for 15 minutes and supernatants discarded. The pellets were washed four times with NP-40 lysis buffer (without protease inhibitors), and spun each time at 5000rpm for 10 minutes. Pellets were resuspended in a total volume of 15µl lysis buffer and 5X loading buffer and boiled for three minutes. Samples, including beads were loaded on polyacrylamide gels, and western blotting carried out as described in section 2.6.6 and 2.6.7.

2.6.6 Gel electrophoresis

Proteins for analysis by Western blotting were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and resolving gels were prepared as

illustrated in table 2.6.6.1. The gels were set in clean 10cm x 8cm gel cassettes, which consisted of a glass plate and an aluminium plate, which were separated by 1mm spacers. The gels resolving were overlaid with 0.1 % SDS or water-saturated butanol whilst polymerising to exclude air and ensure a smooth surface on which to pour the stacking gel. The gel was allowed to polymerise for 1 hour. Once set, the 0.1% SDS was removed and the stacking gel was then added, combs inserted and left to polymerise for 30 minutes.

	Resolving Gel			Stacking Gel	
	7.5%	10%	12%	15%	5%
30%Acrylamide stock	3.8 ml	5.0 ml	6 ml	7.5 ml	0.8 ml
Distilled Water	8.0 ml	6.8 ml	5.8 ml	4.3 ml	3.6 ml
1.875M Tris-HCl pH 8.8	3.0 ml	3.0 ml	3.0 ml	3.0 ml	-----
1.25M Tris-HCl pH6.8	-----	-----	-----	-----	0.5 ml
10% SDS	150 µl	150 µl	150 µl	150 µl	50 µl
10%NH ₄ -(S ₂ O ₃) ₂	60 µl	50 µl	50 µl	50 µl	17 µl
TEMED	9.0 µl	7.5 µl	7.5 µl	7.5 µl	6 µl

Table 2.6.6.1 Recipes for preparation of polyacrylamide-SDS gels

30 % acrylamide stock solution was made by dissolving 29.1 g acrylamide (Sigma, A8887) and 0.9g NN-methylene bis-acrylamide (Sigma, N7256) in 60 ml UHP and made up to 100ml. The solution was stored at 4°C in the dark for up to one month.

Before sample loading, protein was diluted in 5X loading buffer (2.5mL 1.25 M Tris-HCl, 1.0g SDS, 2.5mL mercaptoethanol (Sigma, B6250) 5.8mL glycerol (BDH, 44305) and 0.1% bromophenol blue (Sigma, B8026) and made up to 20mL with distilled water). The molecular weight markers (New England Biolabs, 7708S) and protein samples were boiled for three minutes. Following heating, equal amounts of

protein were added in to each well, and 8 μ l of molecular weight markers were used. The gels were run at 250V and 45mA until the bromophenol blue dye front was found to have reached the end of the gel, or when sufficient resolution of the molecular weight markers was achieved.

2.6.7 Western Blotting

Once electrophoresis had been completed, the SDS-PAGE gel was equilibrated in transfer buffer (25mM Tris (Sigma, T8404), 192mM glycine (Sigma, G7126) pH 8.3-8.5 without adjusting) for 20 minutes. Protein was transferred from the gel to Hybond ECL nitrocellulose membranes (Amersham, RPN 2020D) by semi-dry electroblotting. Seven sheets of Whatman 1mm filter paper were soaked in freshly prepared transfer buffer. These were then placed on the cathode plate of a semi-dry blotting apparatus (Bio-rad) and air pockets removed from between the filter paper by rolling with a glass pipette. Nitrocellulose was also soaked in the transfer buffer and placed over the filter paper on the cathode plate. Air pockets were once again removed. The acrylamide gel was layered gently on top of the nitrocellulose. Seven sheets of transfer buffer soaked filter paper were placed on top of the gel and again air pockets removed. The proteins were transferred from the gel to the nitrocellulose at a constant current of 34mA with 15V for 30 minutes.

When protein had transferred, membranes were removed and stained with Ponceau (Sigma) for 5 minutes or until protein bands were visible. Membranes were washed with PBS until the background was clear, and stained proteins were either photographed or copied using a scanner for future reference. Membranes were destained by washing three times in PBS for 10 minutes.

Membranes were blocked using 5% milk powder (Cadburys; Marval skimmed milk) in TBS (125mM NaCl, 20mM Tris pH 7.5) with 0.1% Tween at room temperature for two hours. Membranes were treated with the appropriate dilution of primary antibody in TBS- 0.1% Tween overnight at 4°C. The antibodies used are listed in

table 2.6.7.1 with the appropriate dilutions. All antibodies were diluted appropriately in TBS-0.1% Tween. A negative control where the gel was exposed to antibody diluent or animal pre-bleed was also performed.

Antibody	Supplier	Dilution	Antibody	Supplier	Dilution
cdk1	Santa Cruz; sc-54	1:1000	caspase-4	Pharmingen; 66171A	1:1000
cdk2	Santa Cruz sc-163	1:1000	caspase-6	Santa Cruz ; sc-1232	1:1000
cdk4	Santa Cruz; sc-601	1:1000	caspase-2	Transduction Labs.; I29120	1:1000
cyclin B	Santa Cruz; sc-245	1:1000	caspase-3	Transduction Labs;C31720	1:1000
cyclin E	Santa Cruz sc-247	1:1000	caspase-7	Transduction Labs;M64620	1:1000
cyclin D	Pharmingen; 14561A	1:1000	caspase-8	Pharmingen; 66231A	1:1000
cdc25B	I.Hoffmann*	2.5µg/ml	caspase-9	Merck Frosst* MF445	1:5000
cdc25C	I.Hoffmann*	2.5µg/ml	Fodrin	Chemicon; MAB1662	1:1000
wee1	Santa Cruz; sc-325	1:1000	PARP#1 (C-210)	Transduction Labs.;SA-250	1:5000
P-glycoprotein (C-219)	Centocor; 800-661	1:40	PARP#2	Boehringer Mannheim; 1 835 238	1:2000
CD-95 (CH-11)	Upstate Biotech.; 05-201	1:1000	PARP#3	Santa Cruz; sc-1561	1:1000

* These antibodies were received as gifts

Table 2.6.7.1 Source of and dilutions of antibodies used in western blotting

Primary antibody was removed after this period and the membranes rinsed three times with TBS-0.1% Tween for a total of 30 minutes. Membranes were incubated with the appropriate secondary antibody (1:000 dilution of anti-mouse IgG peroxidase conjugate (Sigma, A6782) or 1:000 dilution of anti-rabbit IgG peroxidase conjugate (Sigma, A4914) in TBS-0.1% Tween for 1 hour at room temperature. The membranes were washed three times in the wash buffer (TBS containing 0.5% Tween (Sigma, P7949)) for 15 minutes.

2.6.8 Enhanced chemiluminescence (ECL) detection

Immunoblots were developed using an Enhanced Chemiluminescence kit (Amersham, RPN2109). The procedure was carried out according to manufacturers instructions as follows. The nitrocellulose membrane was placed on a plate which was covered with layer of parafilm. 3ml of a 1:1 mixture of ECL reagents were applied to completely cover the nitrocellulose. The ECL reagent mixture was removed after a period of one minute and the membrane wrapped in clingfilm. All excess air bubbles were removed. The nitrocellulose was exposed to autoradiographic film (Kodak, X-OMATS) for various times (from 1 to 15 minutes depending on the signal). The exposed autoradiographic film was developed for 3 minutes in developer (Kodak, LX-24). The film was then washed in water for 15 seconds and transferred to a fixative (Kodak, FX-40) for 2 minutes. The film washed with water for 5-10 minutes and left to dry at room temperature.

2.7 Flow Cytometry analysis of DLKP cells

Cells were set up and treated with doxorubicin as described in section 2.4.1. At the appropriate time medium was removed from flasks and reserved; cells were trypsinised and added to the medium to ensure collection of floating cells. Cells were washed twice in ice-cold PBS. Pellets were resuspended dropwise in 250µl ice-cold ethanol-PBS (70%-30%) and stored at 4°C for up to four weeks until time of analysis. Before analysis, fixed cells were pelleted and resuspended in 40µg/ml propidium iodide (Sigma, P4170) in PBS with 100µg/ml RNase (DNase-free, Boehringer Mannheim, 1 119 915).

Samples were analysed on a FacScan (Becton Dickinson) using 488nm argon ion laser (FL2 detector) measuring forward and orthogonal light scatter with the lysis program to create histograms depicting the cell cycle distribution of the sample.

2.8 Caspase Activity Assays

Caspase enzyme activity was investigated using cytosolic extracts (section 2.6.2) and the ability of the extract to cleave a fluorometric substrate was measured, according to a method described by Martins *et al.* (1997). Caspase activity is represented by the fluorescence of a particular sample.

Cytosolic protein was diluted to 50µg/50µl in buffer C (see section 2.6.2). The protein was then diluted with 225µl of freshly prepared assay buffer (25mM HEPES, pH 7.5, 0.1% CHAPS (Sigma; C3023), 10mM DTT (Sigma, D5545), 100U/ml aprotinin (Boehringer Mannheim A1153, 1mM PMSF)) containing 100µM DEVD.AMC substrate (Calbiochem, 235425). The reaction mixture was incubated for 2 hours at 37°C in darkness. The reaction was terminated by addition of 1.225ml of ice-cold assay buffer. Blanks were set up using 50µl buffer C with 225µl assay buffer incubated at 37°C for 2 hours and then diluted with ice-cold assay buffer.

Fluorescence was measured using an excitation wavelength of 380nm and emission wavelength of 460nm. An AMC standard curve was prepared using 0-3000pmol of AMC (Sigma, A9891) per sample, to determine the amount of fluorochrome released.

2.10 Cdk1 Kinase assay

Cdk1 kinase assays were carried out using a MESACUP cdk1 kinase assay kit (MBL, 5235; Stratech, UK). Cells were set up in 75cm² flasks at 4x10⁴ cells/ml (total 10ml) two days before doxorubicin addition. Cells were drug-treated and samples taken 24, 48 and 70 hours after drug treatment, as described in section 2.4.1. Cell pellets were resuspended in 400µl of ice-cold sample buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5mM EDTA, 2mM EGTA (Sigma, E4738), 0.01% Brij35 (Sigma, P1254), 1mM PMSF (Sigma, P7626) 0.05mg/ml leupeptin (Sigma, L2884), 50mM 2-mercaptoethanol (Sigma, M6250), 25mM betaglycerophosphate (Sigma, M8266), 1mM sodium orthovanadate (Sigma, S6508), and sonicated with ten 0.7sec pulses, 50% power, on ice. Cell extract was separated by centrifugation at 13,000rpm for 1 hour at 4°C in a benchtop microfuge.

200µl cell extract was then incubated with HCK-gel suspension (supplied with kit) gently shaking for 1 hour at 4°C. After incubation, the HCK-gel was washed three times with sample buffer and twice with 2nd wash buffer (25mM HEPES buffer pH7.5, 10mM MgCl₂). The pellets were used as the cdk1 enzyme source for the kinase assay.

The kinase assay was carried out on ice. 5µl sample buffer, 5µl 10x cdc2-reaction buffer, 5µl biotinylated MV peptide and 30µl distilled water were added to the HCK-gel pellets. The phosphorylation reaction was started by the addition of 5µl of 1mM ATP, and the reaction was carried out at 32°C for 45 minutes. The reaction was terminated by addition of 200µl of reaction stop buffer.

The kinase activity was then detected using ELISA. 100µl of terminated reaction mixture was transferred to microwell strips coated with Monoclonal antibody 4A4, which detects the phosphorylated peptide. The microwell strips were incubated at 25°C for 80 minutes, and then washed five times with a wash solution supplied with the kit. 100µl peroxidase (POD)-conjugated streptavidin was added to each well and incubated at 25°C for 25 minutes. The wells were washed five times with wash

solution. Following this step the wells were incubated with 100 μ l POD substrate solution for 3-5 minutes, after which time 100 μ l 20% H₃PO₄ was added. This resulted in a yellow colour development, and absorbance was measured at 492nm.

2.11 Time-lapse videomicroscopy

Time-lapse videomicroscopy was performed using a Nikon Diaphot inverted microscope (Micron Optical, Bray, Ireland) equipped with phase-contrast optics, linked to a Mitsubishi CCD-100 colour video camera (Micron optical, Ireland). Images were recorded in S-VHS format using a Mitsubishi HS-S5600 video recorder with time-lapse capabilities. Recording speed was set at 3.22 sec/field (480hr mode) which resulted in an acceleration factor of 160 when played back at normal speed. Temperature was controlled using a perspex incubator hood (V-Jay Plastics, Dublin) and a Nikon warm air blower.

Cells were set up as described in 2.4.1. A random field in the flask was chosen, and recorded for 72 hours, after which time the tape was played back and kinetics of cell death or division were monitored. Cell death was classified as apoptosis on the basis of apoptotic morphology. Apoptotic events were scored when the classical apoptotic morphology first appeared; *i.e.* when cells rounded up just before chromosome condensation, membrane blebbing and apoptotic body formation. Mitosis was scored at the time of formation of septa between two daughter cells. Typically 100 cells in the field were monitored over 72 hours.

2.12 Detection of Apoptosis

2.12.1 DNA Laddering

Cells were set up as described 2.4.1 and treated with daunorubicin. After 24 hours, cells were trypsinised, washed twice in ice-cold sterile PBS and resuspended in lysis buffer (20mM EDTA, 0.8% (w/v) sodium lauryl sarcosinate, 100mM Tris, (pH 8.0) and 10µl RNase (Boehringer Mannheim, 1 119 915) (10mg/ml), and incubated at 37°C for 18 hours. Proteinase K (Boehringer Mannheim, 161 519) was added and samples incubated for a further 2 hours at 50°C. Samples were resolved on 0.8% agarose gels with EtBr at 50V for 2 hours. DNA in gels was visualised under UV light.

2.12.2 TUNEL analysis

Drug treated cells or untreated (control) cells, as described in section 2.4.1, were trypsinised at the desired times into a single cell suspension, and washed twice with sterile PBS. Cytospins were prepared using a cell suspension of 0.5×10^5 cells/ml. Cytospins were analysed for the presence of apoptotic cells by TUNEL analysis. DNA strand breaks were identified using an In Situ Cell Death Detection Kit with a fluorescein tag (Boehringer Mannheim, 1 684 817).

2.12.3 Phosphatidylserine analysis

The presence of phosphatidyl serine was detected using an antibody specific for phosphatidylserine. DLKP-SQ were set up in 25cm² flasks as described in section 2.4.1. Doxorubicin was added for 2 hours, and washed off. Cells were trypsinised 24 hours later and pooled with the floating cells. Pellets were washed twice in sterile PBS. Cells were resuspended in PBS at a concentration of 1×10^6 /ml, and 50µl cell suspension was transferred to a sterile eppendorf. 50µl of FITC-labelled annexin-V antibody suspension was added to the cell suspension. Tubes were incubated in darkness at 4°C for 30 minutes. Cells were washed three times in PBS by centrifuging at 2000rpm in a benchtop microfuge for 4 minutes to remove unbound antibody. The pellet was resuspended in 50µl PBS and one drop of Vectashield was added. The cells were placed on a glass slide and covered with a coverslip. Slides were viewed using a fluorescent microscope.

2.13 RNA extraction, quantification and analysis by PCR

2.13.1 Total RNA extraction from cultured cell lines.

The four DLKP cell lines were grown in 75cm² flasks until approximately 80% confluent. Media was then removed and 1ml per 75cm² flask of TRI reagent (Sigma, T-9424) was added to the flask for 5 minutes ensuring that all cells are covered with the solution. TRI reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution. It effectively dissolves DNA, RNA, and protein on lysis of cell culture samples. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. To ensure complete dissociation of nucleoprotein complexes, the sample was allowed to stand for 5 minutes at room temperature. 0.2ml of chloroform (not containing isoamyl alcohol or any other additive) per ml of TRI reagent was added to the cell lysate. The sample was covered tightly, shaken vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 12,000rpm for 15 minutes at 4°C in a benchtop microfuge. Centrifugation separated the mixture in to three phases: an organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was then transferred to a fresh tube and 0.5ml of isopropanol per ml of TRI reagent used in sample preparation, added and mixed. The sample was then allowed to stand for 5-10 minutes at room temperature, before being centrifuged at 12,000rpm for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and the bottom of the tube. The supernatant was removed and the RNA pellet washed by adding 1ml of 75% ethanol per 1ml of TRI reagent. The sample was vortexed and centrifuged at 7,500rpm for 5 minutes at 4°C. Samples could be stored for up to one year at -20°C. The RNA pellet was air-dried briefly. Approximately 50ul DEPC treated H₂O was added to the pellet. The RNA was then stored at -80°C until required for PCR analysis.

2.13.2 RNA Quantitation

RNA was quantified spectrophotometrically at 260nm and 280nm. An optical density of 1 at 260nm is equivalent to 40mg/mL RNA. The A_{260}/A_{280} ratio is indicative of RNA purity. Partially solubilised RNA has a ratio of <1.6 (Ausubel *et al.*, 1991). The yield of RNA from most lines of cultured cells is 100-200µg/90mm plate (Sambrook *et al.*, 1989).

2.13.3 Reverse transcription of RNA isolated from cell lines

The following components were used in the reverse transcriptase (RT) reaction for RNA isolated from cell lines:

1µl oligo (dT)₁₂₋₁₈ primers (1µg/ml) (promega, C1101), 1µg of total RNA (1µg/µl), and 3µl of DEPC-H₂O were mixed together and heated at 70°C for 10 min and then chilled on ice to remove any RNA secondary structure formation and allow oligo (dT) primers to bind to the poly (A)⁺ tail on the mRNA. 4µl of a 5X buffer (consisting of 250mM Tris-HCL, pH 8.3, 375mM KCl and 15mM MgCl₂), 2µl of DTT (100mM), 1µl of RNasin (40U/µl) (Promega, N2511), 1µl of dNTPs (10mM each of dATP, dCTP, dGTP and dTTP), 6µl of water and 1µl of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (Gibco; 5108025 SA) was then added to the heat-denatured RNA complex and the mixture incubated at 37°C for 1 hour to allow the MMLV-RT enzyme catalyse the formation of cDNA on the mRNA template. The enzyme was then inactivated and the RNA and cDNA strands separated by heating to 95°C for 2 min. The cDNA was used immediately in the PCR reaction or stored at -20°C until required for analysis.

Typical PCR reactions were set up as 50 μ l volumes using 5 μ l of cDNA formed during the RT reaction (see section 2.13.3). Each PCR reaction tube contained 26.5 μ l of water, 5 μ l 10X buffer (100mM Tris-HCL, pH 9.0, 50mM KCl, 1% Triton X-100), 2 μ l 25mM MgCl₂, 1 μ l of first strand target primer (250ng/ μ l), 1 μ l of second strand target primer (250ng/ μ l), 0.5 μ l of first strand endogenous control primer (β -actin) (250ng/ μ l), and 0.5 μ l of second strand endogenous control primer (β -actin) (250ng/ μ l). 5 μ l of cDNA (pre-heated to 95°C for 3 min. to separate strands and remove any secondary structure if the sample had been stored at -20°C) was added to the above and heated to 94°C for 5 min (reduces non-specific binding of primers to template). 8 μ l of 1.25mM dNTP and 0.5 μ l (1 unit) of Taq DNA Polymerase enzyme (Promega, N1862) was then added to the above. The cDNA was then amplified by PCR using the following program:

35 cycles

54°C for 1.5 min. (anneal primers to cDNA)

72°C for 7 minutes (extension)

93

3.0 Results

3.1 Constitutive protein level of cyclins and cdks in the DLKP cell lines

An investigation was carried out in order to compare protein levels of selected cell cycle-associated proteins in a range of cell lines displaying varying resistance to chemotherapeutic drugs. It has been shown that overexpression or deregulation of some cell cycle proteins frequently occurs in many tumours and can contribute to the malignancy of cancers. Western blotting was carried out to identify cyclins or cdks whose expression may be altered in the resistant variants of DLKP, DLKP-A250 10p #7, DLKP-A2B and DLKP-A5F compared to the sensitive cell line DLKP-SQ, and may contribute to the resistant state. As can be seen from figures 3.1.1 - 3.1.6, there are no significant differences between the protein levels of cyclin E (figure 3.1.2), cyclin B₁ (figure 3.1.1), cyclin D₁ (figure 3.1.3), cdk1 (figure 3.1.4), cdk2 (figure 3.1.5), and cdk4 (figure 3.1.6) in the four cell lines, when protein loading is taken into account. Blots were stained with the reversible stain Ponceau to confirm equal protein loading in each case. From these results it was concluded that levels of protein expression in the four cell lines is equivalent.

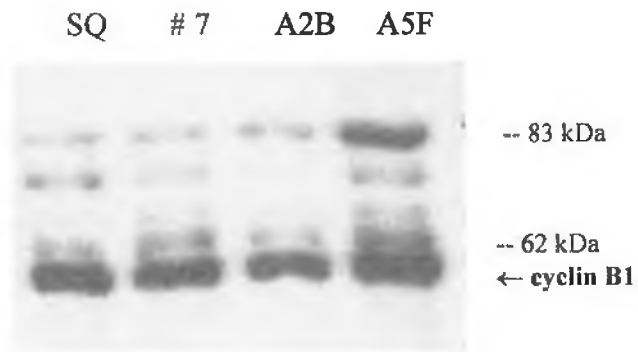


Figure 3.1.1 Cyclin B1 levels in DLKP clones

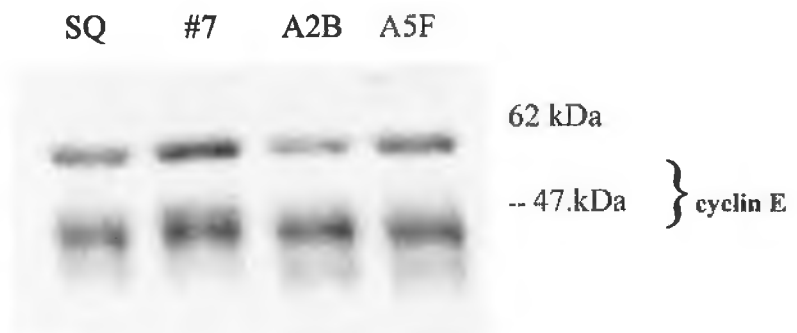


Figure 3.1.2 Cyclin E levels in DLKP clones

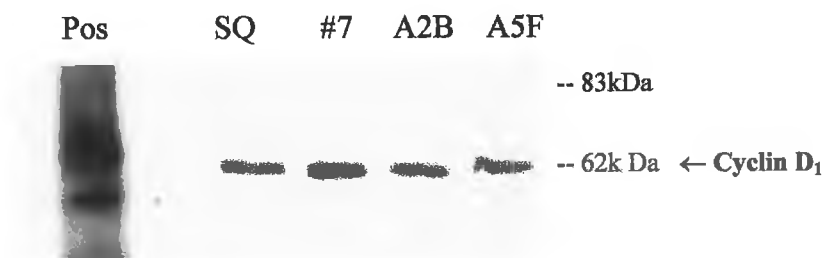


Figure 3.1.3 Cyclin D₁ levels in DLKP clones

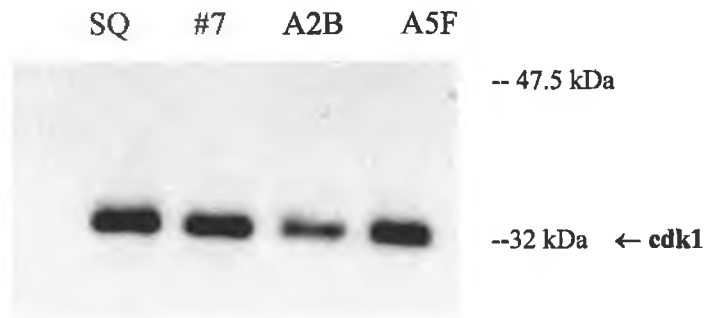


Figure 3.1.4 cdk1 protein levels in DLKP clones

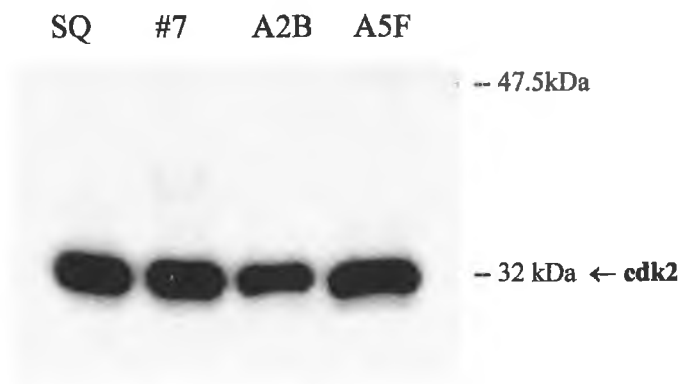


Figure 3.1.5 cdk2 levels in DLKP clones

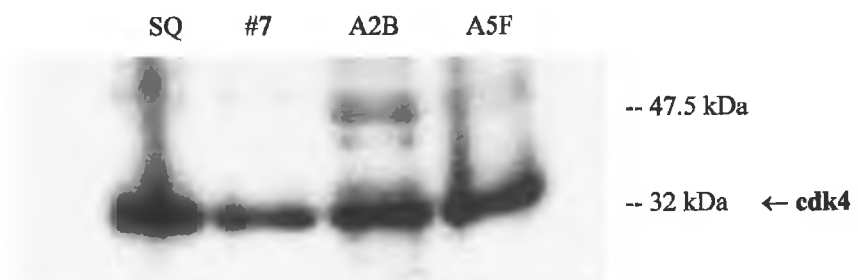


Figure 3.1.6 cdk4 levels in DLKP clones

3.2 Effects of doxorubicin on the cell cycle of DLKP cell lines

Resistant cells have similar cyclins and cdks expression compared to DLKP-SQ. The effect the chemotherapeutic drug doxorubicin exerted on the cell cycle of the DLKP cell lines was studied to determine if sensitive and resistant cells exhibit a comparable response. An equitoxic drug dosage was used which gave approximately 50% kill after 3 days. Growth curves were carried out to determine these values, and representative data are illustrated in figure 3.2.1. The concentrations used are listed in table 3.2.1

CELL LINE	CONC. IC ₅₀
DLKP- SQ	0.6 µg/ml
DLKP- A250 10p#7	2 µg/ml
DLKP- A2B	30 µg/ml
DLKP- A5F	100 µg/ml

Table 3.2.1 IC50 values for the DLKP cell lines after 2 hr Doxorubicin pulse

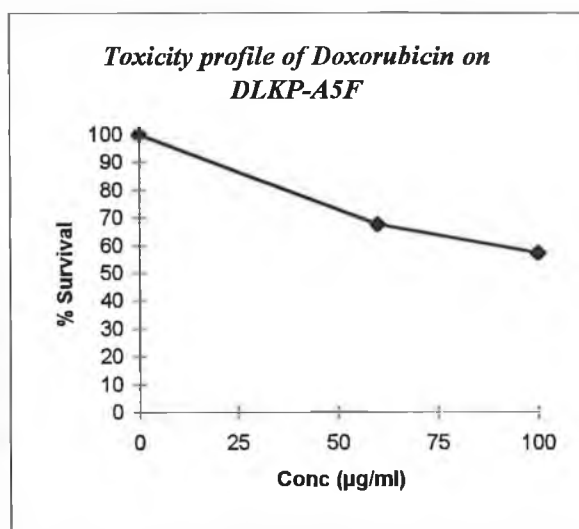
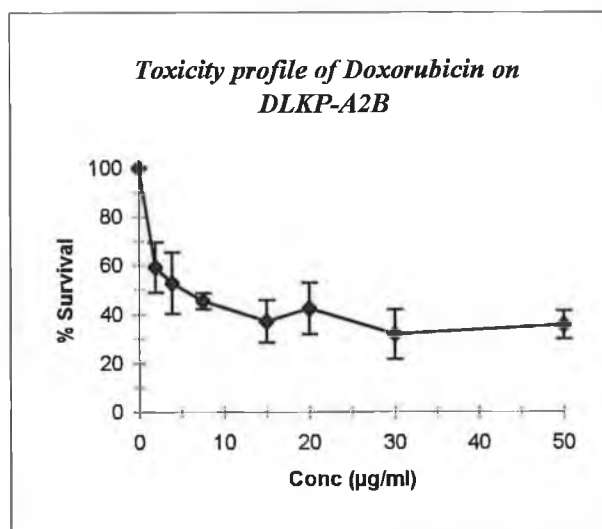
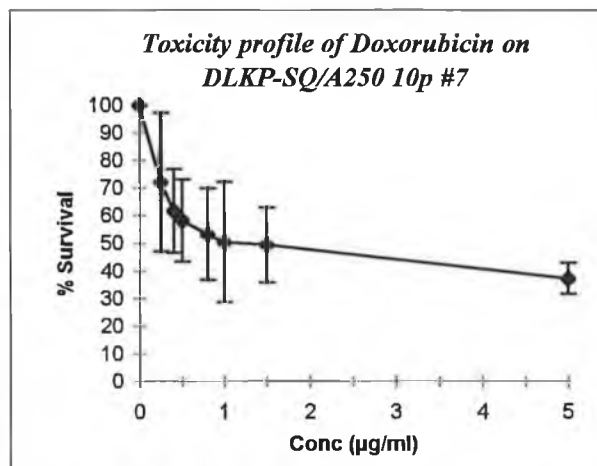
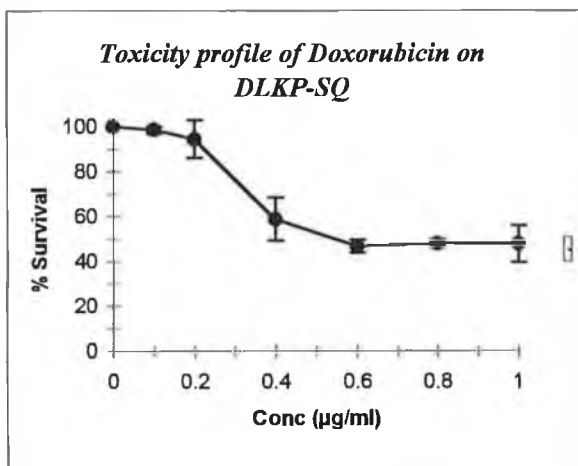


Figure 3.2.1 Toxicity profiles of DLKP cell lines. Cells were treated for 2 hours with doxorubicin, after which time drug was removed and flasks refed. Viability was determined after 72 hours using crystal violet toxicity assay.

3.3.1 Flow cytometric analysis of DLKP cell lines after Doxorubicin treatment

The effect of doxorubicin treatment on the cell cycle of the various cellular populations was initially investigated using flow cytometric analysis. Cell cycle distribution of cells at various times after drug treatment was analysed using propidium iodide (PI), which is a nucleic acid intercalator. Cells in G2 will have twice as much DNA as those in G1 and S, therefore G2/M cells will have a two-fold increase in PI levels over those in G1. S phase cells will have intermediate PI levels according to the amount of DNA synthesised during replication. It is necessary to use clonal populations in order to obtain distinct peaks during analysis since most cancer cell lines contain subpopulations with various levels of ploidy. The effects of doxorubicin treatment on the DLKP cell lines compared to untreated controls are illustrated in figures 3.3.1 - 3.3.8, C refers to untreated control cells and T refers to doxorubicin treated cells at the indicated time following drug treatment. Figure 3.3.1 illustrates the DNA profile of untreated DLKP-SQ cells over three days. The x-axis is a measurement of fluorescence at 488nm and is directly proportional to the DNA content of the cells. The y-axis measures the relative number of cells. 10,000 individual cells were counted for each sample. The left hand peak represents cells in G1 phase of the cell cycle (2n DNA content) and the right-hand peak represents cells in G2 phase having 4n DNA content just before mitosis. S phase is represented by the area between the two peaks. The histograms in figures 3.3.1, 3.3.3, 3.3.5, and 3.3.7 illustrate the unperturbed asynchronous cell cycles of untreated DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F respectively, in which the distribution remains unchanged as the cells progress through the cell cycle.

When DLKP-SQ cells are treated with 0.6µg/ml doxorubicin for 2 hours, alterations in the cell cycle become apparent by 12 hours after treatment, with a slower traverse of the S phase indicated by the increased population in the S phase at T16 and an eventual G2/M arrest at 36 hours (T36) after treatment. Apoptotic cells can be seen by the emergence of a sub G1 population at T36 and T45. These results are illustrated in figure 3.3.2. When the resistant clones were treated with doxorubicin similar results were not obtained. In figure 3.3.4 it can be seen that DLKP-A250 10p#7 cells traverse the S phase much faster than the DLKP-SQ cells and begin to arrest

in G2/M by T16, however this arrest is not permanent because cells begin to accumulate in G1 phase by T36. There is a similar effect seen in DLKP-A2B cells (figure 3.3.6) and DLKP-A5F (figure 3.3.8), in which the cells seem to transiently arrest in G2/M and then bypass the arrest.

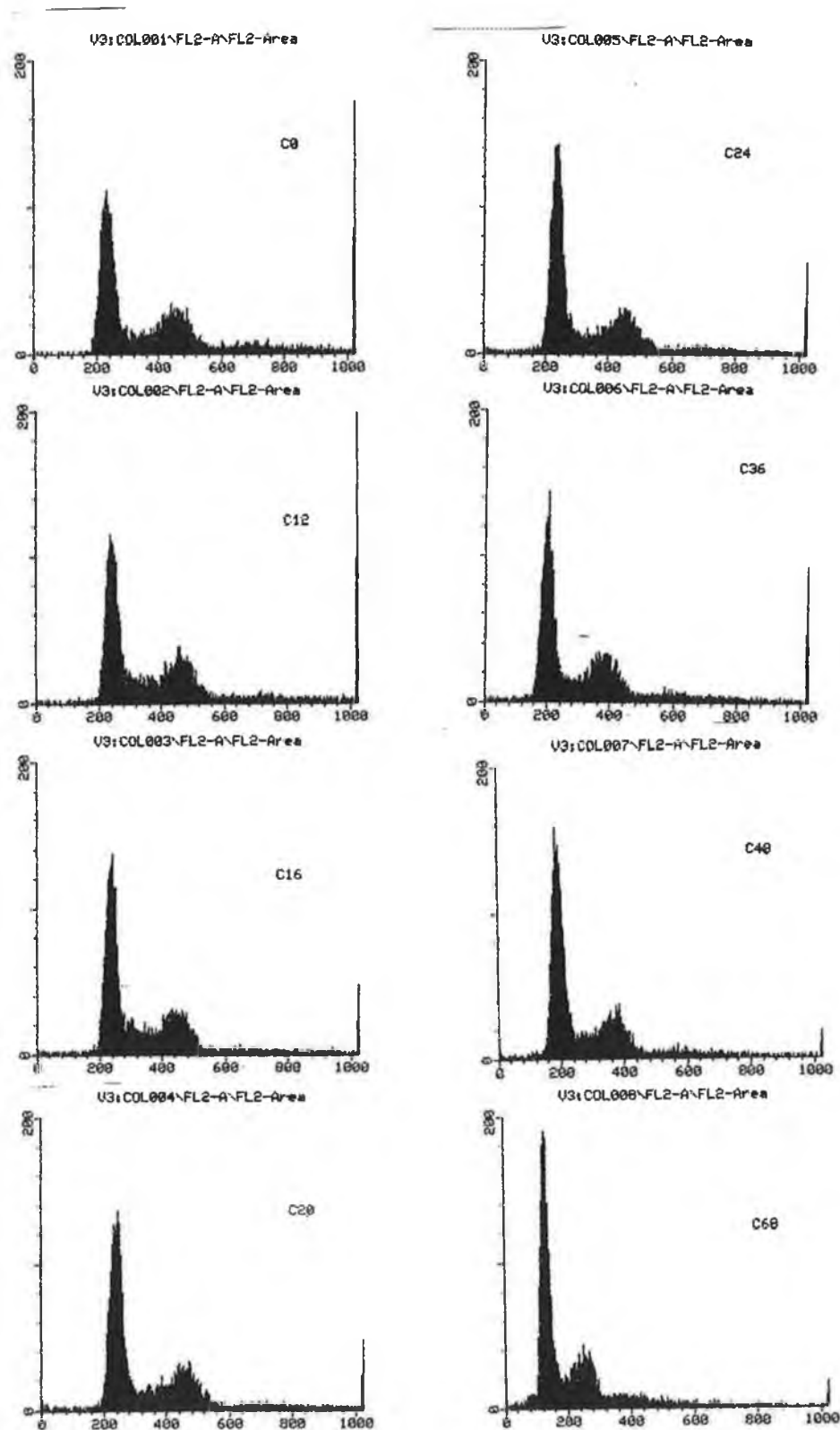


Figure 3.3.1 Cell cycle analysis of untreated DLKP-SQ cells.

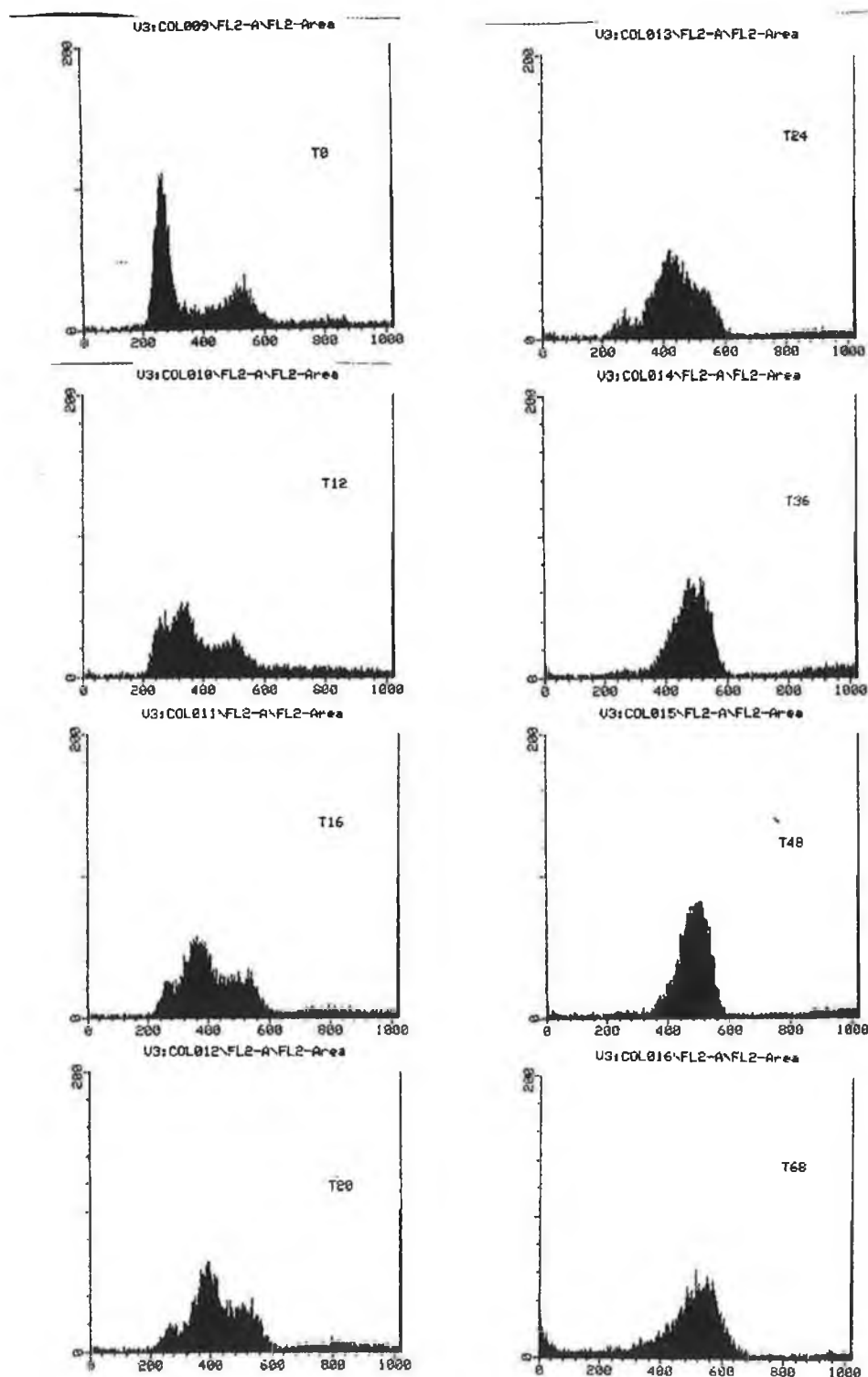


Figure 3.3.2 Cell cycle analysis of DLKP-SQ cells after 0.6µg/ml Doxorubicin 2hr pulse treatment.

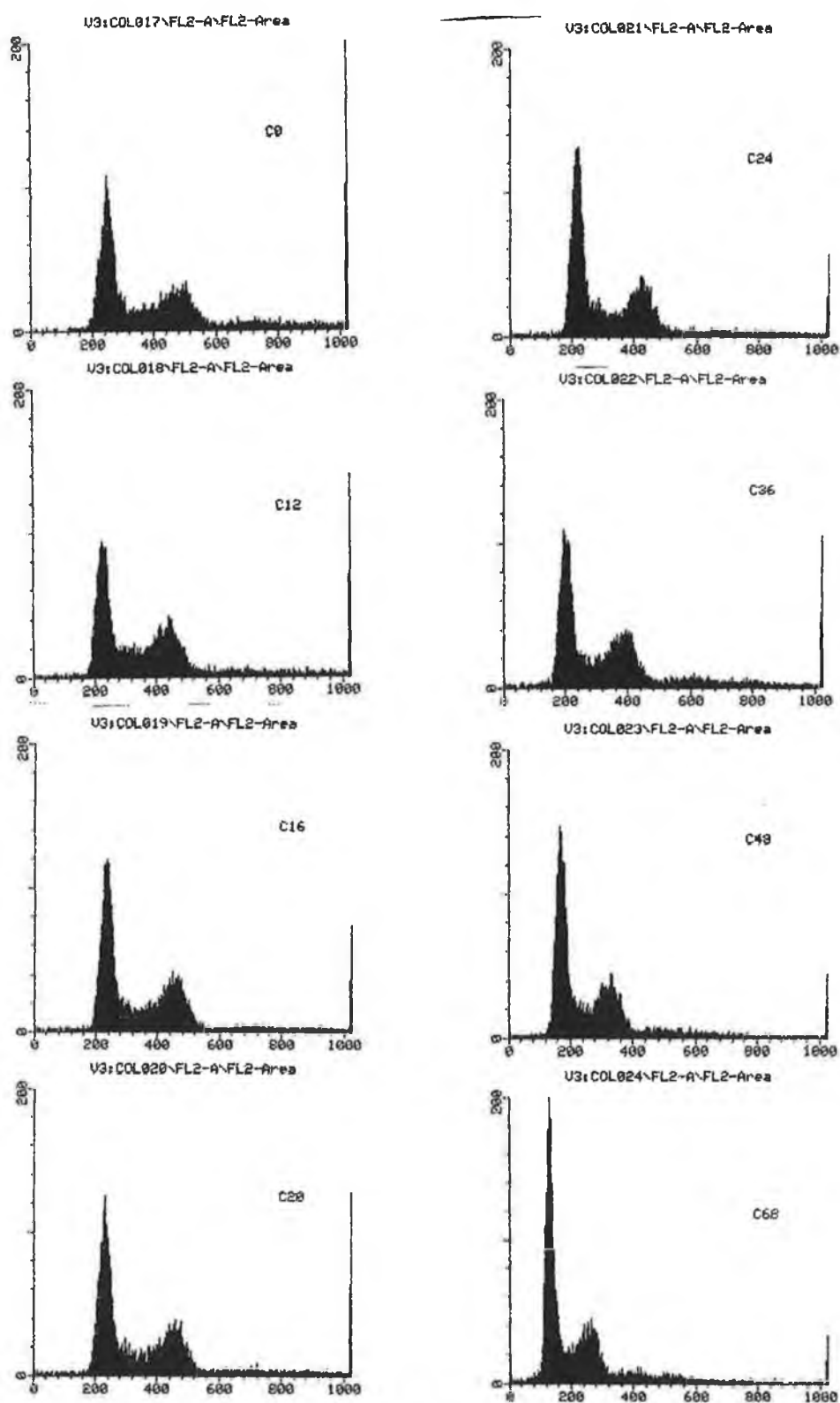


Figure 3.3.3 Cell cycle analysis of untreated DLKP-SQ/A250 10p#7 cells.

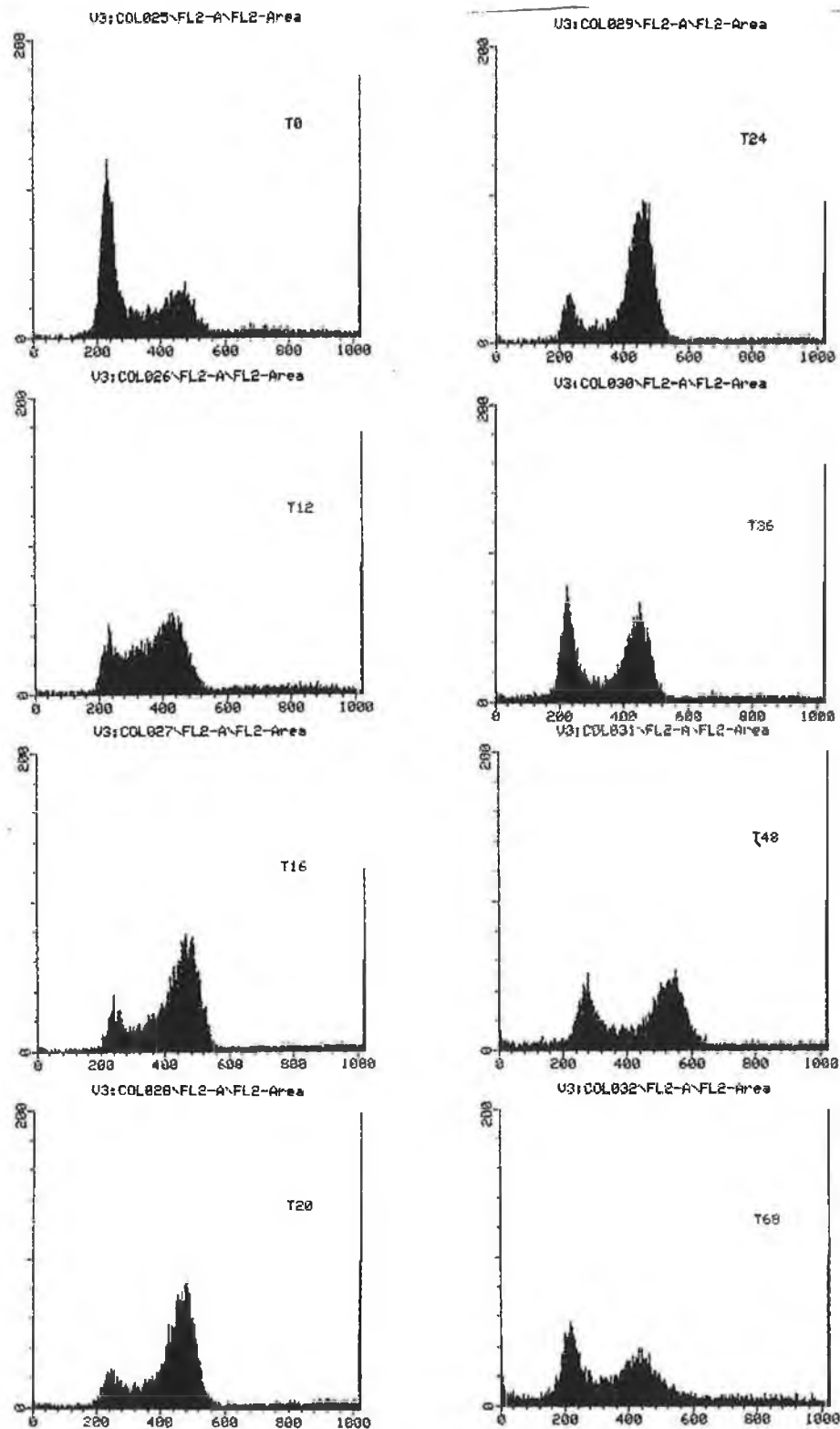


Figure 3.3.4 Cell cycle analysis of DLKP-A250 10p #7 following 2.0 µg/ml Doxorubicin 2 hour treatment

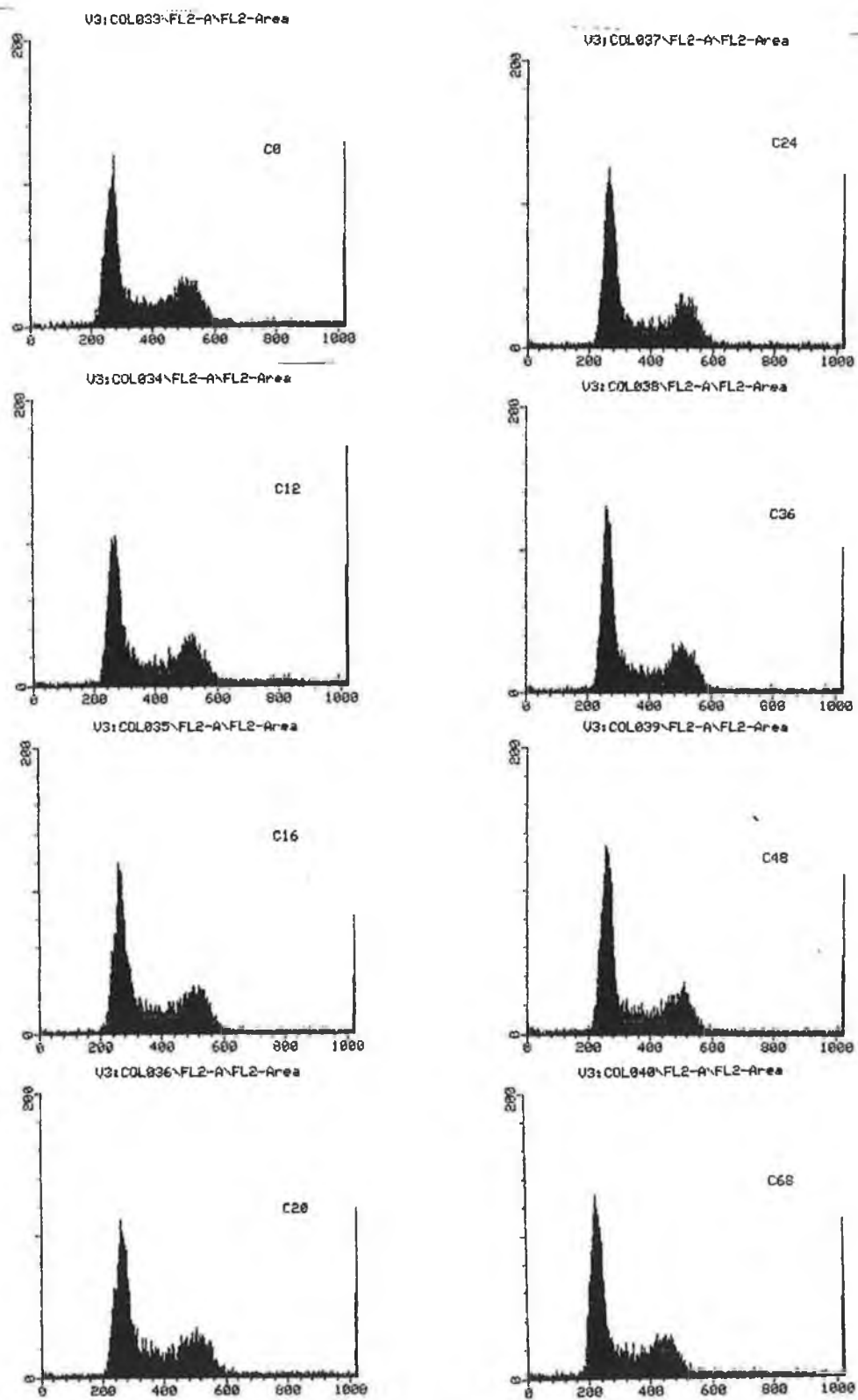


Figure 3.3.5 Cell cycle analysis of untreated DLKP-A2B cells.

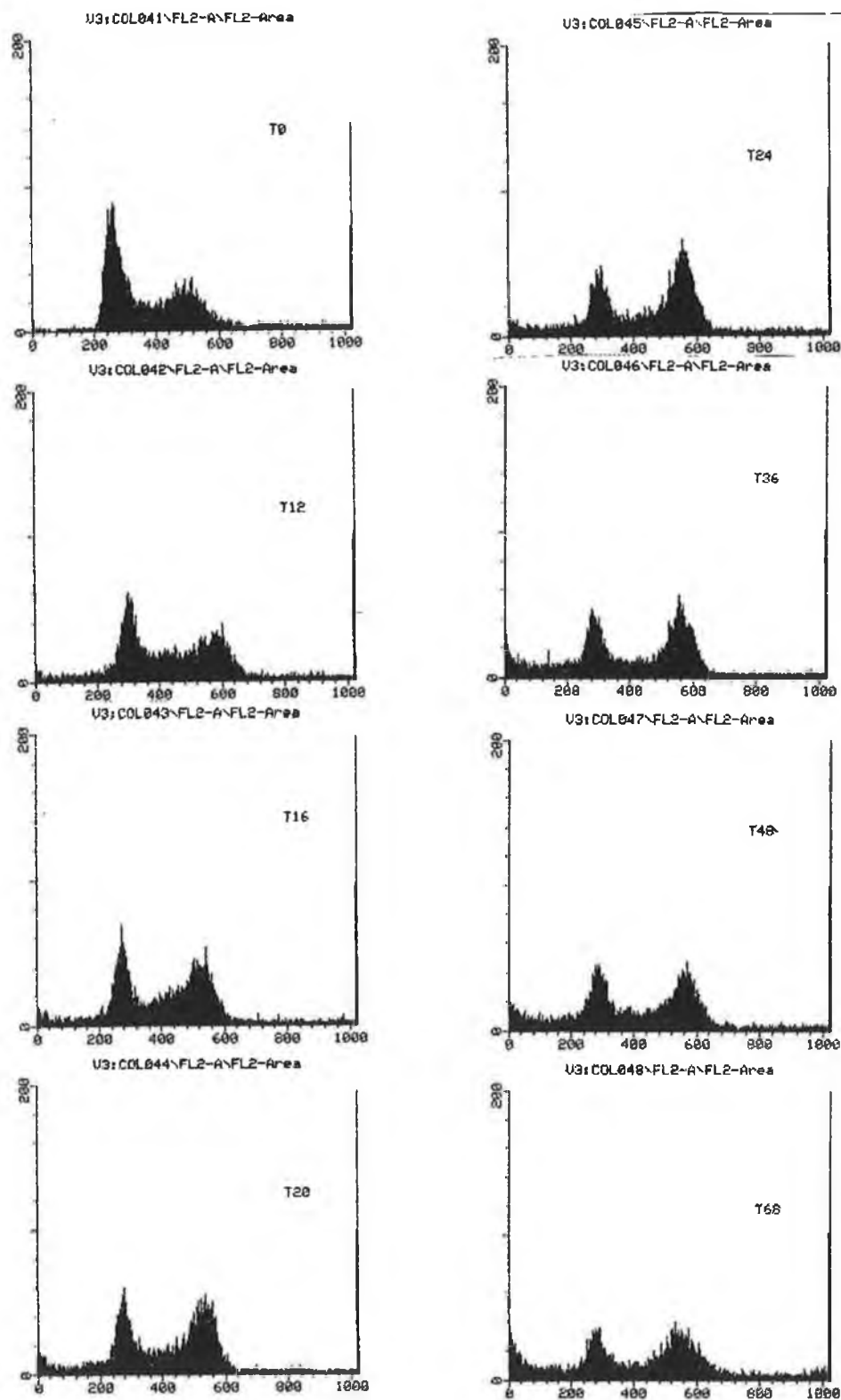


Figure 3.3.6 Cell cycle analysis of DLKP-A2B cells after 30µg/ml Doxorubicin treatment.

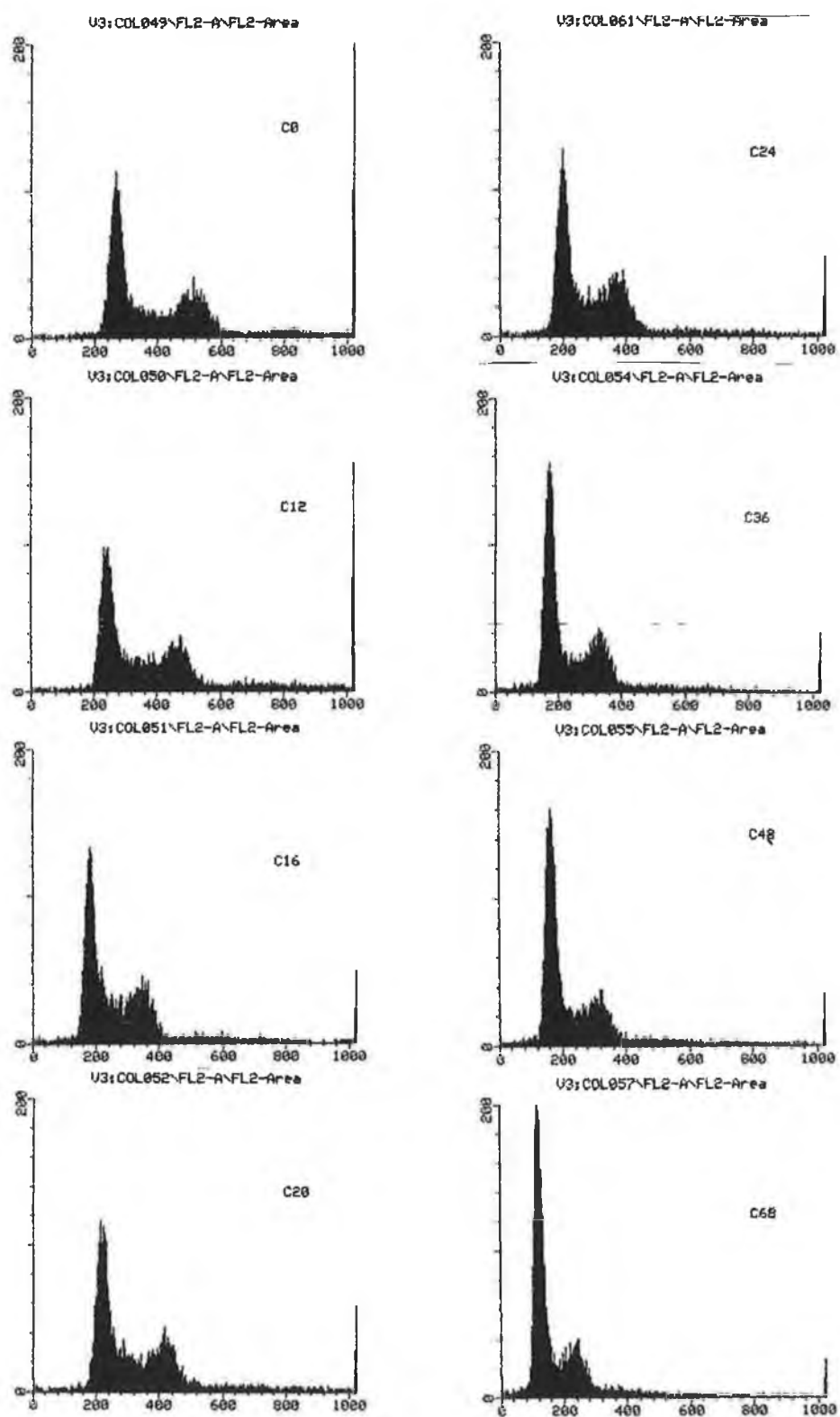


Figure 3.3.7 Cell cycle analysis of untreated DLKP-A5F cells

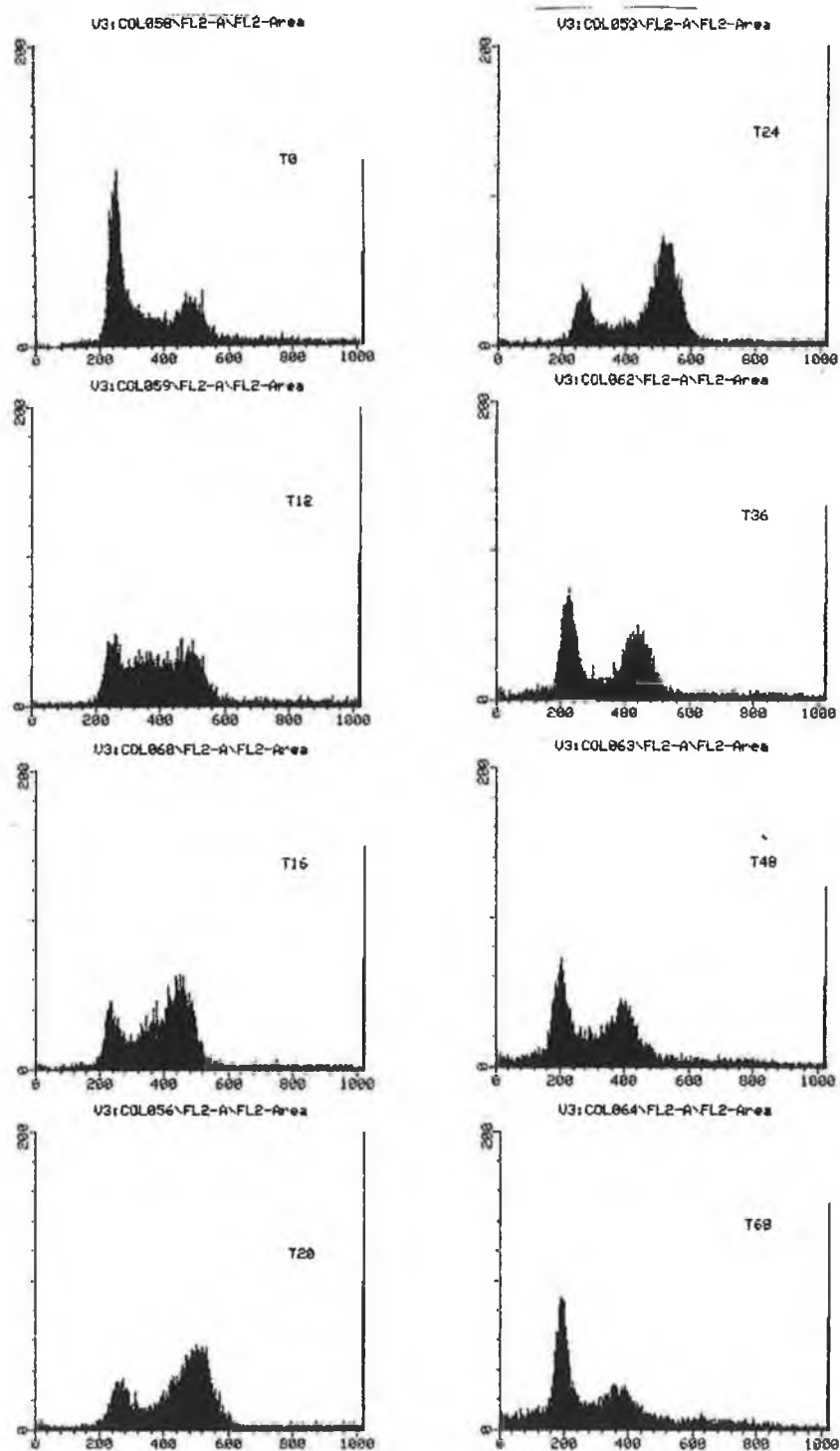


Figure 3.3.8 Cell cycle analysis of DLKP-A5F cells after 100µg/ml Doxorubicin 2 hour pulse treatment

3.3.2 Effect of caffeine on DLKP-SQ cells

Caffeine treatment is a well known means of bypassing a G2/M arrest in cultured cells, and its effect on the doxorubicin-treated DLKP-SQ cell line was investigated to establish if doxorubicin-induced G2/M arrest could be overcome. DLKP-SQ cells were treated with 4mM (non-toxic) caffeine in combination with doxorubicin, with some variations in the timing of caffeine addition in an attempt to mimic the effect seen in the resistant cells. Caffeine treatment alone had no effect on DLKP-SQ cells (seen in figure 3.3.11) and they cycled identically to the untreated control cells, as seen in figure 3.3.9. Doxorubicin 0.6ug/ml treatment arrests the cells in G2/M by T36 in figure 3.3.10. Figure 3.3.12 demonstrates that this arrest can be bypassed when 4mM caffeine is added directly after drug removal at T0, and cells appear to cycle through G1 and G2/M and undergo apoptosis (seen by Time-lapse videomicroscopy). If caffeine treatment is delayed until 18 hours after drug removal the cells have arrested in G2/M, as seen in figure 3.3.13, upon addition of 4mM caffeine cells resume mitotic progression and the G2/M arrest is abrogated. In summary, it appears that DLKP-SQ cells treated with caffeine can mimic the action of the resistant variants after doxorubicin treatment.

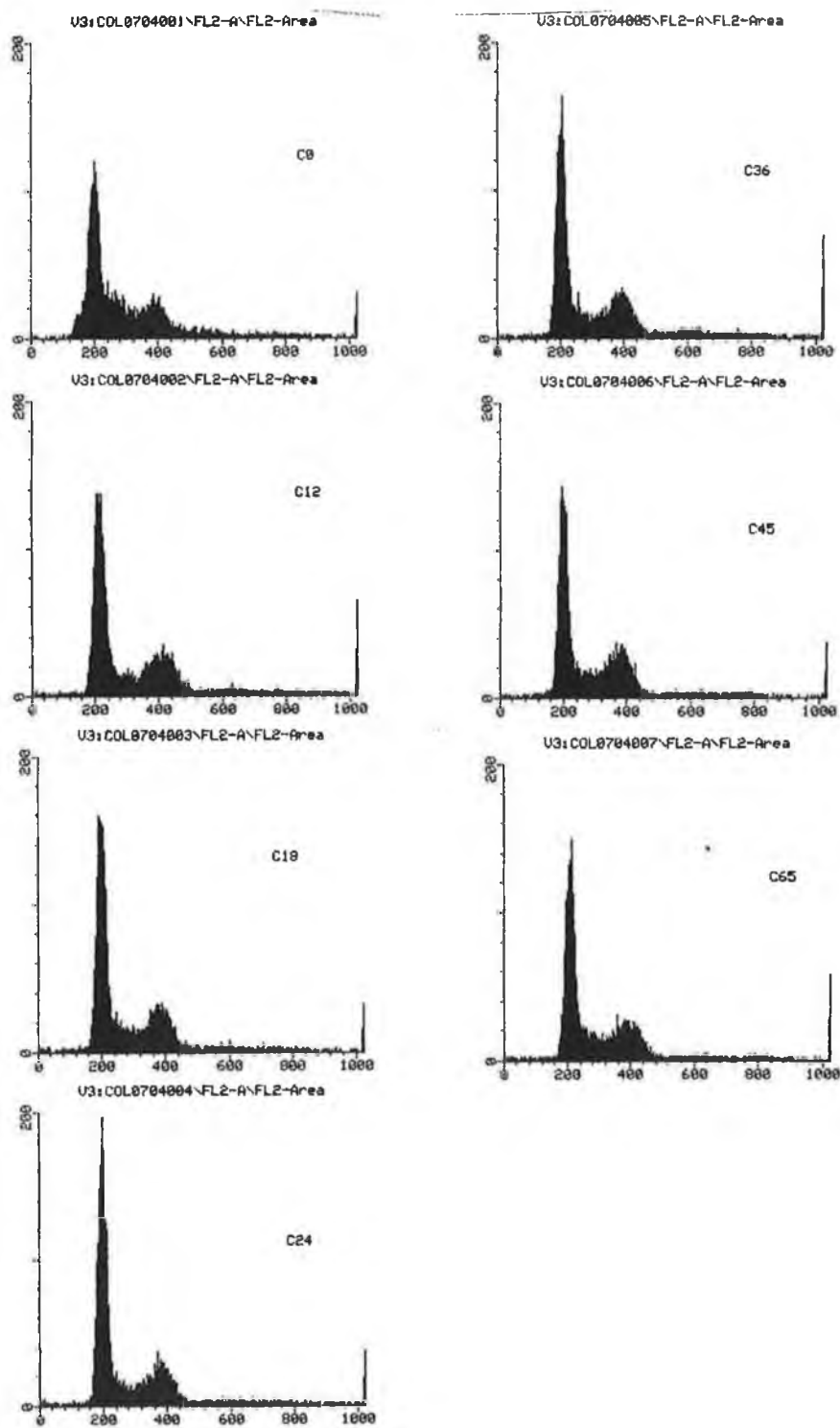


Figure 3.3.9 Cell Cycle Analysis of untreated DLKP-SQ cells

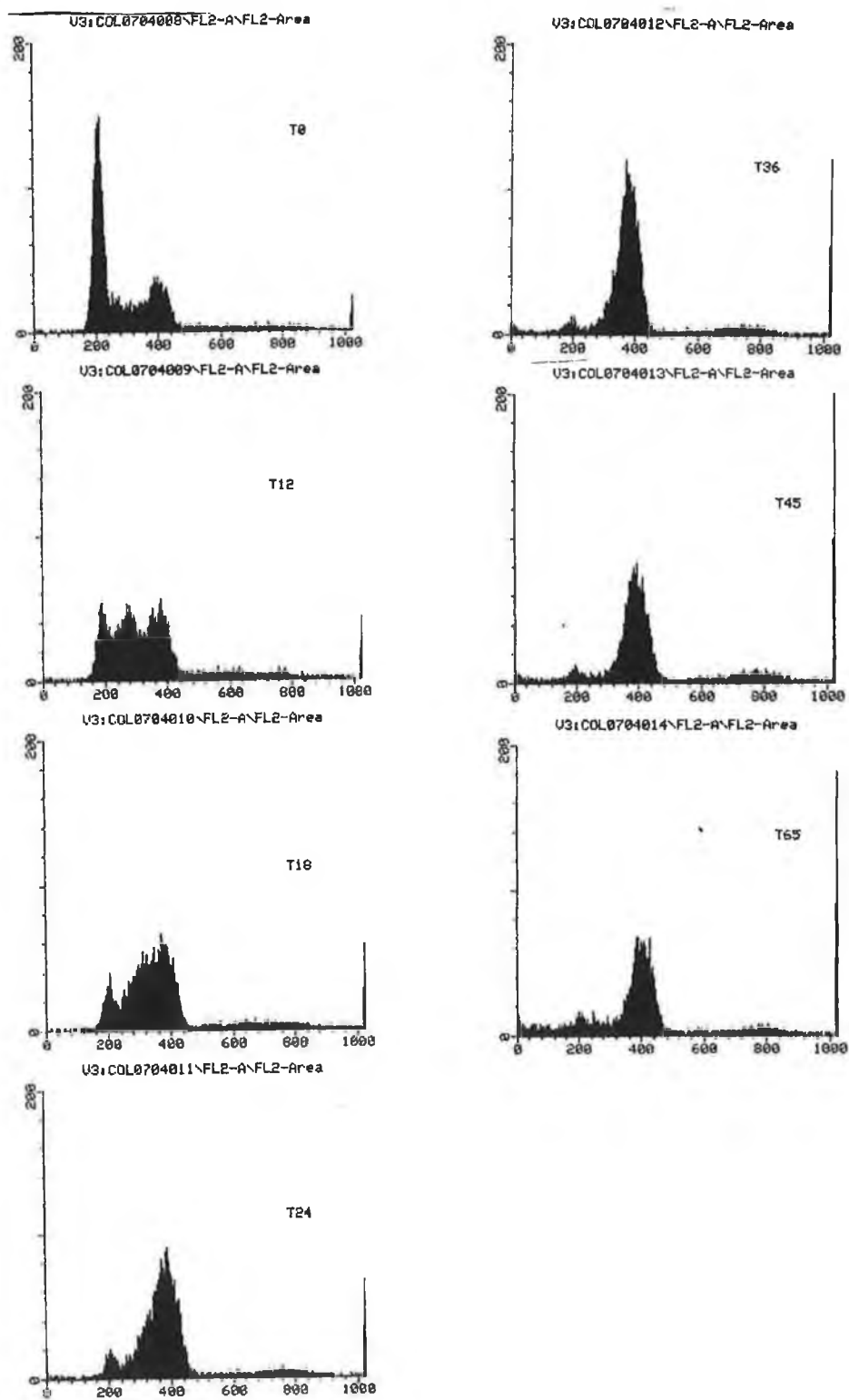


Figure 3.3.10 Cell Cycle Analysis of DLKP-SQ cells after pulsing with 0.6 μ g/ml Doxorubicin for 2 hours.

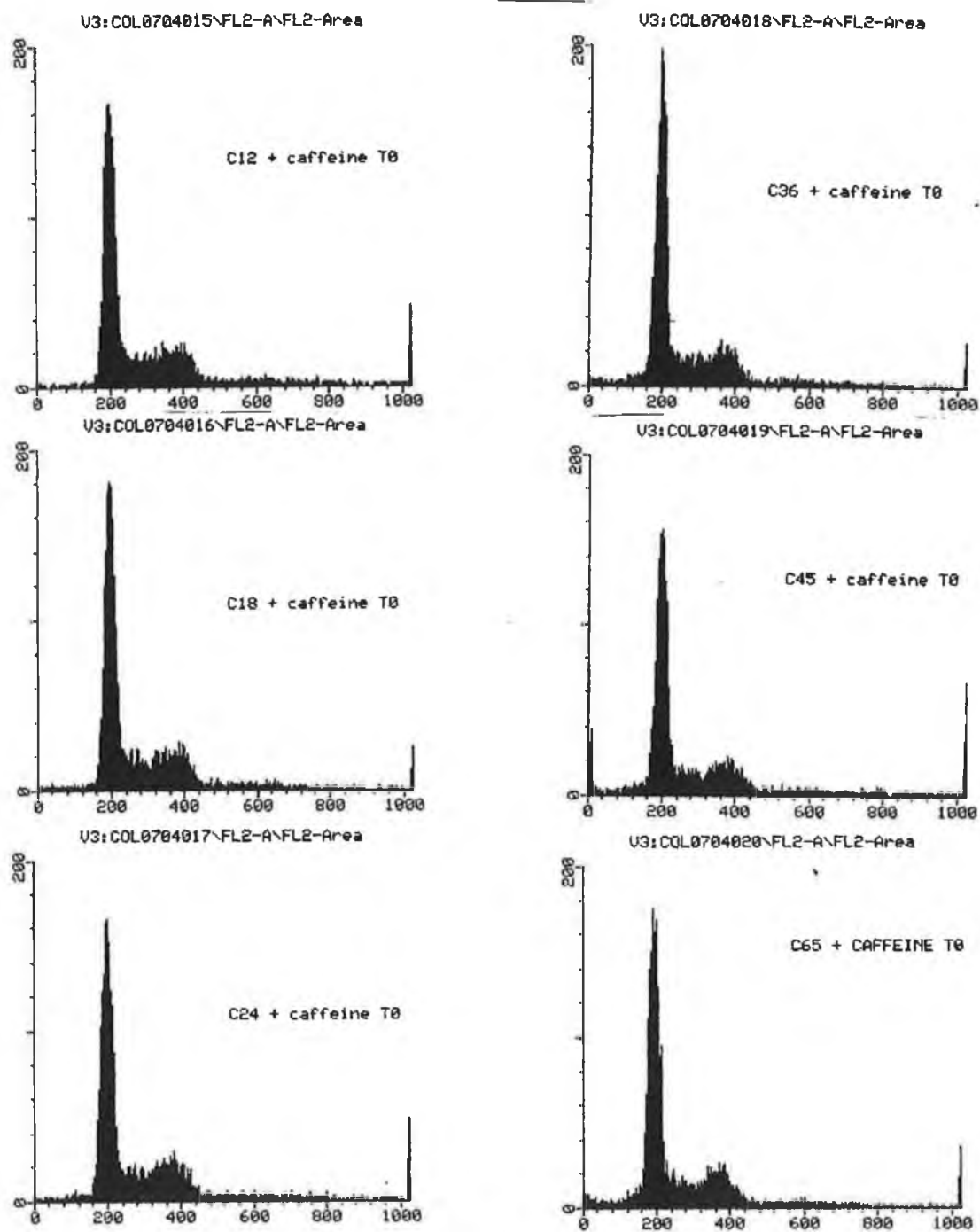


Figure 3.3.11 Cell Cycle analysis of DLKP-SQ cells after treatment with 4mM caffeine, samples were taken at the indicated times.

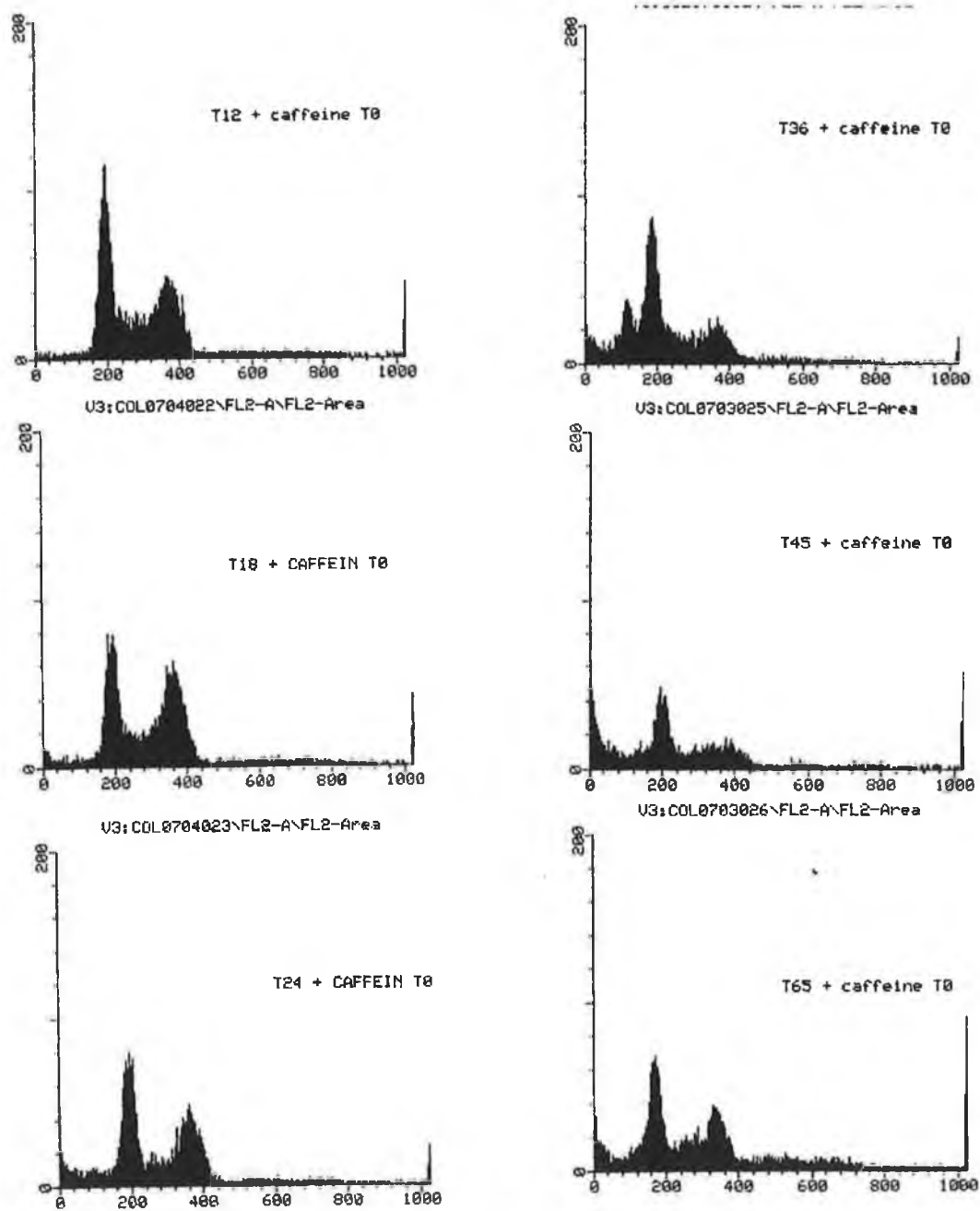
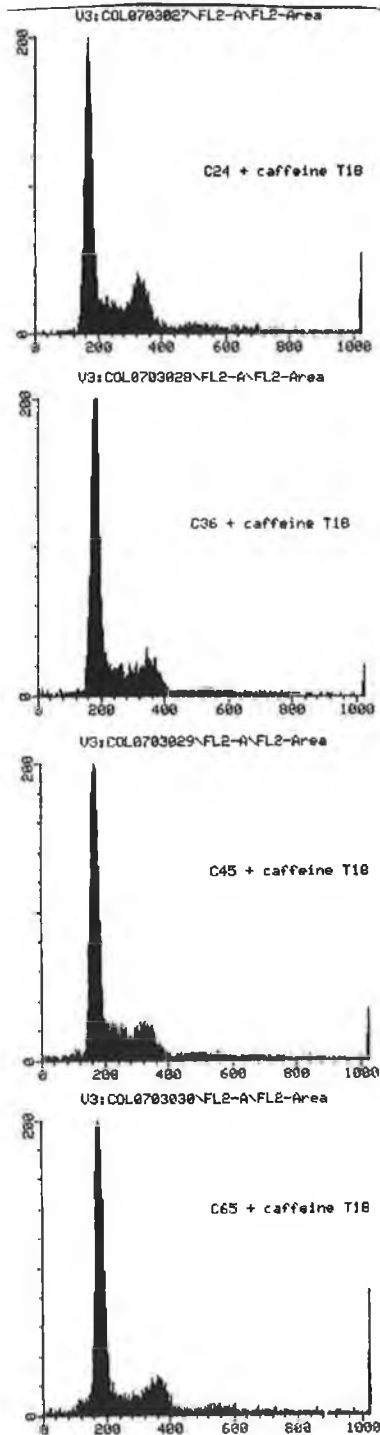


Figure 3.3.12 Cell cycle analysis of DLKP-SQ cells following 0.6 μ g/ml Doxorubicin 2 hr pulse and 4mM caffeine added directly after drug removal.

Controls



Drug treated

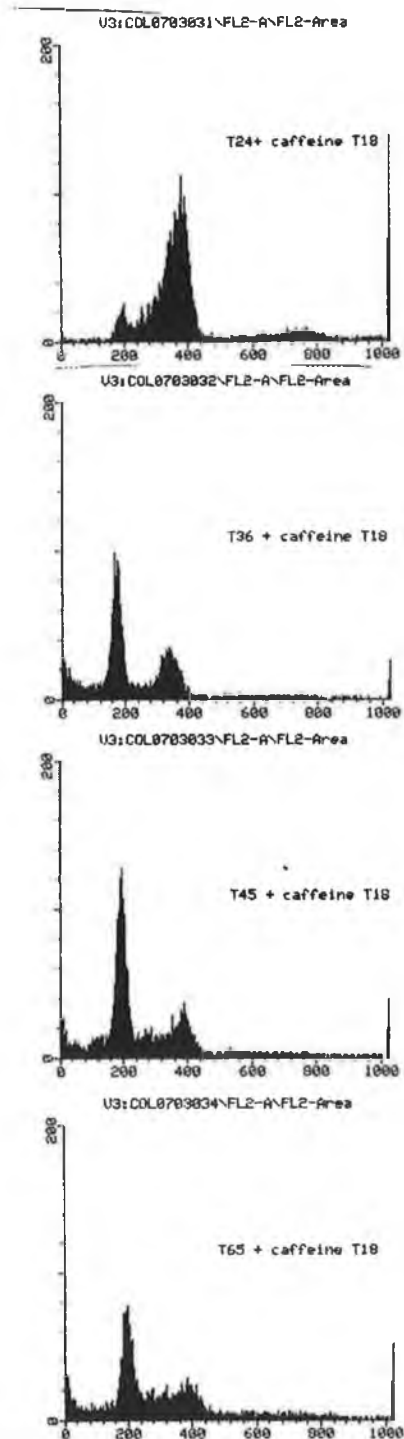


Figure 3.3.13 Cell cycle analysis of DLKP-SQ cells after 0.6µg/ml Doxorubicin 2hr pulse and 4mM caffeine added 18 hrs after drug removal. Controls were treated at 18 hrs with 4mM caffeine

3.4 Time-lapse Videomicroscopy of cells

From section 3.3 it is evident that a certain sub-population of the resistant variants can progress through mitosis after doxorubicin treatment, whilst the sensitive DLKP-SQ cells remain arrested at G2/M. Further evidence for this phenomenon comes from time-lapse videomicroscopy which allows visualisation of dynamic cellular events of cells in culture. Kinetics of cell death and mitosis were monitored in each of the cell lines after drug treatment. 100 cells were randomly chosen in the field and the number of apoptotic and mitotic events were noted (as described in materials and methods). When DLKP-SQ are doxorubicin-treated there is no mitosis observed and cells die by apoptosis, this is seen in figure 3.4.1. In the case of the resistant cells however there is a significant level of mitosis, being highest in DLKP-A5F cells. Figure 3.4.2 illustrates the kinetics of apoptosis and mitosis in DLKP-A20 10p #7 after drug treatment with 60 % apoptosis and 13 % mitosis in 100 cells over three days. DLKP-A2B cells exhibit 73 % apoptosis and 15 % mitosis seen in figure 3.4.3, and DLKP-A5F cells 70 % apoptosis and 58 % mitosis can be seen in figure 3.4.5. Table 3.4.1 summarises these results.

Cell Line	% Apoptosis	% Mitosis
DLKP-SQ	60 %	0 %
DLKP-A250 10p #7	60 %	13 %
DLKP-A2B	73 %	15 %
DLKP-A5F	70 %	58 %

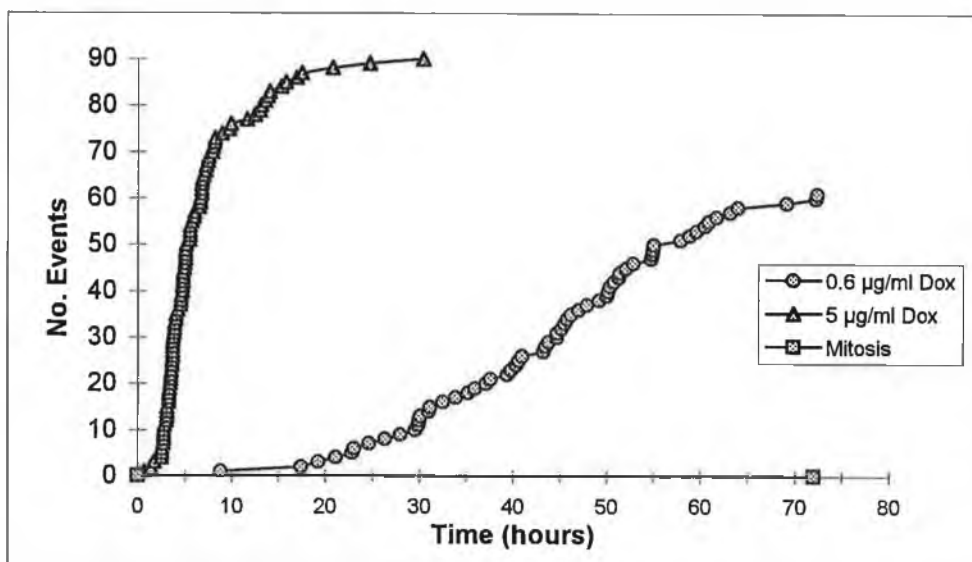


Figure 3.4.1 Timelapse videomicroscopical analysis of doxorubicin 2-hour pulse on DLKP-SQ

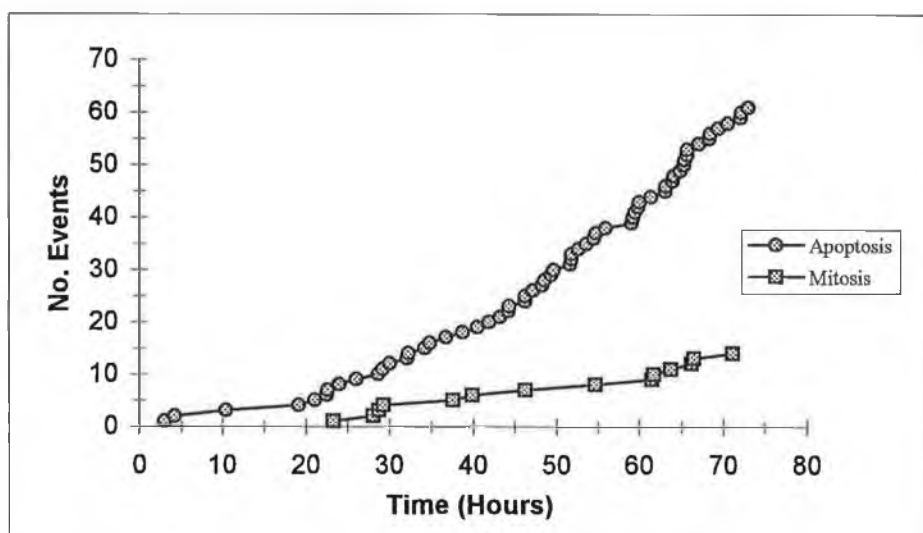


Figure 3.4.2 Timelapse videomicroscopical analysis of 2µg/ml doxorubicin 2 hour pulse on DLKP-A250 10p#7

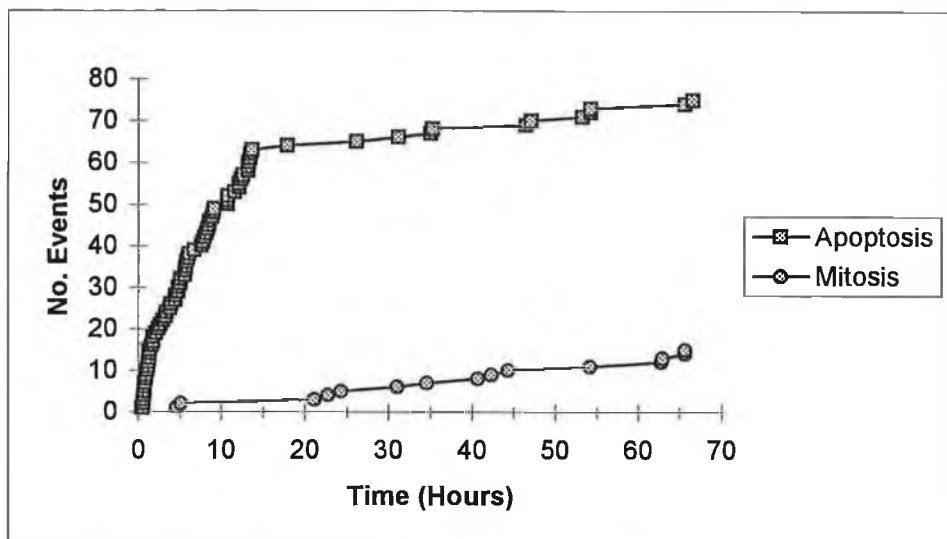


Figure 3.4.3 Timelapse videomicroscopical analysis of 30 µg/ml Doxorubicin 2 hour pulse treatment on DLKP-A2B

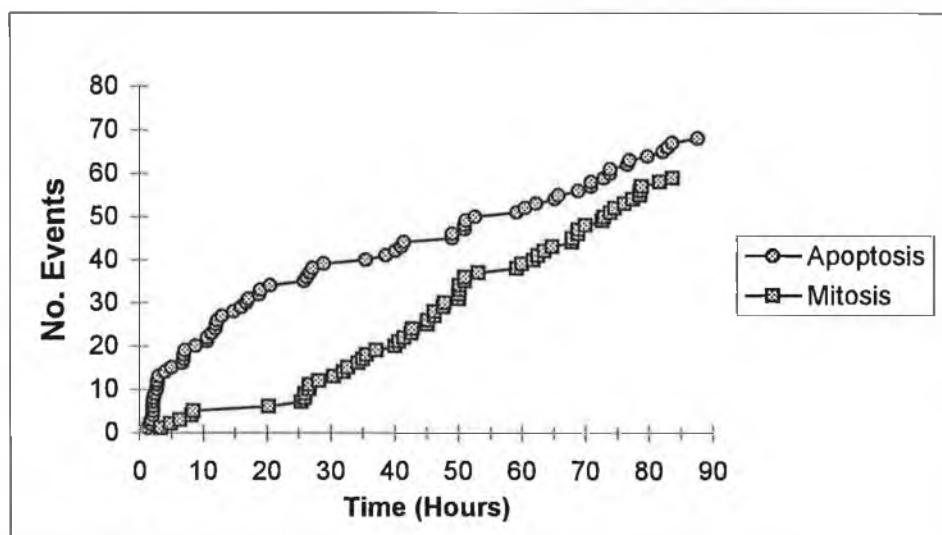


Figure 3.4.4 Timelapse videomicroscopical analysis of 100µg/ml Doxorubicin 2 hour pulse treatment on DLKP-A5F

3.5 Investigation of doxorubicin effect on cyclin and cdk levels

Flow cytometric analysis in section 3.3 demonstrated a G2/M arrest in doxorubicin treated DLKP-SQ cells, and a G2/M bypass in the resistant variants. This raises the possibility of a faulty or inefficient G2/M checkpoint in the resistant variants allowing some cells to over-ride the checkpoint even if their DNA is damaged. This likelihood led to an investigation of the proteins involved in the control of entry to mitosis, the main controller being cyclin B-cdk1 kinase complex. As described in section 1, cyclin B-cdk1 kinase is at the core of a complex control mechanism that allows the cell to sense any DNA damage and halt the cell cycle in order to repair any damage before the cell enters mitosis.

3.5.1 Cyclin B protein expression in DLKP cell lines following doxorubicin treatment

The effect of doxorubicin on cyclin B expression was investigated using Western Blotting. Any decrease in protein levels could indicate reduced protein synthesis or increased degradation rates, and any increase in protein levels could reflect either increased protein synthesis rates or decreased protein degradation. Blots were stained with the reversible protein stain Ponceau to confirm equal loading in each case. Results for cyclin B levels in DLKP-SQ are shown in figures 3.5.1.1 (controls) and 3.5.1.2 (drug treated). Controls are labelled C, at the time samples were taken, and drug-treated samples are labelled T at the time of sampling. There is no change in cyclin B protein levels after drug treatment. Figures 3.5.1.3 and 3.5.1.4, 3.5.1.5 and 3.5.1.6, 3.5.1.7 and 3.5.1.8 illustrate cyclin B protein levels in DLKP-A250 10p #7, DLKP-A2B and DLKP-A5F respectively. Again doxorubicin had no effect on the protein levels of cyclin B in the resistant variants.

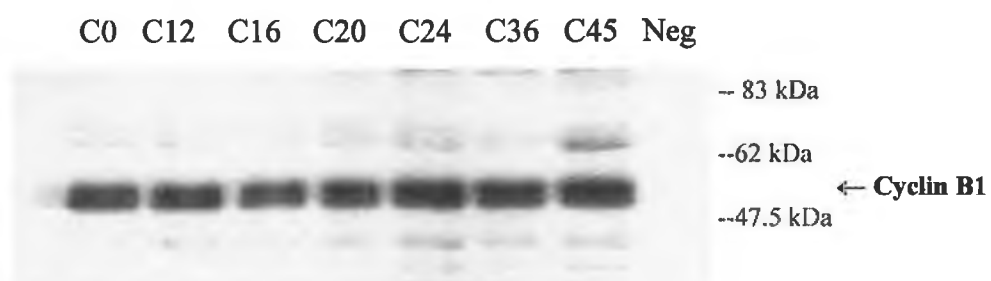


Figure 3.5.1.1 Western Blot showing cyclin B protein levels in DLKP-SQ untreated cells.

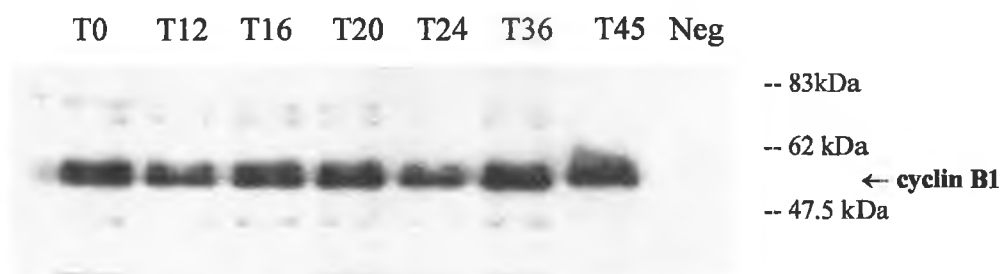


Figure 3.5.1.2 Western Blot showing Cyclin B protein levels in DLKP-SQ cells after 0.6µg/ml Doxorubicin 2hr pulse treatment.

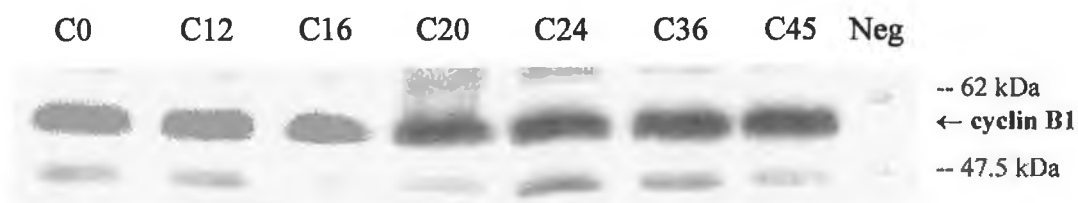


Figure 3.5.1.3 Western Blot showing Cyclin B protein levels in DLKP-SQ/A250 10p#7 untreated cells

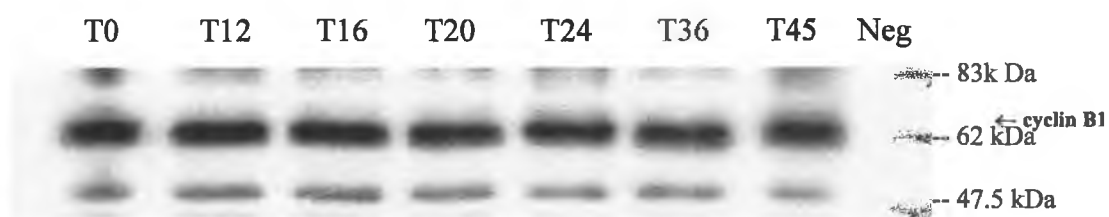


Figure 3.5.1.4 Western Blot of Cyclin B₁ protein levels in DLKP-SQ/A250 10p#7 cells after Doxorubicin 2hr pulse treatment

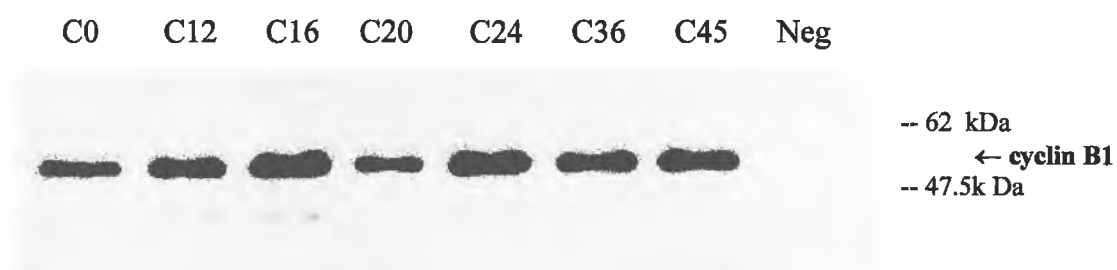


Figure 3.5.1.5 Western blot of Cyclin B protein levels in untreated DLKP-A2B cells

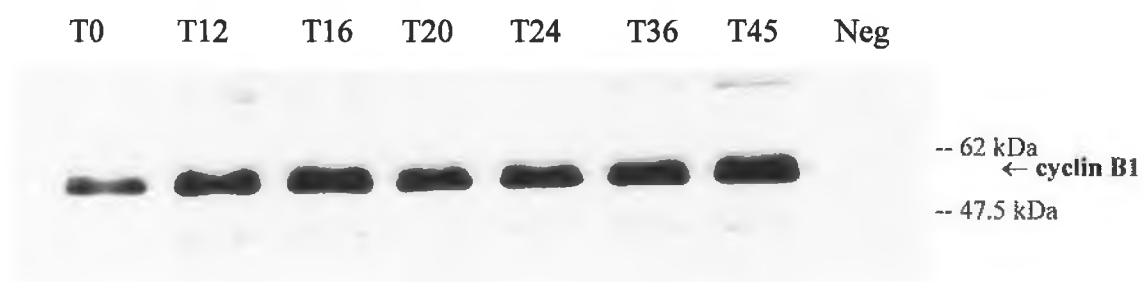


Figure 3.5.1.6 Cyclin B protein levels in DLKP-A2B cells after 30µg/ml Doxorubicin 2hr pulse treatment

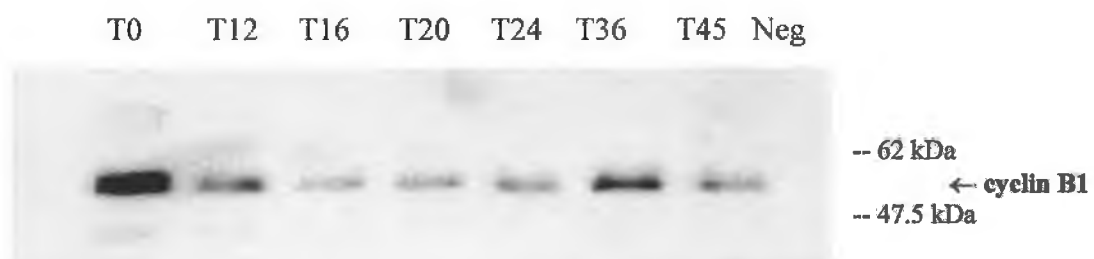


Figure 3.5.1.7 Cyclin B protein levels in untreated DLKP-A5F cells

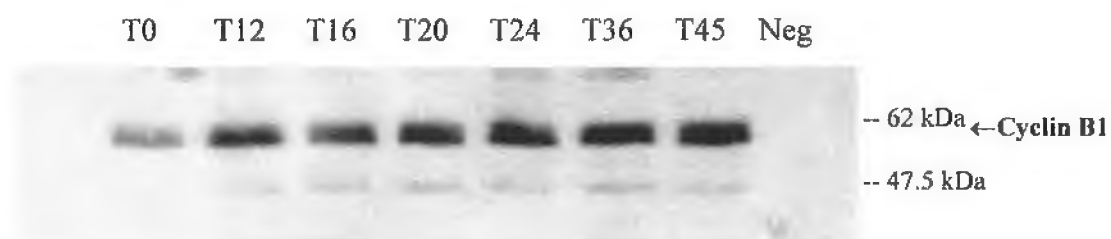


Figure 3.5.1.8 Cyclin B protein levels in DLKP-A5F cells after 100µg/ml Doxorubicin 2hr pulse treatment

3.5.2 Cdk1 expression following doxorubicin treatment

The catalytic partner of cyclin B, cdk1 was studied, in terms of possible altered expression following drug treatment. Western blotting showed no differences in protein levels after drug treatment compared to control cells. Cdk1 levels in DLKP-SQ can be seen in figures 3.5.2.1 and 3.5.2.2. The apparent increase in protein in figure 3.5.2.2 in DLKP-SQ after doxorubicin treatment is due to an unequal protein loading on this blot. DLKP-A250 10p#7 cdk1 levels are shown in figures 3.5.2.3 and 3.5.2.4. DLKP-A2B and DLKP-A5F results are illustrated in figures 3.5.2.5, 3.5.2.6 and figures 3.5.2.7, 3.5.2.8 respectively. In summary doxorubicin treatment does not alter expression of cdk1 protein in either sensitive or resistant DLKP cell lines.

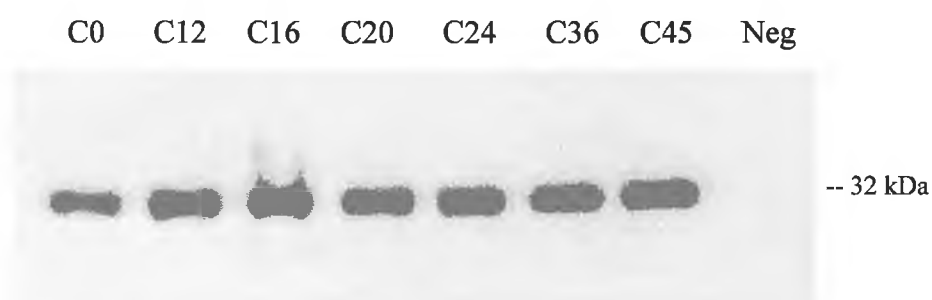


Figure 3.5.2.1 Cdk1 protein levels in DLKP-SQ untreated cells.

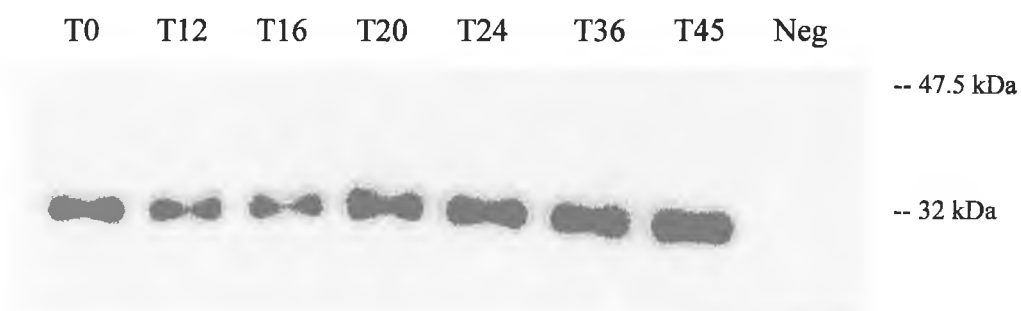


Figure 3.5.2.2 Cdk1 protein levels in DLKP-SQ cells after 0.6µg/ml Doxorubicin 2hr pulse treatment.

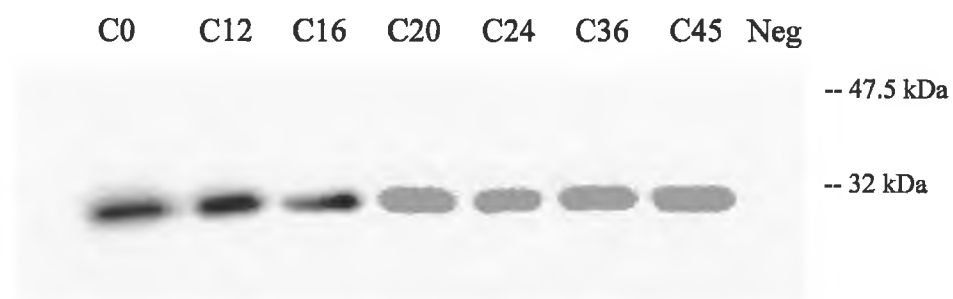


Figure 3.5.2.3 Cdk1 protein levels in DLKP-SQ/A250 10p#7 untreated cells

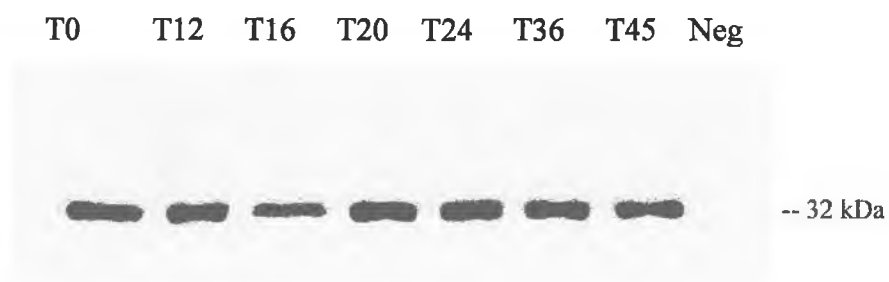


Figure 3.5.2.4 Cdk1 protein levels in DLKP-SQ/A250 10p#7 cells after Doxorubicin 2hr pulse treatment

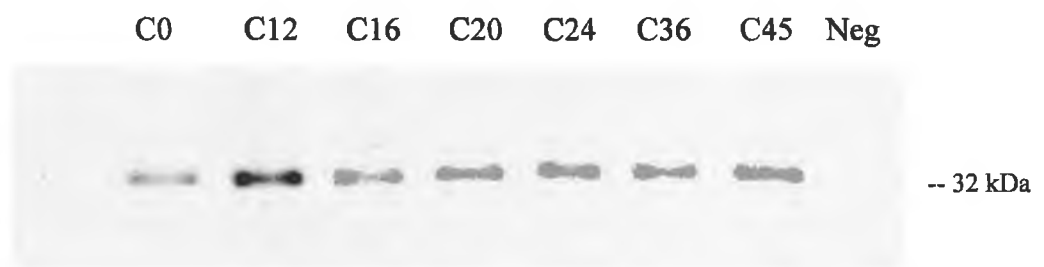


Figure 3.5.2.5 Cdk1 protein levels in untreated DLKP-A2B cells

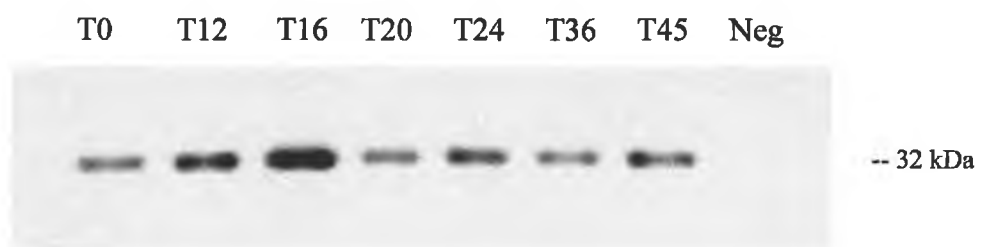


Figure 3.5.2.6 Cdk1 protein levels in DLKP-A2B cells after 30µg/ml Doxorubicin 2hr pulse treatment

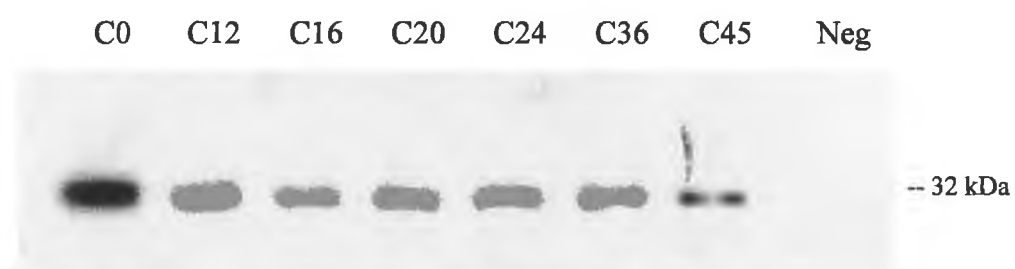


Figure 3.5.2.7 Cdk1 protein levels in untreated DLKP-A5F cells

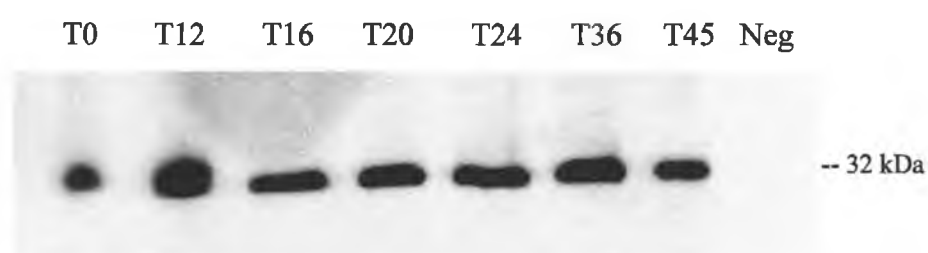


Figure 3.5.2.8 Cdk1 protein levels in DLKP-A5F cells after 100µg/ml Doxorubicin 2hr pulse treatment

3.5.3 Cyclin B-cdk1 complex formation

Section 3.5.1 and 3.5.2 demonstrated that doxorubicin did not affect the protein expression of cyclin B or cdk1 in any of the four DLKP variants. Even though equal protein expression was observed, this does not necessarily indicate that that complex formation was unaffected by drug treatment. If either cyclin B or cdk1 was altered by doxorubicin in such a way as to restrict its binding ability to its partner, yet unaffected protein expression, kinase activity would be prevented, thus arresting the cell cycle before mitosis onset. Conversely aberrant formation of cyclin B-cdk1 complexes, if sufficiently active could be responsible for a G2/M override.

Immunoprecipitations were carried out using an anti-cdk1 antibody. Samples were separated on SDS-page and probed with anti-cyclin B. In this way levels of cyclin B bound to cdk1 could be investigated before and after drug treatment. Figure 3.5.3.1 illustrates results, C0 refers to untreated controls, and T48 refers to doxorubicin-treated samples 48 hours after drug treatment. It can be seen that doxorubicin treatment does not alter levels of cyclin B-cdk1 complex in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B or DLKP-A5F. The alteration in the doxorubicin-induced G2/M arrest seen in resistant variants is not due to deregulated cyclin B-cdk1 complex formation.

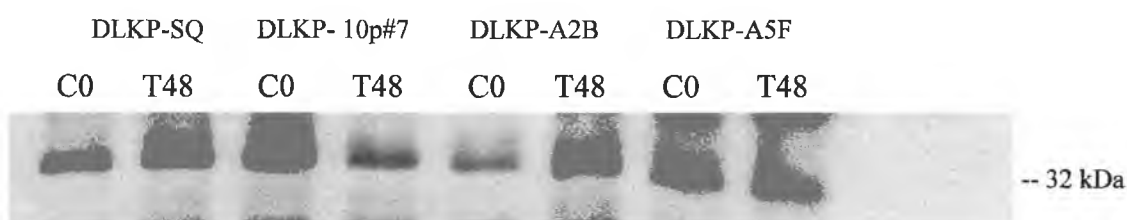


Figure 3.5.3.1 Western blot showing levels of cyclin B-cdk1 complex prior to and following doxorubicin treatment. Co refers to untreated controls, T48 refers to cells 48 hours after drug treatment.

3.6 Investigations on phosphorylation levels of specific residues on cdk1

Section 3.5 demonstrated that doxorubicin treatment did not affect protein levels of cyclin B or cdk1 in either of the cell lines. If these proteins are unaffected at the protein synthesis level, then perhaps the enzyme activity of the complex may be the causative factor accounting for the G2/M over-ride in the resistant variants. The activity of cdk1 is the key regulatory factor in the cyclin B-cdk1 complex, and as described in 1.4.7 is tightly controlled by a phosphorylation and dephosphorylation mechanism. Three main residues are initially phosphorylated on cdk1 when it binds to cyclin B; Thr 14, Tyr 15 and Thr 161. Thr 14 and Tyr 15 are inhibitory sites and when phosphorylated can render the complex inactive, these residues are thought to be phosphorylated by wee1 *in vivo*. The presence or absence of a phosphate residue on the Tyr 15 residue therefore will give an indication as to whether the cdk1 kinase is active (absence of a phosphate on Tyr 15) or inactive (presence of a phosphate on Tyr 15).

Tyr15 (Y15) phosphorylation of cdk1 in DLKP-SQ cells after doxorubicin treatment was looked at using two methods. The first technique involved immunoprecipitation of 100 µg cdk1 protein using an anti-cdk1 antibody. After Western Blotting, an anti-phosphotyrosine antibody was used to visualise the levels of phosphorylated Tyr 15. The blots are shown in figure 3.6.1 and 3.6.2. Negative controls consisted of immunoprecipitated protein exposed to antibody diluent, and IgG controls consisted of 1 µg normal mouse IgG immunoprecipitated instead of cellular protein. As can be seen from the blots, drug treatment had little or no effect on the levels of phosphorylated Tyr 15 compared to the untreated controls. This result is a little surprising because the G2/M arrest seen in flow cytometry could be caused by a decrease in cdc2 kinase activity due to phosphorylation on Y15. But the bands obtained are quite weak, so a definitive comparison is difficult.

A more specific technique involved using a specific anti-cdk1 Y15 monoclonal antibody which recognises the phosphorylated Y15 on cdk1. Results for DLKP-SQ cells are illustrated in figure 3.6.3, and the levels of phosphorylated Y15 seem to

increase after drug treatment. Levels of tyrosine phosphorylation in the resistant variants were also investigated. This was carried out using the specific anti-cdk1 Y15 antibody. There was no change in levels of Y15 phosphorylation in DLKP-A250 10p#7 treated cells compared to control levels (figure 3.6.5 and 3.6.6). This result was also seen in DLKP-A2B doxorubicin-treated cells (figures 3.6.7 and 3.6.8) and in DLKP-A5F doxorubicin treated cells (figure 3.6. 9 and 3.6.10).

To further corroborate these results, samples from the four cell lines were run on one gel together, and results are shown in figure 3.6.11. It can be clearly determined from this blot that DLKP-SQ experiences an increase in Y15 phosphorylation after doxorubicin treatment, and resistant variants seem to have a similar level of Y15 phosphorylation when compared to controls. When analysed using densitometry and taking protein loading into consideration, the DLKP-SQ Y15 phosphorylation increased 1.58-fold compared to the untreated control. The higher protein in the lane marked T48 #7 is due to much higher protein loading in this lane, as concluded from Ponceau staining.

From these results it seems that the G2/M bypass in the resistant cells is due to absence of an increase in Y15 phosphorylation levels. This suggests a consistent cdk1 kinase activity which could push the cells through mitosis. This theory is further investigated by looking at the cdk1 kinase activity in the cell lines following doxorubicin treatment.

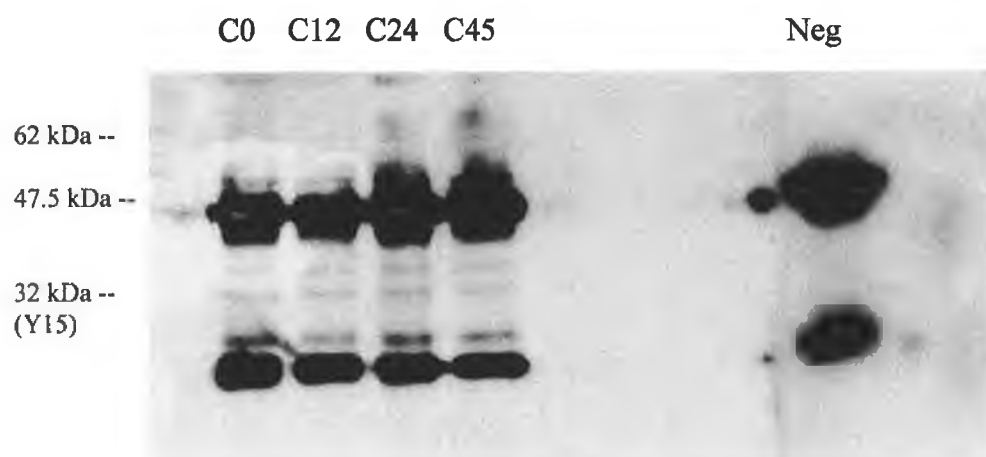


Figure 3.6.1 Untreated DLKP-SQ samples. Immunoprecipitations with anti-cdk1 antibody, and then probed with antiphosphotyrosine to look at levels of Y15 phosphorylation before and after doxorubicin treatment

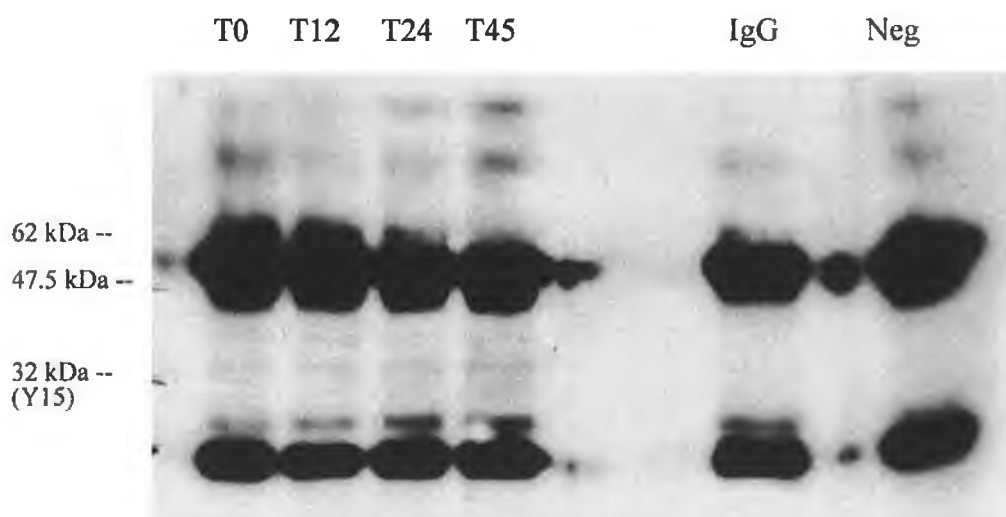


Figure 3.6.2 Doxorubicin-treated samples. Immunoprecipitations with anti-cdk1 antibody, and then probed with antiphosphotyrosine to look at levels of Y15 phosphorylation after doxorubicin treatment

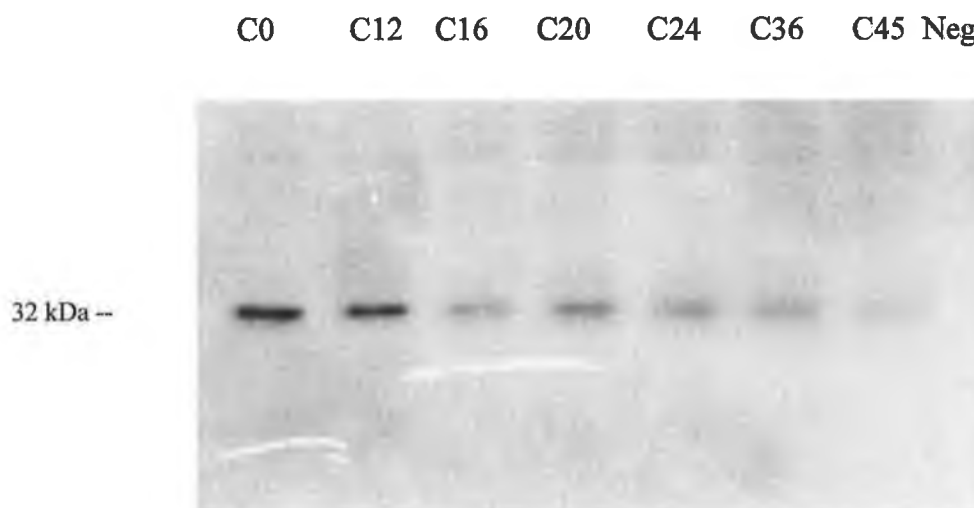


Figure 3.6.3 Untreated controls. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 before doxorubicin treatment in DLKP-SQ cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

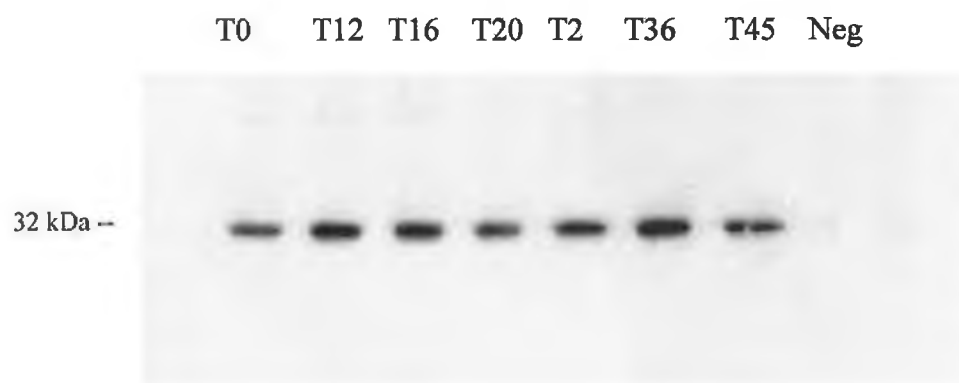


Figure 3.6.4 Western blots showing levels of phosphorylated tyrosine 15 on cdk1 after doxorubicin treatment in DLKP-SQ cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

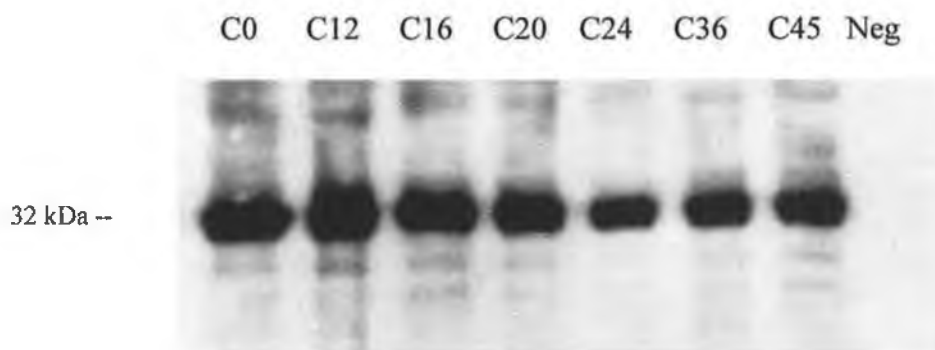


Figure 3.6.5 Untreated DLKP-A250 10p#7 cells. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 before doxorubicin treatment in DLKP-A250 10p#7 cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

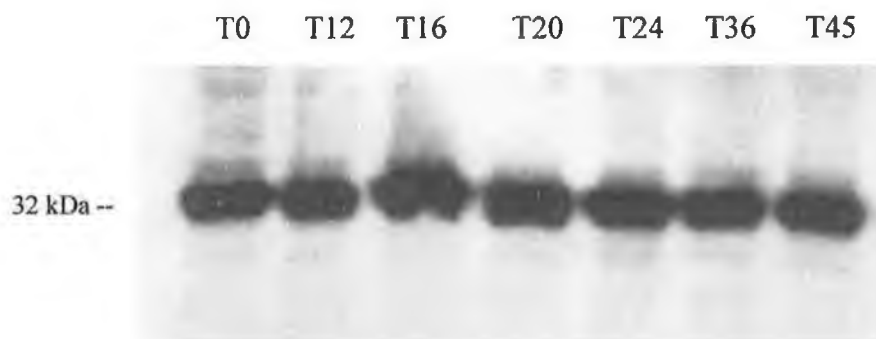


Figure 3.6.6 Doxorubicin treated DLKP-A250 10p #7cells Western blots showing levels of phosphorylated tyrosine 15 on cdk1 after doxorubicin treatment in DLKP-A250 10p#7 cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

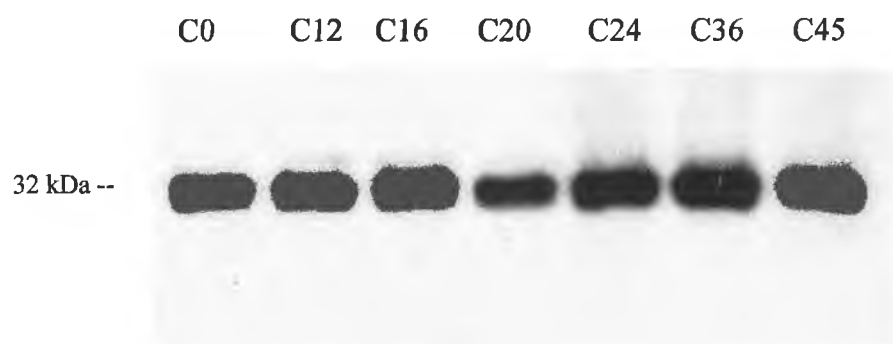


Figure 3.6.7 Untreated DLKP-A2B cells. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 before doxorubicin treatment in DLKP-A2B cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

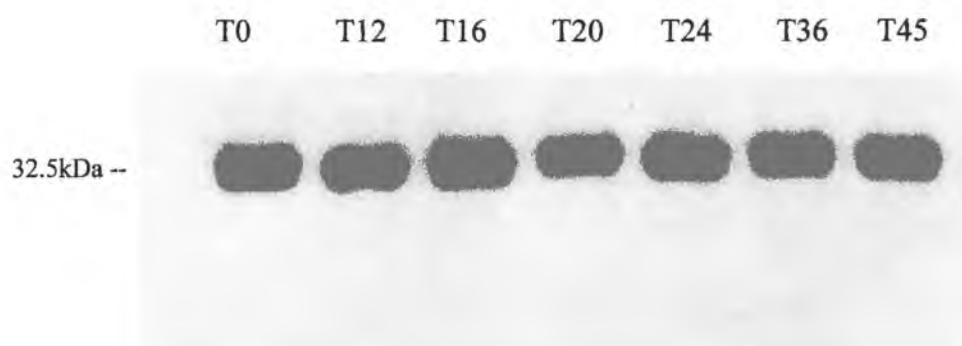


Figure 3.6.8 Doxorubicin treated DLKP-A2B cells. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 after doxorubicin treatment in DLKP-A2B cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

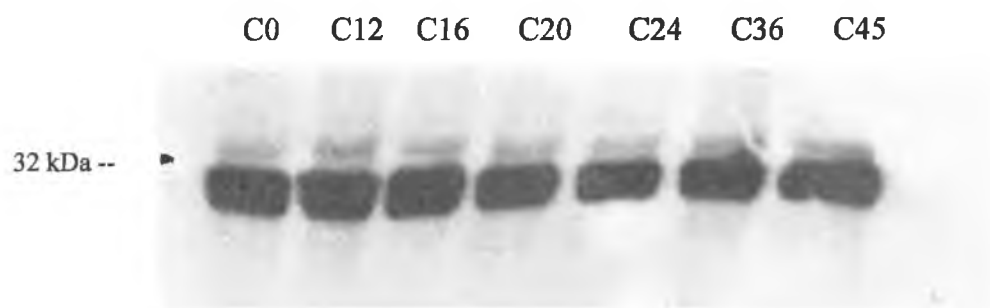


Figure 3.6.9 Untreated DLKP-A5F cells. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 before doxorubicin treatment in DLKP-A5F cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.

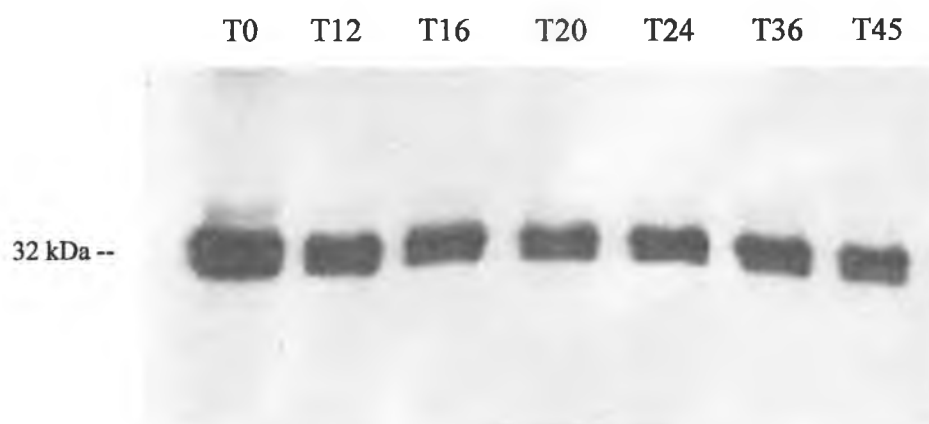
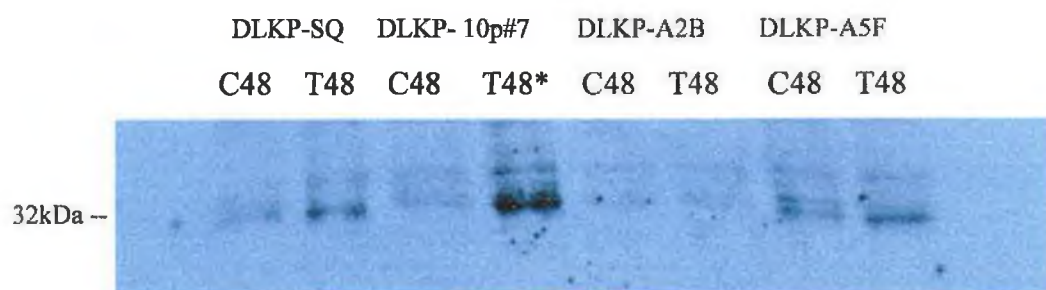


Figure 3.6.10 Doxorubicin treated DLKP-A5F cells. Western blots showing levels of phosphorylated tyrosine 15 on cdk1 after doxorubicin treatment in DLKP-A5F cells. Blots were probed using a cdk1-specific anti-phosphotyrosine 15.



*T48, much higher protein loading, when ponceau staining was carried out.

Figure 3.6.11 Western blot showing levels of Y15 phosphorylation in the four DLKP cell lines. C0 refers to untreated controls, T48 refers to doxorubicin treated samples 48 hours after treatment.

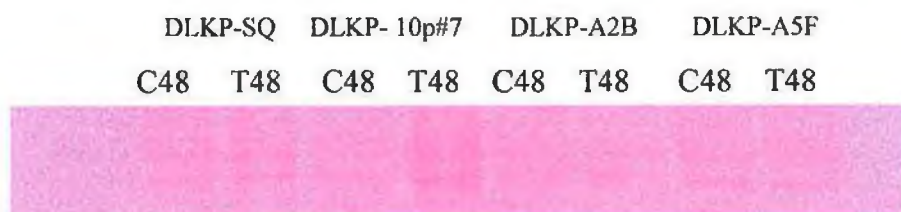


Figure 3.6.12 Ponceau-stained membrane showing levels of protein loading for Y15 phosphorylation Western seen in 3.6.11.

3.7 Cdk1 kinase activity assays

A cdk1 kinase activity assay in the cells was assessed by two methods. A radioactive method was initially employed to look at kinase activity associated with the cyclin B-cdk1 complex after drug treatment. Unfortunately results were difficult to interpret due to high background and uneven loading of beads on the gels. An alternative assay procedure was obtained in the form of an ELISA-based kit. The kit uses a synthetic biotinylated peptide modelled on the phosphorylation sequence surrounding the Y15 phosphorylation site on cdk1. The peptide, when phosphorylated can be picked up by an antibody (which recognises the phosphorylated form of the peptide) coated on the ELISA strip. Streptavidin-labeled peroxidase is then used to detect any bound peptides. Substrate solution is added and the intensity of colour is measured at 492nm.

This ELISA method was used to measure kinase activity in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F. Results are illustrated in figure 3.7.1. The DLKP-SQ cells seem to experience a slight decrease in cdk1 kinase activity following doxorubicin treatment. This observation supports the finding in section 3.6 that cdk1 becomes phosphorylated in DLKP-SQ cells after doxorubicin treatment, coinciding with the G2 arrest seen in section 3.2. The resistant variants, however follow a trend of elevated cdk1 kinase activity following doxorubicin treatment, and this kinase activity rises with increasing time after doxorubicin treatment. This result indicates that a higher kinase activity in the resistant variants is responsible for their ability to overcome the G2/M arrest seen in sensitive cells. The results are not statistically significant but numerous repeat experiments produced similar trends of unaltered or decreased cdk1 kinase activity following drug treatment in DLKP-SQ, with an increase in cdk1 kinase activity in resistant variants following drug treatment.

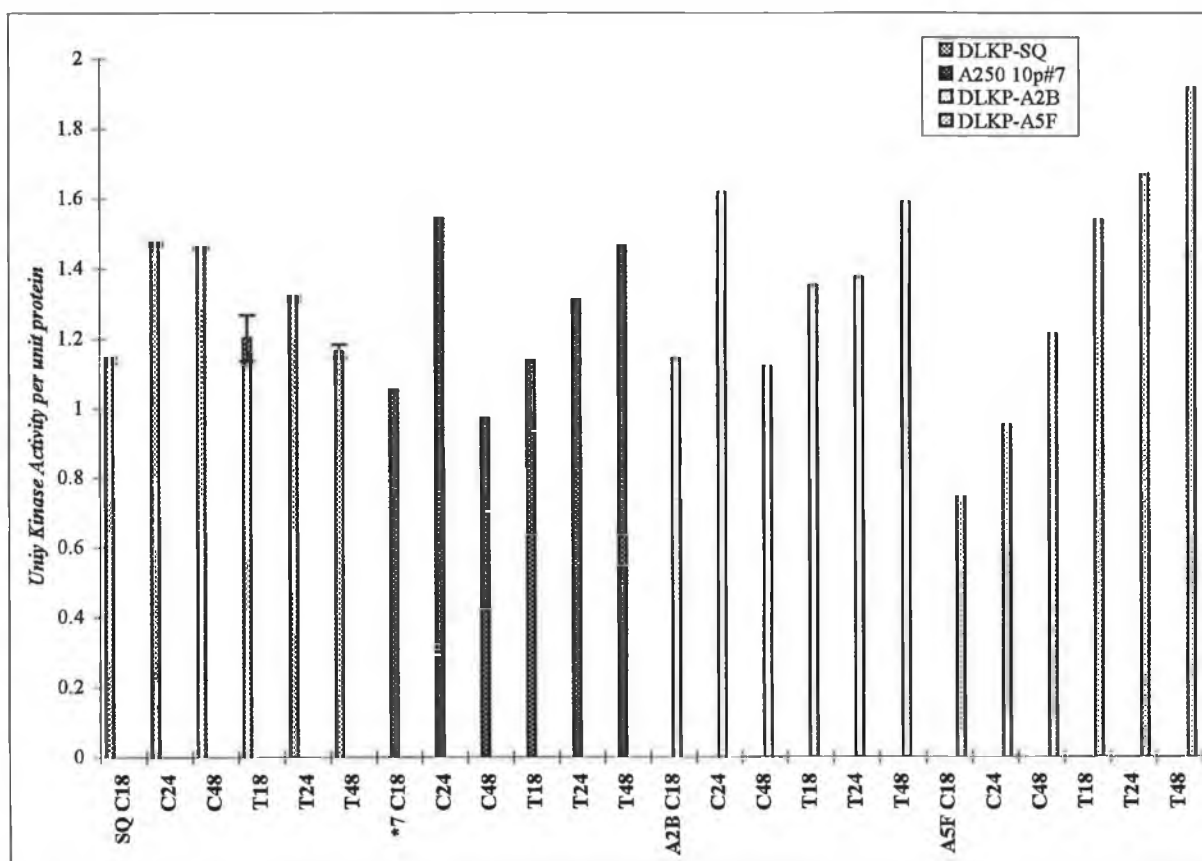


Figure 3.7.1 Cdk1 kinase assay. Four DLKP cell lines were analysed at 18, 24 and 48 hours following doxorubicin treatment. Controls were also analysed at 18, 24 and 48 hours. Cell lines are arranged from left to right, from sensitive to most resistant.

Section 3.8 Analysis of cdc25B and cdc25C in cell lines after drug treatment

Cdc25B and cdc25C are members of the cdc25 phosphatase family, and as described in section 1.4.7 are critically involved in activation of cyclin B-cdk1 and entry to mitosis. The level of expression of cdc25B and cdc25C represents an important control point in the activation of cdk1 kinase. Any overexpression of cdc25C could lead to unscheduled activation of cdk1 and a push past the G2 arrest, ultimately leading to unrestrained cell division and proliferation. Indeed cdc25C overexpression occurs in many human cancers and has been implicated as a potential oncogene (Galaktionov *et al.*, 1995).

Levels of cdc25B and cdc25C were investigated in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F before and after doxorubicin treatment to determine whether or not the G2/M override seen in the resistant variants was associated with an altered expression of cdc25B or cdc25C. Figure 3.8.1 and 3.8.2 show levels of cdc25B protein expression in the cells prior to and following drug treatment. Cdc25B protein expression does not vary after doxorubicin treatment in DLKP-SQ when compared to controls, as illustrated in figure 3.8.1. Equal protein loading was monitored by reversible staining with ponceau. Cdc25B was also investigated in the resistant variants before and after doxorubicin treatment. Results in figure 3.8.2, when protein loading is taken into account, indicate that cdc25B expression is not altered in resistant variants compared to the sensitive cell line, nor is its expression affected by doxorubicin treatment.

Cdc25C was also investigated in the resistant variants, and similar results were found. In figure 3.8.4 it can be seen that there are equal levels of cdc25C in resistant and sensitive DLKP cell lines, and expression is not altered by doxorubicin treatment. From figure 3.8.3 it seems as if there are higher levels of cdc25C expression in DLKP-SQ cells at T24, T36 and T45 but this is due to slightly higher protein loaded in these wells.

The activity of cdc25C increases through phosphorylation, and the phosphorylation can be experimentally detected by altered mobility in SDS-page. Samples were immunoprecipitated using anti-cdc25C before and after doxorubicin treatment, and then run on 15% SDS-polyacrylamide gels. Results for DLKP-SQ are displayed in figure 3.8.5, DLKP-A250 10p#7 in figure 3.8.6, DLKP-A2B in figure 3.8.7 and DLKP-A5F in figure 3.8.8. Inactive hypophosphorylated cdc25C migrates at 55kDa, and when activated (hyperphosphorylated) it migrates at 66kDa. However the 55kDa and 66kDa bands can be seen in all the samples, with no difference in the level of expression of either band following drug treatment. This questions the role of cdc25C in the G2/M override in the resistant cells. Possible reasons explaining this result are discussed in section 4.8.

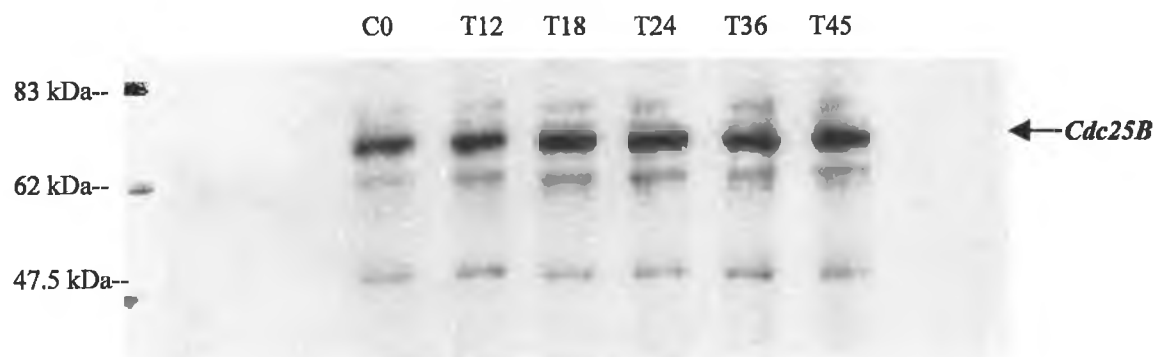


Figure 3.8.1 Effect of doxorubicin on *cdc25B* expression in DLKP-SQ (C refers to controls, T refers to drug-treated samples).

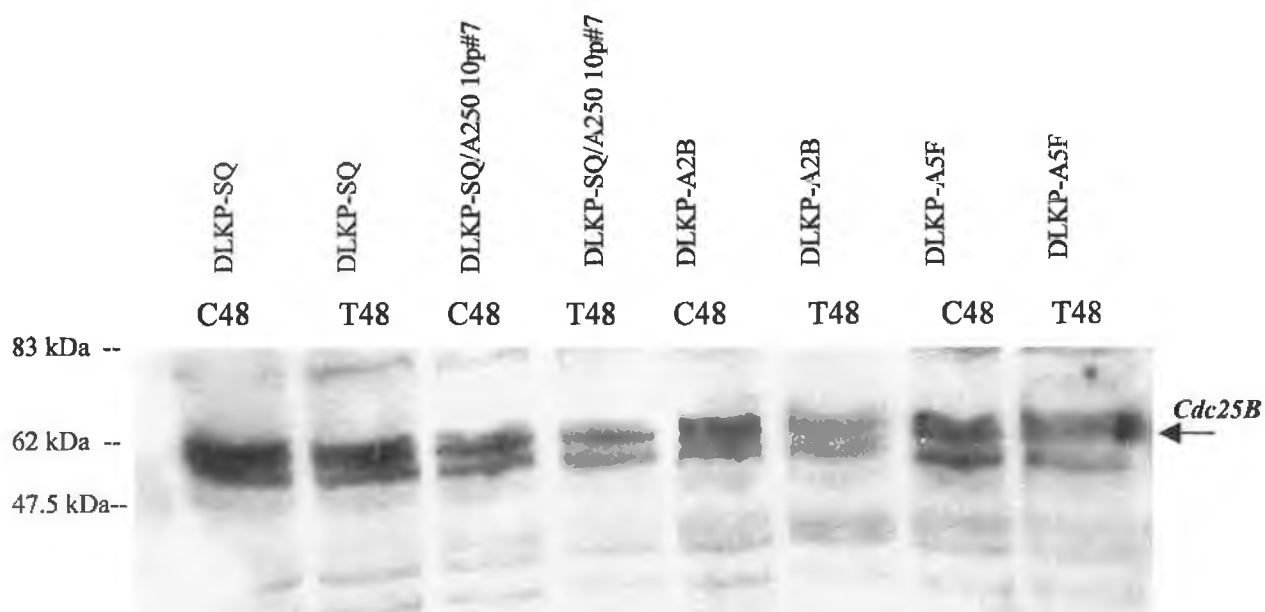


Figure 3.8.2 Effect of doxorubicin on *cdc25B* expression in DLKP cell lines (C refers to controls, T refers to drug-treated samples).

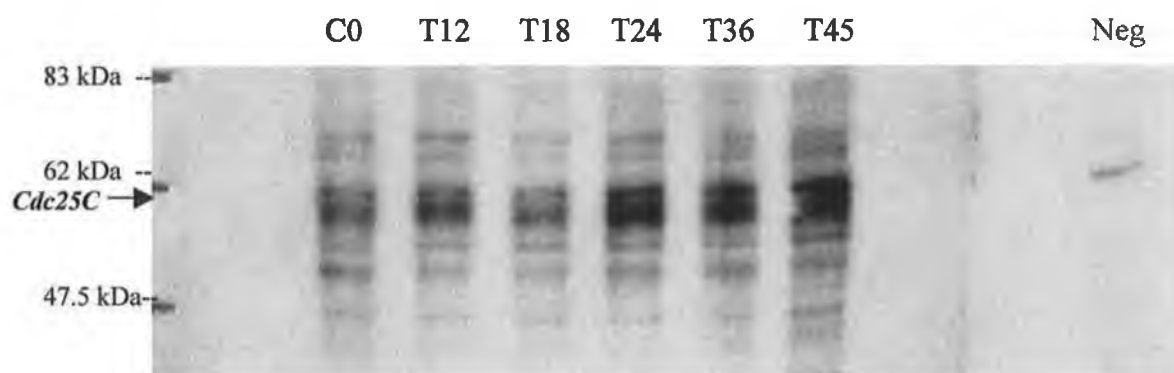


Figure 3.8.3 Effect of doxorubicin on *cdc25C* expression in DLKP-SQ (C refers to controls, T refers to drug-treated samples).

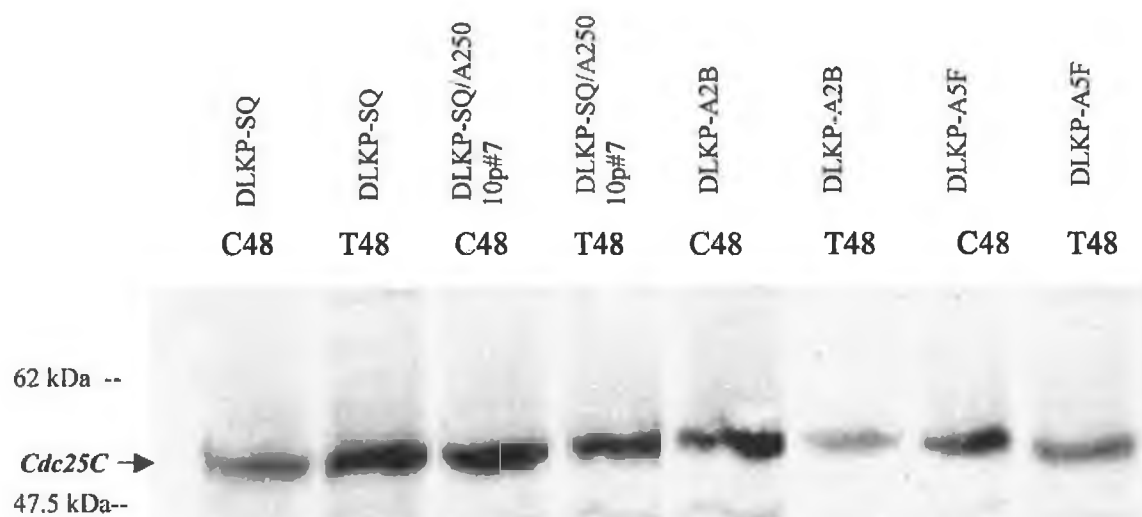


Figure 3.8.4 Effect of doxorubicin on *cdc25C* expression in DLKP cell lines (C refers to controls, T refers to drug-treated samples).

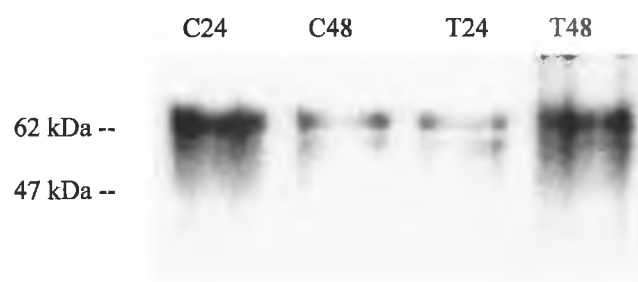


Figure 3.8.5 Immunoprecipitation of cdc25C in DLKP-SQ cells before and after doxorubicin treatment. C24 and C48 refer to untreated controls, T24 and T48 refers to drug-treated cells.

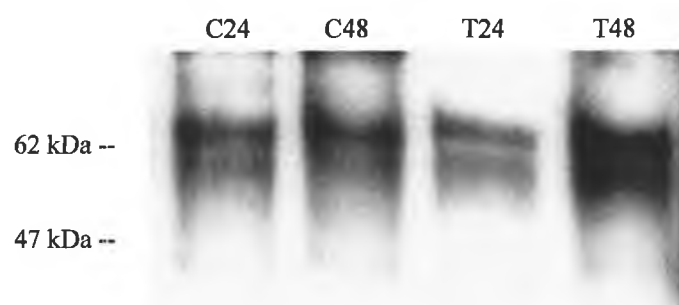


Figure 3.8.6 Immunoprecipitation of cdc25C in DLKP-A250 10p#7 cells before and after doxorubicin treatment. C24 and C48 refer to untreated controls, T24 and T48 refer to drug-treated cells.

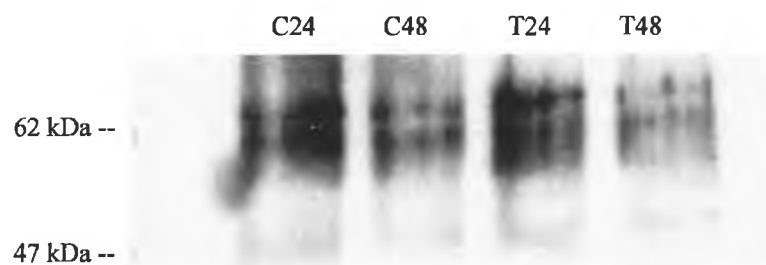


Figure 3.8.7 Immunoprecipitation of cdc25C in DLKP-A2B cells before and after doxorubicin treatment. C24 and C48 refer to untreated controls, T24 and T48 refer to drug-treated cells.

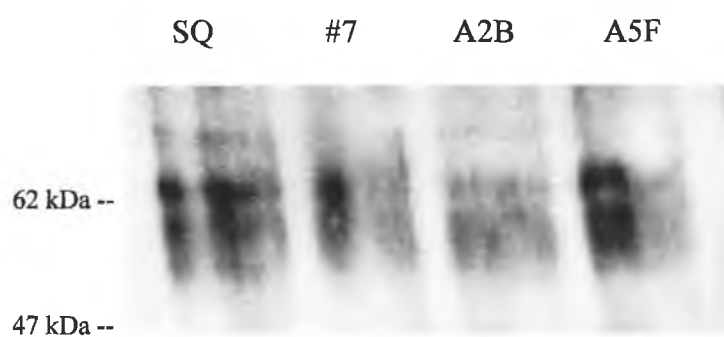


Figure 3.8.8 Immunoprecipitation of cdc25C in DLKP-A5F cells before and after doxorubicin treatment. C24 and C48 refer to untreated controls, T24 and T48 refer to drug-treated cells.

3.9 Investigation of wee1 levels and activation in DLKP cell lines

Wee1 is responsible for the inhibitory phosphorylation of cdk1, and therefore can co-ordinate onset of mitosis by inactivating cyclin B-cdk1 complex activity until required conditions for mitosis are present. In section 3.6, it appears that DLKP-SQ have higher Y15 phosphorylation levels compared to resistant variants after doxorubicin treatment. It was observed in section 3.7 that DLKP-SQ experience a slight decrease in cdk1 kinase activity, when compared to the response of resistant variants following drug treatment. It is necessary to investigate the involvement of wee1 in the G2 arrest of DLKP-SQ, in the override experienced by the resistant variants and to determine if wee1 is responsible for the higher Y15 phosphorylation levels seen in DLKP-SQ. If wee1 is responsible, then it is plausible to suggest that perhaps wee1 activity is lower in resistant variants, thus allowing them to progress through mitosis.

Wee1 protein expression in the four cell lines was investigated to see if wee1 downregulation was associated with the G2/M override seen in resistant variants. Results in figure 3.9.1 demonstrate that wee1 is expressed to a similar extent in resistant and sensitive DLKP cells, suggesting that wee1 expression is not linked to the G2/M override.

The activity of wee1 before and after doxorubicin treatment in each of the four cell lines was investigated by immunoprecipitation with an anti-wee1 antibody, running samples on SDS-polyacrylamide gels and probing with anti-wee1, as described in section 2.6.7. Wee1 runs at approximately 94 kDa, and when inactivated by phosphorylation it should run slightly higher (≈ 5 kDa) on the gel. Results are shown in figure 3.9.2 for DLKP-SQ, 3.9.3 for DLKP-A250 10p#7, figure 3.9.4 for DLKP-A2B and figure 3.9.5 for DLKP-A5F. It can be seen from these results that active wee1 runs at approx. 94 kDa. In DLKP-SQ the upper band is reduced in the drug treated samples, particularly by 48 hours after drug treatment. This suggests that the level of inactive wee1 in these cells is lower, which would tie in well with a G2 arrest. The higher levels of wee1 would be required to prevent cdk1 kinase activity. The wee1 levels in the resistant variants do not vary and therefore must be activated

to an equal extent in all three resistant cell lines, before and after doxorubicin treatment, perhaps suggesting that the unaltered weel is responsible for the unaltered Y15 phosphorylation levels seen in cdk1. The problems associated with using asynchronous cells, and difficulties with detection of changes in weel phosphorylation are discussed in section 4.9.

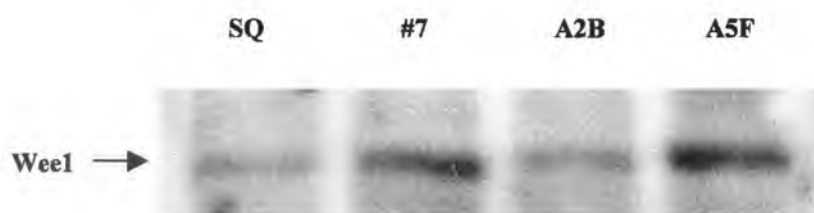


Figure 3.9.1 Levels of weel protein in the four DLKP cell lines. There was slightly less protein loaded in DLKP-SQ and DLKP-A2B.

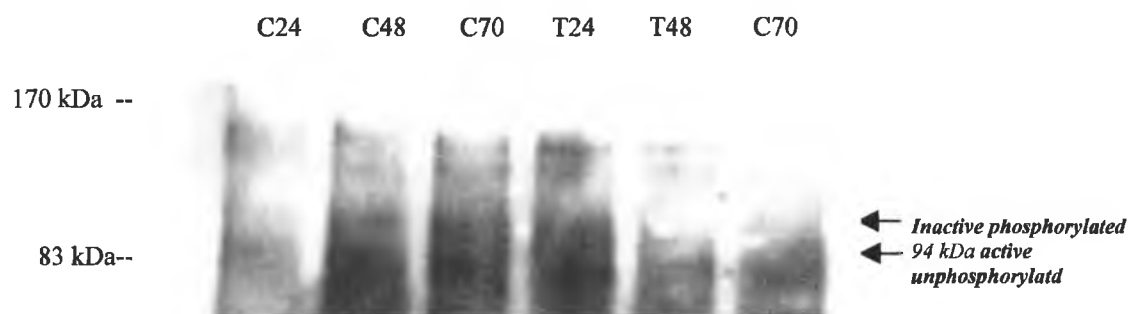


Figure 3.9.2. Immunoprecipitation of weel protein in DLKP-SQ before and after doxorubicin treatment. Two arrows indicate two forms of weel, upper band represents hyperphosphorylated inactive weel, lower band represents active weel.

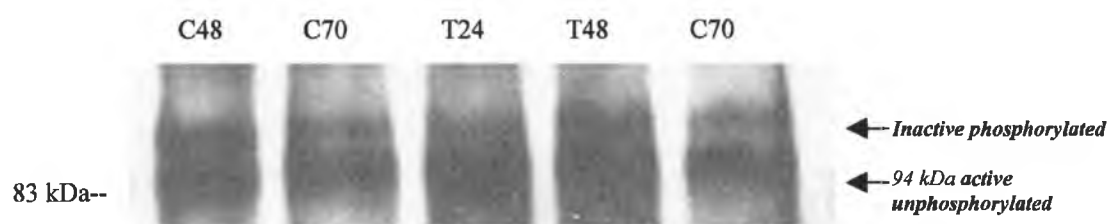


Figure 3.9.3. Immunoprecipitation of weel protein in DLKP-A250 10p#7 before and after doxorubicin treatment. Two arrows indicate two forms of weel, upper band represents hyperphosphorylated inactive wee, lower band represents active weel.

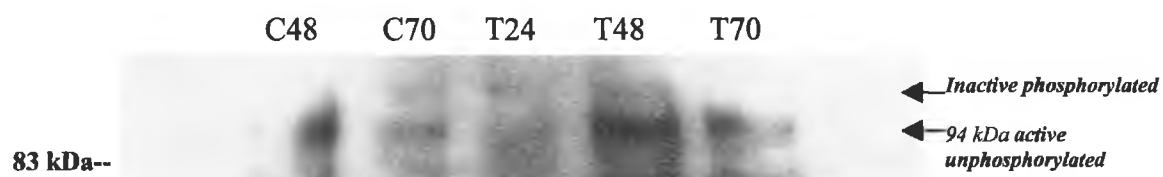


Figure 3.9.4. Immunoprecipitation of weel protein in DLKP-A2B before and after doxorubicin treatment. Two arrows indicate two forms of weel, upper band represents hyperphosphorylated inactive wee, lower band represents active weel.



Figure 3.9.5. Immunoprecipitation of weel protein in DLKP-A5F before and after doxorubicin treatment. Arrow indicates active form of weel.

Section 3.10 MDR characteristics of DLKP cell lines

3.10.1 P-glycoprotein status in DLKP cell lines

Expression of P-glycoprotein (Pgp) in cells is closely associated with the development of multiple drug resistance (MDR). Cells that overexpress Pgp frequently exhibit the MDR phenotype, becoming resistant to a wide variety of structurally unrelated and mechanistically distinct chemotherapy drugs.

The resistant DLKP cell lines were originally selected for resistance by a pulse (DLKP-A250 10p#7) or a continuous (DLKP-A2B, DLKP-A5F) stepwise increase in exposure to doxorubicin. Extensive characterisation has revealed MDR characteristics in these cell lines, including overexpression of Pgp (Heenan *et al.*, 1998). To confirm that these cells are stable in their MDR status, the Pgp levels were compared using western blotting with C219-antibody specific for Pgp, although it may also cross-react with c-erbB2 at around 170kDa. Results are shown in figure 3.10.1. DLKP-SQ cells do not express detectable levels of Pgp because these are a sensitive clonal population isolated from the sensitive parental population (McBride *et al.*, 1997). DLKP-A250 10p#7 express low levels of Pgp; and DLKP-A2B and DLKP-A5F express higher levels of Pgp. The DLKP-A2B and DLKP-A5F display much higher resistance levels than the DLKP-A250 10p#7, and this is mainly conferred through their higher Pgp expression. However, DLKP-A5F are 100-fold more resistant than DLKP-A2B, yet both express a comparable level of Pgp expression, so there must be alternative resistance mechanisms in the DLKP-A5F cell line to account for the increased resistance without an accompanied increase in Pgp expression.

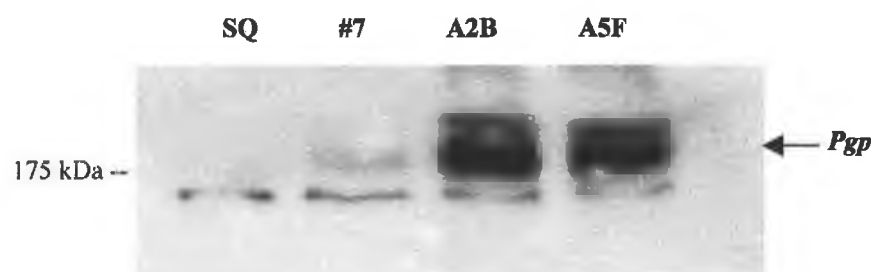


Figure 3.10.1 Western blotting showing P-glycoprotein levels in DLKP cell lines

3.10.2 Doxorubicin toxicity profile on the DLKP cell lines

Doxorubicin was the original selecting drug used in generating the DLKP resistant variants. Resistant cells were developed by exposing the cells to a continuous increasing drug concentration until a resistant population emerged with the ability to proliferate in the presence of high drug concentrations. DLKP-A2B and DLKP-A5F are clonal populations isolated from the original resistant population DLKP-A, which was developed by exposing DLKP to continuous increasing concentrations of doxorubicin (Heenan *et al.*, 1998). DLKP-A250 10p#7 is a clonal population derived from DLKP-A250 10p, as described in materials and methods. DLKP-A250 10p are a cell line exhibiting a low level of resistance which was developed by pulsing the clonal DLKP-SQ cell line 10 times (10p) with 250ng/ml doxorubicin (A250) for four hours (NicAmhlaoibh, 1997). The relative resistance of each of these cell lines to each other in the experimental setting used in this thesis (25cm² flasks, 2 hour drug pulse and viability determined after 72 hours) was examined, and representative data are in figure 3.10.2. It was concluded from numerous toxicity assays that DLKP-A5F is approximately 300-fold resistant, DLKP-A2B is 30-fold resistant and DLKP-A250 10p#7 are 2-3-fold resistant to DLKP-SQ cells. These are at slight variance with previous results of 300-fold, 30-fold and 10-fold respectively to DLKP, but these values represent resistance in 96-well plates after 6 days with continuous drug exposure, compared to 25cm² flasks, 2 hour drug incubation and 72 hour viability estimates being used in this study. Therefore the discrepancies in results can be attributed to different experimental conditions such as cell density, drug exposure times and length of time before viability was determined. Toxicity was assessed after 72 hours because this was the period of time over which the effects of doxorubicin on the cell cycle were monitored. Apoptosis was also monitored over this period of time, and approximately 50% of the cells died over 72 hours.

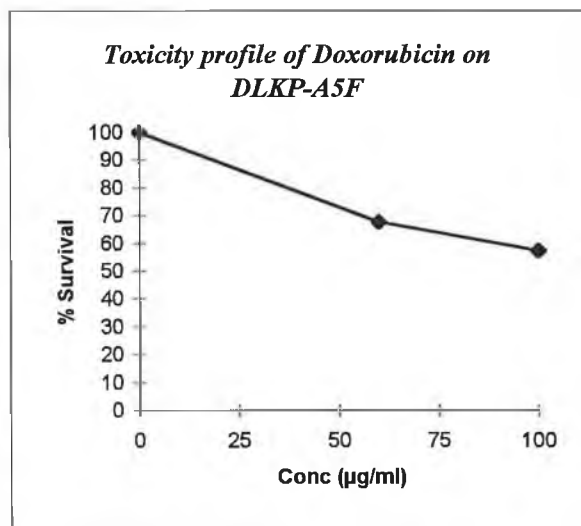
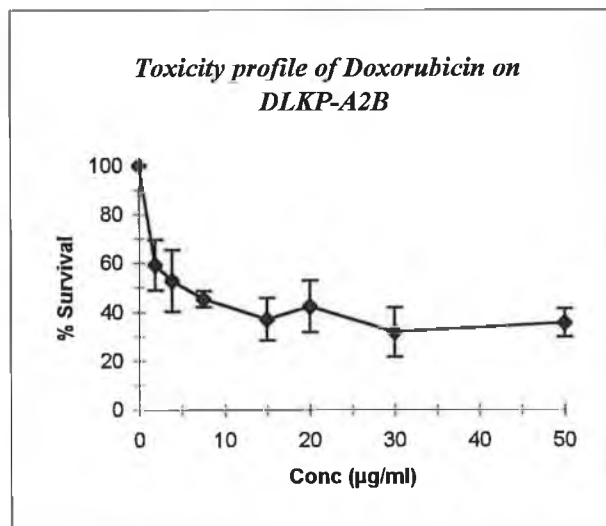
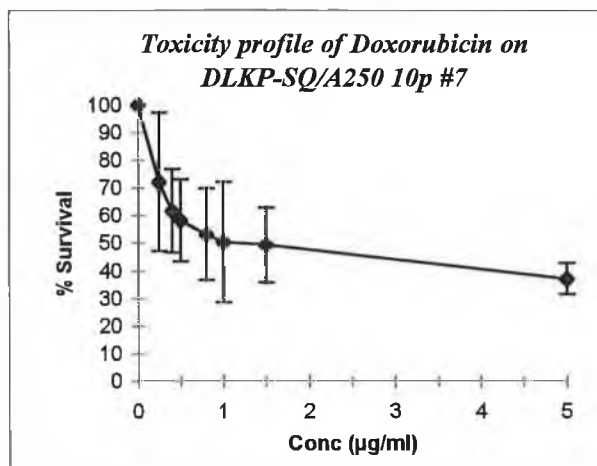
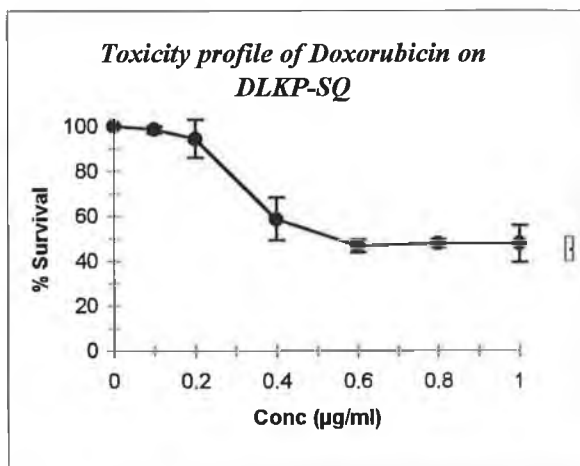


Figure 3.10.2 Toxicity profiles of DLKP cell lines. Cells were treated for 2 hours with doxorubicin, after which time drug was removed and flasks refed. Viability was determined after 72 hours using crystal violet toxicity assay.

3.10.3 Drug Accumulation Analysis in drug resistant cell lines

The principal function of Pgp in MDR is to act as an energy-dependent efflux pump which effectively removes drug from the cell, resulting in a lower concentration of drug in the nuclei of resistant cells. One advantage of using doxorubicin is its innate fluorescence characteristics, it fluoresces under UV light and its cellular location can easily be determined.

DLKP cells were analysed for doxorubicin accumulation by preparing cytopspins, 72 hours after doxorubicin treatment, as described in materials and methods. Cytopspins were examined under FITC filter. TUNEL staining was also carried out on the samples as an indicator of apoptosis, and is described in section 3.12.2.

Some representative photographs are illustrated in figure 3.10.3. The doxorubicin is identified as the yellow-brown staining in the cytoplasms of cells. The nucleus remains as a dark sphere in the resistant variants, with no yellow staining indicating that no drug has entered the nucleus. However, in a subpopulation of the cells, doxorubicin has entered the nucleus and apoptosis has occurred. This situation can be seen in the cells with intense yellow staining in their nuclei, their nuclear material has condensed, characteristic of cells undergoing apoptosis. The intense yellow staining is TUNEL stained cells which stains the fragmented DNA in the nucleus of apoptotic cells. DLKP-A250 10p#7 in particular contain a very good example of this, one cell that has undergone apoptosis as seen by the intense yellow staining of the fragmented DNA in the apoptotic bodies.

These results support the concept of an important role for Pgp in the resistant variants, and its ability to prevent doxorubicin from remaining in the nucleus. The drug concentrations chosen, however induce approximately 50% apoptosis in the cells over 72 hours, so the drug concentration used is sufficient to overcome the efflux activity of Pgp and induce apoptosis, as evidenced by toxicity assays and presence of apoptotic cells in the photographs.

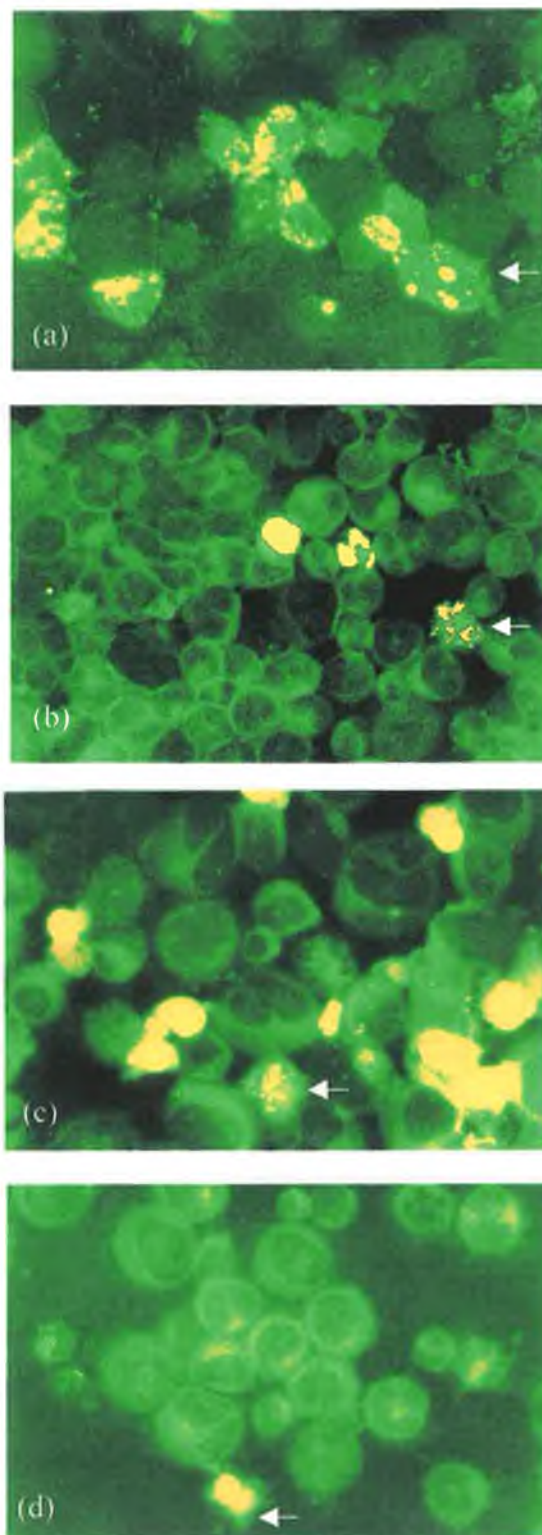


Figure 3.10.3 Accumulation of 2hour doxorubicin in DLKP cells 72 hours after treatment; DLKP-SQ (a), DLKP-A250 10p#7 (b), DLKP-A2B (c) and DLKP-A5F (d). Apoptotic cells (stained with TUNEL) are indicated with a white arrow.

3.10.4 Non-Pgp-mediated apoptosis in DLKP cell lines

5-Fluorouracil is an antimetabolite frequently used in chemotherapy. One of the advantages of 5-FU use in this study is that it is not a substrate of Pgp, and so cannot be effluxed from cells as efficiently as doxorubicin. The toxicity of 5-FU on DLKP cell lines was investigated and results are in figure 3.10.4. The cell lines are equally sensitive to induction of apoptosis by 5-FU, and this is in agreement with previously published reports (NicAmhlaoibh *et al.*, 1999; Heenan *et al.*, 1998).

Another apoptotic stimulus used was serum starvation. Serum starvation-induced apoptosis represents a pathway not involving DNA damage, but rather damage inflicted through absence of growth factors and mitogens. Cells were treated as described in section 2.4.1. Results in figure 3.10.5 indicate that the resistant variants are more resistant to serum-starvation than DLKP-SQ, albeit only 1.3-1.5 fold.

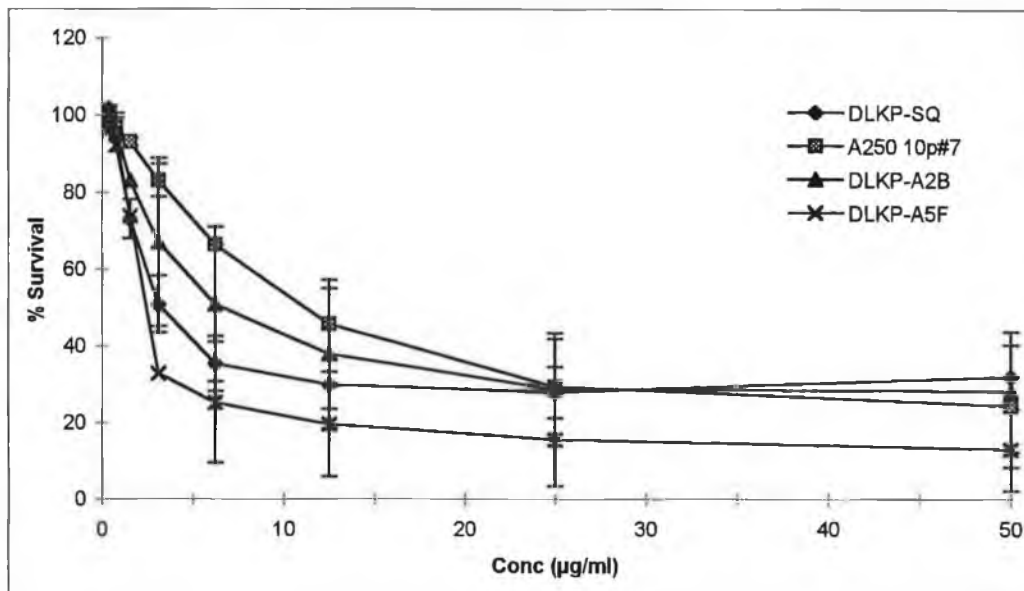


Figure 3.10.4 The effect of 5-Fluorouracil on the four DLKP cell lines. Viability was determined after 72 hours using end point acid phosphatase toxicity assay.

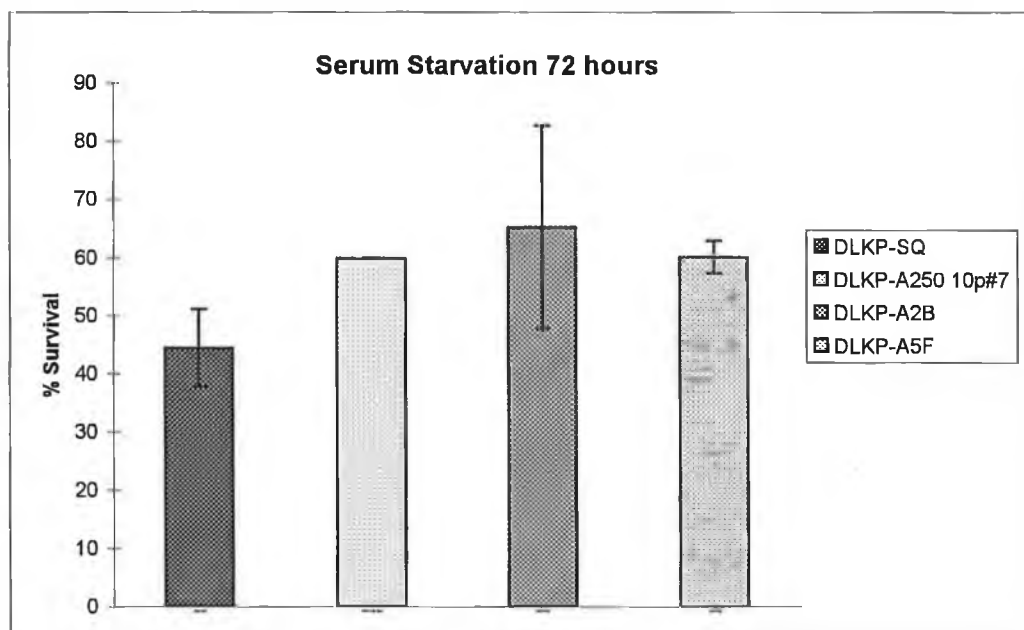


Figure 3.10.5 The susceptibility of DLKP cell lines to cell death induced by serum starvation for 72 hours.

Section 3.11 Kinetics of cell death in DLKP cell lines

Time-lapse videomicroscopy was carried out to monitor the cell death kinetics of the DLKP variants following doxorubicin treatment. Cells were recorded over 70 hours. Cell death events were identified when the classic apoptotic morphology appeared; cell rounding up, membrane blebbing, chromatin condensation and break up into apoptotic bodies. These events usually occurred very rapidly so that it was difficult to distinguish different timing of one event from the next. The time when cells rounded up, as seen by the emergence of a “phase- halo” when viewed using a phase contrast microscope was noted as the time of death for that particular cell. 100 cells were followed in the field for the duration of the experiment. An example of such a field is shown in figure 3.11.1, in which cells are monitored over 72 hours and time of apoptotic onset noted; examples of apoptotic cells are marked with arrows.

The extent of apoptosis is dependent on the drug concentration used, and also the time of exposure of drug to the cells. When DLKP-SQ are treated with varying drug concentrations, the percentage and rate of apoptosis also varies. If DLKP-SQ cells are treated with 5µg/ml Doxorubicin continuous exposure there is a rapid induction of apoptosis with 50% of the cells dead by 5 hours and by 30 hours only 10% of the population remain. At a lower concentration of doxorubicin the rate of apoptosis is slower with 0% dead at 5 hours, 10% dead at 30 hours and 50% viable cells remaining at 72 hours. These results are shown in figure 3.11.2. From these graphs it can be seen that there are no mitotic events in the cell population.

The resistant variants DLKP-A250 10p#7, DLKP A2B and DLKP-A5F also undergo apoptosis when treated with Doxorubicin. Equitoxic drug concentrations were used on the cell lines, a 2 hour Doxorubicin dose which gave approximately 50 % kill after 3 days was used. The growth curves used can be seen in figures 3.10.2. The rates of apoptosis differ between the cell lines however.

DLKP-A250 10p#7 cells have a similar rate of apoptosis to DLKP-SQ after 2 hours treatment with 2 µg/ml Doxorubicin (see figure 3.11.3) when compared to DLKP-SQ apoptosis rate after 0.6 µg/ml 2 hour pulse. A very small percentage of cells have

died by 5 hours, and by 30 hours 10 % cell death has occurred. The apoptosis rate increases after the initial lag and by 72 hours 48 % of the cells are dead.

DLKP-A2B cells have a high initial rate of apoptosis, in the first five hours following treatment up to 25 % of the cells undergo apoptosis, the rate slows at 12 hours when 60 % of the cells have died, and by 72 hours 42 % of the original field remain. This is illustrated in figure 3.11.4. It can also be seen from this kinetic curve that there is mitosis occurring after Doxorubicin treatment (this interesting phenomenon is discussed in section 3.4) and the majority of cells that divide lose at least one of their daughter cells by apoptosis. Quite a high level of mitotic events occur in which the parent cell divides into more than two daughter cells. Up to five daughter cells formed from a parent cell at one instant but all five died within an hour of mitosis.

When DLKP-A5F are treated with 100 $\mu\text{g/ml}$ Doxorubicin less than 50 % kill is seen over three days. Yet when time-lapse is used to analyse cell kinetics it can be seen from figure 3.11.5 that 70 % apoptosis occurs in 80 hours. There is a high rate of mitosis seen also and this compensates for the loss in number due to apoptosis. There were some incidences of post- mitotic apoptosis observed in DLKP-A5F cells, similar to DLKP-A2B cells.

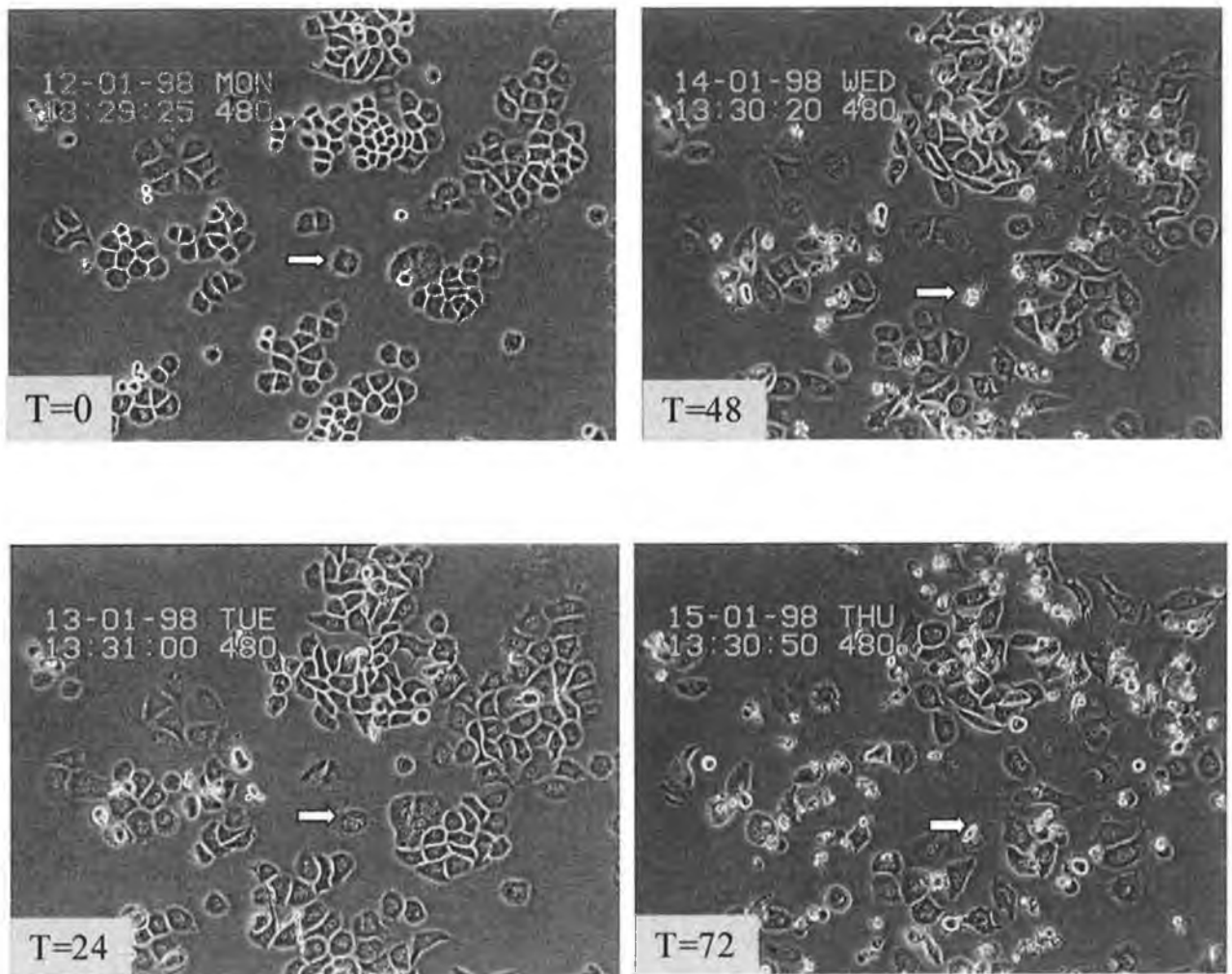


Figure 3.11.1 Timelapse videomicroscopy analysis of apoptosis in DLKP-SQ over 72 hours following 0.6 $\mu\text{g/ml}$ 2 hour Doxorubicin treatment. Examples of individual cells undergoing apoptosis are marked with arrows.

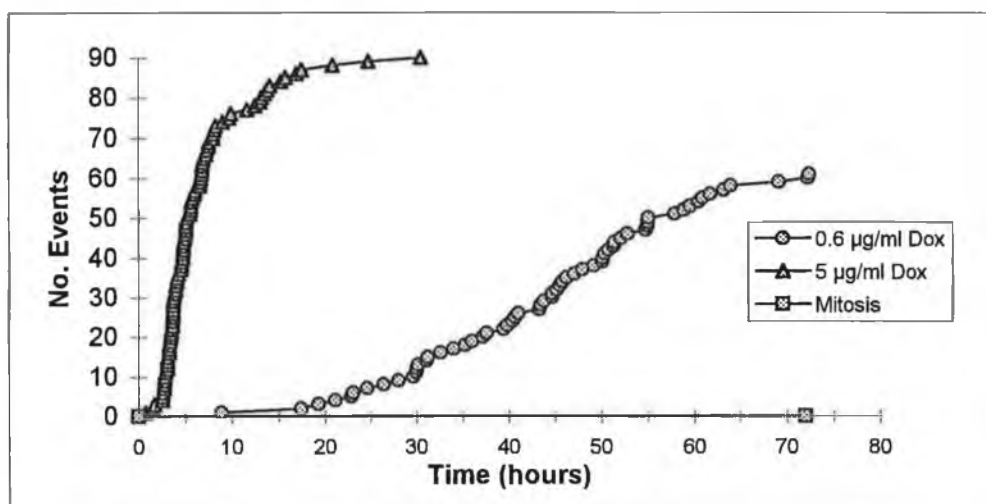


Figure 3.11.2 Timelapse videomicroscopical analysis of 0.6µg/ml doxorubicin 2-hour pulse on DLKP-SQ

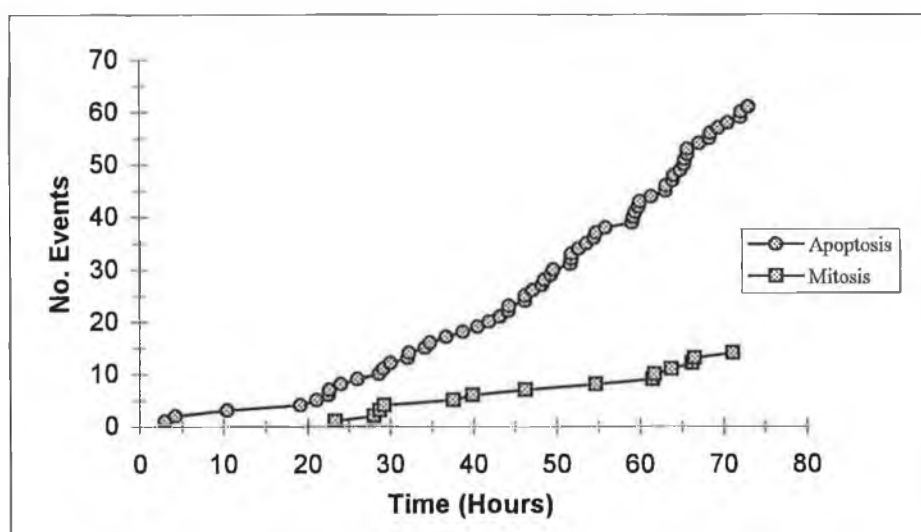


Figure 3.11.3 Timelapse videomicroscopical analysis of 2µg/ml doxorubicin 2 hour pulse on DLKP-A250 10p#7

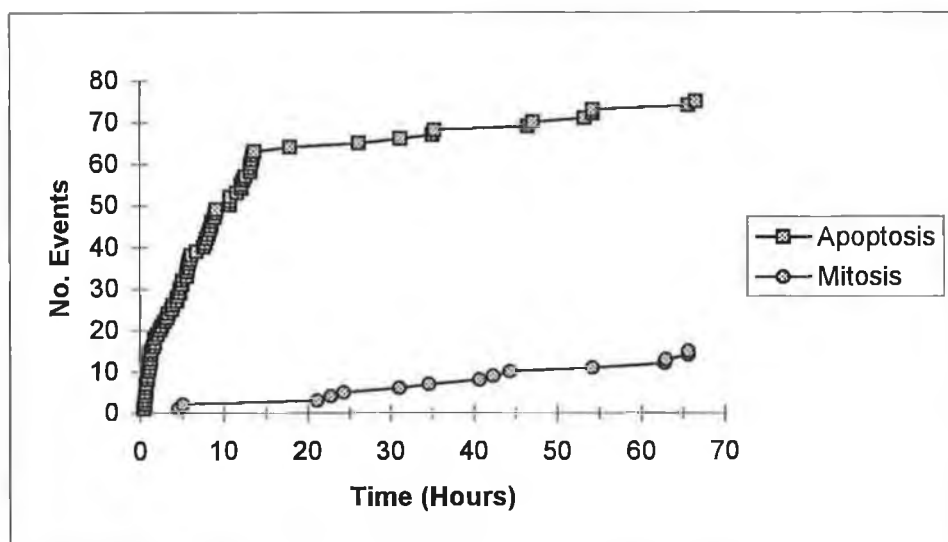


Figure 3.11.4 Timelapse videomicroscopical analysis of 30 µg/ml Doxorubicin 2 hour pulse treatment on DLKP-A2B

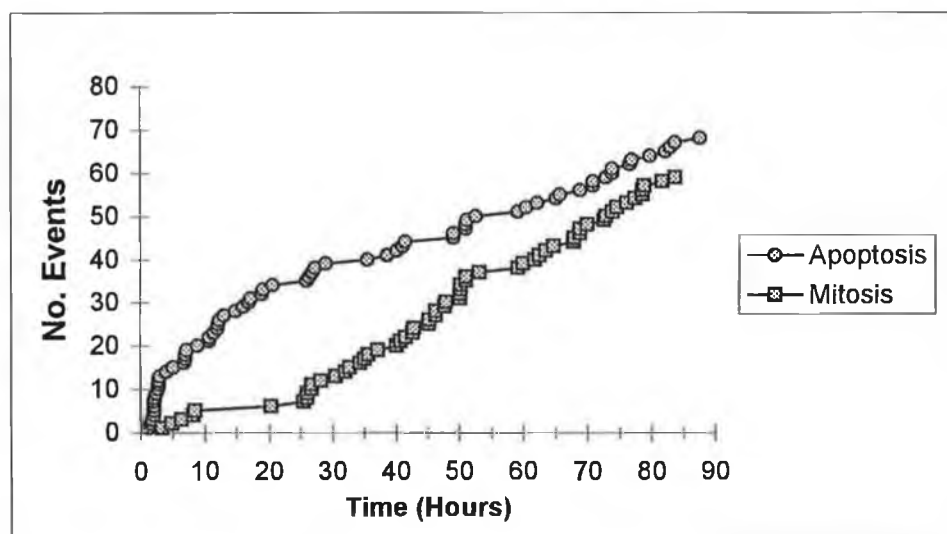


Figure 3.11.5 Timelapse videomicroscopical analysis of 100µg/ml Doxorubicin 2 hour pulse treatment on DLKP-A5F

3.12 Morphological features of apoptosis in DLKP-SQ cells

A number of morphological features specific to apoptotic cells were looked at in DLKP-SQ cells in an attempt to give a comprehensive study of the apoptotic process in DLKP cells. Based on the many morphological and biochemical features in apoptotic cells, there are a variety of techniques that can be utilised to identify cells undergoing apoptosis. Timelapse videomicroscopy was routinely used to confirm that cell death was occurring via apoptosis by identifying the cells with characteristic apoptotic morphology. The morphological features chosen were DNA fragmentation which is assayed using DNA gel electrophoresis, and also TUNEL analysis. The biochemical feature chosen was the externalisation of the membrane phospholipid phosphatidyl serine, using annexin V as an indicator.

3.12.1 DNA fragmentation analysis in Doxorubicin-treated DLKP-SQ cells

One of the main cellular events in apoptosis involves the condensation of chromatin as discussed in section 1. Endonucleolytic activity is responsible for cleavage of the DNA first into large fragments (50 - 300 kb) and then into smaller fragments of 180 bp, or multiples of 180 bp. It is thought that this 180 bp corresponds to the size of linker DNA between two nucleosomes. The 180bp multiples are visible on an agarose gel as "ladders". DLKP-SQ cells were set up as described in materials and methods. A typical DNA gel can be seen in figure 3.12.1. No DNA laddering occurs in the untreated DLKP-SQ control wells (no DNA fragmentation). DLKP-SQ cells treated with varying concentrations of daunorubicin underwent apoptosis and the distinctive ladders were seen.

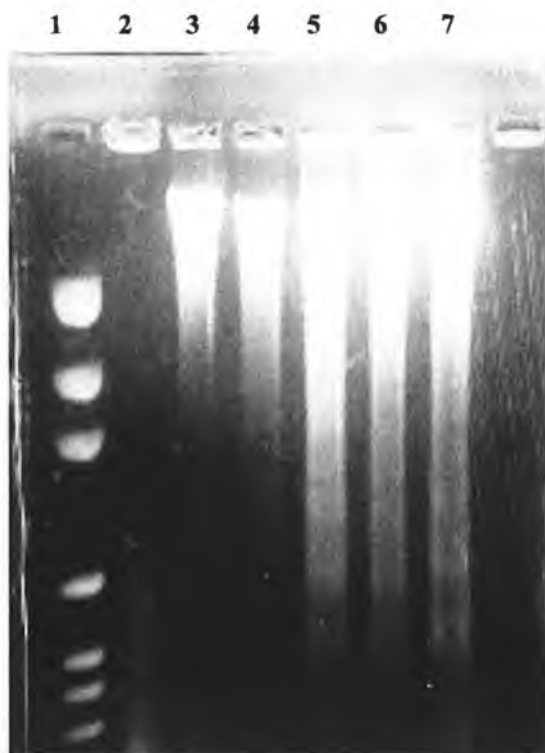


Figure 3.12.1 DNA fragmentation analysis in DLKP-SQ after Daunorubicin treatment. Lanes were loaded as follows; Lane 1: Mr markers. Lane 3 and 4: controls (untreated). Lane 5: 10 μ g/ml. Lane 6: 5 μ g/ml. Lane 7: 1 μ g/ml.

3.12.2 TUNEL analysis

DNA fragmentation can also be detected by staining the 3'-end of the fragments in apoptotic cells. TUNEL staining involves the incorporation of dUTP to the 3'-end of the "nicked" or fragmented DNA formed during apoptosis. It is a sensitive technique that allows visual semi-quantitative detection of apoptotic cells. DLKP cells were set up as described in materials and methods, and treated with equitoxic doxorubicin doses for 2 hours. Samples were taken at 24, 48 and 72 hours after drug treatment and cytopins were prepared as described in materials and methods. Analysis was carried out as in materials and methods, and representative photographs for each cell line can be seen in figures 3.12.2.1, 3.12.2.2, 2.12.2.3 and 2.12.2.4. Each photograph represents a random field of 50-100 cells. It is difficult however to see the background unstained cells in some photographs. Photographs were also taken of untreated control cells, and no TUNEL staining was visible in these (not shown)

TUNEL staining confirmed that all cells died by apoptosis at the doxorubicin concentrations used, and there was negligible background apoptosis in untreated controls. The TUNEL staining shows the classical condensation of the nucleus and its fragmentation. The earlier onset of apoptosis seen by timelapse analysis is also seen by TUNEL staining, a higher level of apoptotic cells are present at earlier times in figure 3.12.3 than in the other cell lines.

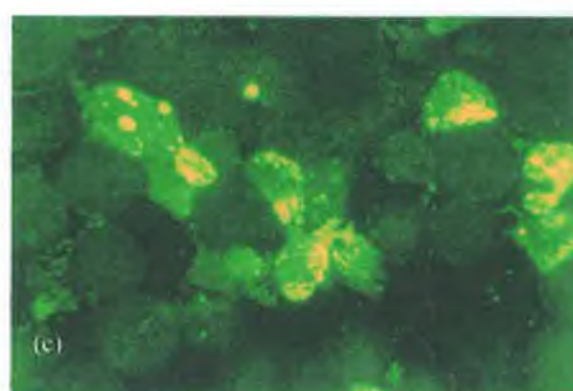
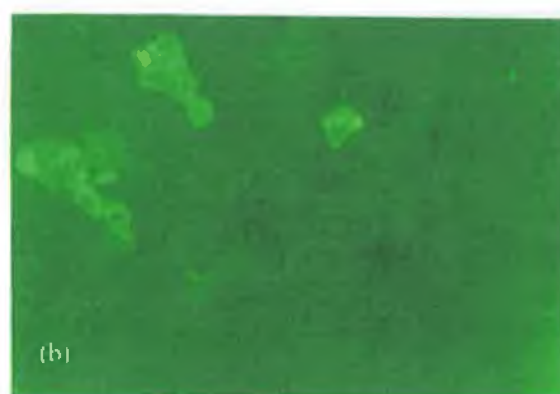
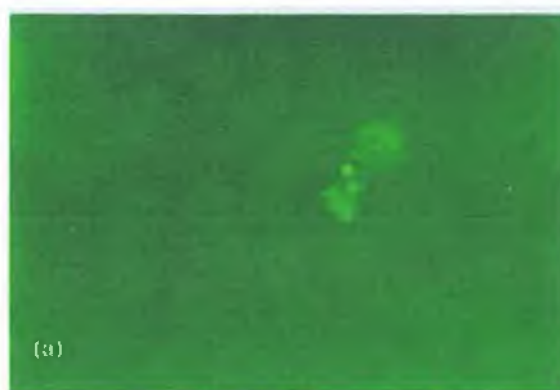


Figure 3.12.2.1 TUNEL analysis of DLKP-SQ cells (a) 24, (b) 48 and (c) 72 hours after doxorubicin treatment.

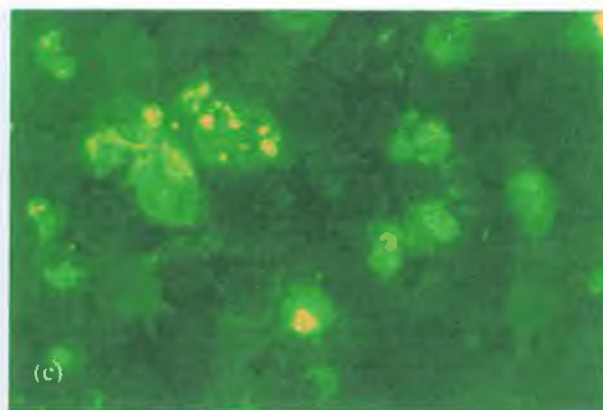
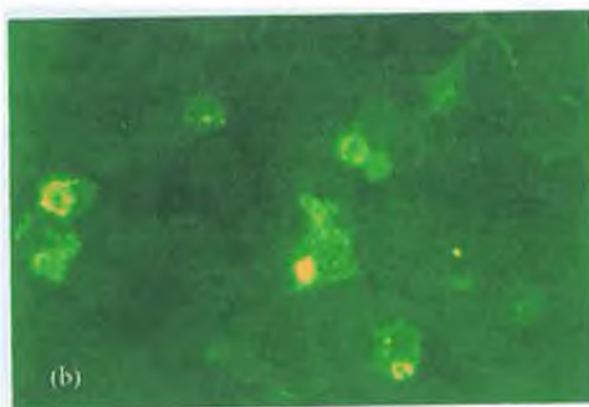
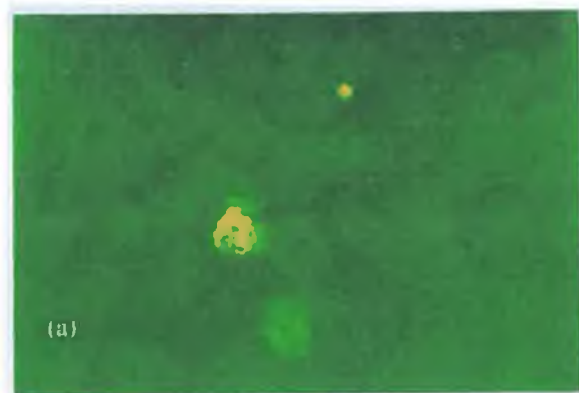


Figure 3.12.2.2 TUNEL analysis of DLKP-A250 10p#7 cells (a) 24, (b) 48 and (c) 72 hours after doxorubicin treatment.

3.12.3 Phosphatidylserine exposure on DLKP-SQ induced by Doxorubicin treatment

Phosphatidylserine is a membrane phospholipid that is transiently exported to the outer plasma membrane during the early stages of apoptosis (Fadok et al. 1992) and this can be monitored using annexin-V-FITC (fluorescein isothiocyanate) which is a Ca^{2+} -dependent phospholipid-binding protein that has high affinity for phosphatidylserine (van Engeland et al. 1996). DLKP-SQ cells were treated with Doxorubicin for 2 hours and monitored for annexin V binding, which is indicative of phosphatidylserine externalisation. Untreated controls exhibited no annexin V binding when looked at under FITC filter as seen in figure 3.12.3.1. Doxorubicin-treated cells however were positive for annexin V staining as seen in figure 3.12.3.2. This is indicated by the intense green staining on the membranes of DLKP-SQ cells about to undergo apoptosis. A low background fluorescence can be seen in the Doxorubicin-treated cells and this is due to the fluorescence of Doxorubicin under FITC filter.

(1)

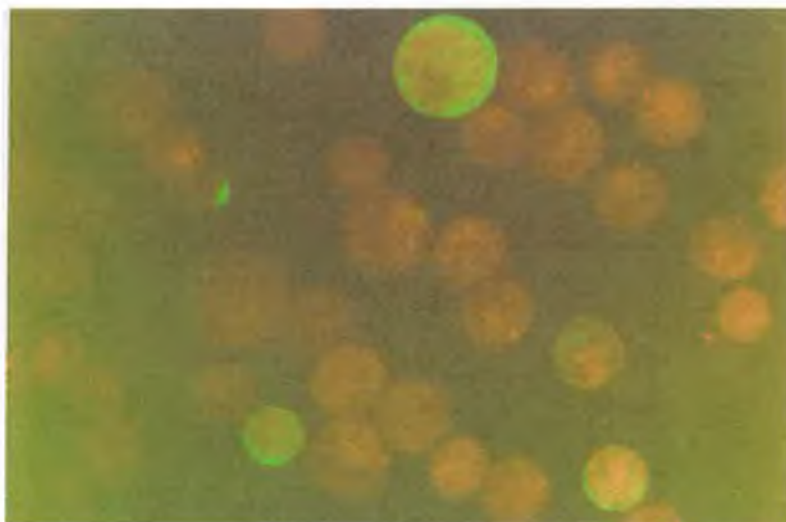


(2)



Figure 3.12.3.1 Untreated DLKP-SQ cells stained with annexin V looked at under (1) FITC filter and (2) white light

(1)



(2)

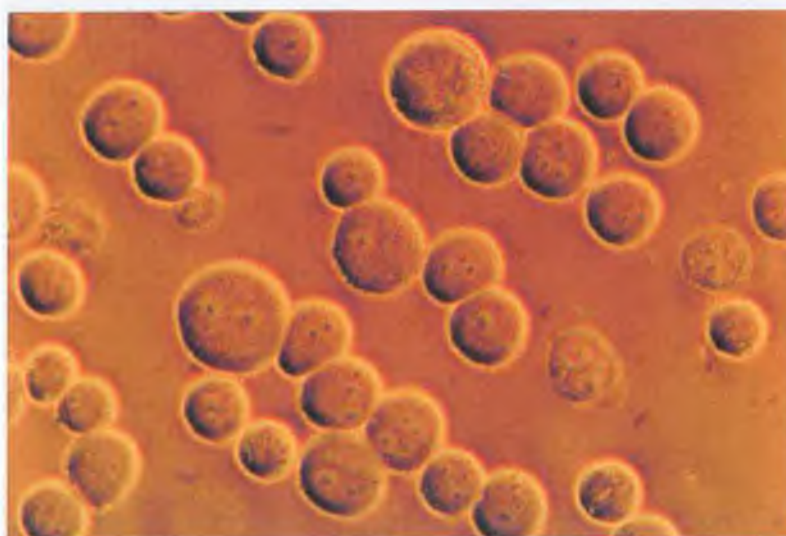


Figure 3.12.3.2 DLKP-SQ cells treated with 0.6 $\mu\text{g/ml}$ Daunorubicin for two hours and labeled with annexin V. Cells were viewed using (1) FITC filter and (2) white light.

3.13 Investigation of Caspase involvement in DLKP apoptosis

Caspases constitute one of the key components in the apoptotic pathway, and their role in apoptosis is discussed in section 1. It is currently proposed that caspases function as part of a cascade and redundancy may exist between a certain subset of the fourteen currently known caspases. Extensive research has been carried out to study the involvement of the various caspases in apoptosis in numerous cell lines. It was therefore decided to investigate which caspases were involved in apoptosis in DLKP cell lines. One other important reason to look at caspase activation in the DLKP cell lines stems from recently published reports in which resistant cell lines have defective caspase activation (Los *et al.*, 1997, Kojima *et al.*, 1998). This raises the possibility that there may be reduced or altered caspase levels, activation or activity in the DLKP resistant variants during apoptosis and this could in part contribute to the resistant state.

3.13.1 Investigation of caspase-2 involvement in DLKP apoptosis

Caspase-2 levels were investigated using an antibody that recognises the proform of caspase-2 at approx. 48 kDa (Transduction Labs.). Human endothelial cell lysate was used as a positive control. Cells were set up as already described and 10 µg protein was loaded on each lane and blots were stained with the reversible protein stain Ponceau to confirm equal loading in each case. Figure 3.13.1.1 illustrate procaspase-2 levels in DLKP-SQ cells before and after a 0.6 µg/ml doxorubicin exposure for 2 hours. Clearly there is no reduction in the levels of pro-caspase-2 after drug treatment. This seems to indicate that caspase-2 does not play a role in DLKP-SQ apoptosis.

Caspase-2 protein levels in the resistant variants were then compared to DLKP-SQ levels. Results are shown in figure 3.13.1.2. Interestingly, very low levels of caspase-2 were detected in control and drug treated DLKP-A250 10p#7, DLKPA2B and DLKP-A5F. 10 µg protein was loaded on each lane and blots were stained with the reversible protein stain Ponceau to confirm equal loading in each case. From the blot it can be seen

that there are high levels of caspase-2 in DLKP-SQ, low levels in DLKP-A250 10p #7, no detectable levels in DLKP-A2B and very low levels in DLKP-A5F.

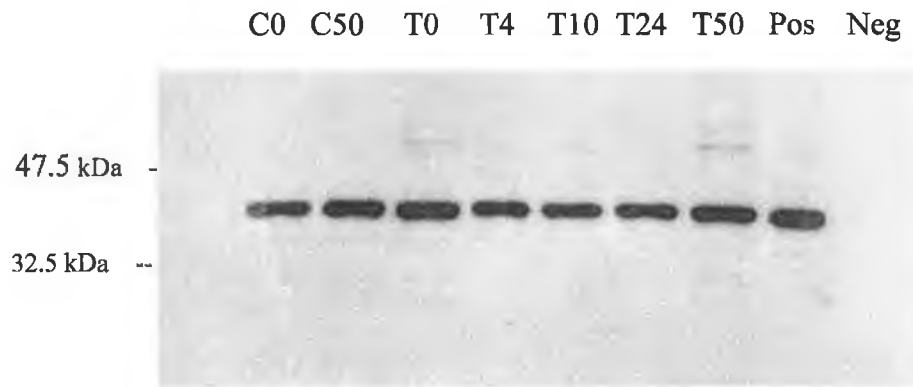


Figure 3.13.1.1 Western blots showing caspase-2 levels in DLKP-SQ before and after doxorubicin treatment

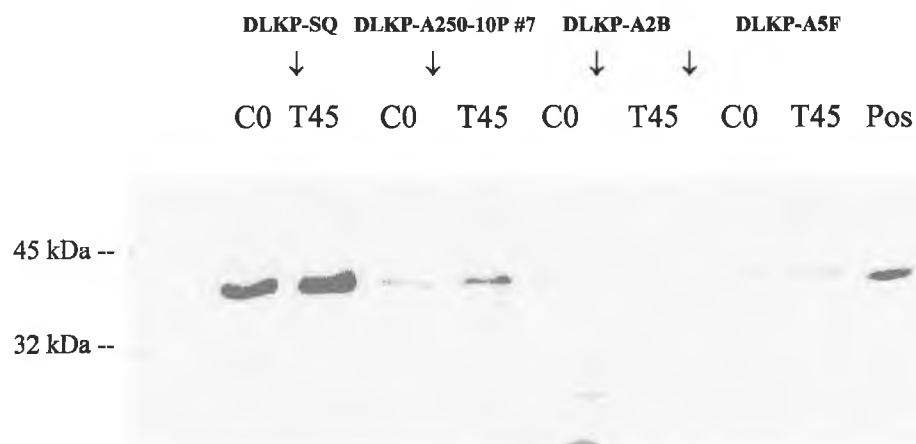


Figure 3.13.1.2 Caspase-2 Levels before and after drug treatment in the four cell lines. C0 refers to untreated cell extracts and T45 refers to drug treated cells 45 hrs after treatment

3.13.2 Investigation of caspase-3 involvement in DLKP apoptosis

Caspase-3 levels were looked at initially in untreated and doxorubicin treated cells using an antibody which recognises pro-caspase-3 at 32kDa. The positive control used was a Jurkat cell lysate (Transduction labs.) 10ug protein was loaded on each lane and blots were stained with the reversible protein stain Ponceau to confirm equal loading in each case. Results are shown in figure 3.13.2.1, and it can be seen that there is no decrease in levels of pro-caspase-3 in apoptotic DLKP-SQ cells. It was shown in section 3.9 and 3.10 that the cells under apoptosis over this timescale, so it appears that pro-caspase-3 is not proteolytically cleaved to form active p17 and p12 subunits in the apoptotic process of these cells.

Figure 3.13.2.2 compares levels of pro-caspase-3 in untreated and drug treated samples from each of the DLKP cell lines. There seems to be an increase in the T45 drug-treated DLKP-SQ sample compared to C0 sample, but using ponceau staining it was obvious that the DLKP-SQ T45 lane had higher protein levels than all the other lanes which were equally loaded. There were low levels of pro-caspase-3 in DLKP-A250 10p #7 similar to the situation for caspase-2. Caspase-3 could not be detected in either DLKP-A2B or DLKP-A5F extracts, yet protein bands of equal intensity were seen on the membrane when stained with ponceau. Perhaps the caspase-3 protein levels in these cell lines are too low to be detected using the ECL detection system.

Caspase-3 levels after doxorubicin treatment in DLKP-A250 10p#7 cells were also investigated over time points as shown in figure 3.13.2.3. The protein loading was approximately equal in this blot except for the lane labelled T4. As can be seen from this result there is no significant decrease in pro-caspase-3 levels after 50 hours in doxorubicin treated cells.

From these results it may be speculated that neither caspase-2 or caspase-3 play a role in doxorubicin-mediated apoptosis in the DLKP cell lines. The possibility that caspase-3 would not have an involvement in doxorubicin-mediated apoptosis is surprising because it is the most widely studied caspase to date and is implicated in apoptosis in the majority of cell lines and *in vitro* models studied.

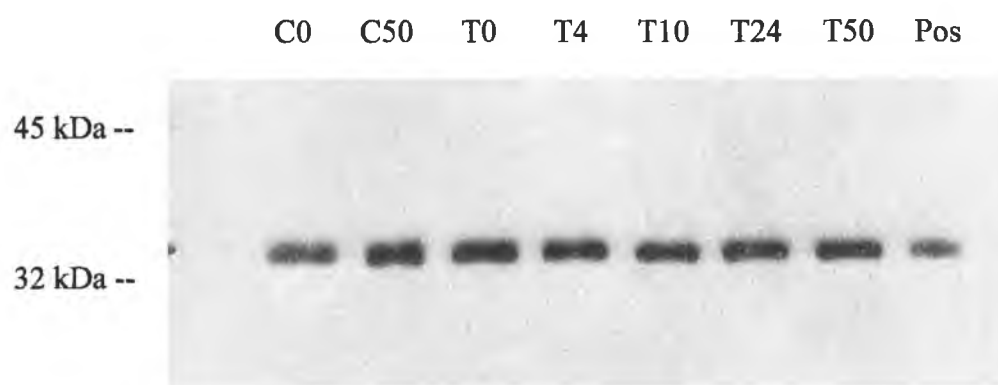


Figure 3.13.2.1 Western blots showing procaspase-3 levels in DLKP-SQ before and after doxorubicin treatment

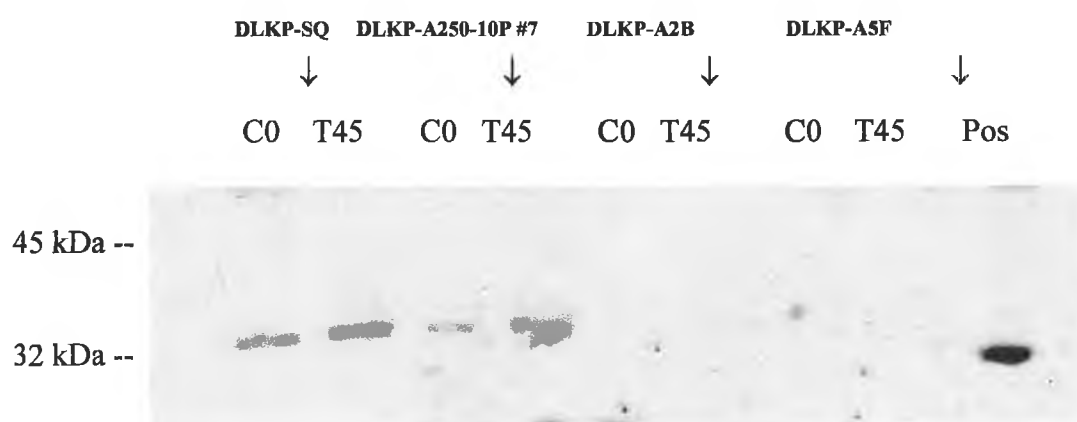


Figure 3.13.2.2 Procaspase-3 Levels before and after drug treatment in the four cell lines. C0 refers to untreated cell extracts and T45 refers to drug treated cells 45 hrs after treatment

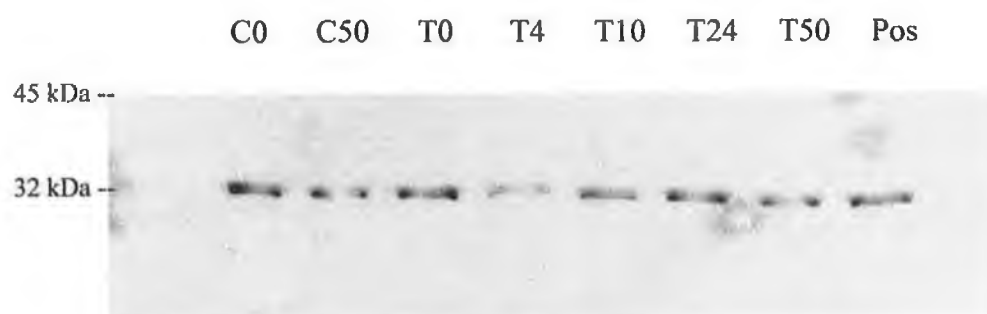


Figure 3.13.2.3 Western blots showing Caspase-3 levels in DLKP-A250 10p #7 before and after doxorubicin treatment

3.13.3 Caspase-7 involvement in apoptosis

Caspase-7 is a group-II caspase, has similar substrate preference to caspase-3 and is involved in apoptosis in various cell lines induced by diverse stimuli. It is a 45kDa protein which is cleaved into 17 kDa and 28 kDa fragments during apoptosis.

Procaspase-7 protein levels were looked at in the four DLKP cell lines, before and after doxorubicin treatment to investigate its role in doxorubicin-induced apoptosis. Looking at treated cells (C0) in figure 3.13.3.1, it can be seen that when protein loading is taken into account, there are approximately equal levels of procaspase-7 in all four cell lines. It seems as if there is a lower level of procaspase-7 in DLKP-A25010p#7 and DLKP-A5F but ponceau staining confirmed that this is due to slightly less protein in these lanes, and repeat westerns confirmed that there are equal levels of procaspase-7 in the four cell lines.

In figure 3.13.3.1, the effect of doxorubicin on procaspase-7 can be seen. There is no reduction in procaspase after doxorubicin treatment in any of the cell lines, the p45 intact caspase remains at comparable levels to the untreated controls. Furthermore there was no detection of p17 fragments in the drug-treated samples, indicating that procaspase-7 is not activated in doxorubicin-induced apoptosis in any of the DLKP cell lines.

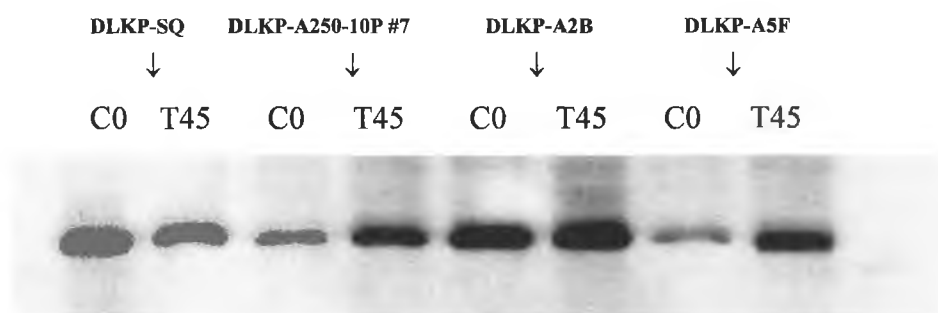


Figure 3.13.3.1 Western blots showing caspase-7 levels in DLKP-SQ before and after doxorubicin treatment

3.13.4 Investigation of procaspase-6 in DLKP apoptosis

Procaspace-6 protein levels before and after drug treatment are shown in figure 3.13.4.1. Procaspase-6 is a 40 kDa protein and the antibody used should pick up both procaspase and the active 24 kDa active fragment. Caspase-6 is the caspase responsible for lamin cleavage during apoptosis, and is a downstream caspase which can be activated by caspase-3 (Orth *et al.*, 1996).

There are relatively equal amounts of procaspase-6 in the four DLKP cell lines, however levels in DLKP-A250 10p#7 C0 seem to be higher on this blot due to unequal protein loading, also DLKP-A2B C0 is less than T45 due to decreased protein loading, when ponceau staining is taken into account. After doxorubicin treatment there is no decrease in the procaspase-6, and active p24 fragments could not be detected using Western blotting (this part of the gel not shown). These results suggest that caspase-6 does not play a role in doxorubicin-induced apoptosis in DLKP cells lines.

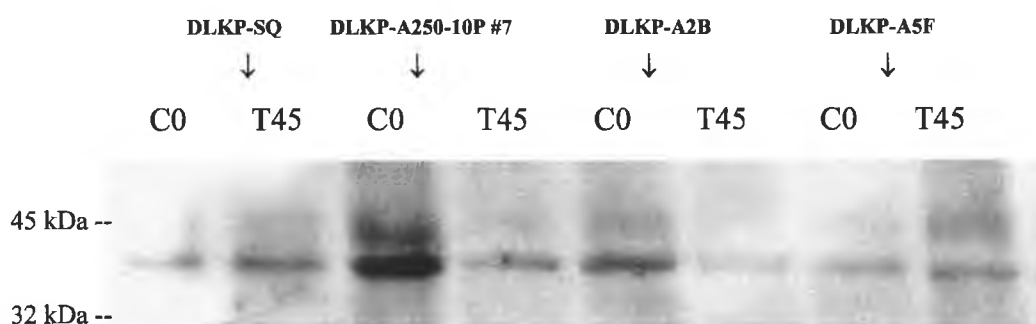


Figure 3.13.4.1 Western blots showing procaspase-6 levels in DLKP cell lines before and after doxorubicin treatment

3.13.5 Investigation of caspase-8 involvement in DLKP apoptosis

Caspase-8 plays an important role in the CD95-mediated apoptotic pathway, being the first caspase to be activated. It had a unique DED domain, absent in other caspases (but also in caspase-10) which allows it to bind directly to FADD, the double adaptor protein, linking it to the CD95 receptor. Given the wealth of evidence existing for doxorubicin-induced activation of the CD95 pathway, the levels and timely activation of procaspase-8 represent critical factors in maintaining an intact CD95 signalling pathway.

Levels of procaspase-8 in sensitive and resistant cell lines is investigated in figure 3.13.5.1. There is equal protein loading on this blot, except for DLKP-SQ T45 lane which has higher protein loaded. There is a definite, though small decrease in procaspase-8 expression in the resistant variants, particularly in DLKP-A5F. This result may have important implications in the ability of these cells to resist apoptotic induction, at doxorubicin concentrations which effectively induce apoptosis in the three other cell lines. Looking at doxorubicin treated cells in figure 3.13.5.1 there was no decrease in procaspase-8 levels, and also absence of caspase-8 activation, judged by the absence of caspase fragments. This result questions the involvement of caspase-8 activation in the doxorubicin-induced apoptosis in DLKP cell lines.

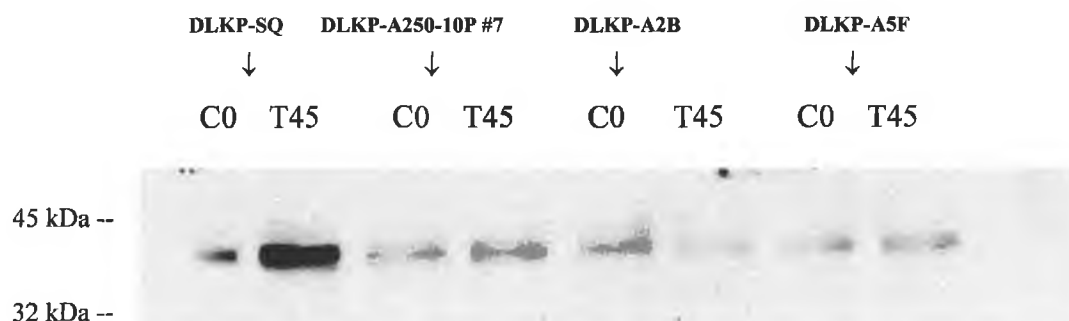


Figure 3.13.5 Western blots showing procaspase-8 levels in DLKP cell lines before and after doxorubicin treatment

3.13.6 Investigation of caspase-4 in DLKP apoptosis

Caspase-4 was investigated also, it has been shown on a number of occasions to be activated and involved in apoptosis induced by such stimuli as CD95 (Kamada *et al.*, 1997). However results in figure 3.13.6 demonstrate that procaspase-4 is not activated in either of the DLKP cells lines during doxorubicin-induced apoptosis. The antibody used picks up the intact procaspase-4 at 43 kDa, and no reduction of this band was seen in the apoptotic samples.

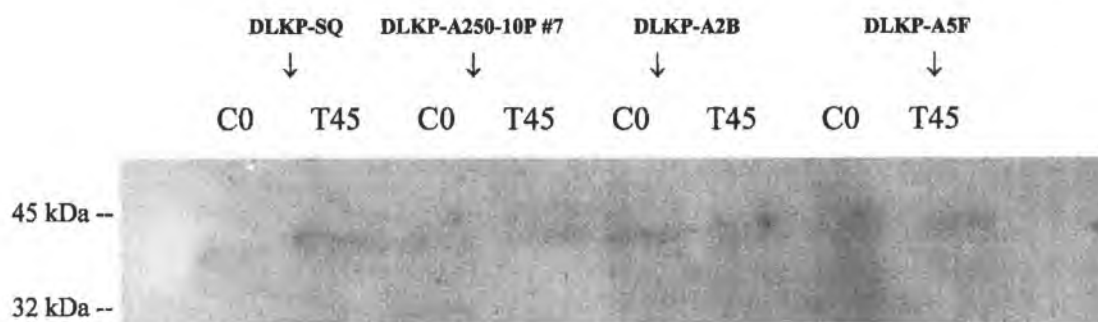


Figure 3.13.6 Western blots showing procaspase-4 levels in DLKP cell lines before and after doxorubicin treatment

3.13.7 Involvement of caspase-9 in DLKP apoptosis

Caspase-9 is a critical factor at the apex of the apoptotic pathway involving cytochrome c release and apaf-1. It is activated through autocatalysis when it binds to apaf-1, cytochrome c and dATP during initiation of apoptosis. Caspase-9, when active, has been shown to activate all the other known caspases (Harte *et al.*, 1999), and so has a key role to play in transducing the death signal to the downstream machinery to bring about destruction of the cell. Any reduction in or faulty caspase-9 could lead to a defective induction of apoptosis. Figure 3.13.7 illustrates levels of procaspase-9 in the four DLKP cell lines. Clearly resistant variants do not have reduced procaspase-9, but the effect of doxorubicin treatment on the cells has not been looked at. Even though the sensitive and resistant variants express caspase-9 to an equal extent, it remains to be seen if the resistant variants have alterations in the activation of procaspase-9.

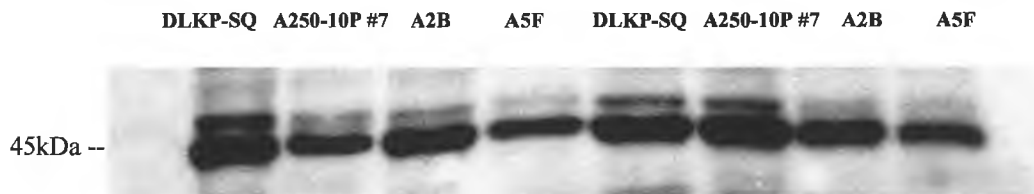


Figure 3.13.7 Western blots showing procaspase-9 levels in DLKP cell lines (untreated)

3.14.1 STAT proteins

STAT proteins are critically involved in many signal transduction pathways, and some recent studies have linked caspase expression to STAT-1 protein expression (Kumar *et al.*, 1997; King *et al.*, 1998). Levels of STAT protein expression were investigated in DLKBP-SQ, DLKBP-A250 10p#7, DLKBP-A2B and DLKBP-A5F using western blotting. Results in figure 3.14.1 indicate that there is equal expression in resistant and sensitive DLKP cell lines. The effect of doxorubicin on STAT-1 expression was also investigated in figure 3.14.1. From the Western blot there is no difference in STAT-1 protein expression in doxorubicin-treated cells compared to the untreated controls.

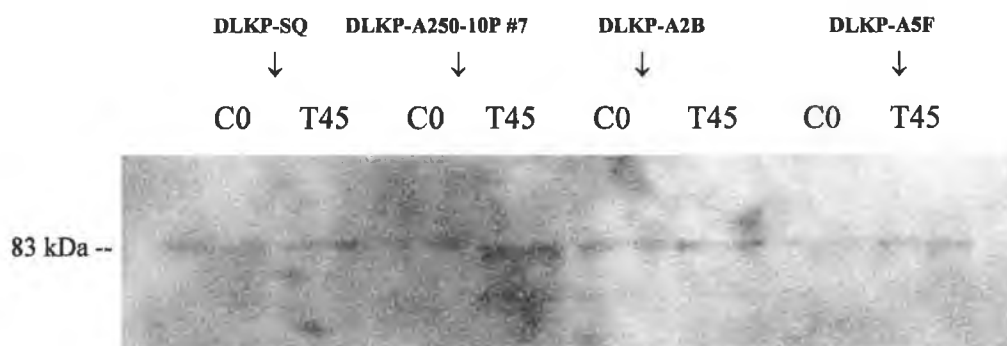


Figure 3.14.1 STAT-1 protein levels in DLKP cell lines, before and after doxorubicin treatment

3.14.2 mRNA levels of procaspase-3

Results in section 3.13.2 indicate that procaspase-3 protein expression is much reduced in the DLKP resistant variants. Therefore it was necessary to also investigate the mRNA levels of procaspase-3 to establish if there was a corresponding decrease in procaspase-3 expression, which would suggest that the procaspase-3 is controlled in resistant cells at a transcriptional level. There is no decrease in procaspase-3 in western blotting, perhaps the activation of procaspase-3 could be masked by increased transcription of the protein in drug-treated cells; analysis of mRNA levels before and after drug treatment would clarify this possibility (this analysis will be carried out in the future).

RT-PCR was carried out to look at mRNA levels of procaspase-3 in the four untreated DLKP cell lines. The forward and reverse procaspase-3 primers used were 5' AAC CAC CAA CCA ACC ATT TCT 3' and 5' AAG GTA TCC ATG GAG AAC ACT GAA AAC 3'. Interestingly results in figure 3.14.2 show equal expression on procaspase-3 in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F. A posttranscriptional control must exist that prevents translation of the mRNA into the protein, or results in increased deregulation of mRNA or protein.

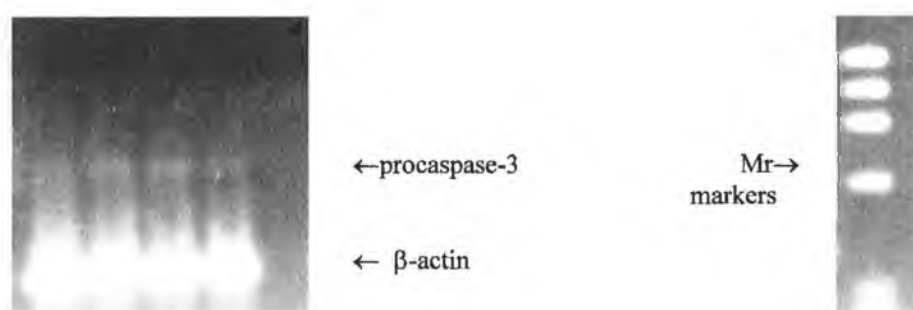


Figure 3.14.2 mRNA levels of procaspase-3 in DLKP-SQ (untreated)

3.15 PARP cleavage - Investigation of caspase substrate cleavage in DLKP cell lines

As described in section 1, there is extensive protein cleavage during apoptosis and many specific substrates of caspases have been identified which are preferentially cleaved by the different subfamilies of caspases. Poly-(ADP)-ribose polymerase is an abundant 116 kDa nuclear protein involved in DNA repair, which is activated by DNA breaks. Lazebnik *et al.* (1994) showed that PARP was cleaved during apoptosis into two signature fragments of 85 kDa and 27 kDa by an ICE-like protease which was subsequently identified as caspase-3. Caspase-7 and caspase-2 may also be candidates for cleaving PARP.

DLKP-SQ cells were treated as previously described in materials and methods and Western blotting was carried out to look at PARP cleavage over time. It can be seen in figure 3.15.1 that the intact 116 kDa band does not decrease over time as the cells are undergoing apoptosis, even at T50 there is no reduction in 116 kDa intact PARP although it can be seen from time-lapse analysis that 40 % cells have undergone apoptosis at this stage. There is a band on the blot corresponding to \approx 85 kDa but this band is present in controls and does not increase as cells die, so perhaps it is an unspecific background band.

HL-60 cells were used as a control for PARP cleavage to validate the detection system, and to confirm that the absence of PARP cleavage is a DLKP-associated phenomenon, and not a result of the experimental techniques used for protein extraction and detection. It has previously been shown that PARP is cleaved from 116kDa to 85kDa in apoptotic HL-60 cells (Lazebnik *et al.*, 1994). In this experiment HL-60 cells were treated with μ g/ml doxorubicin and protein extracted after 0, 1, 2 and 5 hours. These samples were analysed for PARP cleavage by loading 10 μ g protein on each lane and running them on the same gel with apoptotic DLKP-SQ samples. It can be seen in figure 3.15.2 that PARP is cleaved completely in the apoptotic HL-60 cells, even after 1 hour exposure to doxorubicin. However, apoptotic DLKP-SQ cells have intact PARP comparable to untreated controls, even 48 hours after treatment, and at a time when 50% of the cells are apoptotic.

PARP cleavage was also investigated in the resistant variants. DLKP-A250 10p#7 do not show PARP cleavage after doxorubicin treatment, this is evident from the consistent 116 kDa band present in drug treated samples in figure 3.15.3. When DLKP-A2B cells were investigated there was a slight decrease in the 116 kDa intact PARP by 50 hours after Doxorubicin treatment, this is illustrated in figure 3.15.4. DLKP-A5F cells did not show PARP cleavage after Doxorubicin treatment, see figure 3.15.4. These results are surprising because PARP cleavage is seen in the majority of cells undergoing apoptosis. The results do however agree with caspase-3 westerns where pro-forms of caspase-3 are not decreasing after Doxorubicin treatment indicating that caspase-3 is not activated during apoptosis in DLKP cell lines. Taken together these results tentatively suggest that the Doxorubicin induced apoptosis in DLKP cell lines might be caspase-3 independent but further experimental evidence needs to be provided to substantiate this. (described in forthcoming sections)

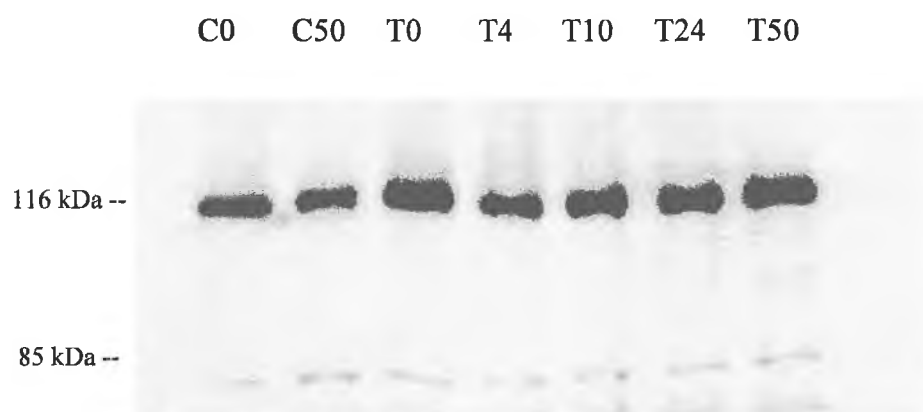


Figure 3.15.1 PARP protein levels before and after Doxorubicin treatment in DLKP-SQ

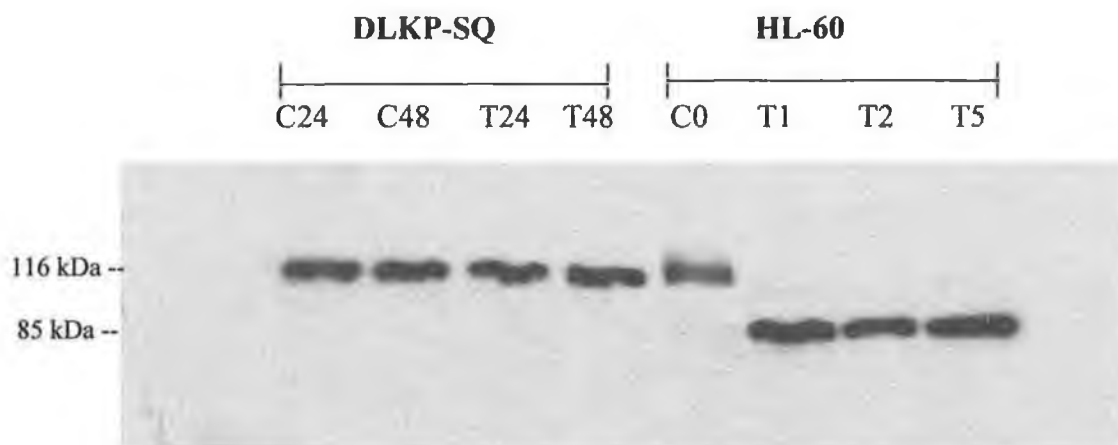


Figure 3.15.2 Analysis of PARP cleavage in DLKP-SQ and HL-60 cell lines after Doxorubicin treatment

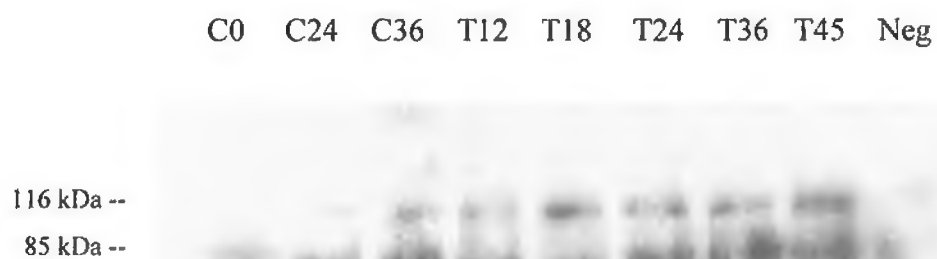


Figure 3.15.3 PARP protein levels before and after Doxorubicin treatment in DLKP-A250 10p #7

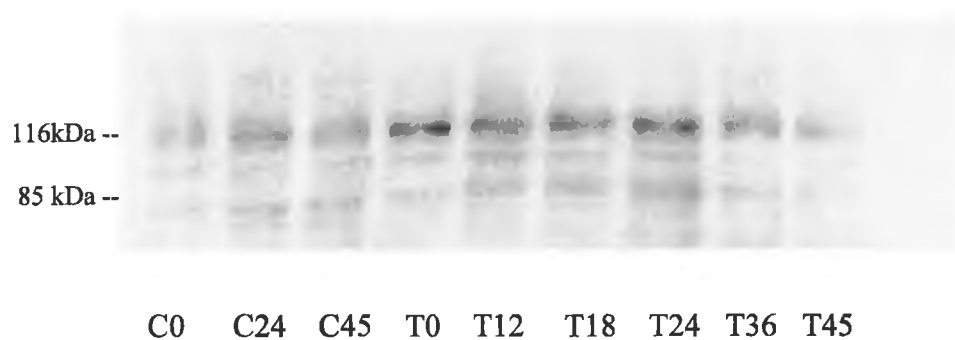


Figure 3.15.4 PARP protein levels before and after Doxorubicin treatment in DLKP-A2B

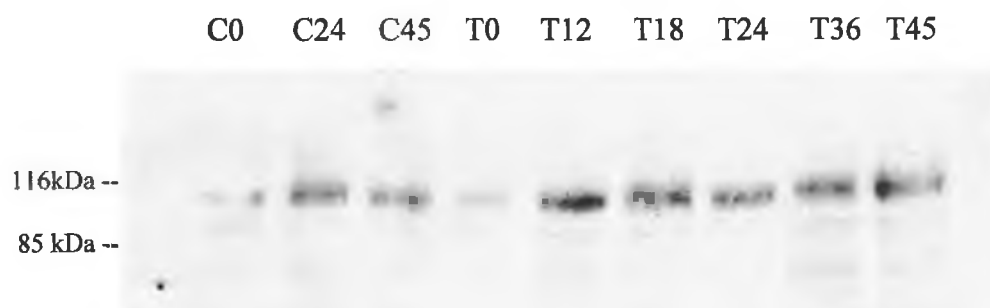


Figure 3.15.4 PARP protein levels before and after Doxorubicin treatment in DLKP-A5F

3.16 Fodrin Cleavage - Investigation of caspase substrate cleavage in DLKP cell lines

Fodrin is a cytoskeletal protein found in all eukaryotic cells and has been shown to undergo cleavage during apoptosis (Martin *et al.*, 1995). Fodrin is a 240 kDa protein and is cleaved to 150 kDa and 120 kDa bands in cells undergoing apoptosis. Caspase-3 recently was shown to be the key protease involved in fodrin cleavage into the 120kDa fragment (Janicke *et al.*, 1998). It is thought that the 150 kDa fragment is generated by calpains.

Fodrin cleavage was compared in untreated and Doxorubicin treated samples from the four DLKP cell lines. Cells were set up as described in materials and methods. Drug treated samples were extracted 50 hours after drug treatment, and control samples were taken from untreated cells growing exponentially. Results are shown in figure 3.16.1. Fodrin is cleaved to some extent in all of the control samples, as seen by the presence of a band at 150 kDa, and a faint band at 120 kDa. The 150kDa band present in all the samples suggests some calpain activity in the cells. The faint 120kDa band is due to background apoptosis. At any instant in an entire cell culture population, at least 5% of the cells will die by apoptosis. These cleavage bands, particularly the 120 kDa band are much weaker in untreated cells than in drug treated cells. There is a strong 120 kDa band in the four drug treated cell lines indicative of fodrin cleavage during Doxorubicin-mediated apoptosis. The extent of cleavage appears equal in each of the four cell lines suggesting that protease activity in the four cell lines is comparable. Fodrin is efficiently cleaved in each of the four cell lines implying activation of caspase-3. Yet active caspase-3 could not be detected in section 3.13.2 as judged by the consistent levels of pro-caspase-3 protein in DLKP-SQ and DLKP-A250 10p#7 cells during apoptosis.

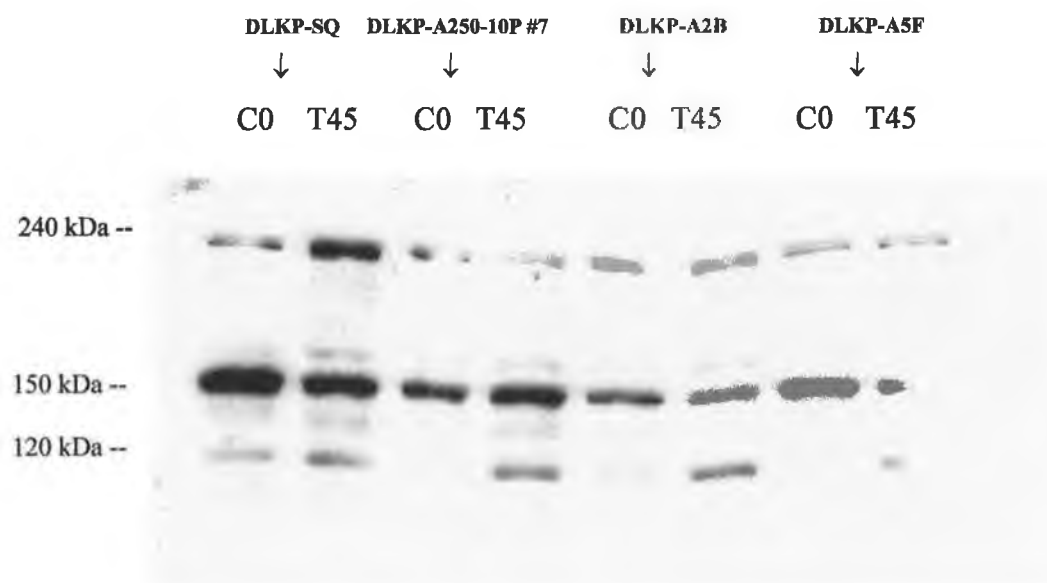


Figure 3.16.1 Fodrin Levels in DLKP-SQ, DLKP-A250 10#7, DLKP-A2B, DLKP-A5F before and after doxorubicin treatment. C0 refers to untreated cells, T45 refers to Doxorubicin treated cells 45 hrs after treatment.

3.17 Effects of caspase inhibitors on Doxorubicin-mediated apoptosis in DLKP cell lines

The development of synthetic caspase inhibitors has allowed extensive research to progress in attempt to further clarify the roles of specific caspases in the apoptosis pathway. In the introduction, 1.5.4, the design of these inhibitors was discussed; in brief if the inhibitor amino acid sequence is identical to the cleavage site in a substrate, then a caspase will bind to this recognition sequence in the inhibitor and further activity is prevented due to sequestration of the active caspase by the inhibitor.

Irreversible inhibitors were chosen to investigate their effect on apoptosis induction in DLKP cell lines. The first inhibitor used was zVAD.fmk, this is a general cell permeable caspase inhibitor having the Asp residue in the P1 position of the cleavage sequence that is common to all caspases. A relatively non-toxic (< 10 % apoptosis) inhibitor concentration of 100 μ M was used in experiments, and cells were pre-incubated with inhibitor to ensure the inhibitor enters the cells prior to exposure to apoptotic stimuli. Three inhibitor treatments were used; the first involved pre-treatment with the inhibitor and removal just before drug addition. The second involved pre-treatment with inhibitor and continuous incubation during drug treatment, and the third consisted of pre-treatment, continuous incubation during drug exposure and a further addition of inhibitor 24 hours after drug treatment. The three different treatments attempted to cover all inhibition possibilities. Pre-incubation alone was carried out with the assumption that the irreversible inhibitor should inhibit all caspase activity in the cell, and this irreversible inhibition would be maintained when the cells were exposed to drug. If drug treatment could induce new caspase activation, this should be blocked by the second treatment, continuous inhibitor presence which should serve to block any new caspase activation. The possibility of inhibitor degradation at 37°C existed however, and to overcome this fresh inhibitor was added to the cells 24 hours after drug treatment. A 24 hour treatment was chosen as the optimum addition time because it was seen in section 3.10.1 that the majority of DLKP-SQ cells did not undergo apoptosis until at least 24 hours after drug treatment. Results of the inhibitor experiments can be seen in figure

3.17.1. All treatments were carried out in duplicate and percentage kill was estimated using the crystal violet elution end point assay. Control cells were untreated and after 3 days were considered to represent 100% viability. The concentration of zVAD.fmk used was 100 μ M, and this alone resulted in 0 - 30 % apoptosis. zVAD.fmk was incubated either for 2 hours or for 72 hours in DLKP-SQ cells as a control for the doxorubicin and inhibitors used. This high level of apoptosis is not ideal, but is nonetheless sufficiently low when compared to the percentage kill seen after Doxorubicin treatment. It can be seen from figure 3.17.1 that zVAD.fmk treatment provided no survival enhancement in any of the combinations used, when standard deviations were taken into account. Continuous Doxorubicin treatment which gave a kill of 80% was also used in combination with zVAD.fmk but no protection from apoptosis was seen. The experiment was repeated and similar results were obtained.

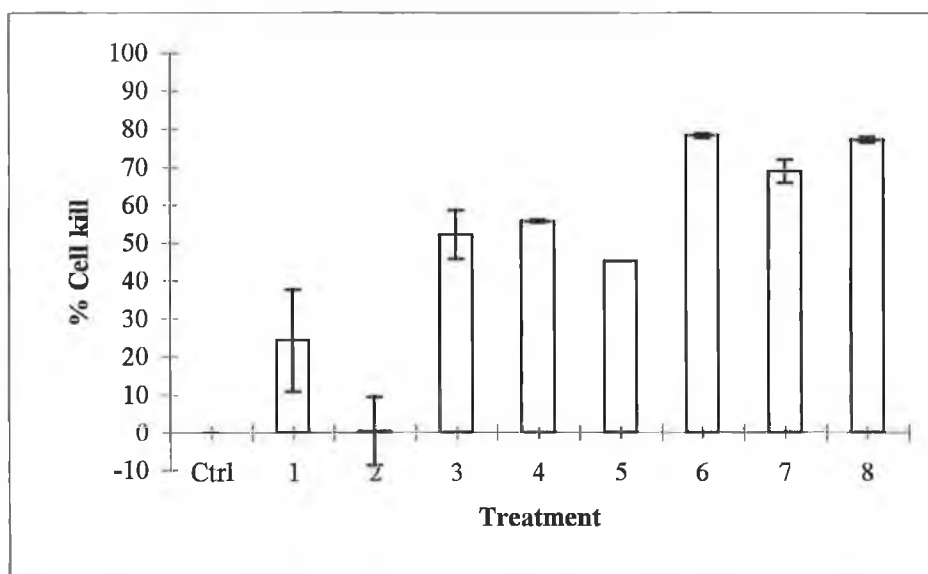
The microtubule inhibitor taxol was then used in combination with zVAD.fmk to look at the effects on apoptosis induction in DLKP-SQ. Taxol was chosen because apoptosis is induced over a much shorter time-scale of 6 hours compared to 24 hours with Doxorubicin. Two taxol concentrations were used, 80ng/ml for two hours resulted in 85% apoptosis and 50ng/ml for two hours resulted in 90% apoptosis after 6 hours. zVAD was again pre-incubated with the cells and then either removed or remained during drug treatment. From figure 3.17.2 it can be seen that the zVAD.fmk had no cytoprotective effect on DLKP-SQ.

zVAD.fmk was then used in combination with doxorubicin treatment to see whether apoptosis could be prevented in DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F, or if similar results to the situation in DLKP-SQ would be obtained. It can be seen in figures 3.17.3, 3.17.4, 3.17.5 and 3.17.6 that the four cell lines respond in a similar manner to doxorubicin treatment and zVAD.fmk; no protection from cell death is achieved.

The ability of the specific inhibitors zDEVD.fmk and YVAD.fmk to block doxorubicin-induced apoptosis was also investigated in the four DLKP cell lines. Again, cells were preincubated for 1 hour with inhibitor before doxorubicin treatment. zDEVD.fmk specifically inhibits the caspase-3-like family of caspases

including caspase-2, -6 and -7, but no protection from cell death occurred in its presence in any of the doxorubicin-treated DLKP cell lines, and results can be seen in figure 3.17.3, 3.17.4, 3.17.5 and 3.17.6. YVAD.fmk is a specific inhibitor of caspase-1 (ICE)-like caspases but failed to inhibit apoptosis induced by doxorubicin in the four DLKP cell lines, as seen by the results in figures 3.17.3, 3.17.4, 3.17.5 and 3.17.6.

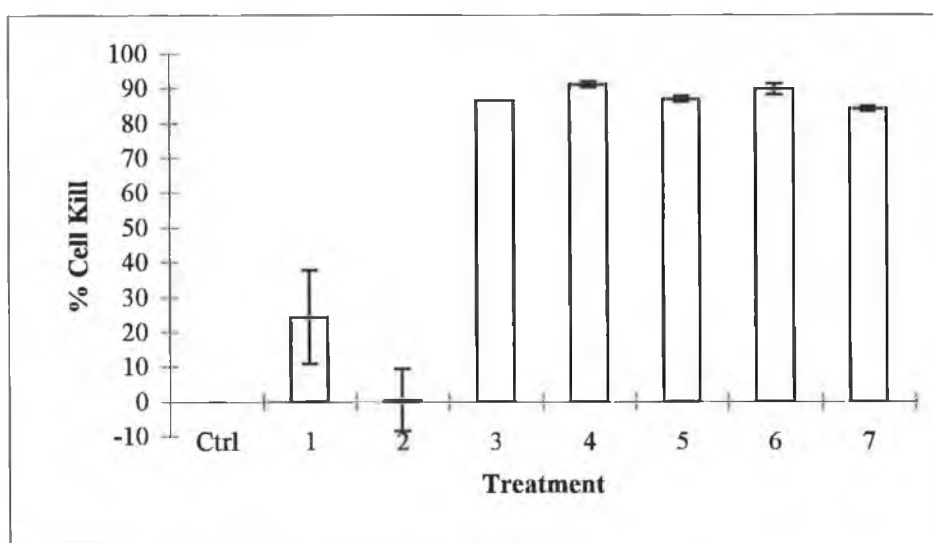
Timelapse videomicroscopy was carried out to analyse the kinetics of apoptosis in DLKP-SQ treated with doxorubicin and the general caspase inhibitor zVAD.fmk. Analysis in figure 3.17.7 revealed a similar rate of apoptosis in the doxorubicin- and inhibitor-treated cells compared to DLKP-SQ treated with doxorubicin alone, indicating that the presence of zVAD.fmk did not alter the rate of apoptosis induced by doxorubicin.



Treatments

- | | |
|----------------------------------|---|
| 1: 100uM zVAD 2hrs | 5: 0.8ug/ml Dox 2hrs + 100uM zVAD (24hrs) |
| 2: 100uM zVAD const | 6: 0.4ug/ml Dox const |
| 3: 0.8ug/ml Dox 2hrs | 7: zVAD const + 0.4ug/ml Dox const |
| 4: zVAD 2hrs + 0.8ug/ml Dox 2hrs | 8: 0.4ug/ml Dox const + 100uM zVAD (24hrs) |

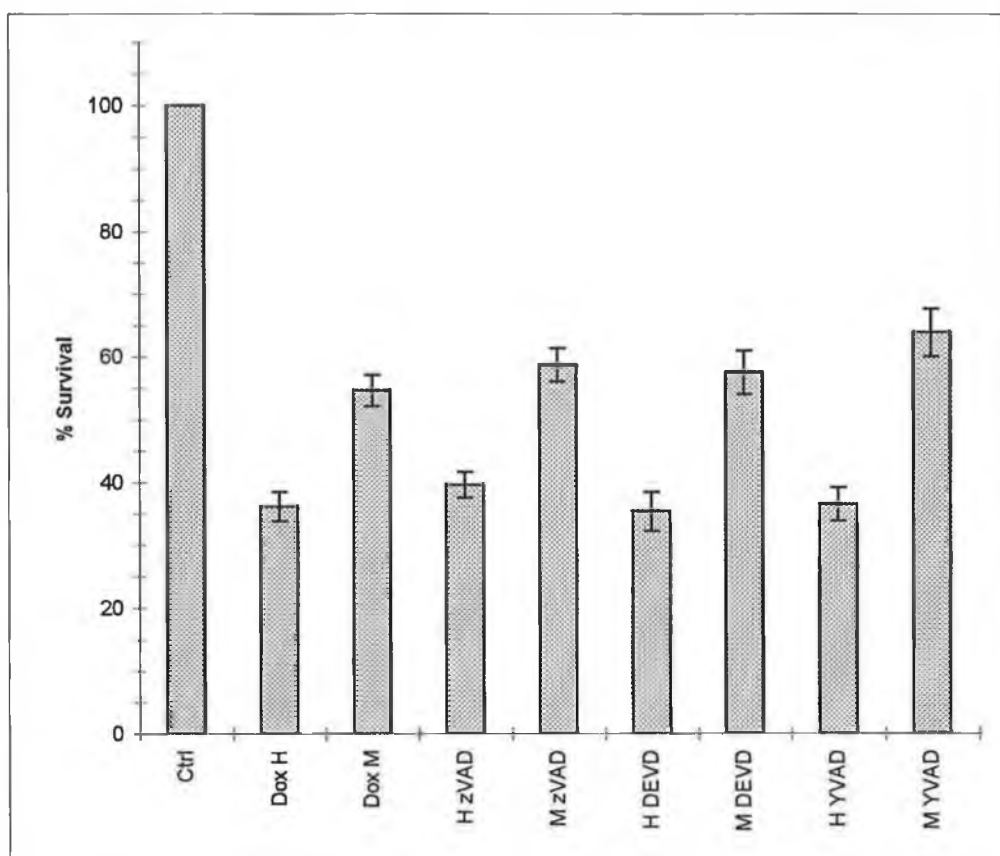
Figure 3.17.1 Effect of zVAD.fmk and doxorubicin on DLKP-SQ



Treatments

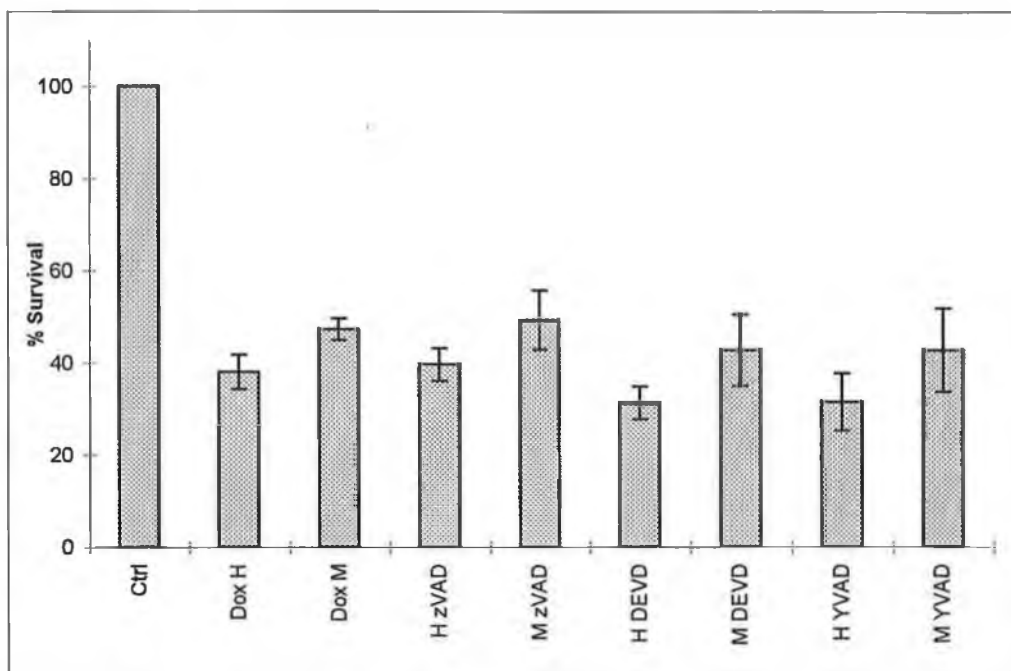
- | | |
|---|---|
| 1: 100uM zVAD 2hrs | 5: 50ng/ml Taxol const |
| 2: 100uM zVAD const | 6: 50ng/ml Taxol const + 100uM zVAD 2hrs |
| 3: 80ng/ml Taxol 2hrs | 7: 50ng/ml Taxol const + 100uM zVAD const |
| 4: 80ng/ml Taxol 2hrs + 100uM zVAD 2hrs | |

Figure 3.17.2 Effect of zVAD.fmk and Taxol on DLKP-SQ



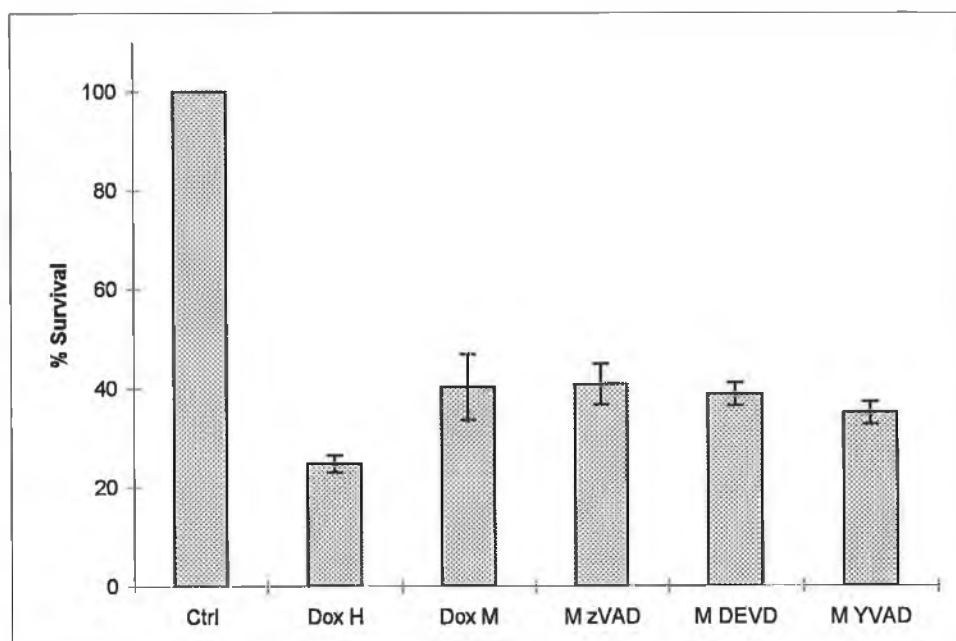
	%Survival	%St Dev
Ctrl	100	0
Dox H	36.13	2.35
Dox M	54.60	2.5
H zVAD	39.59	2.06
M zVAD	58.72	2.64
H DEVD	35.39	3.09
M DEVD	57.54	3.46
H YVAD	36.57	2.65
M YVA	63.87	3.83

Figure 3.17.3 DLKP-SQ treated with doxorubicin and 100µM caspase inhibitors.



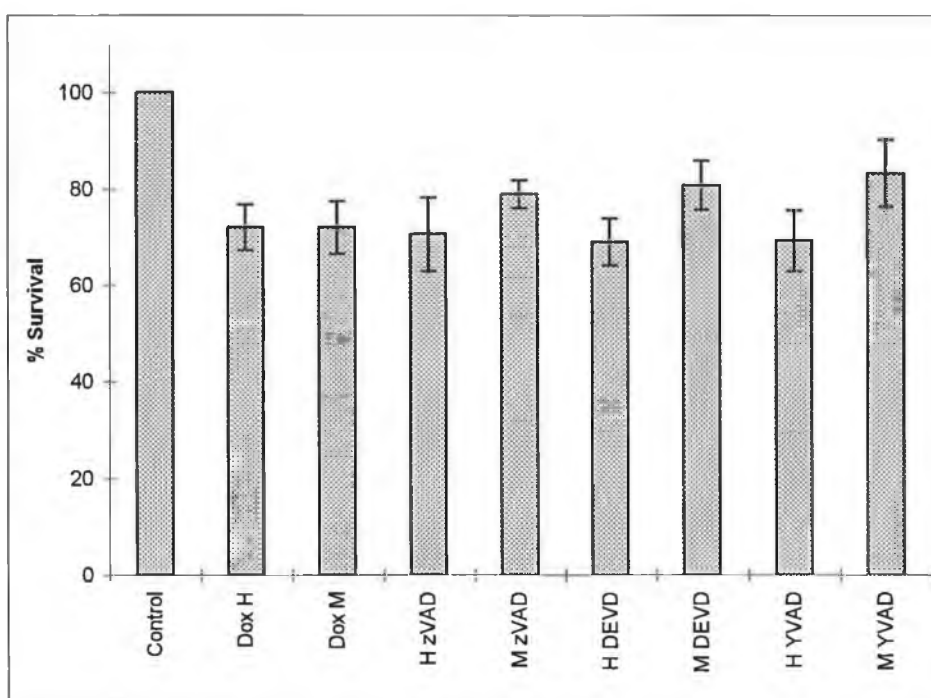
	%Survival	%St Dev
Ctrl	100	0
Dox H	38.02	3.73
Dox M	47.30	2.3
H zVAD	39.60	3.57
M zVAD	49.29	6.43
H DEVD	31.27	3.57
M DEVD	42.78	7.78
H YVAD	31.51	6.27
M YVAD	42.78	9.13

Figure 3.17.4 DLKP-A250 10p#7 treated with doxorubicin and 100 μ M caspase inhibitors.



	%Survival	%St Dev
Ctrl	100	0
Dox H	24.75	1.73
Dox M	40.21	6.62
M zVAD	40.79	4.12
M DEVD	38.67	2.35
M YVA	34.90	2.28

Figure 3.17.5 DLKP-A2B treated with doxorubicin and 100 μ M caspase inhibitors.



	%Survival	St Dev
Control	100	0
Dox H	72.15	4.78
Dox M	72.07	5.48
H zVAD	70.60	7.71
M zVAD	79.01	2.43
H DEVD	69.06	4.86
M DEVD	80.86	5.09
H YVAD	69.29	6.4
M YVA	83.41	6.94

Figure 3.17.6 DLKP-A5F treated with doxorubicin and 100µM caspase inhibitors.

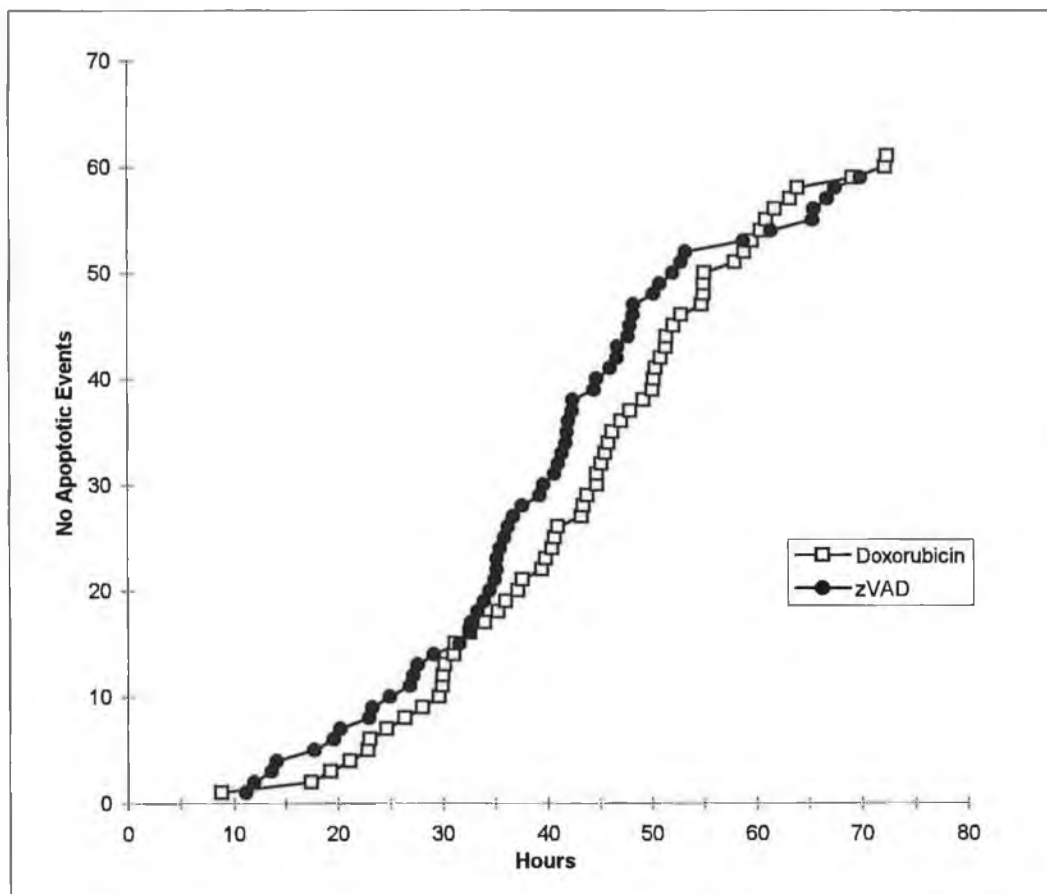


Figure 3.17.7 Timelapse videomicroscopical analysis of apoptosis in DLKP-SQ cells, comparing kinetics of cell death in doxorubicin-treated cells with cells treated with doxorubicin and the caspase inhibitor zVAD. Cells were monitored over 72 hours, and the time of apoptotic induction noted.

3.18.1 Caspase-3 fluorometric assays

Fluorometric substrate assays were carried out in order to measure caspase-3 activity using the specific fluorogenic substrate AcDEVD-AMC. This enzymatic assay quantitates the cleavage of the fluorogenic peptide as a measurement of caspase-3 activation. Cytoplasmic extracts were prepared as described in section 2.x, and protein concentration was estimated using the BCA assay. Figure 3.18 illustrates one set of data from the fluorogenic assay, repeat assays followed similar trends.

In DLKP-SQ there is an increase in caspase-3 activity following doxorubicin treatment of approximately three-fold, but the basal activity in untreated DLKP-SQ cells seems to be active to an equal extent. DLKP-SQ cell extracts which had been treated with the caspase inhibitor zVAD.fmk exhibit a decreased caspase-3 activity indicating that the caspase inhibitor is cell permeable and can efficiently inhibit caspase-3 activity.

DLKP-A250 10p#7 cells exhibit an increase in caspase-3 activity after doxorubicin treatment with highest activity seen 60 hours after treatment. Caspase-3 activity was almost completely abolished in pre-treated zVAD.fmk and doxorubicin-treated extracts. DLKP-A5F also exhibits increased activity, with levels peaking at 45 hours after doxorubicin treatment, but results are not shown in this graph. There is significant decrease in the pre-treated VAD.fmk samples in the resistant cells at 60 hours after drug treatment, in contrast to the sensitive which do not show as much of a decrease in inhibitor-treated extracts.

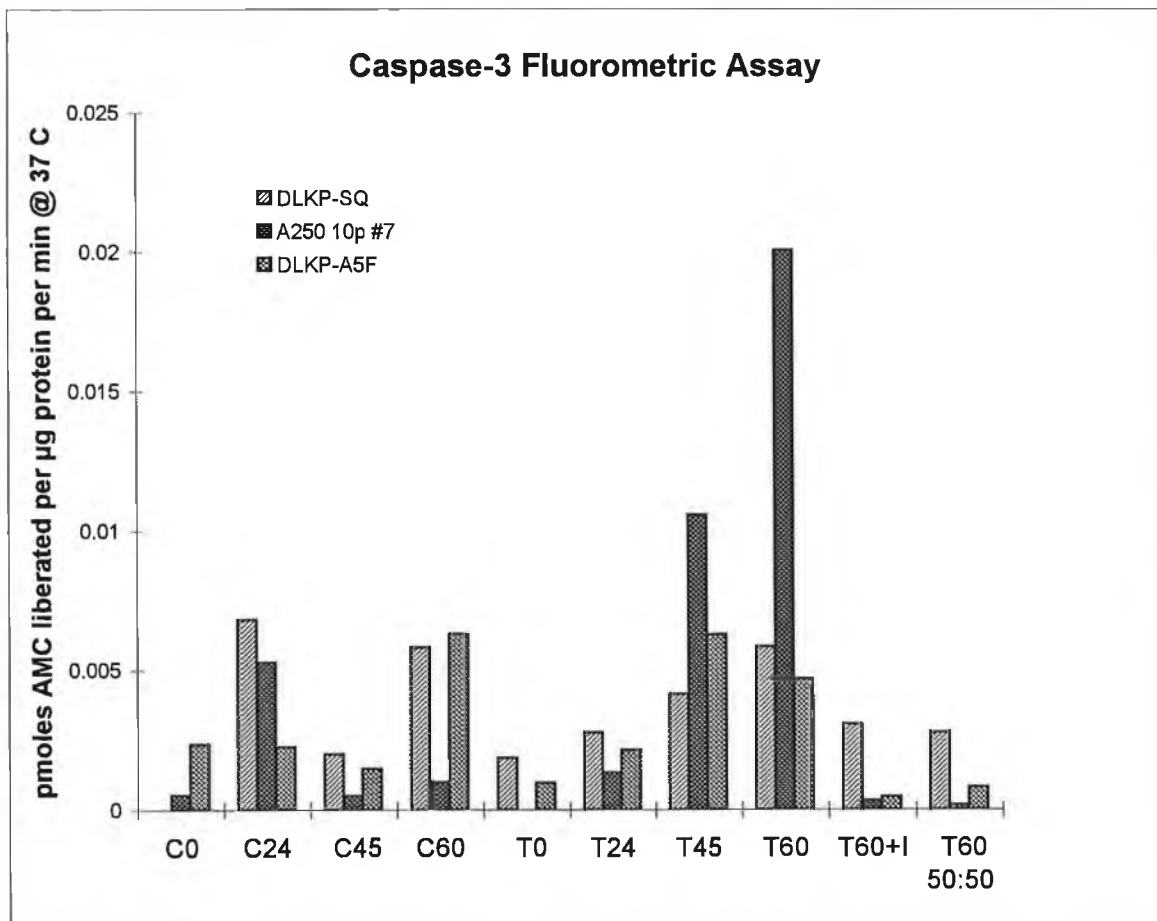


Figure 3.18.1 Caspase-3 enzyme activity as measured using fluorometric DEVD.AMC substrate

3.19 CD95 receptor levels

CD95/CD95-L interaction plays an important role in apoptotic signalling, and is involved in apoptosis induced by chemotherapy drugs, amongst other stimuli. Upon receipt of an apoptotic signal, the CD95 receptors trimerise, allowing CD95-L to bind and a death signal to be transduced. Therefore the amount of CD95 present is an important factor for efficient signalling to occur. It has previously been reported that cells resistant to apoptosis induced either by CD95-L or doxorubicin have altered regulation of CD95, resulting in reduced CD95 expression (Fulda *et al.*, 1998). Also, CD95 expression is upregulated after doxorubicin treatment in some cell lines, such as neuroblastoma cells (Friesen *et al.*, 1998, Fulda *et al.*, 1998).

CD95 receptor levels were looked at using Western blotting in the four DLKP cell lines before and after doxorubicin treatment. In figure 3.19 it can be seen that CD95 is expressed to the same extent in sensitive and resistant cell lines, with no downregulation of CD95 in the resistant variants. After doxorubicin treatment, there is no upregulation of CD95 in any of the cell lines. It had been thought that if after doxorubicin treatment, DLKP-SQ cells upregulated CD95 perhaps the resistant variants would not upregulate CD95 to the same extent, and this failure to upregulate CD95 after doxorubicin treatment could be an additional resistance mechanism in the resistant variants. However results show that there is no upregulation of CD95 in any of the cell lines. After doxorubicin treatment, DLKP-A2B seem to have an upper band, but the significance of this is not known.

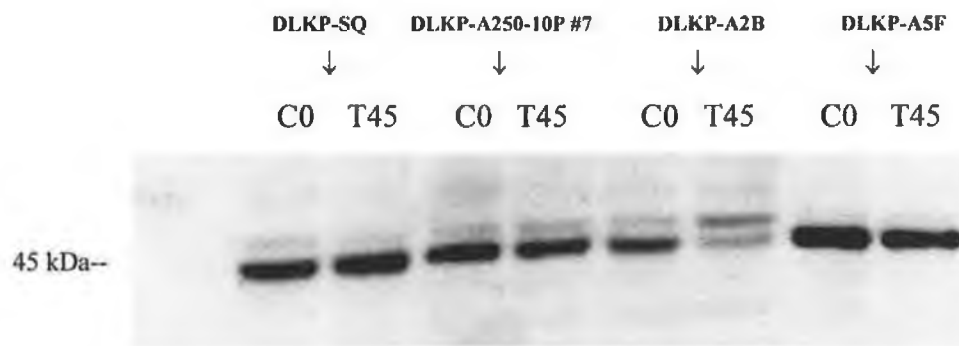


Figure 3.19 Western Blot showing CD95 receptor protein levels in DLKP cell lines before and after doxorubicin treatment

3.20 Effect of CD95 blockers on doxorubicin-induced apoptosis

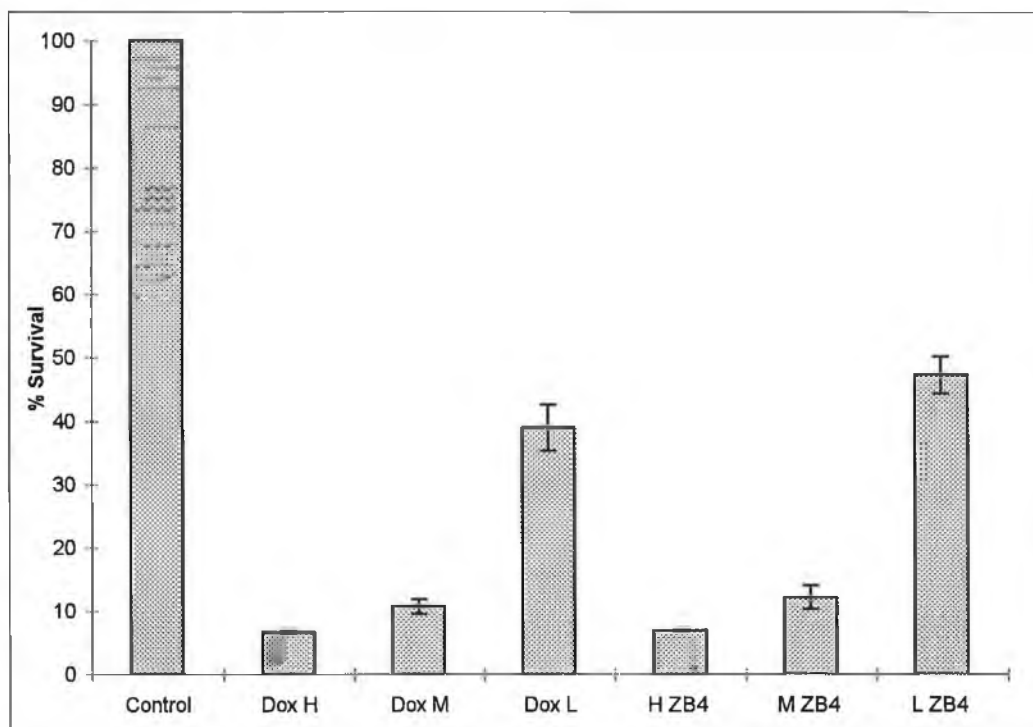
In figure 3.19 it was shown that doxorubicin treatment did not affect the CD95 receptor levels in either sensitive or resistant cell lines. The involvement of the CD95 pathway was further investigated using specific inhibitors of the CD95 pathway. ZB4 is a CD95 antibody that binds to cellular CD95 and prevents CD95L binding, and consequently signal transduction. NOK-2 is a CD95-L antibody which has a neutralising effect on CD95-L action, by binding to CD95-L and preventing its association with CD95. Both these antibodies were used in combination with doxorubicin treatment on DLKP-SQ, DLKP-A250 10p#7 and DLKP-A2B (DLKP-A5F was not used). Three drug concentrations were used for each cell line which gave a high, medium and low cell kill after 72 hours. Cells were preincubated with inhibitor for 1 hour before doxorubicin addition, at which time fresh inhibitor was also added. Cell viability was determined after 72 hours using an *in vitro* acid phosphatase toxicity assay, as described in materials and methods.

Results are shown in figures 3.20.1, 3.20.2 and 3.20.3 for ZB4 and doxorubicin combinations. It can be seen that ZB4 offered no protection to the cells from doxorubicin-induced apoptosis, approximately the same number of cells died when treated with ZB4 and doxorubicin, as those treated with doxorubicin alone. The effect of NOK-2 on doxorubicin-induced apoptosis can be seen in figures 3.20.4, 3.20.5 and 3.20.6 for DLKP-SQ, DLKP-A250 10p#7 and DLKP-A2B respectively. NOK-2 also has no protective effect against doxorubicin-induced apoptosis.

These results, although unexpected, are quite novel because they raise questions about the involvement of CD-95 pathway in doxorubicin-induced apoptosis in the DLKP cell lines. The ZB4 antibody was tested for its ability to block CD95-induced apoptosis, to ensure that it was capable of inhibiting apoptosis mediated by the CD95 signalling pathway and to confirm that the ZB4 antibody is effective in entering the DLKP cell models. Cells were preincubated with ZB4 for 1 hour and then treated with a CD95 activating antibody (CH-11). Results are illustrated in figure 3.20.7. CH-11, even though it had to be used at much higher concentrations than those

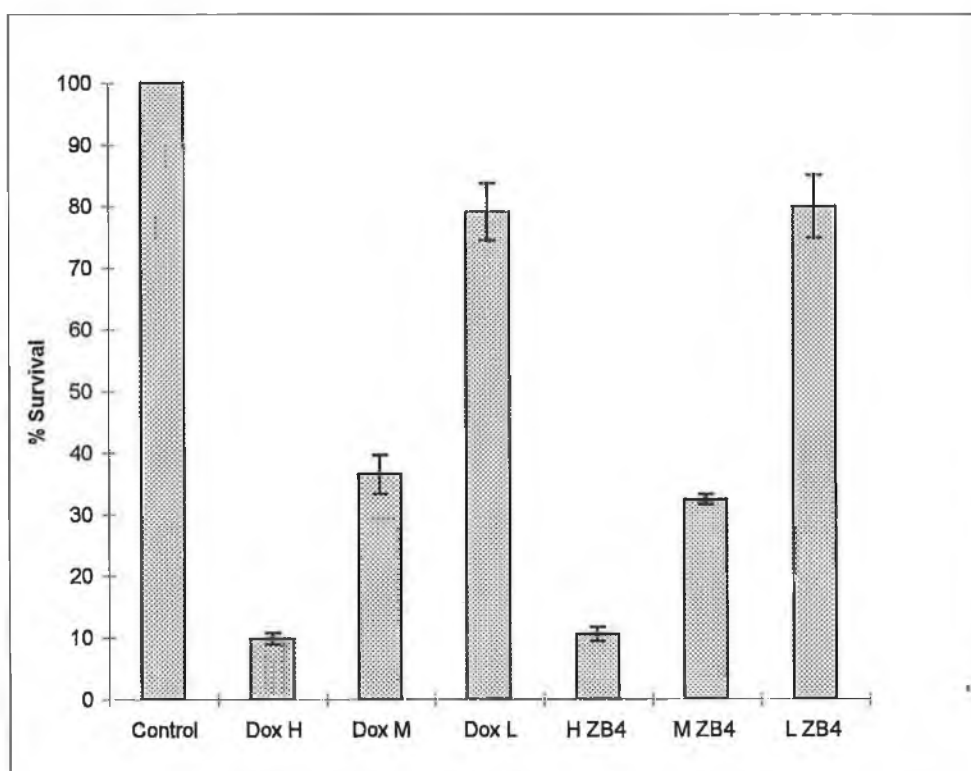
recommended in the literature, induced a low level of apoptosis in DLKP-SQ. When co-treated with the ZB4 blocking antibody the 37% kill decreased to 8 %, indicating that the ZB4 is active in blocking the CD95 signalling pathway.

In section 3.17 cell permeable inhibitors of caspases were tested for their ability to block doxorubicin-induced apoptosis in the DLKP cell lines, without success. The CD95 blockers also failed to inhibit doxorubicin-induced apoptosis, as seen in figures 3.20.1-3.20.6. A further combination was then employed, consisting of the CD95 blocker ZB4 and the general caspase inhibitor zVAD.fmk, in attempt to discover if both inhibitors together could prevent DLKP-SQ from apoptosis. Cells were preincubated with both inhibitors for 1 hour before doxorubicin addition, and again at the time of drug addition. Results in figures 3.20.8, 3.20.9 and 3.20.10 demonstrate that doxorubicin-treated DLKP cells can still die even in the presence of both CD95 blockers and caspase inhibitors. To further corroborate these results, DLKP-SQ cells were closely monitored after this inhibitor and drug combination using timelapse videomicroscopy. The kinetics of apoptosis of the combination treatment were compared to kinetics of apoptosis induced by doxorubicin alone in figure 3.20.11. From the graph in figure 3.20.11 it is clear that the inhibitors have no effect on the rate of apoptotic induction in doxorubicin-treated cells. Apoptosis in inhibitor-treated cells has slightly faster initial rate, if anything, but the two rates closely mirror each other over the 72 hours monitored.



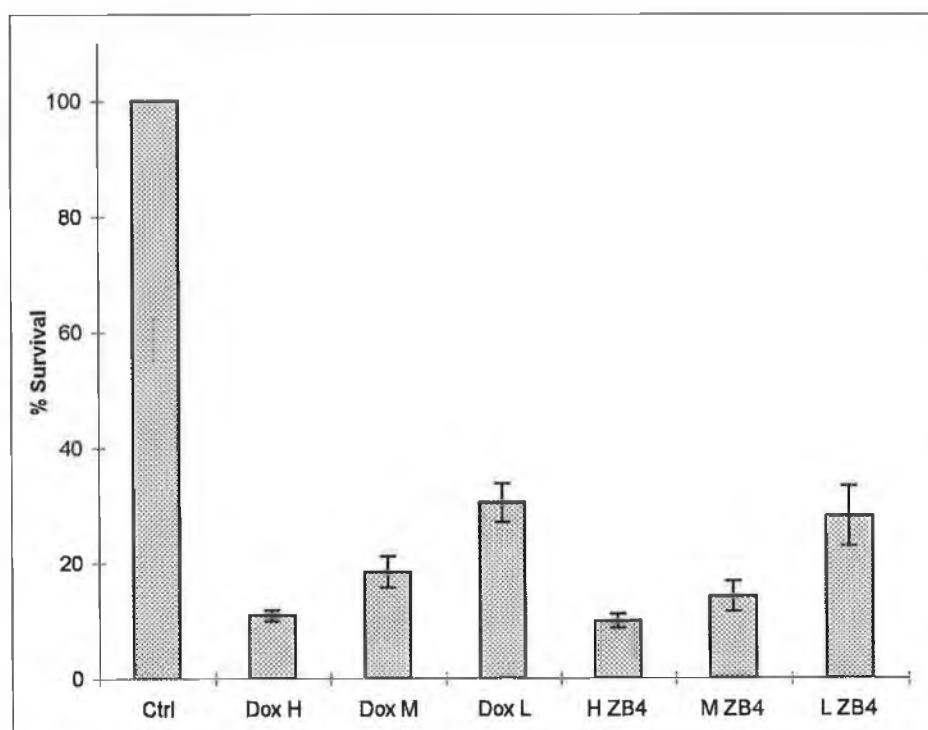
	%Survival	%St Dev
Control	100	0
Dox H	6.59	0.493
Dox M	10.67	1.16
Dox L	38.9	3.63
H ZB4	6.91	0.36
MZB4	12.15	1.83
L ZB4	47.3	2.96

Figure 3.20.1 DLKP-SQ treated with doxorubicin and the CD95 blocker ZB4(250ng/ml)



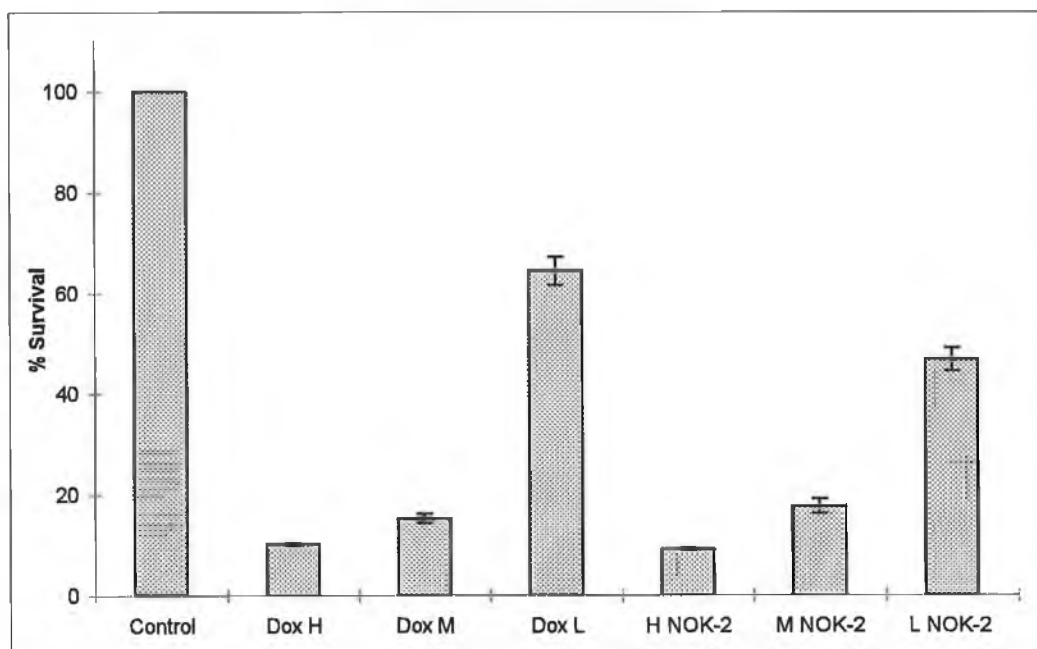
	%Survival	%St Dev
Control	100	0
Dox H	9.74	0.924
Dox M	36.47	3.17
Dox L	79	4.65
H ZB4	10.46	1.12
M ZB4	32.34	0.825
L ZB4	79.9	5.08

Figure 3.20.2 DLKP-A250 10p#7 treated with doxorubicin and the CD95 blocker ZB4 (250ng/ml)



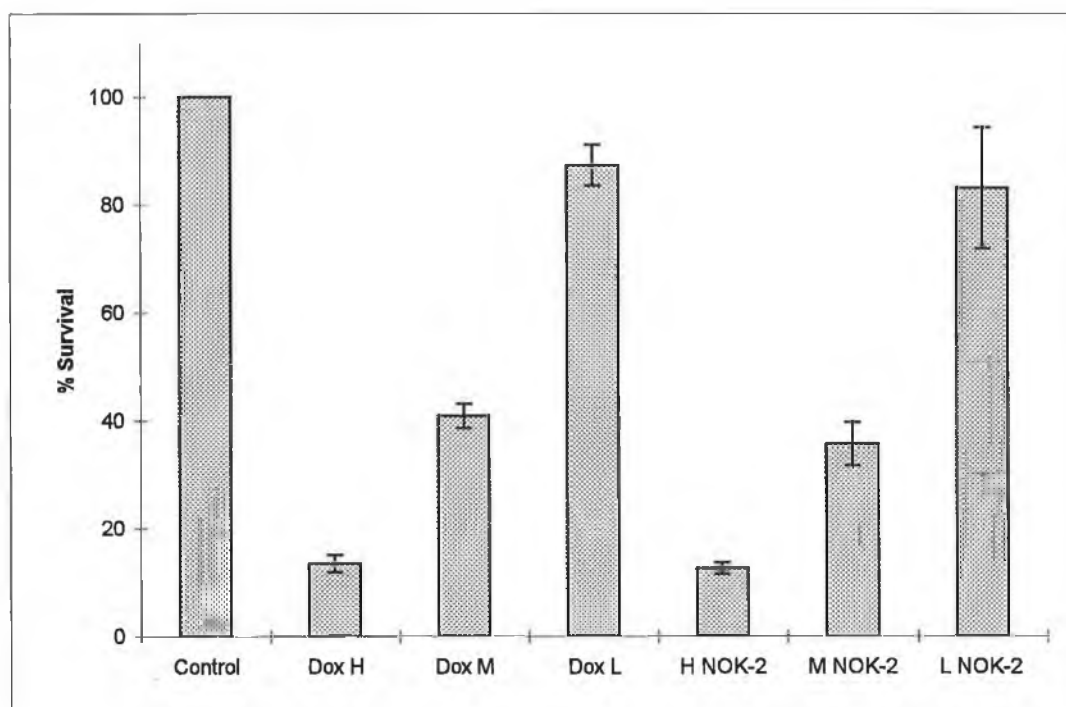
	%Survival	%St Dev
Control	100	0
Dox H	10.9	0.91
Dox M	18.42	2.75
Dox L	30.42	3.3
H ZB4	9.99	1.19
M ZB4	14.2	2.57
L ZB4	28.1	5.22

Figure 3.20.3 DLKP-A2B cells treated with doxorubicin and the CD95 blocker ZB4 (250ng/ml)



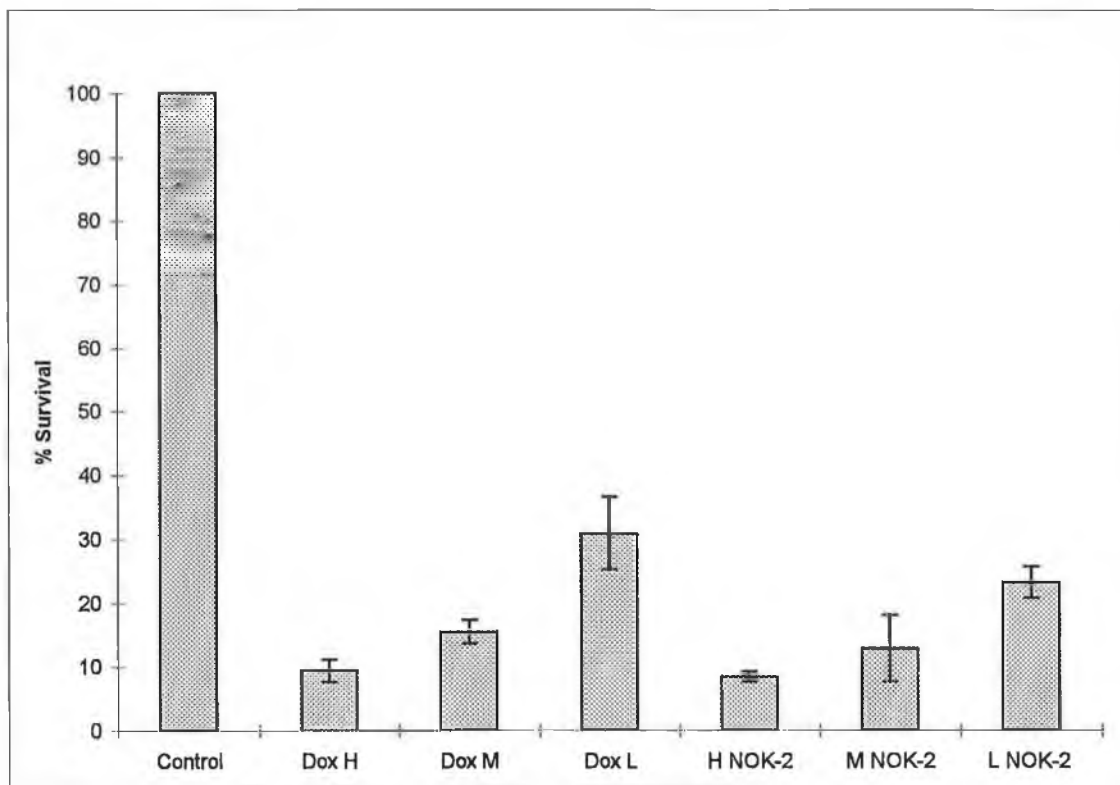
	%Survival	%St Dev
Control	100	0
Dox H	10.15	0.377
Dox M	15.27	0.892
Dox L	64.34	2.78
H NOK-	9.13	0.27
M NOK-	17.67	1.41
L NOK-2	49.77	2.265

Figure 3.20.4 DLKP-SQ cells treated with doxorubicin and the CD95L neutralising antibody, NOK-2 (1µg/ml).



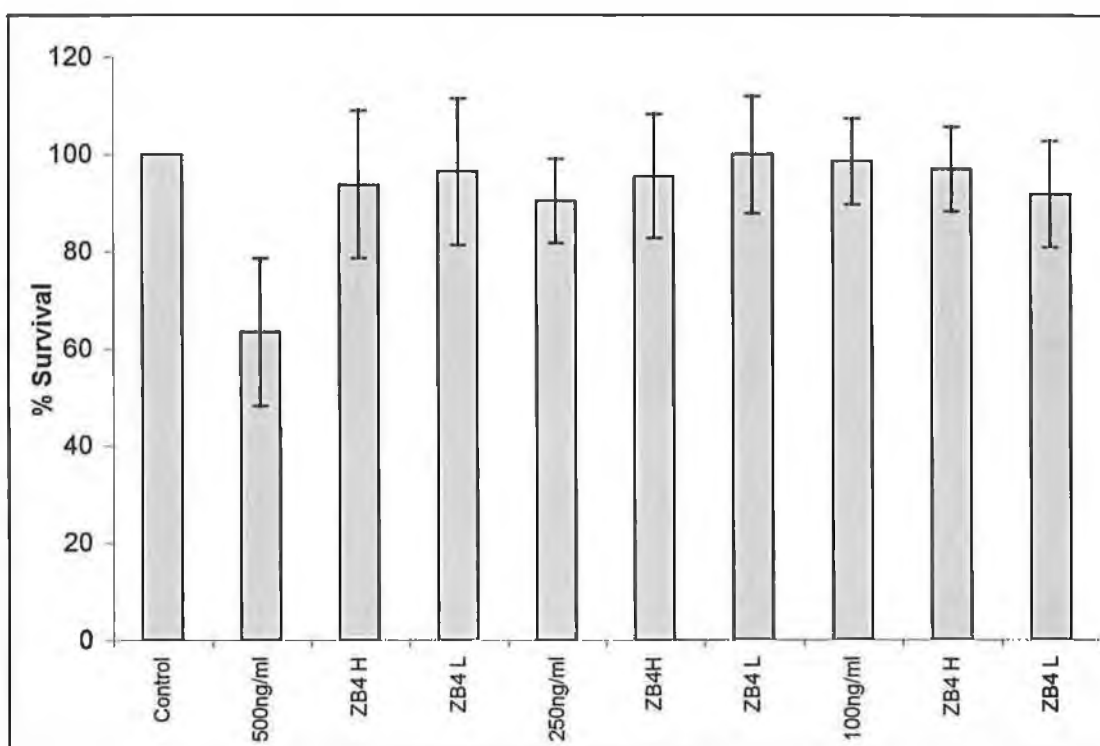
	%Survival	%St Dev
Control	100	0
Dox H	13.39	1.59
Dox M	40.83	2.24
Dox L	87.34	3.79
H NOK-2	12.52	1.03
M NOK-2	35.64	4.02
L NOK-2	83.06	11.25

Figure 3.20.5 DLKP-A250 10p#7 cells treated with doxorubicin and the CD95L neutralising antibody, NOK-2 (1µg/ml).



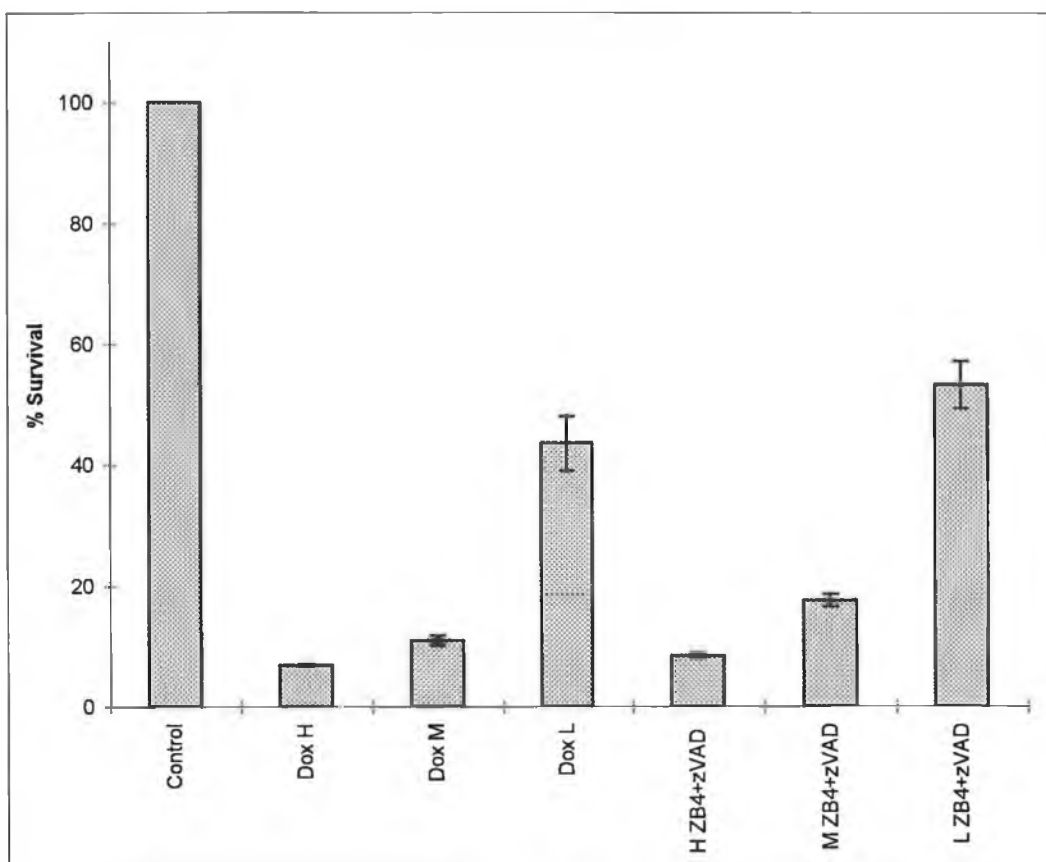
	%Survival	%St Dev
Control	100	0
Dox H	9.38	1.77
Dox M	15.49	1.86
Dox L	30.9	5.67
H NOK-2	8.41	0.79
M NOK-2	12.83	5.22
L NOK-2	23.2	2.48

Figure 3.20.6 DLKP-A2B cells treated with doxorubicin and the CD95L neutralising antibody, NOK-2 (1µg/ml).



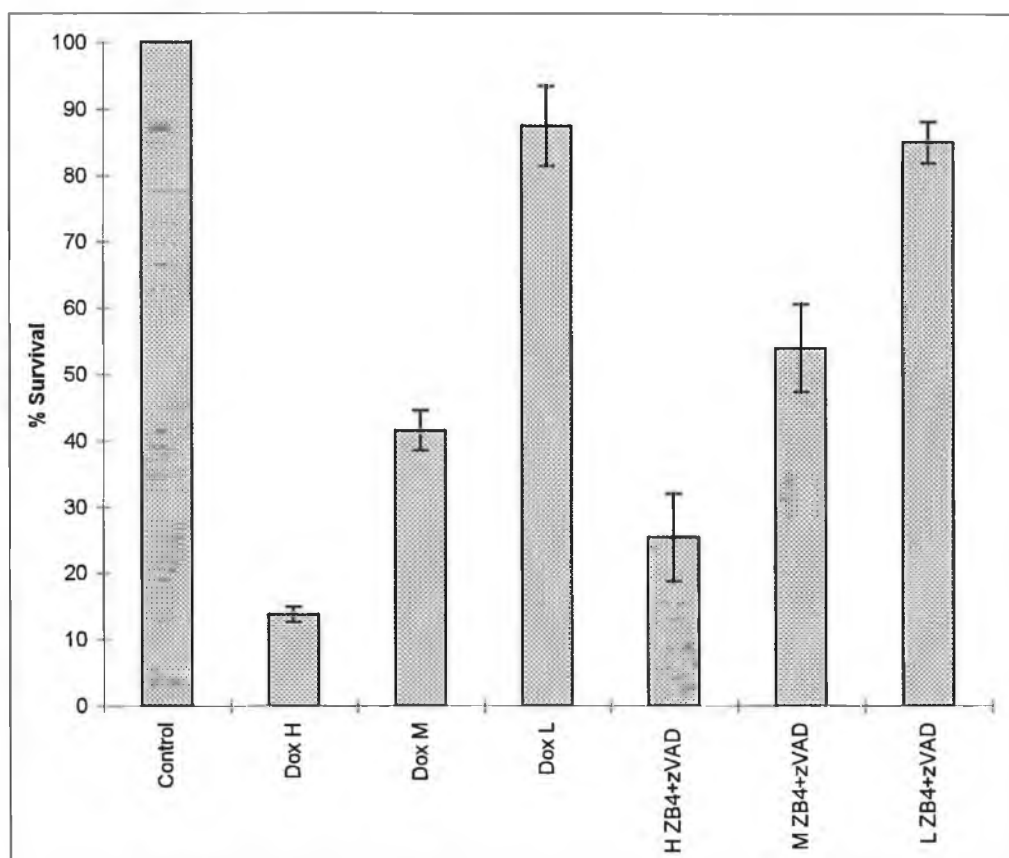
	% Survival	%St Dev
Control	100	0.0
500ng/ml	63.38	15.21
ZB4 H	93.72	15.21
ZB4 L	96.41	15.09
250ng/ml	90.42	8.68
ZB4 H	95.57	12.75
ZB4 L	99.94	12.04
100ng/ml	98.50	8.80
ZB4 H	97.01	8.68
ZB4 L	91.86	10.90

Figure 3.20.7 Effect of ZB4 on CD95 (CH-11)-induced apoptosis after 72 hours. CD-95 concentrations used were 500ng/ml, 250ng/ml and 100ng/ml. ZB4 H was 500ng/ml ZB4 L was 250ng/ml



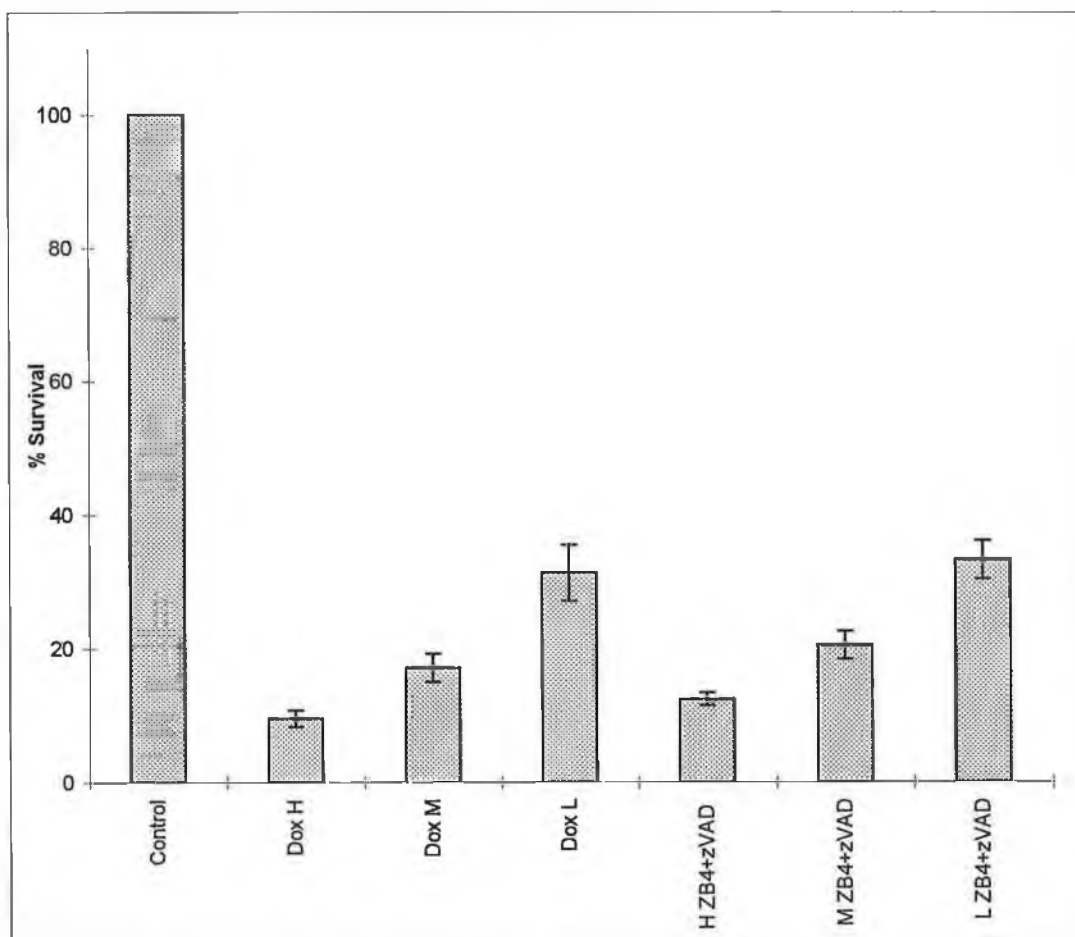
	%Survival	%St Dev
Control	100	0
Dox H	6.83	0.36
Dox M	10.9	0.81
Dox L	43.6	4.47
H ZB4/zVAD	8.37	0.53
M ZB4/zVAD	17.53	1.01
L ZB4/zVAD	53.25	3.9

Figure 3.20.8 DLKP-SQ cells treated with doxorubicin and a combination of caspase inhibitor zVAD (100 μ M) and the CD95 blocker ZB4(250ng/ml)



	%Survival	%St Dev
Control	100	0
Dox H	13.77	1.14
Dox M	41.55	3
Dox L	87.46	6.02
H ZB4/zVAD	25.39	6.61
M ZB4/zVAD	53.98	6.57
L ZB4/zVAD	85.02	3.09

Figure 3.20.9 DLKP-A250 10p#7 cells treated with doxorubicin and a combination of the caspase inhibitor zVAD (100 μ M) and the CD95 blocker ZB4 (250ng/ml)



	%Survival	%St Dev
Control	100	0
Dox H	9.46	1.24
Dox M	17	2.1
Dox L	31.34	4.2
H ZB4/zVAD	12.32	0.95
M ZB4/zVAD	20.35	2.1
L ZB4/zVAD	33.25	2.87

Figure 3.20.10 DLKP-A2B cells treated with doxorubicin and a combination of the caspase inhibitor zVAD (100 μ M) and the CD95 blocker ZB4 (250ng/ml)

4.0 Discussion

4.1 Constitutive protein levels of cell cycle regulatory proteins in DLKP cell lines

The cell cycle is a tightly regulated process governing cellular growth and proliferation, and any defects in cell cycle control may lead to unrestrained growth and proliferation, a feature frequently seen in tumourigenesis. Abnormalities in cyclins and cdks have now been implicated in several human tumours (Bartkova *et al.*, 1997). One of the earliest examples was the finding that the cyclin A gene was the site for integration of the hepatitis B virus in a human hepatocellular carcinoma, but this abnormality appears rarely in liver cancer (Motokura and Arnold, 1997). The cyclin E gene is often dysregulated and overexpressed in a variety of human carcinomas (Keyomarsi *et al.*, 1994). Cdks have also been reported to be overexpressed or dysregulated in many cancers, cdk4 is frequently amplified in glioblastomas (Schmidt *et al.*, 1994) and altered regulation of cdk2 occurs in glioblastoma (Pykett *et al.*, 1997).

Cyclin D₁ has an important role to play in cell cycle progression and proliferation, due to its association with Rb and the E2F family of transcription factors. Therefore any overexpression of cyclin D₁ would have important implications for tumour growth resulting in enhanced transcription of many genes required for the S phase. Cyclin D₁ has been mapped to the 11q13 locus close to the BCL1 region which is frequently amplified in many tumours (Motokura and Arnold, 1997). Abnormalities in the cyclin D gene have been associated with numerous human cancers such as carcinomas of the breast, oesophagus, head and neck, lung, liver and bladder (Jiang *et al.*, 1993; Gillett *et al.*, 1996, Zhang *et al.*, 1993). Overexpression of cyclin D₁ is associated with poor prognosis of patients with breast cancer (Borg *et al.*, 1992, Michalides *et al.* 1996) and human pancreatic carcinoma (Gansauge *et al.* 1997). Recently Arber *et al.* (1997) reported that antisense cyclin D₁ can decrease cyclin D₁ levels and prevent tumorigenicity in human colon carcinoma cells. Hochhauser *et al.*, (1996) investigated the chemosensitivity profiles of human fibrosarcoma cell lines expressing high and low levels of cyclin D₁ with doxorubicin (as well as other chemotherapeutic drugs taxol and cytarbine). It was demonstrated that cells with higher cyclin D₁ protein expression were equally sensitive to Doxorubicin when compared to cell lines with low levels of cyclin D₁, but clones with

high cyclin D₁ levels were more resistant to antimetabolites such as methotrexate. This finding implies that cyclin D₁ levels have no role to play in resistance to Doxorubicin. It was expected therefore that the Doxorubicin-resistant cells and Doxorubicin-sensitive DLKP-SQ should not have any significant differences in cyclin D₁ protein levels and this was the case.

An investigation was undertaken to study the levels of the main cell cycle regulating proteins cyclins and cyclin dependent kinases, in the sensitive DLKP-SQ and also the resistant variants DLKP-A250 10p #7, DLKP-A2B and DLKP-A5F. Cyclin B protein levels in the asynchronous cell lines were of approximately equal intensity. There are no reports of altered cyclin B levels in association with drug resistance, and results in figure 3.3.1 are in agreement with this. Cyclin E protein levels were equal in the four cell lines. It can also be seen that there is an equal level of expression of cyclin D₁ in both resistant and sensitive cell lines.

There was no variation in protein levels of the cyclin dependent kinases cdk1, cdk2, and cdk4 among the four cell lines. It can therefore be concluded that the multidrug resistant cell lines have a similar cell cycle to the sensitive DLKP-SQ with equal levels of the cell cycle regulatory proteins under study.

The fact that protein expression levels of cyclins and cdks do not change does not give a true reflection of the action of these proteins during the cell cycle however. A cyclin-cdk complex must form to initiate cdk kinase activity, which has a profound effect on the subsequent cell cycle events in the cell. Therefore even if protein levels remain at comparable levels, it is still possible that resistant cells may have alterations in activation of cdk complexes or deregulated kinase activity. This assumption holds true for any drug treatments on the cells, the drug may not affect the protein expression, but rather its kinase activity is altered.

4.2 Effect of Doxorubicin treatment on the cell cycle of DLKP cell lines

In section 3.1 it was demonstrated that cell cycle regulatory protein levels in both resistant and sensitive cell lines were comparable. The resistant cell lines DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F differ in their sensitivity to Doxorubicin treatment, and the main contributory factor to this resistance is the presence of p-glycoprotein, the ATP-dependent membrane transport protein whose main function is to act as a drug efflux pump (for review see Germann, 1998). The possibility that cell cycle regulatory proteins may also contribute to this resistance had not been investigated however, and a study was undertaken to clarify this.

Equitoxic concentrations were chosen, rather than equal intracellular concentrations. Some experiments were carried out in an attempt to relate drug dose to intracellular concentration but this was complicated by the fact that Doxorubicin can be sequestered into vesicles and away from their target site in the nucleus (Cleary, I., 1995). DLKP-A5F cells therefore gave an abnormally high doxorubicin intracellular reading even though there are very high levels of p-glycoprotein in this cell line (Heenan *et al.*, 1998).

When compared to DLKP-SQ, drug resistant DLKP-A250 10p#7 cells were approximately 3.5 fold resistant, DLKP-A2B cells were 50 fold resistant and DLKP-A5F cells were 160 fold resistant. These results vary slightly from previously reported values in which the cell lines were compared to the parental DLKP cell line, but this discrepancy is due to different seeding density (25 cm² flasks as opposed to 96-well plates) and different drug exposure times (2 hour pulse compared to continuous exposure over 3 days)

Drug treatment usually results in DNA damage, and the cell is equipped with checkpoints that sense DNA damage, and alter the cell cycle so as to prevent further progression which could be lethal for the cell. Numerous chemotherapy drugs affect the cell cycle, mainly through the instigation of an arrest before entry to mitosis, one of the critical events in the cell cycle. Early studies by Tobey *et al.* (1972) demonstrated that the antitumour drug bleomycin caused chinese hamster ovary cells to arrest in G2. Tobey *et al.* (1975) subsequently identified a number of other chemotherapeutic drugs which could

inhibit cell cycle traverse and cause an accumulation of cells in G2. Krishan and Frei, (1976) carried out a study investigating the effects of exposure of CCRF-CEM cells to doxorubicin, and concluded that cells accumulated in G2 phase and cell cycle traverse was inhibited. A G2 arrest occurs irrespective of the phase the cells are in during doxorubicin exposure, for instance cells exposed in late S and G2 experience an immediate arrest, cells in G1 will arrest more slowly in G2, but if they do manage to divide in M, their progeny will arrest in the subsequent G2 phase (Barlogie and Drewinko, 1978). Many other chemotherapy agents cause cells to arrest and accumulate in G2 and M, and these are summarised in table 2.1.

CHEMOTHERAPEUTIC AGENT	REFERENCE
Actinomycin D	Dewey and Highfield, 1976; Kimler <i>et al.</i> , 1978
Azacytidine	Tobey <i>et al.</i> , 1972
Bleomycin	Tobey, 1972;
Camptothecin	Tobey <i>et al.</i> , 1972; Tsao <i>et al.</i> , 1992
Cis-diamminedichloroplatinum (II)(CDDP)	Nishio <i>et al.</i> , 1993
Doxorubicin	Krishan and Frei, 1976, Barlogie <i>et al.</i> , 1976
Etoposide	Barlogie and Drewinko, 1978; Lock and Ross, 1990
Irradiation	Lock and Ross, 1990
Lucanthone	Kimler <i>et al.</i> , 1978
Melphalan	Barlogie and Drewinko, 1978
Mitomycin C	Kimler <i>et al.</i> , 1978

Table 2.1 Chemotherapeutic agents capable of inducing G2/M arrest

The effect of Doxorubicin treatment was initially investigated using flow cytometry. Doxorubicin or adriamycin is a member of the anthracycline group of chemotherapeutic drugs and exerts its effects by DNA intercalation and topoisomerase II inhibition (Pratt *et al.* 1994). The effect of doxorubicin treatment on DLKP-SQ cells was investigated. At 12

hours after treatment there is an increased number of cells in S phase, and this increases at 16 hours and 20 hours. An accumulation in late S and G2 phase is seen at 24 hours with a definite G2/M arrest by 36 hours. Apoptotic cells are evident by the sub-G1 peak at 68 hours. Doxorubicin is widely reported to cause a G2/M arrest in cycling cells. Krishan and Freii (1976) demonstrated that Doxorubicin treatment resulted in a G2/M arrest in asynchronous CCRF-CEM cells, and the situation in DLKP-SQ cells mirrors these results.

Doxorubicin treatment of DLKP-A250 10p#7 cells resulted in the cells accumulating in G2/M phase at 12 hours, peaking at 24 hours. After this time cells can be seen to increase in G1 phase with a decrease in G2/M population. The G1 cells seem to arrest at this point, and there is no increase in the S phase population. It is apparent from the histograms that the DLKP-A250 10p#7 cells cycle much faster than the DLKP-SQ cells. DLKP-A2B cells also exhibit a G2/M arrest, but the effect is not as prominent as for the DLKP-A250 10p#7 cells and there seems to be a partial block in G1 in a small percentage of cells as evident by the small G1 peak still present at 24 hours. DLKP-A5F cells accumulate in G2/M by 16 hours, again evidence for a faster cell cycle traverse in resistant cell lines compared to DLKP-SQ. By 24 hours the G2/M arrest has peaked and after this time the increase in G1 peak occurs, as previously seen in DLKP-A250 10p #7 and DLKP-A2B.

Barlogie *et al.* (1976) concluded that a G2 arrest was dependent on the doxorubicin concentration used and drug exposure time, with higher concentrations and longer exposure times causing a G1 arrest, delay in S phase transit and an irreversible G2/M arrest. The effect in DLKP-SQ cells reflects this "classic" effect (with arrest seen only at very high Doxorubicin concentrations) and contrasts sharply with the weak G2/M arrest, override of this arrest and subsequent G1 accumulation seen in the resistant variants. This difference is not due to a concentration effect because equitoxic doses were used to minimise any variation due to the higher concentrations used in resistant cells. Exposure times used for the four cells lines were of equal duration thus ruling out the possibility of the difference in effect being due to unequal exposure times. Therefore the possibility of a drug-specific effect being responsible for the G2/M override in the resistant variants seems unlikely.

It is obvious from these results that there must be some alteration in the controller complex cyclin B-cdk1 whose activity is critical for entry of the cells to mitosis. The activation and regulation of this complex is tightly controlled by phosphorylation levels (Parker *et al.*, 1992). The resistant cells that bypass the G2/M arrest may have deregulated control of entry to mitosis, or may not be able to sense the damage that the sensitive DLKP-SQ experience; whatever the difference, it is significant because it allows the resistant variants to over-ride the G2/M arrest in response to doxorubicin treatment.

When taken together, these results imply that resistant cells must possess a defective checkpoint which empowers them to override the G2/M arrest seen in sensitive cells after doxorubicin treatment. The G2/M arrest seen is due to a cell cycle checkpoint that delays progression from G2 into mitosis in response to DNA damage, to allow time for repair of any damaged DNA before entry to mitosis. DLKP cells were investigated for p21 induction following doxorubicin treatment but no p21 could be detected, suggesting that wild type p53 is absent in these cells. Current studies suggest that the G2 checkpoint occurs independently of p53; p53 is responsible for inducing p21 and consequently a G1/S arrest, and it is thought that p53 does not play a role in the DNA-damage-induced G2/M arrest. p53 is involved in bringing about a G1/S arrest in response to DNA damage (Westphal, 1997). Furthermore, Waldman *et al.* (1996) reported that cells lacking p53 did not arrest in G1, but could arrest in G2 following doxorubicin treatment. The current model for the G2/M checkpoint is outlined in figure 4.1.

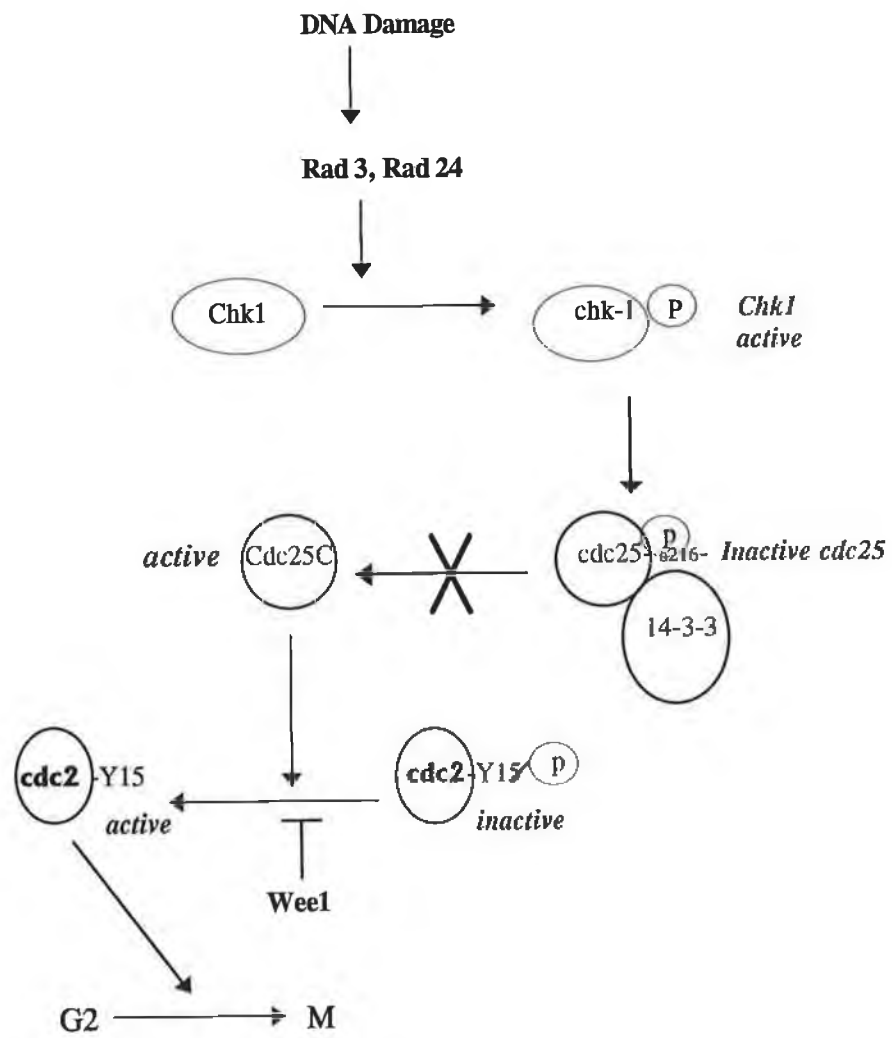


Figure 4.1 Outline of the G2/M Checkpoint

The G2/M checkpoint is discussed in detail in section 1.4.12. Briefly, when DNA damage is inflicted, the DNA damage-sensor chk1 protein kinase is activated through phosphorylation. This active kinase then phosphorylates cdc25C phosphatase on a specific residue (serine 216 in humans), resulting in the sequestration of the phosphorylated cdc25C protein by 14-3-3 proteins, resulting in inhibition of its phosphatase activity. This inhibition of cdc25C phosphatase activity in turn prevents removal of the inhibitory phosphate on Tyr15 of cdk1, and thus inhibits cdk1 kinase activity and cell cycle arrest, to allow for DNA repair. From this checkpoint model it can be seen that there are various proteins whose activity is crucial for an intact G2/M checkpoint and arrest to occur and any disruption of these proteins would result in a bypass of G2/M.

There are a number of possibilities to explain why the resistant variants can override the arrest. These can be arranged in levels according to their proximity to the cdk1-cyclin B complex, as outlined in figure 4.2. The first level includes the components of the complex, cyclin B and cdk1 protein expression after drug treatment, and their ability to form an active complex. Continuing from this is the presence of inhibitory phosphate on Tyr15 of cdk1, and consequently kinase activity of the active complex, the effectors of phosphorylation are particularly important; cdc25C and wee1. Following these levels are 14-3-3 protein and phosphorylation levels, and co-immunoprecipitation with cdc25C (i.e. 14-3-3-cdc25C complex) and finally chk1 levels after drug treatment and its phosphorylation levels. Some of these G2/M checkpoint components were investigated in an attempt to explain the G2/M over-ride seen in the resistant variants following doxorubicin treatment, and are discussed in sections 4.5, 4.6, 4.7, 4.8 and 4.9.

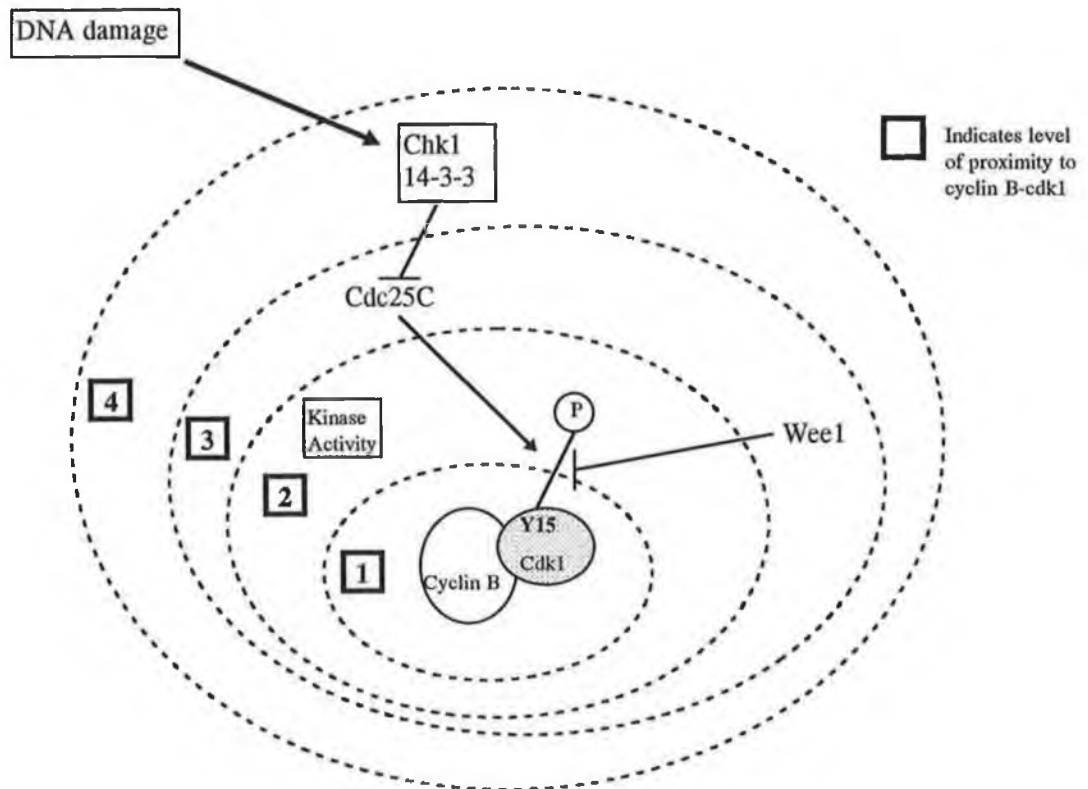


Figure 4.2 Components of the G2/M checkpoint possibly involved in the doxorubicin-induced arrest in DLKP cell lines

4.3.1 Attenuation of cell cycle checkpoints - Cellular Implications

Cells which have an intact checkpoint will arrest and carry out any required DNA repair so to maintain integrity of the DNA in each round of cell cycle. Proliferating cells normally respond to DNA damage by delaying the transitions from G1 to S or from G2 to M. By delaying progression through the cell cycle, cells may gain additional time for repair processes to remove any potentially mutagenic or lethal lesions before the genome is replicated or divided. Cells without an intact checkpoint will continue to divide with DNA damaged or incorrectly repaired, and become further unstable and contain chromosomal deletions, rearrangements and multiplications perhaps further advancing their genomic instability. This instability is a key feature of cancer cells. It is possible that a cell with enhanced genomic instability would progress rapidly to form a metastatic tumour. If cell cycle control is missing or defective, progression towards malignancy is accelerated, and also increasingly chemoresistant phenotypes have potential to develop.

The G2 checkpoint is particularly important due to the segregation of the genome in mitosis. Progression past M phase in the presence of incompletely repaired DNA would result in the acquisition of damaged genomic material in the progenitor cells. Kaufmann *et al.* (1995) investigated the possibility that attenuation of the G2 checkpoint may contribute to the genomic instability of immortal human cell lines. It was concluded that the G2 checkpoint attenuation preceded immortalisation, and plays a role in immortalisation of human fibroblasts. Immortal fibroblasts display aneuploidy and genetic instability and attenuation of the G2 checkpoint could allow cells to enter mitosis with levels of DNA damage that would normally arrest cells with a functioning G2 checkpoint. Therefore cells with the ability to bypass the DNA damaged-induced G2 checkpoint would be expected to have higher levels of chromosomal aberrations and aneuploidy. Indeed DLKP cells have shown tendencies to be aneuploid (McBride, 1995).

Defective G2/M checkpoints occur in many familial cancer syndromes such as Li-Fraumeni syndrome and AT (Paules *et al.*, 1995). Some cells do however die by

apoptosis, but those that do not die can continue to cycle and carry out DNA synthesis and mitosis resulting in proliferation of very unstable cells. So could resistant cells, when treated with doxorubicin become even more tumorigenic, by having highly unstable cells which have the ability to bypass G2/M checkpoint?

One important question is that of the cell cycle effect of doxorubicin treatment in the initial resistance selection of the cells. Resistant and sensitive cells have similar doubling times when growing exponentially, also cyclin and cdk protein levels are not altered in resistant cells. When sensitive cells are first exposed to doxorubicin, they arrest in G2/M as seen in DLKP-SQ. Resistance develops with continuing doxorubicin presence, and apart from MDR changes, the cell cycle in drug-treated cells also changes. Initially, doxorubicin-treated cells irreversibly arrest in G2/M, but after development of resistance the functional G2/M checkpoint is dismantled in some way. Perhaps in their development to resistance, their cell cycle has evolved so that the G2/M checkpoint is compromised allowing some of them to continue to divide in an attempt to create a survival advantage mechanism.

4.3.2 Effect of caffeine on DLKP-SQ cells

Caffeine belongs to the methylxanthines which are known to sensitise cells to DNA damage (Busse *et al.*, 1978). Musk *et al.* (1990) demonstrated that methylxanthines can induce an override of the G2/M block in human tumour cells after ionising radiation. Little evidence is available to date on the mechanisms of caffeine action on cell cycle delay, but it is thought that caffeine prevents the G2/M checkpoint from arresting the cell cycle in order to repair damaged DNA and this results in higher levels of cell kill.

Caffeine treatment on DLKP-SQ cells in various combinations with doxorubicin was investigated for its ability to overcome the G2/M arrest seen with doxorubicin alone. 4mM caffeine treatment alone had no obvious effect on the cell cycle of DLKP-SQ cells. The doxorubicin-induced G2/M arrest in DLKP-SQ can be prevented by addition of 4mM caffeine and if G2/M arrest has already occurred it can be reversed by caffeine addition. Ilialis *et al.* (1986) investigated the effects of doxorubicin on Chinese hamster V79 cells with caffeine and found that caffeine reduced doxorubicin-induced arrest, similar to the situation in DLKP-SQ cells. From these results it appears that DLKP-SQ cells can mimic the resistant variants in their response to doxorubicin treatment. Perhaps caffeine affects the G2 cell cycle checkpoint of DLKP-SQ cells in a similar mechanism to that of doxorubicin in the resistant variants, or the resistant variants may already have the cellular defects that caffeine produces in DLKP-SQ cells, thus enabling them to override the G2/M arrest seen in the sensitive DLKP-SQ.

Dunphy (Keystone Meeting, 1998) suggested that caffeine's effect of bypassing the G2/M arrest may be due to its action on the G2/M checkpoint. The most likely candidate to be affected is chk1 protein in some as yet unknown way. When chk1 is inhibited, cdc25C is not phosphorylated on ser 216 and remains active, thereby resulting in unscheduled removal of the inhibitory phosphate on Tyr15 on cdk1. The results discussed here provide further evidence for a faulty checkpoint as the cause of the G2/M override by the resistant variants.

4.4 Time-lapse Videomicroscopy analysis of Doxorubicin-induced mitosis and apoptosis

As seen in flow cytometry histograms in section 3.3.1, DLKP resistant variants have the ability to bypass the G2/M arrest seen in the sensitive cells after doxorubicin treatment. These results are further strengthened by the use of time-lapse videomicroscopy which allows visualisation of cell death and cell division events. It is possible to follow the dynamics of a certain population continuously over time. In section 3.4 the graphs indicate that mitosis occurs in the resistant cells but not in the sensitive cells, this was assessed by monitoring the morphological changes that took place after doxorubicin treatment.

In DLKP-A250 10p#7 cells there is 13 % mitosis, 15 % mitosis occurs in DLKP-A2B and a high 58 % mitosis occurs in DLKP-A5F cells, and this occurs over a 70 hour period following exposure of the cells to a concentration of doxorubicin that induced 50% cell kill, as determined by miniature *in vitro* toxicity assays. The high rate of mitosis in DLKP-A5F is surprising but the majority of post-mitotic cells rapidly undergo apoptosis just after mitosis. This is also seen to a lesser extent in DLKP-A2B and DLKP-A250 10p#7 cells in which at least one of the daughter cells dies by apoptosis. The phenomenon of post-mitotic apoptosis lends further weight to the suggestion that the resistant cells undergo mitosis with damaged DNA without an intact G2/M checkpoint. The cells with impaired DNA after division cannot survive and die by apoptosis. If these cells had a functional checkpoint the cells would possibly not be pushed into lethal mitosis.

4.5 Cellular Effects of Doxorubicin in DLKP cells

4.5.1 Doxorubicin effects on cyclin B levels

Cyclin B and cdk1 are critical elements of the G2/M transition in eukaryotic cells, and their regulation is important in maintaining proper control of the cell cycle. As cells progress through S and G2 phase, cyclin B protein levels begin to accumulate. When a certain threshold level of cyclin B is reached, it can form a complex with cdk1. Cyclin B accumulation is essential for activating cdk1 and for cells to progress into mitosis. Any treatment that prevents cyclin expression would therefore result in reduced cdk1-cyclin B complex formation, if the levels of cyclin B were insufficient, and ultimately lead to a G2 arrest. However if cyclin B levels are sufficiently high to activate cdk1, and a G2 arrest still occurs, then the possibility of an additional regulatory control exists, such as inhibition of cdk1 kinase activity.

It has been previously shown that cyclin B accumulation decreases due to DNA damage induced by ionising radiation (Datta *et al.* 1992, Muschel *et al.* 1997). Muschel *et al.* (1993) investigated the effects of irradiation on cyclin A and cyclin B expression in HeLa cells, and found that expression of cyclin B mRNA and protein is delayed after irradiation and levels of cyclin B protein do not accumulate to the same extent in irradiated cells compared to controls. Studies investigating the effects of irradiation on HeLa cells revealed that lower cyclin B levels after irradiation may contribute to the G2/M block (Bernhard *et al.*, 1994). Induction of high cyclin B levels through use of an inducible expression vector shortens the G2 delay after irradiation. This induction, however, does not eliminate the G2 block suggesting that the cyclin B depression contributes to the G2 delay but other regulatory factors must exist (Kao *et al.*, 1997). Recently Innocente *et al.* (1999) reported that cyclin B expression can be regulated by p53 during the G2/M arrest after DNA damage. They demonstrated that in the presence of p53, cyclin B protein levels are decreased due to attenuation of the cyclin B promoter.

In contrast to these reports, Nishio *et al.*, (1993) demonstrated that during a CDDP-induced G2 arrest, cyclin B protein levels and degradation were not affected. In addition,

Shimizu *et al.* (1995) reported that camptothecin treatment of HL-60 cells does not alter cyclin B protein levels. Camptothecin treatment of HeLa cells results in a G2 arrest and minimally affects cyclin B protein synthesis, and high levels of cyclin B accumulate in conjunction with the G2 arrest (Tsao *et al.*, 1992). High cyclin B levels are most likely due to a reduced rate of degradation, consistent with the observation that cyclin B is rapidly degraded only after mitosis. Furthermore, Ling *et al.* (1996) demonstrated that cyclin B1 levels increased relatively constantly after Doxorubicin treatment, due to induction of cyclin B synthesis and inhibition of its degradation in synchronised P388 cells, and a constant cyclin B protein level was seen using western blotting.

The influence of doxorubicin on cyclin B expression in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F was investigated in section 3.5.1. Doxorubicin treatment did not induce changes in cyclin B protein levels in any of the cell lines when compared to control levels in DLKP-SQ. Cyclin B protein levels remained constant over 45 hours after drug treatment, by which time a G2/M arrest had occurred. Results were similar for DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F. These results are similar to Ling's (1996) results in that the cyclin B levels remain constant when compared to controls (but rates of synthesis were not looked at in this thesis).

4.5.2 Doxorubicin effects on cdk1 levels

Levels of cdk1 do not oscillate enormously during the cells cycle, and the protein relies on the presence of cyclin B for its kinase activity, thus the cyclin acts as the regulatory subunit of the complex. Ling *et al.* (1996) reported that doxorubicin treatment did not influence cdk1 protein synthesis in synchronised P388 cells which were G2/M arrested. The topoisomerase I inhibitor camptothecin has no effect on cdk1 protein levels in HL-60 cells when accompanied by a G2/M arrest (Shimizu *et al.* 1995).

Cdk1 protein levels in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F before and after doxorubicin treatment were monitored in section 3.5.2. Clearly there is no change in cdk1 protein expression after doxorubicin treatment when compared to

controls. These results are similar to the published reports mentioned previously. Therefore the altered G2/M transition in the resistant variants is not due to deregulation of cyclin B or cdk1 proteins, and cyclin B and cdk1 in sensitive and resistant cells are unaltered in their responses to doxorubicin treatment.

4.5.3 Effects of Doxorubicin on cyclin B-cdk1 complex forming ability

The ability of cyclins and cdks to form complexes represents one of the fundamental regulatory events in the cell cycle. Cdks are the catalytic subunits of the cyclin-cdk complex, and they require cyclins for activation. If cyclin is not present the cdk remains catalytically inactive so when cdk1 is not complexed to cyclin B it remains inactive, and entry to mitosis will not occur. The complex-forming ability of DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F was not affected by doxorubicin treatment, levels of cyclin B-cdk1 complex after drug treatment were not altered, indicating that alterations in cell cycle response in the resistant variants are not due to deregulated cyclin B-cdk1 complex formation, but may lie downstream from this.

4.6 Phosphorylation of Tyr 15 on cdk1

The kinase activity of cdk1 is the key regulatory factor in the cdk1-cyclin B complex and is controlled tightly by phosphorylation and dephosphorylation mechanisms. As discussed in section 1.4.2, 1.4.3 and 1.4.7., Thr 14 and Tyr 15 (Y15) are inhibitory sites, and the presence of a phosphate on Tyr 15 generally confers inhibition of the cdk1 kinase activity. Numerous reports have demonstrated G2/M arrest caused by inhibition of cdk1 kinase activity as a result of decreased Y15 dephosphorylation. Failure to dephosphorylate Y15 has been reported after treatment with various chemotherapy agents; camptothecin (Tsao *et al.*, 1992; Shimizu *et al.*, 1995) and etoposide (Lock *et al.*, 1992) induced a G2 arrest, which was mediated through a failure to dephosphorylate Y15. G2 arrest induced by irradiation in HeLa cells was found to be due to an increase in phosphorylation on cdk1, and consequently a decrease in cdk1 kinase activity (Metting and Little, 1995). The study by Lock *et al.* (1992) looked initially at asynchronous CHO cells after etoposide treatment and found a decrease in cdk1 kinase activity and a G2 phase arrest. They subsequently studied synchronous cells to facilitate further analysis of the effect of Y15 dephosphorylation in relation to the decrease in kinase activity. This report suggests that the use of synchronised cells would eliminate the difficulties associated with asynchronous cells. Ling *et al.* (1996) demonstrated that doxorubicin-induced G2/M phase arrest in synchronised murine leukaemia p388 cells is mediated by deregulation of cdk1 kinase activity through prevention of Y15 dephosphorylation.

An investigation into the observed G2/M arrest in DLKP cell lines and its association with levels of Y15 phosphorylation on cdk1 was carried out in an attempt to explain the altered G2 response seen in the resistant variants. Two methods of detection were employed to detect Y15 in the DLKP cell lines before and after doxorubicin treatment. Initially immunoprecipitated DLKP-SQ cells (with anti-cdk1 antibody) were probed with anti-phosphotyrosine, and the second method involved using a specific anti-cdk1 Y15 antibody. Doxorubicin treatment influenced the levels of Y15 in DLKP-SQ cells (section 3.6); there seems to be a slight increase in Y15 phosphorylation after drug treatment. This reflects the situation seen in the flow cytometry analysis; 45 hours after doxorubicin treatment the cells experience a distinct G2/M arrest and Western blotting analysis reveals

a corresponding increase in Y15 phosphorylation levels compared to controls. The control levels of Y15 in untreated cells should remain constant because in an asynchronous population of exponentially growing cells, there should be a relatively constant number of cells undergoing mitosis at any instant, and this effect was also seen in control DLKP-SQ cell extracts.

The resistant variants did not show any changes in Y15 phosphorylation levels after doxorubicin treatment. Samples from all four cell lines before and after doxorubicin treatment were run on one gel to compare Y15 levels to untreated and drug-treated on one gel, a ponceau-stained photograph of the gel is illustrated in figure 3.6.12 for comparison in protein loading. When an untreated sample is compared to an extract from doxorubicin-treated cells, it can be seen that when the protein loading is taken into account, the DLKP-SQ experience a slight increase in Y15 phosphorylation levels, to a greater extent than the resistant variants, with an increase of 1.58 when analysed using densitometry.

These are the expected results for the resistant variants, when comparing them to the results seen by flow cytometrical analysis of cell cycle progression following doxorubicin treatment. The resistant cells can bypass the G2/M arrest, therefore the cdk1 kinase activity should be active in order to allow the cells pass through mitosis and Y15 should be dephosphorylated to a similar level as control cells, as was seen in this study.

As already mentioned in section 3.6, one important point for consideration is the experimental design. The cells are asynchronous, so at any instant in cycling cells only a small percentage of cells would be mitotic, would have active kinase activity and in addition possess Y15 phosphorylation. Y15 phosphorylation only occurs during G2 phase however. Parker *et al.* (1992) demonstrated that Y15 is only phosphorylated by wee1 when cdk1 and cyclin B are in a complex, and this complex only occurs during G2 when cyclin B levels rise above a threshold and bind to cdk1 in anticipation of mitosis. Based on this theory G2/M arrested DLKP-SQ cells should have a higher Y15 phosphorylation level, and the resistant cells which can continue to cycle should have similar levels of Y15 phosphorylation to untreated asynchronous cells. This assumption is in agreement with

actual results found in section 3.6. The above findings indicate that the cdk1 kinase activity is the key component allowing the resistant cells to over-ride the G2/M arrest. Therefore further analysis is necessary to elucidate the exact mechanism that effectively reduces the levels of Y15 phosphorylation on cdk1 and triggers the resistant variants to enter mitosis. Further analysis would involve investigation of wee1 kinase activity and cdc25C phosphatase activity to determine their role in the regulation of cdk1 kinase activity in resistant variants following doxorubicin treatment.

4.7 Cdk1 Kinase Activity

Activation of cdk1 kinase requires both cyclin B binding and removal of the inhibitory phosphates on cdk1. Alterations in both of these regulatory components may contribute to the diminished cdk1 activity seen after irradiation or treatment with DNA damaging agents. Numerous studies report decreased cdk1 kinase activity due to the presence of inhibitory phosphates associated with a G2/M arrest induced by such chemotherapeutic drugs as doxorubicin (Ling *et al.*, 1996), etoposide (Lock and Ross, 1990) and camptothecin (Lock *et al.*, 1995; Tsao *et al.*, 1992; Shimizu *et al.*, 1995). Particularly relevant to this thesis is the study by Ling *et al.* (1996) on the effects of doxorubicin on p53-mutant murine leukaemia P388 cells. Findings indicated that doxorubicin induces a G2/M arrest due to a reduction in cdk1 kinase activity, as a result of failure to remove the inhibitory phosphates on cdk1. In addition cdk1 protein levels remained unchanged after doxorubicin treatment. In a cell-free system, doxorubicin did not affect *in vitro* cdk1 kinase activity indicating that the reduction in kinase activity was not due to a direct drug effect on the cdk1 enzyme.

Initial attempts to look at cdk1 kinase activity of DLKP-SQ and resistant variants before and after doxorubicin treatment using the well known radioactive histone H1 kinase assay were unsuccessful. A level of kinase activity was identified in DLKP-SQ untreated cells, but drug treated samples were difficult to interpret due to high background levels and seemingly unequal loading of beads, even though equal protein amounts were immunoprecipitated. In hindsight, the antibody used to immunoprecipitate cdk1 may not have been ideal, judging from the high background obtained.

An alternative non-radioactive ELISA-based procedure was then employed to investigate the effects of doxorubicin on the DLKP cells in an attempt to discover the mechanism of G2/M over-ride in resistant cells. The cdk1 kinase assay kit is based on enzyme-linked immunosorbent assay (ELISA) that utilises a synthetic peptide which is specifically phosphorylated by active cdk1 kinase, and a monoclonal antibody recognising the phosphorylated form of the peptide.

DLKP-SQ cells after doxorubicin treatment exhibited an unaltered or slight decrease in cdk1 kinase activity. The control cells had quite a high kinase activity but this was probably due to the asynchronous populations being used in this study. In an asynchronous population, at any one instant at least 5% of the cells will be dividing. This number could be higher depending on the growth phase of the cells, so they will have a certain level of active cdk1 kinase activity. Doxorubicin treatment results in a slight reduction in kinase activity in DLKP-SQ cells compared to doxorubicin treatment in the resistant variants. This result suggests a G2 arrest before entry to mitosis. This result is in keeping with previous observations that DLKP-SQ cells arrest in G2/M using flow cytometry analysis and the cdk1 is phosphorylated on inhibitory Y15. Therefore the arrest in the sensitive cells is most likely due to the higher inhibitory phosphorylation on cdk1 with a subsequent decrease in kinase activity.

The situation in the resistant variants is however quite different. Doxorubicin treatment on the resistant variants seems to induce a slightly elevated kinase activity compared to untreated controls. The asynchronous resistant variants also have a significant cdk1 kinase activity before doxorubicin treatment, probably due to a small percentage of these cells undergoing mitosis. After drug treatment, an increase in cdk1 kinase activity was observed, most significantly in the most resistant cell line DLKP-A5F. Taking earlier results into consideration, this amplified kinase activity is not due to enhanced cyclin B or cdk1 protein expression, nor is it due to higher cyclin B-cdk1 complex levels. The principle factor governing the higher kinase activity in the doxorubicin-treated resistant variants may be the unchanging levels of Y15 inhibitory phosphorylation, seen by Western blotting. This higher kinase activity in drug-treated resistant cells versus the drug-treated sensitive cells must be sufficient to push the cells into mitosis.

Taken together, these results prompted an investigation into mechanisms that control cdk1 kinase activity. There are a number of possible control points in cdk1 activation and inhibition; complex-forming ability of cdk1 with cyclin B (also cyclin B levels), activity of the weel kinase which is responsible for the inhibitory phosphorylation on Y15 and activity of cdc25C, the phosphatase responsible for removal of the inhibitory phosphate and subsequent activation of the kinase. One other regulatory element possibly involved

could be CAK (cyclin H-cdk7) which is responsible for the activating phosphate on Thr 161 of cdk1. Inability of cdk1 to form complexes with cyclin B can be precluded from this investigation because both sensitive and resistant variants are equally capable of forming cyclin B-cdk1 complexes after doxorubicin treatment (detailed in section 3.5.3).

4.8 Analysis of cdc25B and cdc25C phosphatase activity after Doxorubicin treatment

An early study by Strausfeld *et al.* (1991) identified a cdc25 gene product as a novel phosphatase that acts directly on cdk1-cyclin B complex to induce its activation by removing inhibitory phosphate groups on tyrosine, presumably Y15 in cdk1. Since this initial finding, further studies have provided evidence that phosphatase cdc25 plays a key role in the activation of cdk1-cyclin B complex by dephosphorylation of cdk1 at Y15 (Kumagi and Dunphy, 1991; Dunphy and Kumagi, 1991). Cdc25 phosphatase is activated directly by phosphorylation in mitotic cells, and activation depends on an active cyclin B-cdk1 complex. When inactive, cdc25 remains underphosphorylated, and becomes active through phosphorylation. The cdc25 family comprises three homologues, cdc25A, cdc25B and cdc25C, with cdc25B and cdc25C being involved in onset of mitosis. Cdc25C plays an important role in maintaining a positive feed back loop; cdc25C is first phosphorylated and activated by a small amount of active cyclinB-cdk1. The cdc25C can then phosphorylate and activate the cellular pool of cyclin B-cdk1 kinase. In this way cdc25C can amplify cdk1-cyclin B activity. Cdc25C also plays a role in the G2/M checkpoint (Hoffmann and Karsenti, 1994).

The importance of cdc25C in G2/M arrest and checkpoint was revealed by a study that demonstrated G2/M arrest due to an inactive cdc25C. O'Connor *et al.* (1994) found that cells arrested in G2/M by nitrogen mustard had very high levels of phosphorylated inactive cdk1-cyclin B complexes, and this was due to a failure to activate cdc25C. Cdc25C remained in its inactive underphosphorylated form and suppressed activation of cdk1 kinase and mitosis onset. Barth and Kinzel (1994) reported that reduction in cdc25C activity was responsible for G2 delay in synchronised HeLa cells after treatment with the phorbol ester TPA. A decrease in cdc25C activity coincided with dephosphorylation of the enzyme. Cdc25C of mitotic cells is fully activated through phosphorylation by cdk1 and this hyperphosphorylated form of cdc25C migrates as a 65 kDa band in SDS gels (Hoffmann *et al.*, 1993), whereas the dephosphorylated cdc25C (inactive) migrates at 55kDa. From this study, cells arrested in G2 would be expected to have an inactive cdc25C and an increase in the 55 kDa form. Cells undergoing mitosis on the other hand

should have constant levels of active 65 kDa phosphorylated cdc25C. Cdc25B is also involved in activation of cdk1 in human cells (Hoffmann, pers. commun., Keystone Meeting, 1998) and when active is phosphorylated and migrates at 62 kDa in SDS gels, and a lower 50 kDa band when inactive, although this is only readily observed in SDS gradient gels (pers. commun., Hoffmann, 1998).

The effect of doxorubicin on cdc25C protein expression has previously been investigated by Ling *et al.* (1996) who found that doxorubicin treatment did not affect expression of cdc25C compared to control cells. However the activity status of cdc25C was not investigated in this study. The expression and activity state of cdc25B and cdc25C proteins were investigated in doxorubicin-treated DLKP-SQ in attempt to establish their role in the G2/M arrest. Western blotting was carried out to look at protein expression levels, and immunoprecipitation followed by Western blotting was utilised to determine the phosphorylation state of cdc25C. Immunoprecipitation was carried out to concentrate the cdc25C protein, which is present at very low levels in the cell. The expression of cdc25B and cdc25C before and after doxorubicin treatment were investigated, and it can be concluded from results that doxorubicin had no effect on the level of expression of either cdc25B or cdc25C in the DLKP cell lines. This result is similar to the findings by Ling *et al.* (1996).

Hoffmann *et al.* (1993) and O'Connor *et al.* (1994) demonstrated that asynchronous cells have mostly inactive cdc25C, and migrate at 55kDa with a weak band (sometimes absent) at 66kDa, indicative of the small percentage of cells in mitosis in the asynchronous population. Using synchronised populations they concluded that cells arrested in G2 phase have minimal active cdc25C and migrate in SDS gels at 55kDa with a weak 66kDa band. However cells arrested in mitosis have largely active cdc25C and so will migrate in SDS gels at 66kDa. These findings are illustrated in figure 4.2.

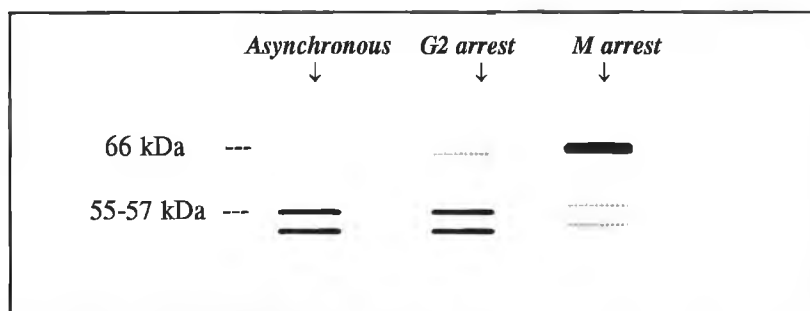


Figure 4.2 Mobility of *cdc25C* in SDS gels

With these observations in mind, the phosphorylation state of *cdc25C* was investigated in the DLKP cells before and after doxorubicin treatment, using immunoprecipitation followed by western blotting. Results show no differences in phosphorylation of *cdc25C* in control compared to drug-treated DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B or DLKP-A5F cells, thus questioning the role of altered *cdc25C* as a possible mechanism for the G2/M override seen in the resistant variants.

In putting these results in context it should be remembered that the DLKP cells are asynchronous so the cells are in different stages of the cell cycle and this will immediately reduce the clarity of the specific 55kDa or 66kDa bands that would be seen if the entire population were in interphase or mitosis, respectively, i.e. a synchronous population. It was expected that asynchronous control cells would be mainly in interphase and are represented by a 55kDa band, with a small proportion in mitosis, as seen by a faint 66kDa band. Resistant variants that over-ride the G2/M arrest following drug treatment begin to accumulate in G2, and then a small percentage enter mitosis, so this situation would also be represented by a strong 55kDa band (G2 cells) and a faint 66kDa (mitotic cells) band. A strong 66kDa and 55-57kDa band were detected in all four cell lines before and after doxorubicin treatment. The band intensity for resistant cells is equal and does not alter following doxorubicin treatment. Perhaps a different experimental setting utilising synchronous DLKP populations might reveal more distinct alterations in the sensitive compared to resistant variants.

4.9 Wee1 involvement in DLKP response to doxorubicin

Initial studies in fission yeast by Paul Nurse identified *wee1* as a gene that controlled the onset of mitosis (Nurse and Thuriaux, 1980), and *wee1* could effectively prevent entry to mitosis in fission yeast (Russell and Nurse, 1987). The *wee1* gene in fission yeast encodes a protein kinase with serine-, threonine- and tyrosine phosphorylating activities (Parker *et al.* 1993), but the human homologue, *wee1HU* encodes a tyrosine-specific protein kinase (Parker and Piwnica-Worms, 1992). The human homologue directly inhibits the cdk1-cyclin B complex by phosphorylating cdk1 on Y15, and this inhibition can be reversed by human *cdc25C* (McGowan and Russell, 1995; Parker and Piwnica-Worms, 1992). Wee1 is negatively regulated by the mitotic induced Nim1 kinase, which phosphorylates *wee1* and inactivates it (Parker *et al.* 1993). When *wee1* is phosphorylated, its ability to phosphorylate cdk1 on Y15 is inhibited; therefore Nim1 kinase can promote mitosis by inactivating *wee1* kinase (Wu and Russell, 1995).

Wee1 is thought to co-ordinate the transition between S phase and mitosis by inhibiting any cdk1 in the nucleus until G2/M, when cyclin B and *cdc25C* enter the nucleus in preparation for mitosis onset (Heald *et al.* 1993). Watanabe *et al.* (1995) revealed that in human cells *wee1* activity is increased during S and G2 phases, and during mitosis *wee1* activity is strongly suppressed through transient hyperphosphorylation, coinciding with the activation of cdk1 kinase. Therefore negative regulation of *wee1* is required for cdk1-cyclin B activation to occur through the action of *cdc25C* (McGowan and Russell, 1995). It has been reported that during G2 arrest, *wee1* activity increases, and an abrogation of this G2 arrest was associated with a decrease in *wee1* protein levels and a lower *wee1* kinase activity. The possibility that this situation may also occur in DLKP cell lines displaying a G2 override prompted investigation of *wee1* in the DLKP cell lines.

The levels of *wee1* protein were examined in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F to determine if a decrease in *wee1* protein was associated with the resistance in these cells. The fact that resistant cells can override the G2 arrest suggested that perhaps the higher cdk1 kinase activity may be due to lower *wee1* levels. However this was not the case. Both sensitive and resistant DLKP cells have similar *wee1*

expresssion.

Wee1 activity is decreased by phosphorylation, and this can be visualised by an altered mobility on SDS gels. Therefore the activity of wee1 in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F was investigated before and after doxorubicin treatment using immunoprecipitation followed by Western blotting. In DLKP-SQ, both treated and untreated cells possess an active wee1, as seen by the unchanging 94kDa band, but inactive wee1 is also present as the upper band. However this upper band seems to decrease over time after doxorubicin treatment in DLKP-SQ, suggesting a decrease in inactive wee1 with perhaps a concomitant increase in active wee1. The doxorubicin-treated cells are arrested in G2, when wee1 activity should be high, so the elevated wee1 activity may be necessary to maintain the inactivation of cdk1 kinase and G2 arrest.

Again the problem of using asynchronous cells arises here; the control cells also have active wee1, presumably due to inhibition of mitosis during interphase, so it is difficult to distinguish between the G2-arrested population and the asynchronous population. In earlier discussion it was mentioned that the arrested cells have higher levels of Y15 phosphorylation, so it was speculated that the arrested cells would have a higher levels of active wee1 responsible for this inhibitory phosphorylation, and the immunoprecipitations of wee1 in DLKP-SQ seem to agree with this. A wee1 kinase assay, which directly looks at its kinase activity *in vitro* could be used to confirm this observation.

The DLKP resistant variants exhibited similar levels of 94kDa wee1 compared to untreated controls, indicating that the activity was similar before and after drug treatment. It was seen earlier that resistant variants had similar amounts of Y15 phosphorylation on cdk1 before and after doxorubicin treatment, indicating that wee1 may be active to an equal extent. Inhibition of wee1 activity was not observed using immunoprecipitation and altered mobility; such inhibition might have been anticipated in the resistant variants since they can override the G2/M arrest. Perhaps the level of inhibition was below the sensitivity of detection on the western blots, especially given that the population was asynchronous. A definitive conclusion could be obtained by using an *in vitro* wee1 kinase assay to directly measure the kinase activity of wee1.

4.9.1 Other mechanisms responsible for G2/M override

Investigations into the levels and activity of the principal cdk1 regulators, cdc25C and wee1 failed to provide a complete explanation of the mechanism of G2 override in the DLKP resistant variants. Another possible mechanism is that involving localisation of cyclin B during the cell cycle, perhaps cyclin B in resistant variants have altered localisation during G2 arrest promoting cdk1-cyclin B kinase activity and entry to mitosis.

Entry to mitosis can be regulated by the subcellular localisation of cdk1-cyclin B. During late S and G2 cyclin B accumulates in the cytoplasm due to a specific sequence in its N-terminus called a cytoplasmic retention signal (CRS) (Pines and Hunter, 1994). Cyclin B contains in its CRS a nuclear export signal (NES) which can mediate nuclear export of the protein. At the onset of mitosis cyclin B-cdk1 complexes are shuttled to the nucleus where they can act on substrates that promote mitosis. Therefore the exclusion of cyclin B-cdk1 from the nucleus until mitosis acts as a control mechanism to limit its effects before mitosis. Cyclin B with a defective NES is restricted to the nucleus, and inhibition of NES-mediated transport can result in accumulation of cyclin B in the nucleus.

In response to DNA damage and a G2 arrest, cyclin B accumulates in the cytoplasm (Smeets *et al.* 1994; Toyoshima *et al.* 1998) and this exclusion of cyclin B from the nucleus may possibly prevent cells from entering into premature mitosis in G2-arrested cells. To test this hypothesis, Toyoshima *et al.* (1998) developed a mutant cyclin B with a disrupted NES (i.e. cyclin B cannot be exported from the nucleus) and found that in the presence of caffeine (caffeine alone did not abrogate the etoposide-induced G2 arrest in this case) the G2/M arrest was abrogated. Treatment of cells possessing normal cyclin B with the NES inhibitor leptomycin B also resulted in an abrogation of the etoposide-induced G2/M arrest, but again only in the presence of caffeine. The requirement of caffeine suggests that there might be other mechanisms active in the DNA damage-induced G2 checkpoint. In agreement with this report Jin *et al.* (1998) observed that nuclear targeting of cyclin B was effective in overcoming DNA damage-induced G2 arrest, and the G2 arrest was almost completely abrogated.

Taking these observations into consideration, it is plausible that a similar situation may be partly responsible for the doxorubicin-induced G2 arrest override seen in DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F. It may be possible that the resistant variants have altered NES sequences in their cyclin B protein, that restricts the protein to the nucleus after doxorubicin-induced DNA damage, resulting in the activation of cdk1 kinase and consequently onset of mitosis. The sensitive DLKP-SQ, on the other hand, could perhaps actively export cyclin B from the nucleus following doxorubicin treatment, and thus premature activation of cdk1 kinase cannot occur. This hypothesis would explain the higher cdk1 kinase activity seen in the resistant variants following doxorubicin treatment, given that differences in activation of cdc25C phosphatase and weel kinase could not be detected.

4.9.2 Chemotherapy effects - links between apoptosis and cell cycle

Since many chemotherapeutic agents that induce apoptosis also affect and perturb cell cycle progression, apoptosis may be linked to the cell cycle proteins affected during drug treatment. Numerous studies implicate inappropriate activation of cyclin B-cdk1 in apoptotic cell death following treatment with anticancer agents.

Preliminary studies by Lock and Ross (1990) demonstrated a decrease in inhibition of cdk1 kinase activity during an etoposide-induced G2 arrest, and an increase in cdk1 kinase activity during the period in which cells underwent apoptosis. Shimizu *et al.* (1995) reported an unscheduled activation of cdk1 kinase which was correlated with the DNA damage-induced apoptosis in leukaemia cells. Cdk1 kinase activity transiently increased following camptothecin treatment and then rapidly decreased during onset of mitosis. Shi *et al.* (1994) demonstrated that premature activation of cdk1 was required for apoptosis to occur, by using temperature-sensitive mutants, but Martin *et al.* (1995) concluded that cdk1 activation was not required for apoptosis to occur, but may be a consequence of apoptosis. Furthermore Yao *et al.* (1996) demonstrated that cdk1 kinase is a critical determinant of cell death mediated by Fas and caspases, and that Fas-induced apoptosis induced caspase activity as well as cdk1 activation. Taxol treatment of HeLa cells also induced cdk1 kinase activity which did not correlate with the timing of the G2/M arrest observed, but coincided with the onset of apoptosis (Donaldson *et al.* 1994).

There have also been reports linking cyclin A with apoptosis. Upregulation of mRNA levels and activation of cyclin A-cdk1 occurred during apoptosis (Meikrantz *et al.* 1994; Hoang *et al.* 1994). A study by Huang *et al.* (1997) revealed an increase in cdc25C phosphatase activity as the responsible factor for an inappropriate activation of cdk1 during apoptosis induced by a podophyllotoxin.

Taken together, these reports generally implicate cdk1 kinase as having a role in apoptosis, although this may be an essential or consequential one. The substrates acted on by cdk1 kinase during mitosis are usually structural components of the cytoskeleton, which must be fragmented in order for cytokinesis to occur during mitosis, but these

substrates may also be acted upon in apoptosis to promote structural degradation of the cells. However, the significance of this in the apoptotic process still remains undetermined.

4.10 Cellular effects of chemotherapy and implications for resistance development

Treatment of cancer with chemotherapeutic drugs frequently results in the development of multiple drug resistance (MDR), in which the tumour cells exhibit resistance to a wide range of structurally and functionally unrelated chemotherapy drugs. In many cases the MDR phenotype is mediated by P-glycoprotein (Pgp). Pgp is 170kDa plasma membrane protein and is an energy-dependent pump with ability to transport cytotoxic agents out of the cell, resulting in reduced intracellular concentrations of drug and failure of the drug to reach an intracellular target. However recent identification of other mechanisms of drug resistance such as MRP and LRP demonstrate the ability of a tumour to adapt to diverse cytotoxic stimuli.

There is a lot of evidence that chemotherapeutic drugs, radiation and other clinically relevant therapies can induce apoptosis in their cellular targets. Any disruption of this cell death pathway may contribute to a failure to undergo apoptosis, resulting in continued proliferation, ultimately leading to tumour progression and metastasis. If tumour progression selects for apoptosis resistance, it is conceivable that apoptosis resistance may be an important cause of drug resistance.

Studies by Frankfurt *et al.* (1994) and Kataoka *et al.* (1994) demonstrated that cells with drug resistance, following exposure to a variety of cytotoxic agents, exhibit resistance to drug-induced apoptosis. Therefore deregulation of the apoptotic response may be an important evolutionary occurrence in development of drug resistance in tumours. Furthermore Landowski *et al.* (1997) reported that selection of CEM and human myeloma cells for drug resistance results in resistance to apoptosis mediated by the CD95 system. These results indicate that as tumour cells develop mechanisms of drug resistance, they also develop mechanisms of resistance to physiological signals for apoptosis.

The Bcl-2 family is critically involved in the modulation of the apoptotic response, as described in section 1.5.9. Selection of tumour cells with chemotherapeutic drugs often results in modification of antiapoptotic Bcl-2 family members. Overexpression of Bcl-2

can interfere with diverse triggers for apoptosis, including chemotherapeutic drugs and radiation. Ibrado *et al.* (1996) demonstrated that overexpression of Bcl-X_L can interfere with caspase-3 activation.

4.10.1 P-glycoprotein levels in DLKP cell lines

Expression of p-glycoprotein (Pgp) in cells is closely associated with the development of multiple drug resistance (MDR). Cells that overexpress Pgp frequently exhibit the MDR phenotype, becoming resistant to a wide variety of structurally unrelated and mechanistically distinct chemotherapy drugs. The resistant DLKP cell lines used in the work described in this thesis were originally selected for resistance by a pulsed (DLKP-A250 10p#7) or a continuous (DLKP-A2B, DLKP-A5F) stepwise increased exposure to doxorubicin. Extensive characterisation has revealed MDR characteristics in these cell lines, including overexpression of Pgp (Heenan *et al.*, 1998; NicAmhlaoibh *et al.*, 1999).

The resistance in the DLKP cells can be overcome by interfering with the efflux activity of Pgp using the calcium channel blocker, verapamil. The principal mode of action of verapamil is through inhibition of the binding of various chemotherapeutic drugs to Pgp. Verapamil can effectively block Pgp activity, resulting in higher intracellular drug concentrations capable of reaching the cellular target, ultimately leading to a restoration of original sensitivity in the cells. Other Pgp blockers such as MRK-16 and UIC2 are anti-Pgp antibodies which can bind to and inhibit the action of Pgp.

Western blotting analysis detailed in section 3.10.1 investigated levels of Pgp in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F. DLKP-SQ cells do not express detectable levels of Pgp. This result is as expected because these are a sensitive clonal population isolated from the sensitive parental population (McBride *et al.*, 1997). DLKP-A250 10p#7 express low levels of Pgp, and DLKP-A2B and DLKP-A5F express higher levels of Pgp. The difference in expression of Pgp

between the DLKP-A250 10p#7 and DLKP-A2B/DLKP-A5F correlates with the levels of resistance of these cell lines to doxorubicin. The DLKP-A2B and DLKP-A5F display much higher resistance levels than the DLKP-A250 10p#7, and this is mainly conferred through their higher Pgp expression. However, DLKP-A5F are 100-fold more resistant than DLKP-A2B, yet both express a comparable level of Pgp as detected by Western blot, so there is not an exact correlation between the P-170 protein levels and the resistance to doxorubicin. This discrepancy suggests that there must be alternative resistance mechanisms in the DLKP-A5F cell line to account for the increased resistance without an accompanied increase in Pgp expression. It is possible that these alternative resistance mechanisms also occur in the DLKP-A250 10p#7 and DLKP-A2B, but the Pgp-mediated resistance probably overshadows any other mechanisms present. Further analysis of the apoptotic pathway should identify any alterations that exist in the three resistant variants compared to the sensitive DLKP-SQ.

Smyth *et al.* (1998) investigated the possibility that Pgp may, in addition to its drug transport function, generally inhibit cell death. They demonstrated that Pgp could specifically inhibit caspase activity and apoptosis in drug-selected CEM cell lines that expressed Pgp. Inhibition of Pgp with verapamil or MRK-16 restored cleavage and activation of procaspase-3 on addition of an apoptotic stimulus. Furthermore cells expressing Pgp were resistant to CD95-mediated apoptosis, but this resistance was not due to a defect in the CD95 pathway given that resistant cells did not have alterations in CD95 receptor. Resistance to CD95-mediated apoptosis could be reversed by inhibiting Pgp function with verapamil or MRK-16. Caspase-independent forms of cell death such as perforin-induced apoptosis were not inhibited by Pgp. These results suggest that at least in some cell backgrounds, caspase-dependent apoptotic pathways may in some way be inhibited by Pgp function.

Recently this study was extended by Johnstone *et al.* (1999) to demonstrate that the protective effect of Pgp was not restricted to drug-selected cells. Pgp was introduced by retroviral transfer of the MRD1 gene. Drug-selected and retroviral-transduced Pgp⁺ cells were resistant to CD95-mediated, caspase-dependent DNA fragmentation and membrane

lysis. They were also resistant to UV-irradiation and TNF-mediated apoptosis. Yet when Pgp action was inhibited by verapamil or MRK-16, the cells were rendered sensitive to apoptosis induced by these stimuli. Several possible mechanisms of antiapoptotic action of Pgp have been suggested; It is possible that Pgp presence may alter intracellular ATP levels, ATP is a critical component required for caspase-9 activation (Li *et al.* 1997). Pgp is an ATPase, so perhaps it acts by decreasing the intracellular ATP pool to below a level sufficient for caspase-9 activation. However the Pgp inhibitor verapamil may also decrease intracellular ATP levels perhaps by increasing the ATPase activity of Pgp (Ambudkar *et al.* 1992; Broxterman *et al.* 1988), so perhaps this hypothesis is not feasible. Another theory stems from the fact that Pgp expression is associated with intracellular alkalinisation (Thiebaut *et al.* 1990), but apoptosis induced by chemotherapeutics, UV-irradiation, serum starvation and CD95-ligation is preceded by cellular acidification (Gottlieb *et al.* 1996; Li *et al.* 1995). DNA laddering can be prevented by increasing intracellular pH (Sarin *et al.* 1997). Furthermore cells can be made resistant to CD95-mediated or serum starvation-induced apoptosis by increasing intracellular pH (Robinson *et al.* 1997; Gottlieb *et al.* 1996). Therefore it is possible that Pgp alters intracellular pH, and inhibits caspase activity, causing the cell to become resistant to caspase-dependent apoptosis. In support of this theory, verapamil has been shown to cause acidification in Pgp-expressing cells, thus lowering pH sufficiently to allow caspase activation and apoptosis to occur (Robinson *et al.* 1997).

4.10.2 Toxicity of doxorubicin in DLKP cell lines

The anthracycline doxorubicin was used throughout this thesis to treat the four DLKP cells lines in question, and its level of toxicity varies between cell lines. DLKP-SQ are most sensitive being a clonal population isolated from the parental DLKP (McBride *et al.* 1998). DLKP-A250 10p#7 are approximately 3-fold resistant compared to DLKP-SQ. When DLKP-A250 10p#7 were isolated they were determined to be 8-10 fold resistant compared to DLKP-SQ, but a different experimental setting was used; 96-well plate format and toxicity determined after 7 day drug-exposure. In these studies toxicity profiles were carried using a 2 hour doxorubicin pulse in 25cm² flasks and toxicity

determined after 72 hours, so the difference in fold resistance must be due to the differences in experimental conditions. DLKP-A2B were approximately 45-fold resistant and DLKP-A5F were 150-fold resistant to DLKP-SQ, compared to 30-fold and 100-fold resistant to DLKP in 96-well plates (Heenan *et al.* 1998). Toxicity was determined after 72 hours because the effect of doxorubicin on the DLKP cell lines was monitored over 72 hours. Drug treatment induces cellular alterations in two parallel pathways, in the cell cycle and also in apoptosis induction. Therefore the effects of doxorubicin on the DLKP cell lines was investigated in respect to apoptosis induction over the same period of time.

4.10.3 Doxorubicin accumulation in DLKP cell lines

Drug resistance can alter drug uptake and cellular accumulation, and reduced cellular accumulation is often an indicator of drug resistance. Doxorubicin accumulation was investigated in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F to determine if the drug accumulation correlated with the resistant state of the cells. Sensitive cells can accumulate doxorubicin in their nuclei, as is seen in DLKP-SQ, and the doxorubicin can exert its cytotoxic effects resulting in chromatin condensation and nuclear fragmentation, a hallmark of apoptotic cells. The resistant variants accumulate less doxorubicin, and it is restricted to the cytoplasm of these cells. However the drug concentration used in the resistant cells was much higher than for DLKP-SQ, and is capable of inducing apoptosis. This was clearly seen in the resistant cells that were unable to withstand such a high drug insult and apoptosis occurred. This data indicates that an important mechanism of resistance in the DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F is mediated through decreased intracellular drug accumulation, but this does not exclude the possibility of alternative resistance mechanisms being active also.

4.10.4 Toxicity of 5-FU and serum starvation to DLKP cells

The toxicity of 5-FU on DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F was also investigated. 5-FU is not transported by Pgp, and therefore resistance to 5-FU is

not associated with the Pgp status of the cell, but is due to alternative mechanisms of resistance. The resistant cell lines are equally sensitive as DLKP-SQ to 5-FU treatment, indicating the existence of an identical apoptotic pathway in these cell lines that is triggered by 5FU. However the effects of serum starvation reflect a different apoptotic induction pathway. DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F are more resistant to apoptosis induced by serum starvation than DLKP-SQ and results were shown in figure 3.10.5. Even though the extent of resistance is quite low, it is nevertheless significant and demonstrates that the resistant variants may have apoptotic resistance mechanisms other than Pgp-mediated resistance. In support of this finding, Robinson *et al.* (1997) reported that expression of the MDR1 gene in chinese hamster ovary fibroblasts was associated with resistance to apoptosis induced by serum starvation. Pgp must therefore be involved in additional mechanisms that promote the survival of cells.

4.11 Kinetics of Apoptosis in DLKP cell lines

Doxorubicin treatment results in a G2/M arrest and apoptosis in the DLKP-SQ cell line. The resistant variants have an altered cell cycle response to doxorubicin, as was discussed in preceding sections, but the resistant variants still retain the ability to undergo apoptosis. Apoptosis was characterised by the apoptotic morphology seen using time-lapse videomicroscopy. The typical events were rounding up of the cell, chromatin condensation, membrane blebbing and break up into apoptotic bodies. Many chemotherapeutic drugs such as melphalan, cisplatin and 5-fluorouracil induce apoptosis in cultured cell lines (Huschtscha *et al.* 1996). Doxorubicin has previously been reported to induce apoptosis in tumour cells by Sklandowski and Konopa (1993). All four DLKP cell lines undergo apoptosis after Doxorubicin treatment but with varying rates of apoptosis. As previously mentioned equitoxic doxorubicin concentrations are being used to induce apoptosis in the cells. This treatment was chosen so that an equivalent situation is present in each of the cell lines, that allows direct comparison to each other. The rate and extent of apoptosis depends on the drug concentration used and exposure time, this can be seen in figure 3.11.1 with DLKP-SQ cells.

Barlogie *et al.* (1976) previously demonstrated that doxorubicin has concentration-dependent effects on cells. A high doxorubicin concentration of 5µg/ml continuous exposure results in rapid induction of apoptosis with 50% of the cells dead within 5 hours, compared to a lower concentration of 0.6µg/ml, for a 2 hour exposure which resulted in a much slower rate of apoptosis with 1 % dead at 5 hours and 60% by 72 hours. DLKP-A250 10p#7 cells, when treated with 1 µg/ml doxorubicin for 2 hours have a slow initial rate of apoptosis similar to DLKP-SQ (when treated with 0.6µg/ml Doxorubicin), a small percentage of cells die by 5 hours, but by 72 hours 48% apoptosis has occurred. In contrast DLKP-A2B have a rapid induction of apoptosis with up to 25% cells dead by 5 hours, the rate slows at 12 hours when 60% cells have died. DLKP-A5F cells also have a high initial rate of apoptosis for the first 10 hours after which time it levels off. An important finding here was the occurrence of mitosis after doxorubicin treatment in the three resistant variants, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F, and absence of mitosis in the sensitive DLKP-SQ. It was this unusual observation that prompted the

subsequent detailed investigation into the doxorubicin-induced G2/M arrest in DLKP cell lines. Mitotic events occurred concurrently with apoptosis in all of the resistant variants, and a high percentage of the resulting daughter cells underwent apoptosis, a phenomenon previously reported as post-mitotic apoptosis by Vidair *et al.* (1996). It seems as if cells are pushed into mitosis with incompletely repaired DNA after doxorubicin treatment, and then cannot survive with defective DNA so undergo apoptosis, perhaps contributing to the genetic instability of the cells..

4.12 Morphological Features of Apoptosis in DLKP-SQ cells

It was seen in section 3.11 that DLKP-cells readily undergo apoptosis after Doxorubicin treatment. The induction of apoptosis was further investigated in section 3.12 in which morphological and biochemical features were examined.

4.12.1 DNA fragmentation in DLKP-SQ

During the effector phase of apoptosis, genomic DNA is cleaved between nucleosomes, thus forming 180bp fragments, the size of DNA between one nucleosome and the next. Caspase activity has been linked to this fragmentation. DNA fragmentation factor (DFF) or ICAD usually exists in an inactive form but during apoptosis caspases can cleave DFF/ICAD, and once cleaved its protease action on DNA is unleashed resulting in DNA cleavage (Liu *et al.* 1997). DNA fragmentation into nucleosomal fragments is one of the most recognisable biochemical features of apoptosis (Wyllie, 1980). Detection of internucleosomal DNA fragmentation is widely used as one of the hallmarks of apoptosis. However in some cases DNA fragmentation may not occur during apoptosis, but this is dependent on the situation; the cell lines used and the apoptosis-inducing drug (Huschtscha *et al.* 1996).

Daunorubicin induced apoptosis along with DNA fragmentation in DLKP-SQ cells when used at concentrations from 1µg/ml to 10µg/ml, control cells did not give a DNA ladder. Daunorubicin has previously been shown to induce internucleosomal DNA fragmentation in acute myeloid cell lines such as HL-60 (Quillet-Mary *et al.* 1996). However doxorubicin-induced DNA fragmentation was not investigated in DLKP cell lines. DNA fragmentation was also analysed in the DLKP cells after doxorubicin treatment using TUNEL staining to detect the fragmented DNA. DNA fragmentation occurs in DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F after doxorubicin treatment. The extent of DNA fragmentation varies with the rate of apoptosis, it can be seen that DLKP-A2B have quite a lot of DNA fragmentation at 24 hours compared to DLKP-SQ, reflecting the differences in apoptotic rates seen in timelapse videomicroscopy.

4.12.2 Phosphatidylserine exposure in DLKP-SQ cells during Doxorubicin-induced apoptosis

Phosphatidylserine is a membrane phospholipid that normally localises on the internal surface of cellular plasma membranes, and is transported to the outer membrane of apoptotic cells (Fadok *et al.* 1992).

Phosphatidylserine expression during apoptosis was demonstrated in section 3.12 using annexin V staining. This is further evidence for an intact apoptotic mechanism in DLKP-SQ cells. Only a small percentage of cells stained with annexin V as illustrated in figure 3.12.3.2, but this was expected because phosphatidylserine externalisation is a transient event in the apoptotic induction pathway. The same field of cells under white light identified which cells in the population have externalised phosphatidylserine. It can be seen from figure 3.12.3.2 that the cells positive for annexin V have not lost their membrane integrity, compared to some apoptotic cells in the field which have no annexin V staining. This has been previously been reported by Koopman *et al.* (1994), Gudas *et al.* (1986), and more recently by Martin *et al.* (1996) who looked at Jurkat cell apoptosis and found that loss of membrane integrity only occurred 8-12 hours after phosphatidylserine detection on the outer membrane of the plasma membrane. Recently Naito *et al.* (1997) demonstrated that phosphatidylserine externalisation is a downstream event of caspase activation during apoptosis in U937 cells, and its externalisation coincides with caspase activation. Pervaiz *et al.* (1998) provided further evidence for this in a study demonstrating that caspase-3 inhibition results in inhibition of phosphatidylserine externalisation. Thus phosphatidylserine externalisation is closely linked to caspase activation, and the fact that it occurs in DLKP-SQ cells undergoing apoptosis is evidence of active caspases in these cells.

4.13 Role of caspases in Doxorubicin-induced apoptosis in DLKP

It is now apparent that caspases play an important role in apoptosis. The initial discovery by Yuan *et al.* (1993) that ICE had high homology to the CED-3 proteins in *C. elegans* led to the identification of fourteen proteins constituting the caspase family. Caspases are synthesised as proenzymes and are activated by proteolytic cleavage, which generates two fragments of ≈ 20 kDa and ≈ 10 kDa. At present (as discussed in section 1) evidence exists for several specific pathways in which caspases exert their proteolytic activity resulting in the destruction of the cell. These proposed pathways comprise a cascade of caspases and caspase substrates, which can be caspases or other cellular proteins. There is a possibility that all caspases may be expressed in apoptotic cells but as to whether all of these participate in apoptotic events or not is currently under investigation.

Numerous reports demonstrate specific caspase activation during apoptosis and caspases activated vary according to cell line and apoptotic stimulus. Greidinger *et al.* (1996) reported three distinct ICE-like activities in Jurkat cells induced to undergo apoptosis by Fas ligation, and the differences were based on the substrate cleavage and distinct inhibition profiles. Martins *et al.* (1997) investigated activation of caspases during etoposide-induced apoptosis in HL-60 cells and discovered multiple active caspases with structural homology to caspase-1 (ICE) and these caspases had distinct enzyme activities. They also reported caspase-3 activation during etoposide-induced apoptosis whilst pro-enzyme levels of caspase-1 and caspase-2 did not decrease, implying that caspase-3 is selectively activated in HL-60 cells in etoposide-induced apoptosis even though other caspases are present in their pro-enzyme form. Polverino and Patterson (1997) examined caspase activation in HL-60 cells and found that caspase-3 is selectively activated during apoptosis in HL-60. More recently Grossmann *et al.* (1998) demonstrated presence of pro-caspase-1, -10, -6 and -3 in normal epithelial cells, and selective sequential activation of caspase-6 and caspase-3 during apoptosis. Based on these reports it was important to determine which caspases are present in DLKP cell lines, and to characterise their activation in doxorubicin-induced apoptosis.

A further incentive to investigate caspase activation in DLKP cell lines comes from recent

reports linking caspase deficiency with drug resistance. Los *et al.* (1997) investigated defects in caspase-3 activation in Fas-resistant and doxorubicin-resistant CEM cell lines. They found defective caspase-3 activation in both cell lines after Fas ligation implying that defective activation of caspases could be a mechanism to overcome drug resistance. Nagane *et al.* (1998) recently reported that suppression of cisplatin-induced apoptosis was mediated by overexpression of Bcl-X_L and inhibition of caspase-3 activity in cisplatin-resistant cell lines. Kojima *et al.* (1998) also investigated cisplatin resistance and subsequent caspase activation during cisplatin-induced apoptosis in a human SCC-25 squamous carcinoma cell line. They found that resistance to apoptosis could be mediated by deficient caspase-3 activation. The resistant variant of SCC-25 overexpressed Bcl-X_L and there was no detectable cytochrome c release. U937 cells which were resistant to doxorubicin were also resistant to cisplatin-induced apoptosis and were shown to have Bcl-X_L overexpression, high Pgp levels and deficient caspase-3 activation (Kojima *et al.* 1998).

Analysis of caspase expression was carried out using western blotting on cellular extracts taken before and after doxorubicin treatment. It is important to remember that active caspases have a short half-life in the cell, and therefore it can be difficult sometimes to pick up active fragments of caspases. Sometimes researchers use cell extracts and induce caspase activation *in vitro*, and monitor caspase activation by ³⁵S-methionine incorporation followed by electrophoresis and autoradiography which effectively detects the fragmented protein, and perhaps this technique would clarify the results obtained using western blotting.

Caspase-2

Caspase-2 was originally identified as Nedd2, a homologue of a mouse gene that when overexpressed, induces apoptosis in mammalian cells (Kumar *et al.* 1994). Caspase-2, because of its long prodomain and its similarity to caspase-9, may play a role in apoptotic induction. Susin *et al.* (1999) recently reported caspase-2 release from the mitochondria, along with caspase-9 release during the apoptotic process. They found an increase in processed caspase-2 in the cytosol of apoptotic cells, therefore caspase-2 may actively participate in the apoptotic process.

Caspase-2 involvement in Doxorubicin-induced apoptosis in DLKP-SQ was investigated in section 3.13.1 using a mouse monoclonal antibody (Transduction Laboratories) . There was no reduction in the proform of caspase-2 over 50 hours as cells were undergoing apoptosis. Similar results using the same antibody were found by Polverino and Patterson (1997) in that they could not detect a decrease in procaspase-2 during induction of apoptosis in HL-60 cells. However when they used a different antibody from Santa Cruz Inc., they did observe a decrease in procaspase-2 with a corresponding increase in a 12kDa fragment, but the results were ambiguous due to the non-specific cross-reactivity of the Santa Cruz antibody. Results seem surprising in that no detectable levels of procaspase-2 were found in DLKP-A2B and barely detectable levels in DLKP-A5F compared to DLKP-SQ, but in subsequent Western blots, low levels of procaspase-2 were detected in the resistant variants. In the less resistant DLKP-A250 10p#7 low levels of procaspase-2 were detected compared to DLKP-SQ, but nevertheless higher than in DLKP-A2B and DLKP-A5F.

Caspase-3

Resistant variants have lower caspase-3 protein expression levels than DLKP-SQ, and the reduction is most notable in the most resistant cells DLKP-A2B and DLKP-A5F. In section 3.13.2 caspase-3 proenzyme levels in DLKP-SQ were examined over time following Doxorubicin treatment. It can be seen that there is no caspase-3 processing in either DLKP-SQ or DLKP-A250 10p#7. When procaspase-3 levels are compared in the four cell lines before and after drug treatment, procaspase-3 levels do not change, following a similar trend to procaspase-2 levels. These results are completely unexpected given that the majority of reports published implicate caspase-3 activity in apoptosis. DNA damaging agents such as Ara-C, cisplatin, etoposide, camptothecin (Datta *et al.*, 1996), CPT-II (Suzuki *et al.*, 1996) and MC540 (Pervaiz *et al.*, 1998) have been shown to activate caspase-3 activity. One explanation for this is that if procaspase-3 is expressed in abundance and caspase-3 processing and activation occurs at a minimal level, then the high levels of pro-caspase-3 could mask any slight decrease in pro-caspase-3 which therefore may not be detected on Western blots. The antibody used to detect caspase-3 does not pick up the processed form of the enzyme, so a reduction in

proenzyme levels is the only available measure of enzyme processing. Simizu *et al.* (1998) recently demonstrated the requirement of caspase-3 for apoptosis (mediated by hydrogen peroxide production) induced by camptothecin, vinblastine, inostamycin and adriamycin. Keane *et al.* (1997) have also shown that caspase-3 activity is essential for apoptosis to occur after staurosporine treatment in astrocytoma and neuronal cells. Therefore the situation in DLKP cells is very unusual; *i.e.* the cells can apparently undergo apoptosis without activation of caspase-3. Nagane *et al.* (1998) demonstrated suppression of caspase-3 activity in drug resistant human glioblastoma cells. Cisplatin resistant squamous carcinoma cells fail to activate caspase-3 when compared to parental cells (Kojima *et al.*, 1998). Therefore the resistance of DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F could be due in part to the very low levels of expression and failure to activate caspase-3 in the cells.

Caspase-7

Caspase-7 shares over 50% sequence similarity with caspase-3 and shares substrate specificity (Lippke *et al.* 1996). Overexpression of caspase-7 can induce apoptosis in various cell lines (Duan *et al.* 1996; Lippke *et al.* 1996), Fas and TNF can trigger its activation resulting in the induction of apoptosis (Duan *et al.* 1996). Caspase-7 is an effector caspase and can be processed by caspase-3 (Fernandes-Alnemri *et al.* 1995) and also by caspase-8 (Muzio *et al.* 1998). In some cases its involvement in apoptosis is implicated rather than caspase-3. During lovastatin-induced apoptosis in the prostate cancer cell line LNCaP procaspase-7 mRNA is upregulated, and the proenzyme is activated by proteolytic processing, resulting in PARP cleavage and apoptosis while procaspase-3 is not activated (Marcelli *et al.* 1998). Therefore procaspase-7 is capable of implementing further downstream events to induce apoptosis, without a contribution from caspase-3.

Procaspace-7 protein levels were looked at in the four DLKP cell lines, before and after doxorubicin treatment to investigate its possible role in doxorubicin-induced apoptosis. Using western blotting it was concluded that there are approximately equal levels of procaspase-7 in all four cell lines indicating that sensitive and resistant

DLKP cells express procaspase-7 to the same extent. The antibody used was raised to the p17 fragment in procaspase-7, and therefore should detect the proenzyme, and the active p17 fragment. Doxorubicin treatment does not induce activation of procaspase-7 and there is no reduction in procaspase after doxorubicin treatment in any of the cell lines. Furthermore there was no detection of p17 fragments in the drug-treated samples, confirming that procaspase-7 is not activated in doxorubicin-induced apoptosis in any of the DLKP cell lines.

Caspase-6

Procaspace-6 is a 40kDa protein which shares some substrate specificity with caspase-3. It is a downstream caspase which can be activated by caspase-3 (Orth *et al.*, 1996) and caspase-6 is responsible for lamin cleavage during apoptosis (Orth *et al.* 1996) The antibody used should pick up both procaspase and the active 24kDa active fragment. There are relatively equal amounts of procaspase-6 in the four DLKP cell lines, and after doxorubicin treatment there is no decrease in the procaspase-6, and active p24 fragments could not be detected using western blotting. These results suggest that caspase-6 is not activated in doxorubicin-induced apoptosis in DLKP cell lines, and may not play a role in the apoptotic pathway in DLKP cells.

Caspase-8

Caspase-8 plays an important role in the CD95-mediated apoptotic pathway, being the first caspase to be activated. It has a unique DED domain, absent in other caspases (also present in caspase-10) which allows it to bind directly to FADD, the double adaptor protein, linking it to the CD95 receptor.

Treatment of neuroblastoma cells with doxorubicin results in cleavage of caspase-8 to active 18kDa fragments, 41kDa and 43kDa intermediate inactive fragments, and also cleavage of procaspase-3 to its active form (Fulda *et al.* 1998b). Neuroblastoma cells treated with doxorubicin induce apoptosis by activating the CD95 pathway, and following doxorubicin treatment the cells upregulate CD95 and CD95L which

mediate the death signal to the downstream apoptosis machinery (Muller *et al.* 1997; Fulda *et al.* 1997, 1998b).

The main effector of caspase-8 activation is the CD95 system, although caspase-8 can also be activated by cytochrome c release (described in section 1.5.7). In some cellular systems, such as betulinic acid treatment of neuroblastoma cells, caspase-8 can be activated independently of CD95 and CD95L induction. This then triggers activation of the effector phase involving caspase-3 cleavage which ultimately leads to PARP cleavage (Muller *et al.*, 1997). Caspase-8 can also be activated directly by cytochrome c, resulting in cleavage of its proform to active fragments in Jurkat cells (Ferrari *et al.*, 1998). Doxorubicin can efficiently induce caspase-8 activation in CD95-sensitive and CD95-resistant cells, so caspase-8 activation is not restricted to apoptosis mediated by death receptors, but can also be induced by proapoptotic stimuli in a CD95-independent pathway (Ferrari *et al.* 1998). Activation of caspase-8 in the CD95-mediated pathway is ATP-independent, but in the absence of a functional CD95 (in CD95-resistant cells) system, caspase-8 requires ATP to become proteolytically cleaved, probably involving activation by apaf-1 which requires ATP for its activity.

Therefore caspase-8 activation is a key event in signal transduction from the initiator phase to the effector phase, and given the wealth of evidence existing for doxorubicin-induced activation of the CD95 pathway (Fulda *et al.* 1997, 1998a, 1998b; Friesen *et al.* 1997), the levels and timely activation of procaspase-8 represent critical factors in maintaining an intact CD95 signalling pathway. Another important point to consider is that resistant cells have the potential to develop a defective apoptotic signalling pathway, so perhaps the DLKP resistant variants could have alterations in caspase-8-mediated signalling. Perera and Waldman (1998) evaluated the modulation of expression profiles of genes associated with apoptotic pathways induced by activation in human monocytes. Interestingly, they found that activated monocytes that were resistant to apoptosis also had downregulated caspase-8 mRNA, thus inhibiting the CD95 signalling pathway. This resistance however depended on the stimulus used, when monocytes were activated, they became were

resistant to etoposide and CD95-induced apoptosis and yet remained sensitive to UV- and staurosporine-induced apoptosis. This report highlights the specificity of resistance to different forms of apoptosis induction such as signalling by death receptors, DNA damage, cytochrome c and apaf1 involvement.

Using Western blotting to examine procaspase-8 levels, it can be seen from figure 3.13.5.1 that there is a definite decrease in procaspase-8 expression in the resistant variants. This result may have important implications on the ability of these cells to resist apoptotic induction. Reduction in procaspase-8 protein levels may limit the ability of these cells to undergo apoptosis via the CD95-mediated apoptotic pathway, because procaspase-8 is the principle apical caspase activated by CD-95. Looking at doxorubicin treated cells, there was no decrease in procaspase-8 levels, and also absence of caspase-8 activation, judged by the absence of caspase fragments. This result questions the involvement of caspase-8 activation in the doxorubicin-induced apoptosis in DLKP cell lines. The fact that procaspase-8 activation did not occur suggests that the CD95 pathway may be defective in some way.

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Caspase-4

Caspase-4 has 55% sequence similarity to caspase-1 and shares similar substrate preference (Cohen, 1997). It has been shown on a number of occasions to be activated and involved in apoptosis. It is thought that caspase-4 may have a role in activation of caspase-3 and could be the link between caspase-8 and caspase-3 in some cellular models, such as Fas-mediated apoptosis in NIH3T3 cells (Kamada *et al.* 1997). However procaspase-3 can be directly activated *in vivo* by caspase-8 (Stennicke *et al.* 1998) and recently Slee *et al.* (1999) demonstrated that caspase-4 can be activated by caspase-9, thus questioning a general role for caspase-4 in apoptosis induction. Results demonstrated that procaspase-4 protein levels do not vary among the sensitive and resistant DLKP variants, and is not activated in any of the DLKP cells lines during doxorubicin-induced apoptosis.

Caspase-9

Caspase-9 is a critical factor at the apex of the apoptotic pathway involving cytochrome c release and apaf-1. The search for the human homologue of CED-4 led to the discovery of apoptotic protease activating factors (apafs). Apaf-1 is the human homologue of CED-4, apaf-2 is cytochrome c and apaf-3 was discovered to be procaspase-9 (Liu *et al.* 1997). During an apoptotic signal transduction, cytochrome c is released from the mitochondria, and in the presence of dATP it binds to apaf-1. Binding of cytochrome c to apaf-1 is thought to promote clustering of apaf-1, to which procaspase-9 can bind. The close proximity of procaspase-9 molecules facilitates activation through autocatalysis. Caspase-9, when active, has been shown to activate all the other known caspases (Slee *et al.* 1999), and so has a key role to play in transducing the death signal to the downstream machinery to bring about destruction of the cell.

Caspase-9^{-/-} (double knockout) mice die at the embryo stage, and caspase-9 deficiency also results in brain malformation, due to reduced apoptosis in the absence of caspase-9 (Kuida *et al.* 1998; Hakem *et al.* 1998). These results reinforce the important role of caspase-9 during development. Caspase-9 is required for activation of caspase-3 *in vivo*, indicating that caspase-9 is upstream of caspase-3. It is also required for an effective apoptotic response (including caspase-3 activation and DNA fragmentation) in thymocytes exposed to etoposide, γ -irradiation and dexamethasone. However apoptosis induced by CD95 or UV-irradiation can still occur in thymocytes isolated from caspase-9^{-/-} mice (Hakem *et al.* 1998; Kuida *et al.* 1998) suggesting that redundant apoptotic pathways are utilised depending on the stimulus presented to the cell.

A recent report by Kuida *et al.* (1998) demonstrated that mice defective in caspase-9 had defective caspase-3 activation; therefore it appears vital to examine caspase-9 levels in the cell lines, because caspase-9 may be a critical factor in the activation of caspase-3 through the cytochrome c mediated pathway. Furthermore Hu *et al.* (1998) reported that Bcl-X_L protein can inhibit the association of apaf-1 with caspase-9 and thus prevent caspase-9 processing. This is a significant finding particularly relevant to the DLKP cells resistant

cell lines, because the resistant cell lines overexpress Bcl-X_L, and this could be a potential key to the absence of caspase-3 activity seen in the resistant cell lines.

Any reduction in, or faulty caspase-9 could lead to a defective induction of apoptosis. DLKP resistant variants do not have reduced procaspase-9 compared to sensitive DLKP-SQ indicating that the resistant state of the cells is not due to downregulated caspase-9. But the effect of doxorubicin treatment on the cells has not been looked at. Fearnhead *et al.* (1998) demonstrated that cells with a mutant caspase-9 which was unable to undergo autocatalytic processing were resistant to drug-induced apoptosis, and this was mainly due to the prevention of caspase-9 activation . Even though DLKP-SQ, DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F express caspase-9 to an equal extent, it remains to be seen if the resistant variants have alterations in the activation of procaspase-9.

4.14 STAT1 protein expression in DLKP variants

Signal transducers and activators of signal transduction (STAT) are involved in many cellular signalling pathways, particularly those involving signal transduction pathways of interferons, cytokines and growth factors (Darnell, 1997). STATs have membrane-associated domains as well as DNA binding domains. Cells lacking STAT1 protein are resistant to TNF α -induced apoptosis (Kumar *et al.* 1997). The study by Kumar *et al.* (1997) revealed that these cells lacked caspases-1, -2 and -3 mRNA and protein expression. Absence of caspase expression was directly linked to the STAT-1 levels in the cells. Re-introduction of STAT-1 to the cells restored caspase-3 mRNA and protein expression to normal levels. STAT-1 therefore may play a role in mediating expression of caspases, and also sensitivity to apoptosis induction.

DLKP resistant variants have reduced levels of procaspase-3 protein expression (figure 3.13.2.2). Considering the findings by Kumar *et al.* (1997) it was important to establish if there was a link between this reduced procaspase-3 expression and STAT-1 levels in the DLKP cells, so that the possibility of STAT1 involvement in drug resistance could be clarified. STAT-1 protein expression in DLKP cells was determined using Western blotting with an antibody specific for the alternative splice forms of STAT-1 (α and β). Results in 3.14.1 demonstrated that STAT-1 expression is equal in the four cell lines. From these results it can be concluded that STAT-1 expression in DLKP cell lines is not linked to reduced procaspase-3 levels in the resistant DLKP variants.

There is no difference in STAT-1 protein expression in doxorubicin-treated cells compared to the untreated controls. However, King and Goodburn, (1998) demonstrated STAT-1 cleavage from 91kDa to an 81kDa product in HeLa cells undergoing apoptosis induced by dsRNA, cycloheximide and etoposide, and this cleavage was mediated by a caspase. The caspase responsible for this specific cleavage of STAT-1 may be caspase-3, because recombinant caspase-3 was capable of cleaving STAT-1 into the 81kDa form, and a this cleavage could be inhibited by the caspase inhibitor zVAD. The absence of STAT1 cleavage in DLKP cell lines

reinforces the theory that caspase-3 does not play a major role in doxorubicin-induced apoptosis in DLKP cell lines.

4.15 PARP Cleavage in DLKP cell lines

Poly (ADP)ribose polymerase is an abundant nuclear enzyme involved in the formation of ADP ribose polymers during DNA damage, which have a role in DNA repair (Sato *et al.* 1992). PARP has been shown to be specifically cleaved from 116 kDa to 85 kDa and 25 kDa signature fragments during apoptosis induced by a variety of chemotherapeutic agents (Kaufmann *et al.* 1993). The cleavage site in PARP occurs at DEVD ↓ GVDE (Nicholson *et al.* 1995) and this (DEVD↓x) is the preferred cleavage site for a number of caspases including caspase-3, -7 and -2 (Nicholson and Thornberry, 1997). Therefore it is plausible that PARP may be cleaved by any of these caspases.

Tewari *et al.* (1995) and Nicholson *et al.* (1995) reported that PARP is cleaved *in vitro* by caspase-3/cpp32. Caspase-6 (Mch2) can cleave PARP into the 85 kDa and 25 kDa signature fragments (Orth *et al.* 1996) as well as caspase-7 which can also cleave PARP into the signature apoptotic fragments (Fernandes-Alnemri *et al.* 1995). Other caspases such as caspase-1, -2 and -4 are also capable of cleaving PARP *in vivo*, but cleavage only occurs when these caspases are expressed at high intracellular concentrations (Gu *et al.* 1995). Shah *et al.* (1995) developed a simple detection technique for PARP cleavage using Western blotting, and PARP is now a readily accepted indicator of apoptosis and caspase activity.

PARP was therefore examined in the four DLKP cells lines undergoing apoptosis (see section 3.15). PARP cleavage does not occur after Doxorubicin treatment in any of the four cell lines. There is however a slight decrease in intact PARP in DLKP-A2B cells by 50 hours but no corresponding increase in the 85 kDa fragment. Though unusual, these results do however agree with the unchanging procaspase-3 levels after Doxorubicin treatment. The experiment was repeated numerous times with three different antibodies and similar results were obtained.

HL-60 cells undergoing doxorubicin-induced apoptosis were analysed for PARP cleavage as a comparison to the situation in the DLKP cells. It is very clear from the Western blots that PARP is cleaved from 116kDa to 85kDa in the apoptotic HL60 cells, yet PARP

remains completely intact in the apoptotic DLKP-SQ cells. This is definitive evidence that the absence of PARP cleavage in the DLKP cell lines, whilst undergoing doxorubicin-induced apoptosis is not a result of the detection system employed, but rather an intrinsic feature of the cells.

It has been established that apoptosis can take place in the absence of PARP cleavage (Adjei *et al.* 1996), and also in the absence of PARP protein itself (Leist *et al.* 1997), thus questioning the necessity of PARP cleavage in the apoptotic process. Leist *et al.* (1997) demonstrated that apoptosis can occur in PARP-deficient mice, and that PARP-deficient mice were equally susceptible to apoptosis induced by a variety of apoptotic stimuli. These results suggest that PARP cleavage may have a non-essential role in apoptosis. Adjei and co-workers (1996) investigated the role of PARP cleavage in CPT-induced apoptosis in Hep 3B cells, and found that PARP was not cleaved during apoptosis in these cells. Absence of PARP cleavage was associated with very low expression of procaspase-3 protein and reduced caspase-3 enzyme activity during apoptosis. This finding might explain the situation in the DLKP cells which have very low caspase-3 expression accompanied by very low caspase-3 activity during Doxorubicin-induced apoptosis. However, the fact that other caspases are capable of cleaving PARP does not fully explain the results in DLKP, given that caspase-7 is equally expressed in the cells. However the level of caspase-7 in the cells following doxorubicin treatment is unclear. PARP cleavage can still occur *in vivo* in the absence of caspase-3 expression (Janicke *et al.* 1998) indicating that caspase-3 is not required for PARP cleavage to occur.

PARP can still be cleaved even in the absence of caspase-3, -2, -6 and -7. Knockout mice which have a deficiency for these caspases still retain the ability to cleave PARP, thus casting doubt on the significance of its cleavage in apoptosis (Kuida *et al.* 1996; Tim Zheng, pers. Comm.).

Additionally, Friesen *et al.* (1997) reported that PARP was not cleaved after Doxorubicin treatment in leukemia cells which were resistant to apoptosis due to a defective CD95 signalling pathway. Absence of PARP cleavage may also delay the

onset of apoptosis (Olivier *et al.* 1998). Therefore DLKP cells which are resistant to Doxorubicin may possess a supplementary resistance mechanism in the form of defective caspase activation and absence of PARP cleavage.

4.16 Fodrin Cleavage in DLKP cells during doxorubicin-induced apoptosis

The membrane-associated cytoskeletal protein fodrin is cleaved from 240kDa to 150kDa and 120kDa fragments during apoptosis (Martin *et al.* 1995). Fodrin cleavage is mediated by caspase activity. Vanags *et al.* (1996) demonstrated that this cleavage was mediated by cpp32/ICE-like proteases, and the caspase inhibitor VAD.cmk could inhibit fodrin cleavage in Jurkat cells, but only partially inhibit cleavage in TNF-treated U937 cells, indicating that the inhibition was cell and stimulus-specific. Janicke *et al.*, (1998) demonstrated that fodrin cleavage is mediated by caspase-3 and not by caspases-1 and -2, using the caspase-3-deficient MCF-7 cell line. They also confirmed that caspase-3 is required for fodrin cleavage to 120 kDa, whereas the 150 kDa fragment can be formed in the absence of caspase-3. It is thought that 150 kDa cleavage is mediated by calpain activity (Nath *et al.* 1996). Consistent with these findings Zheng *et al.* (1998) demonstrated that fodrin is only cleaved in caspase-3^{+/+} hepatocytes but not in caspase-3^{-/-} cells confirming the requirement of caspase-3 in fodrin cleavage. However Cryns *et al.* (1996) reported that fodrin is cleaved by a protease distinct from the one responsible for cleaving PARP. Apoptosis and fodrin cleavage in Fas-treated Jurkat cells could be prevented in the presence of YVAD.cmk, a tetrapeptide inhibitor specific for ICE-like caspases. DEVD.cho, a tetrapeptide inhibitor specific for cpp-32-like (caspase-3-like) caspases could not inhibit fodrin cleavage, yet effectively inhibited apoptosis in the cells. This finding suggested that fodrin cleavage can even be uncoupled from the apoptotic pathway. Conversely, PARP cleavage was completely inhibited by YVAD.cmk and DEVD.cmk. The dramatically different sensitivities of fodrin and PARP suggest that these cleavage events are mediated by different caspases, and the fodrin-cleaving protease is not a cpp-32-like protease (i.e. caspase-3, -7, -6), because it is not inhibited by DEVD-cho.

It is clear from the results in figure 3.16.1 that fodrin is effectively cleaved during doxorubicin-induced apoptosis in all four cell lines. From the reports by Janicke *et al.* (1998) and Zheng *et al.* (1998) it could be concluded that caspase-3 is activated in DLKP cells lines during apoptosis, as judged by the appearance of the 120 kDa cleavage product in the apoptotic cells. These results are in contrast to the caspase-3 processing Westerns, in which caspase-3 is not seemingly activated during apoptosis. Very low levels of

caspase-3 were detected in the resistant lines even though fodrin was shown to be cleaved in these cell lines. Figure 3.16.1 provides conclusive evidence for the existence of caspase-3-like activity in all four cell lines, but perhaps this activity is at a level which is undetectable as a change in proenzyme levels by Western blotting. If current reports are accurate then caspase-3 activity is required for fodrin cleavage. In DLKP cell lines the detection of fodrin cleavage in the absence of PARP cleavage represents an unusual situation. Fodrin is clearly cleaved indicating that there must be caspase-3 activity, but then why does caspase-3 not cleave PARP into the apoptotic signature fragments? One plausible explanation is that these two substrates are differentially localised in the cell, fodrin is a membrane-associated cytoskeletal protein, whereas PARP is a nuclear protein, so perhaps fodrin is more accessible for caspase-3 cleavage than PARP.

However if the report by Cryns *et al.* (1996) is considered, the situation in DLKP cell lines might be viewed differently. There may be a distinct protease active in apoptotic DLKP cells, capable of cleaving fodrin, and no PARP cleavage occurs due to the lack of caspase-3 activation. This hypothesis would explain the cleavage of fodrin together with absence of PARP cleavage. Looking at the timing of apoptosis onset in DLKP cells, it is quite a lengthy process, so in the intervening time before nuclear condensation, cytosolic caspase-3 may have adequate time to cleave fodrin, whereas the nuclear condensation events are rapid, and PARP might remain intact. However, the nucleus also contains active caspases during apoptosis (Martins *et al.* 1997) that are activated at a similar rate to cytosolic caspases, so the action of these caspases on PARP cannot be excluded. In addition, HL-60 cells undergo apoptosis with rapid nuclear condensation (not shown) and exhibit PARP cleavage, thus questioning the theory of slow apoptotic rate as a reason for absence of PARP cleavage.

Another possibility for absence of PARP cleavage may be the existence of a noncleavable mutant of PARP in the DLKP cells. It was also seen in Western blotting that the procaspase-3 does not decrease during apoptosis in DLKP, so perhaps the active caspase-3 is not present in sufficient amounts to carry out PARP cleavage, but is adequate to carry out fodrin cleavage.

4.17 Role of Caspase Inhibitors in Doxorubicin-induced Apoptosis

Previous sections have elucidated the role of caspases in doxorubicin-induced apoptosis in DLKP cell lines, by looking directly at caspase processing and caspase-substrate cleavage. An alternative method of investigation involves the use of peptide inhibitors modelled on the cleavage site of caspase substrates. The tripeptide inhibitor zVAD.fmk is thought to be a general caspase inhibitor and is reversible and cell permeable. DEVD.fmk is an inhibitor based on the PARP cleavage sequence, DEVD ↓ , and can inhibit caspases which normally cleave PARP, namely the cpp32 family comprising caspase-3, caspase-2 and caspase-7 among others. The YVAD inhibitor is modelled on the cleaved sequence in pro-interleukin 1B, YVAD ↓ and inhibits caspases such as caspase-4 and caspase-1. Numerous reports demonstrate caspase inhibition by zVAD.fmk, and a subsequent inhibition of apoptosis as judged by absence of the morphological and biochemical features of apoptosis. Slee *et al.* (1996) demonstrated that zVAD.fmk inhibited apoptosis in THP1 cells and Jurkat cells by inhibition of caspase-3 processing. zVAD was used in combination with doxorubicin to assess its effects on apoptotic induction in DLKP-SQ cells. The chosen end point was viability of cells after doxorubicin treatment, and results were not as expected. zVAD treatment provided no survival enhancement in any of the combinations used (detailed in section 3.17). The percentage kill after 72 hours in the presence of zVAD.fmk equalled the percentage cell kill due to doxorubicin alone. The various combinations of preincubation, co-incubation and re-addition after 24 hours failed to protect the cells from apoptosis induced by doxorubicin. This phenomenon was previously reported by Lotem and Sachs (1996), who found that myeloid leukaemic cells when treated with zVAD.fmk were afforded no protection from apoptosis induced by doxorubicin.

Taxol-induced apoptosis was also investigated; the concentration used (50-80ng/ml) resulted in rapid apoptotic induction over 6 hours compared to the slower timescale of 24 hours with doxorubicin. Again no cytoprotective effect was observed by the use of zVAD.

Considering these results, it was decided to carry out an investigation looking at the

ability of the other, more specific caspase inhibitors, DEVD.fmk and YVAD.fmk to protect the DLKP cells from doxorubicin-induced apoptosis. The three different inhibitors were used in combination with two different doxorubicin concentrations which gave a high and medium level of cell kill. Results were consistent with the previous findings; again, no increased viability was seen in the presence of the caspase inhibitors. Looking at photographs of DLKP-SQ treated with either doxorubicin alone or in combination with one of the three inhibitors, it can be seen that there is approximately equal cell kill in inhibitor/doxorubicin-treated cells and doxorubicin-treated cells alone over 72 hours. To further investigate any difference in the rate of apoptosis, or a possible delay in induction of apoptosis in the presence of inhibitors timelapse videomicroscopy was carried out to compare kinetics of cell death in doxorubicin-only and doxorubicin-zVAD treated cells. Analysis revealed a similar rate of cell death in both situations, and the mechanism of cell death was confirmed to be apoptotic, based on the appearance of the characteristic morphological alterations in the cells, so zVAD does not affect the timing of apoptosis onset in DLKP-SQ.

These results suggest the possibility of a caspase-independent pathway in doxorubicin-induced apoptosis. Adjei *et al.* (1996) found that apoptosis induced by the topoisomerase I inhibitor, camptothecin occurs independently of caspase-1 and caspase-3 activity in Hep3B hepatocarcinoma cells. Yet Datta *et al.* (1996) reported that chemotherapy drugs such as cisplatin, etoposide and camptothecin induce activation of caspase-3. It is obvious that the caspase-dependence or independence depends very much on the cell lines being used and the agent used to induce apoptosis. Results suggest a possibility where doxorubicin may induce apoptosis via a pathway independent of caspase-3 activation, but as yet this pathway remains elusive in DLKP-SQ cells.

Cytotoxicity levels in the caspase inhibitor experiments were measured after 72 hours. One could argue that this timepoint is too late in the process, and at this stage any effects the inhibitors may have on apoptosis would be bypassed. However time-lapse videomicroscopy over this time revealed that the cells died at a similar rate to cells which were just treated with doxorubicin, and exactly the same percentage viability resulted after 72 hours, indicating that zVAD has no effect on the cells, so the time of viability analysis

is of little consequence to the overall findings.

4.18 Fluorogenic Caspase-3 Assays

Using the fluorogenic caspase-3 assays provides a direct method for measuring caspase enzyme activity. Using the caspase-3 specific fluorogenic peptide DEVD.AMC, caspase-3 activity was monitored in each of the cell lines following the method described by Martins *et al.* (1997) before and after doxorubicin treatment. Actual fluorescence readings from each independent assay (four cell lines) varied from day to day but the resulting trend remained the same for each of the cell lines, so statistical analysis was not feasible. Representative results can be seen in figure 3.18.1. It can be seen that there is a high basal activity in DLKP-SQ untreated cells compared to cells undergoing apoptosis and from this result caspase-3 seems to be only marginally activated during apoptosis in DLKP-SQ.

In contrast to this, caspase-3 activity in DLKP-A250/10p#7 cells increases to quite high levels in apoptotic cells, with highest activity seen at 60 hours. DLKP-A5F also exhibits an increase in caspase-3 activity but not to the extent seen in A250 10p#7 cells. DLKP-A2B also exhibited an increase in caspase-3 activity parallel to that of DLKP-A5F but the data is not included on this graph.

The effect preincubated zVAD.fmk had on caspase-3 activity was also investigated. In DLKP-SQ there was a reduction in caspase-3 activity, indicating that the inhibitor is entering the cell. The caspase-3 activity was almost completely inhibited in DLKP-A250 10p#7 at 60 hours in the presence of zVAD.fmk, and this effect is also seen in DLKP-A5F.

When all results are combined, it can be concluded that there is a detectable basal caspase-3 activity in DLKP-SQ cells and this does not significantly increase during Doxorubicin-induced apoptosis. DLKP-A250 10p#7 on the other hand experience an increased activation of caspase-3 activity, and furthermore this activity is inhibited by zVAD.fmk. A similar but not as pronounced effect is seen in DLKP-A2B and DLKP-A5F.

4.19 CD95 receptor Levels

The possibility of a caspase-3 independent apoptotic pathway led to investigation of the CD95 system in DLKP cells. CD95/CD95-L interaction plays an important role in the apoptotic signalling pathway, and are involved in apoptosis induced by chemotherapy drugs, amongst other stimuli. Doxorubicin and other cytotoxic drugs are thought to induce apoptosis through induction of CD95 and CD95L with subsequent activation of caspase-8.

Upon an apoptotic signal, the CD95 receptors trimerise, allowing CD95-L to bind and a death signal to be transduced. Therefore the amount of CD95 present is an important factor for efficient signalling to occur. It has previously been reported that cells resistant to apoptosis have altered regulation of CD95. Friesen *et al.* (1997) demonstrated that doxorubicin-resistant cells have downregulated CD95 receptor compared to sensitive cells, and resistant cell lines neither upregulate CD95L, or activate caspase-3, resulting in inhibition of PARP cleavage. Together these defects contribute to a defective signalling pathway. Doxorubicin treatment can cause upregulation of CD95 expression in some cell lines, such as neuroblastoma cells (Fulda *et al.* 1998) and it is thought that this upregulation can enhance the death signal by interacting with CD95L.

CD95 receptor levels were examined using Western blotting in the four DLKP cell lines before and after doxorubicin treatment. CD95 is expressed to the same extent in sensitive and resistant cell lines, with no downregulation of CD95 in the resistant variants. After doxorubicin treatment, there is no upregulation of CD95 in any of the cell lines. It had been thought, that if after doxorubicin treatment DLKP-SQ cells upregulated CD95 perhaps the resistant variants would not upregulate CD95 to the same extent, and this failure to upregulate CD95 following doxorubicin treatment could be an additional resistance mechanism in the resistant variants. However results show that there is no upregulation of CD95 after doxorubicin treatment in any of the cell lines.

4.20 Inhibitors of CD95/CD95L

Results using caspase inhibitors indicate that there could be another mechanism by which the apoptotic pathway could operate, perhaps bypassing the need for caspase activation. The CD95 pathway does require caspase activity to initiate the apoptotic response, but only at a downstream level, after the receptors have transduced the apoptotic stimulus from the external environment to inside the cell.

The interaction between CD95- and drug-induced apoptosis has received a lot of attention over the past few years, with many conflicting reports of independence and interdependence between the signalling pathways activated by the two sets of stimuli. Fulda *et al.* (1997) found that treatment of neuroblastoma cells with such drugs as doxorubicin, cisplatin and VP-16 induced apoptosis, which was accompanied by an induction of CD95 receptor and CD95L. CD95-resistant neuroblastoma cells were also resistant to chemotherapeutic drug-induced apoptosis. Los *et al.* (1997) also demonstrated that cells resistant to doxorubicin-induced apoptosis were also resistant to CD95-induced apoptosis. Cross-resistance between drug-induced apoptosis and the CD95 pathways gives important insights into the mechanism of drug sensitivity and resistance in tumour cells. In the development of resistance, a common element of the apoptotic pathway must be damaged in some way, resulting in resistance to diverse stimuli which can activate different branches of the pathway involving either CD95 or drugs.

However evidence for drug-induced apoptosis independent of the CD95/CD95L system also exists. Many studies showing that CD95/CD95L inhibitors fail to inhibit drug-induced apoptosis suggest that there is a pathway independent of the CD95 system (Eischen *et al.* 1997; Villunger *et al.* 1997; Micheau *et al.* 1999). These independently-activated pathways converge on a common downstream pathway involving caspase-3 activation. Eischen *et al.* (1997) concluded that CD-95 resistant Jurkat cells are not resistant to drug-induced apoptosis and also that the CD95-blocking antibody ZB4, cannot prevent drug-induced apoptosis in CD95-sensitive Jurkat cells. This is clear evidence that there are distinct pathways activated by CD95 and cytotoxic drugs.

In section 3.17 cell permeable inhibitors of caspases were tested for their ability to block doxorubicin-induced apoptosis in the DLKP cell lines, without success. It can also be seen in section 3.20 that ZB4 offered no protection to the cells from doxorubicin-induced apoptosis, approximately the same number of cells died when treated with ZB4 and doxorubicin, as those treated with doxorubicin alone. However ZB4 could reduce CD95-induced apoptosis in DLKP-SQ indicating that it is capable of inhibiting CD95-mediated apoptosis. The effect of NOK-2 on doxorubicin-induced apoptosis was also investigated and it was concluded that NOK-2 has no protective effect against doxorubicin-induced apoptosis in DLKP cell lines.

As previously mentioned, cytotoxic drugs such as cisplatin, doxorubicin and fludarabine can induce apoptosis in the presence of ZB4 and NOK-2 in CEM lines (Villunger *et al.*, 1997) thus questioning the involvement of the CD95/CD95L signalling in drug-induced apoptosis. Doxorubicin has previously been shown to induce apoptosis in Jurkat cell lines, and to do so even in the presence of the CD95 blocker ZB4, and etoposide treatment of the cells displayed similar characteristics (Eischen *et al.*, 1997) These results raise the possibility that chemotherapeutic drugs can induce apoptosis independently of the CD95 pathway. Furthermore, Micheau *et al.* (1999) recently reported on the inability of ZB4 to prevent drug-induced apoptosis in a variety of human cell lines. They suggest that cytotoxic drugs can induce apoptosis through a pathway involving caspase-8 and FADD, independent of CD95L action, and found that downregulation of FADD expression was associated with resistance to drug-induced apoptosis.

Considering that the DLKP cells can still die by apoptosis in the presence of either caspase inhibitors or CD95 blockers, a combination of inhibitors was employed, consisting of the CD95 blocker ZB4 and the general caspase inhibitor zVAD.fmk, in an attempt to discover if both inhibitors together could prevent DLKP-SQ from apoptosis. Cells were preincubated with both inhibitors for 1 hour before doxorubicin addition, and again at the time of drug addition. However even the combination could not prevent apoptosis from occurring. Analysis of the kinetics of apoptosis of cells exposed to the combination treatment using timelapse videomicroscopy, reveals a

similar rate to kinetics of apoptosis induced by doxorubicin alone, suggesting that the inhibitors have no effect on the rate of apoptotic induction in doxorubicin-treated cells. Apoptosis in inhibitor-treated cells occurs at a slightly faster initial rate, if anything, but the two rates closely parallel each other over the 72 hours monitored.

These results suggest that DLKP cells can induce apoptosis in a seemingly novel manner, independent of the caspase- or CD95-dependent signalling pathways. A possibly significant characteristic of the resistant DLKP cells is the overexpression of Pgp and Bcl-X_L. Ha *et al.* (1998) recently provided evidence of a caspase-3-independent apoptotic pathway in Bcl-2 overexpressing cells. Using polyamine analogues which resulted in G2/M arrest and apoptosis, they observed activation of caspase-3, DNA fragmentation, PARP cleavage and cytochrome c release. Yet when bcl-2 was overexpressed all the apoptotic features were inhibited but the cells still underwent apoptosis suggesting the existence of a caspase-independent pathway. The overall conclusions and future experimental work are detailed in section 4.21.

5.0 Conclusions and Future work

5.0 Conclusions and Future work

In this thesis the effects of doxorubicin on the cell cycle and on apoptotic pathways were investigated in variants of a human lung carcinoma cell line DLKP. Particular emphasis was placed on identification of any alterations that may exist in resistant variants compared to sensitive DLKP-SQ. DLKP-A250 10p#7, DLKP-A2B and DLKP-A5F exhibit varying levels of resistance to doxorubicin and other anticancer drugs. In contrast to much of the literature which uses identical drug concentrations to investigate apoptosis in wild-type versus resistant cells, equitoxic doxorubicin concentrations were used to treat the cell lines, and the cell cycle response and induction of apoptosis were monitored. Cell cycle conclusions are outlined first, followed by conclusions drawn from the apoptosis-related results.

There were no alterations in cyclins (-B, -D, -E, -A) or cdk1 (-1, -2, -4, -6) expression in resistant variants compared to sensitive DLKP-SQ cells, indicating that the resistant cells do not experience alterations in expression of cell cycle regulators as a mechanism or consequence of their resistant state. Doxorubicin treatment of DLKP-SQ cells induced a G2/M arrest by 24 hours; interestingly, it was at this time that apoptosis occurred, suggesting that the cells halt at the G2/M checkpoint, and then undergo apoptosis. In contrast the resistant variants did not completely arrest in G2/M; some cells only transiently arrest in G2/M or can bypass the G2/M arrest and accumulate in G1. This suggests that resistant cells may have a defective G2/M checkpoint that allows the cells to overcome the G2/M arrest, even if they have experienced DNA damage. Further investigation was carried out to identify the cellular alterations in the resistant variants that allow a G2/M override. The activity and regulation of cyclin B-cdk1 kinase was looked at in detail in sensitive and resistant cells before and after doxorubicin treatment. There is no difference in cyclin B or cdk1 expression following doxorubicin treatment and sensitive and resistant cells equally retain the ability to form a kinase complex. However levels of inhibitory tyrosine 15 phosphorylation seem to be higher in DLKP-SQ after doxorubicin treatment, which would account for the G2/M arrest, and the cells also experience a slight decrease in cdk1 kinase activity. The resistant cells have an elevated cdk1 kinase activity after doxorubicin treatment; perhaps this is a cellular response to enable them to pass into mitosis. This G2/M bypass would result in cells with increased genomic instability, if they divide with damaged

or incompletely replicated DNA. Caffeine treatment in combination with doxorubicin treatment, allows the DLKP-SQ cells to bypass the G2/M arrest in a similar fashion to the resistant variants. This suggests that the resistant variants may possess cellular alterations similar to those induced by caffeine in the DLKP-SQ cells, which allows the cells to override the G2/M checkpoint. One possibility is that caffeine may alter chk1 kinase activation in the G2/M checkpoint, which leads to inappropriate activation of cdc25C and by pass the G2/M checkpoint.

The regulators of Tyr15 phosphorylation on cdk1, cdc25C phosphatase and weel, were also investigated. Cdc25C protein levels and phosphatase activity did not change following drug treatment in resistant or sensitive cells, suggesting that it may not be the critical factor responsible for the G2/M override seen in the resistant variants. The activity status of cdc25C was investigated using Western blotting to measure the phosphorylation status by altered mobility in SDS-PAGE and gave largely inconclusive results, perhaps due to the experimental system in use.

Wee1 is a negative regulator of cdk1 kinase, and is responsible for the inhibitory phosphorylation on Tyr15. Wee1 expression levels did not vary after drug treatment, but phosphorylation levels did change slightly in DLKP-SQ compared to the resistant variants. When phosphorylated, wee1 is inactive, and this results in a decreased phosphorylation on Tyr15 of cdk1. DLKP-SQ appear to experience a decrease in wee1 phosphorylation, following doxorubicin treatment, indicating that wee1 is fully active, perhaps maintaining the Tyr15 phosphorylations on cdk1, and the G2/M arrest. Therefore wee1 in the resistant cells may not be fully active, allowing cells to bypass the G2/M arrest.

Further investigation of the altered cell cycle response to doxorubicin in resistant variants may lead to discovery of a unique cell cycle effect in resistant cells. Initial studies would involve a study of cdc25C phosphatase activity. This would involve an *in vitro* phosphatase assay, using purified cyclin B-cdk1 complex, and measuring the ability of cdc25C to dephosphorylate cdk1 on tyr15. The resistant variants may have a higher phosphatase activity following doxorubicin treatment, resulting in removal of the inhibitory phosphorylation on Tyr15 of cdk1, and entry to mitosis.

The involvement of wee1 kinase in the G2/M override could also be further investigated. From mobility in SDS-PAGE gels, wee1 seems to be more active in DLKP-SQ following drug treatment, thereby preventing the DLKP-SQ cells from entering mitosis. If the activity of wee1 is reduced in the resistant variants after drug treatment, it would allow them to bypass the G2/M arrest. Kinase activity of wee1 could be studied using an *in vitro* kinase assay, looking at the ability of immunoprecipitated wee1 to phosphorylate cyclin B-cdk1 complex *in vitro*. Both regulators of cdk1 need to be examined in detail, because they are closely involved together in the activation and inhibition of cdk1 kinase. The regulator of wee1, nim1 could also play a role in inactivation of the wee1 kinase in the resistant variants, and its kinase activity could also be investigated using *in vitro* kinase assays.

The precision and accuracy of the DNA damage-induced checkpoint is extremely important in maintaining genomic stability of cells, as discussed earlier. The integrity of the DNA damage-induced checkpoint must be investigated because it may be a key factor in the G2/M override in the resistant variants. Chk1 kinase is activated in response to DNA damage; chk1 kinase activity in DLKP cells would define the response to the drug treatment. Perhaps the chk1 kinase response is attenuated in the resistant variants, allowing them to continue in the cell cycle without halting in response to the checkpoint.

The fact that the G2/M override is only seen in the resistant variants suggests that this response may be linked to the resistant state of the cells. All the resistant variants express Pgp, as shown by Western blotting. An interesting experiment to investigate this possible link would involve deletion or inactivation of Pgp through transfection of an MDR ribozyme in a resistant variant, and monitoring the cell cycle response of these Pgp-deficient cells to doxorubicin. This would confirm whether the altered cell cycle response is restricted to Pgp-expressing cells. It could also uncover a novel role for Pgp in the cell cycle, perhaps implicating Pgp in promoting cell cycle progression, either through its effects on abrogating the DNA damage-induced G2 checkpoint, or through inappropriate mitosis.

Many recent studies have confirmed the importance to cyclin B localisation in the G2 arrest response to DNA damage. Cyclin B localisation will be investigated in the sensitive compared to the resistant cells. Perhaps cyclin B in resistant variants is not exported from

the nucleus as efficiently as in the sensitive variants; residual cyclin B in the nucleus could therefore promote premature entry to mitosis. Localisation of cyclin B can be studied using immunocytochemistry, with specific fluorescent-labelled antibodies.

p21 expression could not be detected after drug treatment in the DLKP cells, even though the cells are visibly undergoing apoptosis (results not shown) and presumably have experienced DNA damage. This points to the p53 status in these cell lines. The cells do not arrest in G1 at the concentrations of Doxorubicin used, only a G2/M arrest is seen. It is known that cells which are defective in p53 do not have an ability to arrest in G1, and subsequently arrest in G2/M. It would be interesting to determine the p53 status of DLKP cells in order to further characterise their response to drug treatment.

The DLKP cells used throughout the course this thesis were asynchronous. This is a very important experimental parameter, that has many consequences on the results following drug treatment. Asynchronous cells were chosen because the effects to be monitored after doxorubicin treatment were solely drug effects, and it was thought that cell synchronisation could hamper these results. Cell synchronisation is usually carried out using various chemicals such as nocodazole and hydroxyurea, or by serum starvation or thymidine starvation. However, all these treatments induce cellular alterations, which could interfere with the doxorubicin-induced effects, and the overall effect seen might not be a true reflection of the situation in the cell. In light of the results obtained, especially those in the *cdc25* and *wee1* sections, the asynchronous cells could possibly mask any differences that might be present. The control cells are asynchronous, with approximately 5% in mitosis; this is a similar situation to G2-arrested cells in that there are very few cells in mitosis, so it is difficult to differentiate between the drug-induced G2-arrested cells and the resistant variants that continue to cycle in a similar fashion to the control cells. Cell synchronisation must therefore be carried out; this should give more clear-cut results, and cells that arrest in G2 will be easily distinguished from those that continue to cycle. In future experiments cell synchronisation could be carried out using centrifugal elutriation (non-chemical treatment), if possible. FACS (fluorescent activated cell sorting) could also be used to separate the cells according to their DNA content. The cells would be drug treated as before. Then the cell cycle responses would be monitored, and findings should explain altered cell cycle response in the resistant DLKP cells more clearly.

A summary of the events in the apoptotic pathway that occur, as reported in the literature, following doxorubicin treatment can be seen in figure 4.4. Also included (in italics) are the findings in the DLKP cell lines. From the results, the situation concerning doxorubicin-induced apoptosis in DLKP appears quite unusual. Apoptosis occurs and cells exhibit classical morphological features of apoptosis such as DNA fragmentation, nuclear condensation and phosphatidylserine externalisation, confirming that the end result of the signalling pathways activated by doxorubicin is apoptotic cell death. In addition preliminary results suggest that cytochrome c is released from mitochondria after doxorubicin treatment (not included in results section, needs to be further investigated).

A significant level of caspase activation was not seen, however, and this observation is puzzling because caspases constitute the critical components of the apoptotic pathway in all cells. However this may be due to the antibodies used in Western blotting; they may be unable to pick up the active fragments due to the very short half-life of active caspases in the cell. Caspase-3 expression is significantly reduced in resistant variants, suggesting that caspase expression may be linked to drug resistance. Perhaps a decrease in caspase expression may be an attempt by a resistant cell to alter or dismantle its apoptotic signalling pathway to increase its chances of survival. The parental cell line and resistant variants express procaspase-3 mRNA to an equal extent, suggesting that there must be some post-transcriptional control which prevents translation of the procaspase-3 mRNA. Future experiments on the effects of doxorubicin treatment on procaspase-3 mRNA expression will reveal any altered regulation of the procaspase-3. Translation regulation of procaspase-3 gene expression might play a role in controlling the protein expression of procaspase-3. One interesting aspect would be to investigate the regulation of translation of procaspase-3, perhaps there may be some alterations in the components governing procaspase-3 translation, such as the translation initiation factor, eIF-4E. If procaspase-3 mRNA is upregulated following doxorubicin treatment, resulting in increased protein synthesis, this would explain the failure to detect any decrease in procaspase-3 expression during apoptosis.

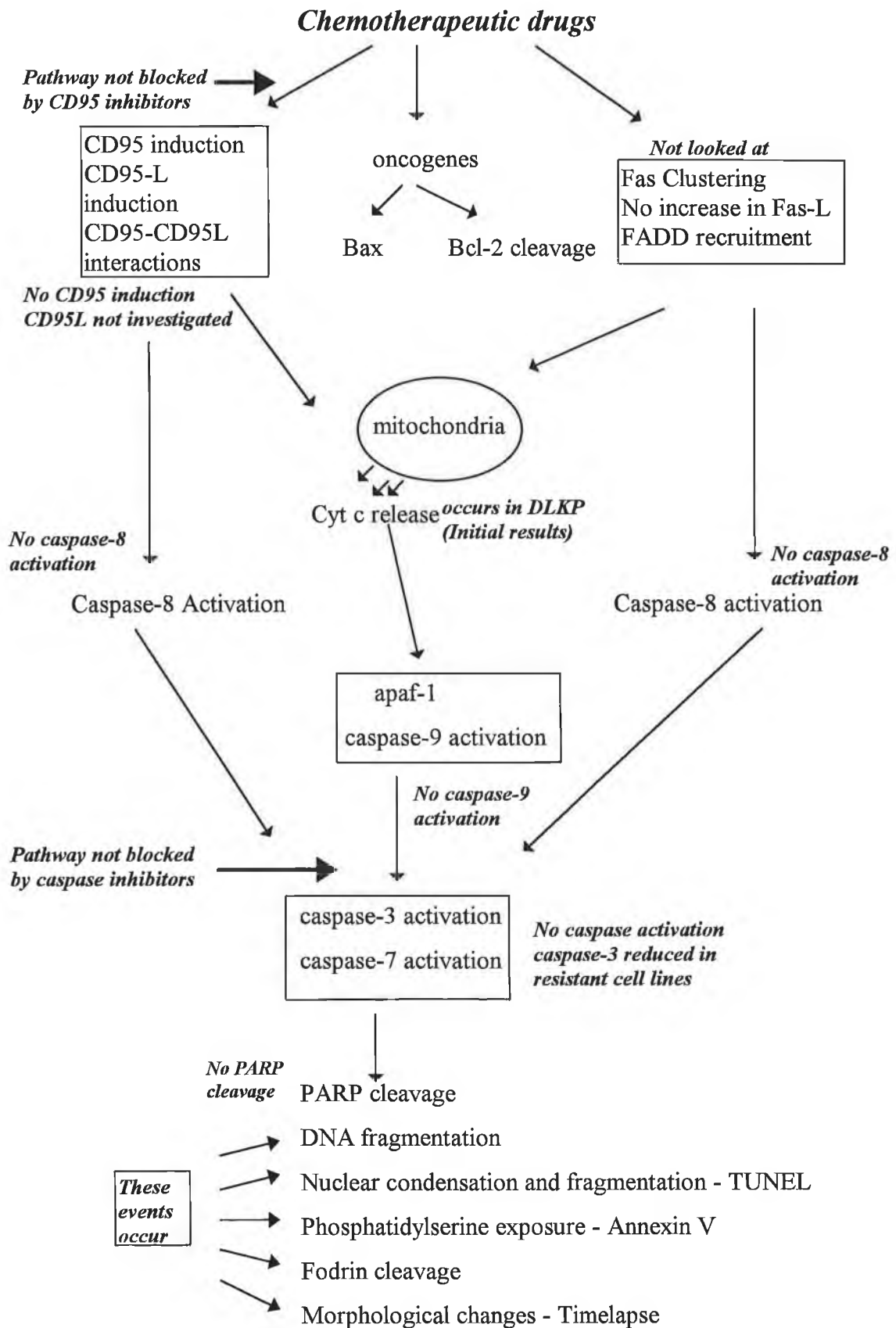


Figure 4.4 Summary of apoptotic pathway in DLKP

Another unusual finding is the inability of cell permeable caspase inhibitors and CD95 inhibitors to prevent cell death in the DLKP cell lines. It is possible that an alternative pathway exists in the DLKP cell lines which is independent of CD95 and caspase activation, but still results in apoptotic cell death, but this does not concur with the currently accepted view of apoptosis. Another observation not in line with the literature on apoptosis is that fodrin is cleaved in the absence of PARP cleavage, suggesting that PARP cleavage is not a marker for apoptosis in the DLKP cell lines. Overall the findings suggest that DLKP cells, when treated with doxorubicin die by apoptosis, and some but not all of the characteristic apoptotic alterations occur in these cells. Further investigation of these cell variants may lead to the discovery of novel apoptotic pathways.

Apoptosis in DLKP cell lines appears to occur in the presence of both caspase and CD95 inhibitors, and control experiments confirmed that the inhibitors were permeating the cells. One theory arising from the inability of synthetic inhibitors to prevent doxorubicin-induced apoptosis in DLKP cells is that the inhibitors suppress activity of a subset of caspases, but perhaps some caspases exist whose activity is not prevented by the synthetic inhibitors zVAD.fmk, DEVD.fmk and YVAD.fmk. This raises the possibility of redundancy amongst the 14 known caspases, an assumption that is quite plausible. It would be interesting to see if the downstream substrates of caspases are still cleaved, in the presence of inhibitors. This experiment would confirm if the inhibitors just prevent activation of a subset of caspases, allowing other caspases to continue in the apoptotic signalling pathway.

The involvement of Pgp in resistance of the cells to apoptosis will also be investigated. The resistant variants have much reduced procaspase-3 protein expression, and also express high levels of Pgp. This observation raises the possibility that Pgp expression may result in reduction of procaspase-3 expression. To test this hypothesis, levels of procaspase-3 protein expression and activation will be examined in a resistant cell line, which has been transfected with an MDR-ribozyme, and thereby knocking out Pgp expression. The Pgp status in the DLKP cells could also be linked to the inability of synthetic inhibitors to prevent doxorubicin-induced apoptosis. DLKP-SQ express very low levels of Pgp, and higher levels of Pgp are expressed in DLKP-A250 10p#7,

DLKP-A2B and DLKP-A5F. Perhaps the Pgp can actively export the synthetic inhibitor out of the nucleus, and prevent the inhibitory actions on the apoptotic signalling pathway from taking place. One method to further investigate this possibility would involve co-incubation of the Pgp inhibitor verapamil with the caspase inhibitor before doxorubicin treatment. If this treatment prevented apoptosis, then it could be concluded that Pgp expression effectively reduces the ability of synthetic caspase inhibitors to prevent apoptosis induction.

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