Gene Expression in the Development of Multidrug Resistance

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

Molecular processes involved in the development of malignancy have a role to play in the establishment of the multidrug resistant (MDR) phenotype. Analysis of oncogene expression, at the mRNA level using RT-PCR, in resistant variants of the human squamous lung cell line, DLKP, and the human ovarian carcinoma cell line, OAW42, was carried out in this study. Analysis of gene expression, by RT-PCR and *in situ* hybridisation, on tumours from breast cancer patients was also carried out on fresh and formalin-fixed paraffin-embedded tissue.

Altered expression of c-*erb*B-2, c-Ha-*ras*, c-*myc* and c-*fos* and the *bcl-2* family members, *bcl-*x_L and *bax*, were seen in resistant variants of the DLKP cell line developed by continuous exposure to adriamycin, carboplatin and VP-16. Ribozyme technology was used to down-regulate the expression of the c-*fos* gene in the adriamycin-selected variant, DLKP-A, and the effect on adriamycin toxicity was analysed.

Drug-resistant cell lines developed by continuous exposure to drug for prolonged periods are frequently resistant to drug concentrations above those achieved in the clinic. As patients can develop drug resistance after only a few courses of chemotherapy, a more clinicallly relevant *in vitro* model of drug resistance was developed in this thesis by pulse exposure of clonal populations of DLKP, DLKP-SQ and DLKP-I, to clinically attainable levels of adriamycin for a short period of time. The resulting resistant variants, DLKP-SQ/A25010p and DLKP-I/A25010p, exhibited a classical MDR phenotype in that increased resistance to adriamycin, vincristine and VP-16 was demonstrated. However, no alteration in 5-fluorouracil sensitivity was seen. The cell lines were found to over-express the *mdr1* gene and decreased adriamycin accumulation was observed in the resistant variants.

Characterisation of the resistant variant of DLKP-SQ revealed increased expression, using RT-PCR, of the *bcl-*x_L, *bax*, c-*erb*B-2 and c-*fos* genes relative to the sensitive parental line. To counter-act the effect of *bcl-*x_L over-expression in the DLKP-SQ/A25010p, the antagonistic *bcl-*x_S gene was introduced into the resistant cell line, which resulted in increased sensitivity to adriamycin, VP-16 and vincristine but not to 5-fluorouracil.

Attempts were made throughout the course of this thesis to determine the initialising events in the establishment of the MDR phenotype in the pulse-selected model. Results found indicate that *bcl-*x_L and c-*fos* may be important initialising events in this process. Molecular studies of gene expression pattern in the pulse-selected variant of DLKP-SQ suggested that this may be a novel system amenable to the search for inhibitors of resistance development. Initial studies have been carried out in this thesis to determine if known inhibitors/circumventors of the MDR phenotype or oncogene signalling cascades affected the development of resistance using the DLKP-SQ model.

Buíochas

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Tiomnaím an tráchtas seo, le fíor bhuíochas agus grá,
do mo thuismitheoirí, Seán agus Caitlín.....

"Is aoibhinn beatha an scoláire"

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1. Introduction

1.1 Introduction

In cellular systems the balance of life and death is paramount, with control of cell number under tight genetic regulation; any alteration to this control can have catastrophic effects for the organism as a whole. The control of cell number involves mechanisms for regulating cell proliferation and cell death. Malignancy is seen as a loss of normal control of cell growth whereby accumulation of multiple events leads to the propagation of a mass of cells that ignore any normal inhibitory messages and have the propensity to grow indefinitely if left undetected or untreated. Cancer, therefore, is fundamentally a result of inappropriate cell proliferation.

Studies of malignant cells have identified molecular mechanisms whereby an appropriately regulated cell is transformed into a potentially immortal cell that has the ability to ignore cellular control. The mechanism which regulates this transformational process is governed by oncogenes, dominant mutated forms of normal cellular gene products which encode proteins that are involved in basic cellular signal transduction cascades. These signal cascades transduce growth-regulatory messages from outside the cell to the cell's replication workshop in the nucleus; any alteration to these stringently controlled pathways can result in the development of neoplasia (for review see Bishop, 1991). To date over 100 oncogenes have been identified.

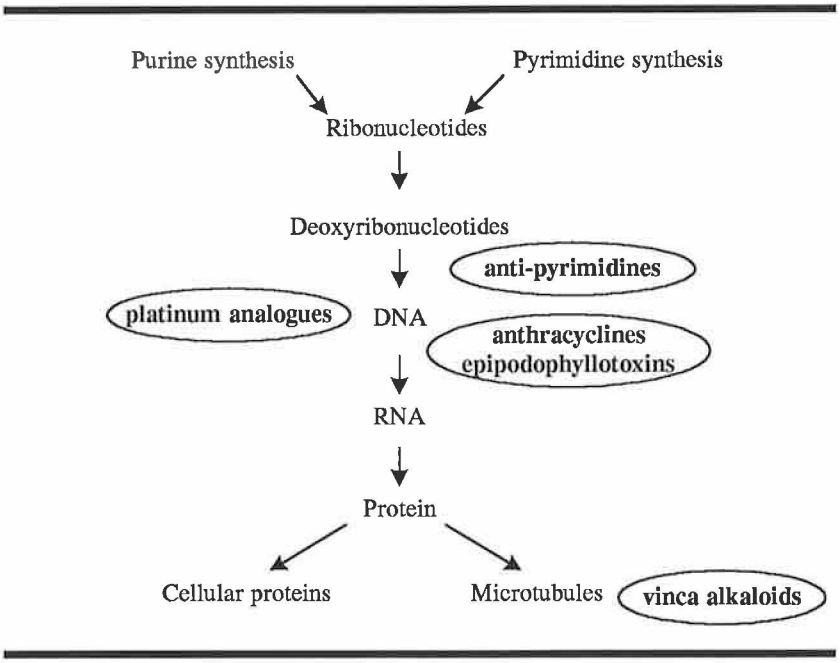
1.2 Treatment of Cancer

Currently, cancer is treated either by surgery, radiotherapy, immunotherapy, hormone treatment or chemotherapy. The aim of any of these regimens is to achieve optimal kill of malignant cells while sparing their normal neighbours. Chemotherapy is most useful against tumours with a high proportion of proliferating cells, such as leukaemia and lymphoma. The more common solid tumours (colorectal, lung, breast) have a low proportion of dividing cells and are largely refractory to chemotherapeutic drug treatment. Some normal tissues, such as bone marrow and cells lining the gastrointestinal tract, also have a high proportion of proliferating cells and therefore are also targetted by chemotherapeutic drugs. Clinical doses of chemotherapeutic drugs administered *in vivo* are limited by these facts.

The original strategy employed in the design of chemotherapeutic drugs which are used to

treat cancer, involved the development of agents which prevented malignant cells from dividing. The commonly used chemotherapeutic drugs include the anthracyclines, vinca alkaloids, epipodophyllotoxins, antipyrimidines and the platinum analogues. These drugs act at many sites to interfere with cell growth and division (Figure 1.2.1)

Figure 1.2.1 Sites of action of Cytotoxic Drugs



1.2.1 The Anthracyclines

The anthracyclines are a class of anti-tumour antibiotics derived from the *Streptomyces* species (Tannock and Hill, 1992). Included in this family are adriamycin (also called doxorubicin), daunorubicin, epirubicin and an ever-increasing number of semi-synthetic variants. Adriamycin is one of the most important agents used in the treatment of human cancer (Tritton and Yee, 1982). Clinical effectiveness is seen in the treatment of acute myelogenous leukaemia, acute lymphoblastic leukaemia, carcinomas of the bladder, breast, ovaries, lung, bronchi and thyroid, Ewing's sarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, neuroblastoma and many others (Carter *et al.*, 1987). Adriamycin exhibits a tri-phasic plasma decay curve with half-lives of 12 minutes, 3.3 hours and 30 hours (Pratt and Ruddon, 1979). The standard dosage of adriamycin is 30-60mg/m^2 intravenously to a total cumulative dosage of 550mg/m^2 (Carter *et al.*, 1987).

Adriamycin treatment is not without its faults, however, as serious side-effects are seen

with use of this drug. The drug is carcinogenic and mutagenic and causes immunosuppression. Damage to heart muscle is a major side-effect and incidence increases with increasing cumulative dose of drug (Tannock and Hill, 1992).

The primary mechanism of action of adriamycin in the cell is due to the drug's ability to bind DNA and RNA. Intercalation between adjacent pairs in the double helix results in the inhibition of replication, transcription and translation. The drug stabilises topoisomerase II-DNA complexes (Chan *et al.*, 1993a) and exerts free radical mediated cytotoxicity (Pasada *et al.*, 1989) which may be responsible for the cardiotoxicity of adriamycin (Pratt *et al.*, 1994).

1.2.2 The Epipodophyllotoxins

The plant *Podophyllum peltatum* synthesises a compound known as podophyllotoxin which is a mitotic inhibitor that acts by binding to tubulin (Pratt and Ruddon, 1979) thereby preventing the formation of microtubules by blocking polymerisation of tubulin (Long, 1991). Semi-synthetic derivatives of podophyllotoxin, namely etoposide (VP-16) and teniposide (VM-26), have shown efficacy in the treatment of some human cancers, including testicular and small cell lung cancer (Carter *et al.*, 1987; Van Maanen *et al.*, 1988). Administration is by intravenous injection or orally (Smyth *et al.*, 1985). The plasma decay of etoposide is bi-phasic (Carter *et al.*, 1987) with a terminal half life of 4 - 9 hours. Haematologic toxicity is usually dose-limiting in the treatment of cancer patients with epipodophyllotoxins and gastrointestinal side-effects are frequently seen.

Originally it was believed that VP-16 exerted its effect on proliferation by inhibiting microtubule function. It has since been shown that the main target of this epipodophyllotoxin is the topoisomerase II protein, an enzyme that facilitates DNA unwinding (Van Maanen *et al.*, 1988).

1.2.3 Microtubule Inhibitors

The *Vinca alkaloids* are derived from the periwinkle plant *Catharanthus rosea*. The two drugs in common clinical use are vincristine and vinblastine. Vincristine exhibits a relatively low toxicity for normal bone marrow cells and is used to treat acute leukaemia, Hodgkin's lymphoma, aggressive non-Hodgkin's lymphoma, small cell lung cancer, Wilm's tumour, neuroblastoma, rhabdomyosarcoma and Ewing's sarcoma (Pratt *et al.*,

1994). This drug, administered as a bolus intravenous injection, exhibits tri-phasic decay kinetics. The dose-limiting toxicity of vincristine is pre-dominantly peripheral neuropathy. Vincristine arrests cells in the mitotic phase of the cell cycle by disrupting the mitotic spindle by interrupting microtubule polymerisation (Pratt *et al.*, 1994).

Another microtubule inhibitor is taxol. Taxol was first isolated from the bark of the slow-growing yew tree, *Taxus brevifola*, and now synthetic derivatives are on the market. The importance of microtubules as a target for taxol was suggested by its ability to induce cell cycle arrest at G_2/M with minimal effect on RNA, DNA or protein synthesis. Taxol treatment stabilises cytoplasmic microtubules and the formation of abnormal bundles of microtubules. Taxol is administered either as short (1-6 hour) or long (24 hour) infusions, at doses of 200-300mg/m^2. The drug appears to have bi-phasic kinetics with a terminal half-life of about 5 hours. Clinical effectiveness is seen in the treatment of refractory ovarian cancer and metastatic breast cancer. Dose-limiting side-effects include bone marrow depression, hypersensitivity reaction, peripheral neuropathies and cardiac arrhythmias (Pratt *et al.*, 1994).

1.2.4 The Antimetabolites

The antimetabolites are drugs that are chemically related to naturally occurring compounds and interfere with cellular metabolic pathways, especially those involved in the synthesis of DNA. This group of cytotoxic drugs is divided into folate antagonists, pyrimidine analogues and purine analogues (for review see Skovsgaard *et al.*, 1994).

Methotrexate, an analogue of folic acid, is the most important anti-folate and since its introduction in the clinic in 1948 is the best understood antineoplastic compound to date. Methotrexate exerts its cytotoxic effect by competitive inhibition of the cytosolic enzyme dihydrofolate reductase (DHFR) (Waltham *et al.*, 1988) which is the key enzyme in the biosynthesis of pyrimidines and purines. Methotrexate is used in the treatment of acute lymphocytic leukaemia of children, osteosarcomas, head and neck cancers, and is also used effectively in combination for the treatment of breast cancer (Pratt *et al.*, 1994). The two major sites of methotrexate toxicity are the bone marrow and the endothelium of the oropharynx and gastrointestinal tract. Methotrexate is administered by oral, intravenous, intraarterial or intrathecal routes. The plasma disappearance curve of methotrexate has been characterised as tri-phasic with half-lives of 0.75, 3.5 and 27 hours.

5-Fluorouracil, an anti-pyrimidine, is one of the most widely used agents in the treatment

of human cancer and is effective in the treatment of the most commonly occurring solid tumours, including colorectal, breast, head and neck, gastric and pancreatic cancers (Pratt *et al.*, 1994). Other anti-pyrimidines include cytosine arabinoside which is widely used in antileukaemic therapies. 5-Fluorouracil is usually administered parenterally as a continuous infusion. 5-Fluorouracil is more toxic to proliferating than non-proliferating cells leading to toxic side-effects in tissues of the gastrointestinal tract and bone marrow. 5-Fluorouracil represents one of the few examples of antineoplastic drugs synthesised on a rational basis. 5-Fluorouracil is a simple derivative of uracil where the hydrogen at position 5 is replaced by a fluorine atom. The rationale used in the design of this drug was that rat hepatomas utilised more uracil than non-malignant tissues. 5-Fluorouracil is a direct inhibitor of thymidylate synthase, a key enzyme in the production of thymidine nucleotides; the drug can also be incorporated into RNA and DNA and consequently inhibits transcription, translation and the intracellular distribution of mRNA (Pratt *et al.*, 1994).

1.2.5 Covalent DNA-Binding Drugs

The first successful systemic application of cancer chemotherapy was demonstrated in 1942 when Gilman, Goodman, Lidskog and Dougherty used nitrogen mustard to treat a patient suffering from lymphosarcoma (Pratt *et al.*, 1994). Since then many alkylating agents have been tested for their usefulness as anti-cancer agents. DNA-binding drugs have proven effective in a variety of different tumours and many are used commonly in modern chemotherapeutic regimens. Cyclophosphamide, another nitrogen mustard, is the most commonly used alkylating agent. The drug is used in combination to treat a wide variety of cancers, including lymhoid tumours, myeloma, osteogenic disease, neurobastoma, retinoblastoma and carcinomas of the breast, lung, ovary and endometrium. It is administered either orally or intravenously. Dose-limiting toxicities include immunosuppression, bone marrow suppression and long-term treatment can be followed by the development of bladder cancer (Pratt *et al.*, 1994). The alkylating agents are capable of cross-linking an alkyl group with cellular constituents such as DNA. Alkylation of the DNA decreases its ability to act as a template for DNA synthesis and results in DNA damage and potential cell kill.

The platinum complexes were first discovered by Rosenberg and co-workers and were shown to cross-link DNA in a manner distinct from alkylation. The platinum compunds

exert their toxicity by binding to base pairs of DNA, resulting in the formation of intra-strand cross-links and adducts, and ultimately leads to the disruption and unwinding of the DNA double helix. Cisplatin is currently one of the most effective anticancer agents, used in the treatment of ovarian, testicular, head and neck, non-small cell lung and brain tumours (Rosenberg, 1985). Dose-limiting toxicity is nephrotoxicity which may ultimately lead to renal failure. Other side effects include gastrointestinal toxicities, myelosuppression, tinnitus and loss of hearing. To avoid these side-effects other platinum compounds, including carboplatin, have been designed. Carboplatin produces considerably less nephrotoxicity than cisplatin and is often used in preference to cisplatin for the treatment of ovarian and lung carcinomas (Gore *et al.*, 1989; Raghaven *et al.*, 1994). The platinum compounds are usually injected intravenously and following administration are rapidly and tightly bound to proteins. The clearance of total drug (free and bound) has been described as tri-phasic with a prolonged terminal half-life of 58 to 73 hours.

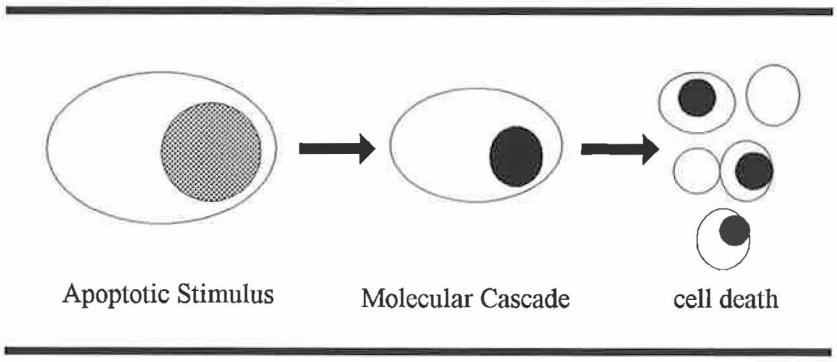
1.3 Chemotherapy and Cell Death

The metabolic processes targeted by chemotherapeutic drugs are thought to be more critical for the viability of rapidly dividing tumour cells than their normal counterparts. However, non-cycling tumour cells, such as the ones that accumulate in chronic lymphocytic leukaemia, are also targeted effectively by the same agents (Orrenius, 1995). Recently it has been shown that chemotherapeutic drugs act not only to prevent proliferation by targetting cellular DNA or inhibiting cell division but also succeed in their task by promoting an active form of cell death, namely apoptosis (Skladanowski and Konopa, 1993). Why chemotherapeutic agents are more toxic to cancer cells than normal cells is unknown but a clue may lie in the mechanisms whereby drugs with different cellular targets, result in a common cell death pathway.

1.3.1 Apoptosis

Apoptosis is a mechanism of cell death whereby a cell actively participates in its own destruction. "Apoptosis" was a term used by Kerr *et al.* (1972) to describe a series of morphological changes shared by dying cells in various biological systems. These morphological changes include cell shrinkage, membrane blebbing, chromatin condensation and endonucleolytic cleavage of DNA into nucleosomal-length fragments, and finally the production of budding "apoptotic bodies". The process does not induce an inflammatory response.

Figure 1.3.1.1 The Apoptotic Pathway



Since those initial findings, a tightly controlled molecular pathway has emerged which ultimately leads to the demise of the cell. Most of the early research on apoptosis was

carried out on the nematode, *Caenorhabditis elegans*. This organism has a defined cell lineage of 1090 somatic cells, 131 of which are programmed to die during the development of the organism making it an ideal system to study programmed cell death. Three cell death abnormal (*ced*) genes (*ced3, ced4* and *ced9*) were identified as playing a role in the regulation of cell death in the nematode (for review see Uren and Vaux, 1996). Two of these genes, *ced3* and *ced4*, are required for all programmed cell deaths in *C. elegans*. The *ced3* amino acid sequence shows significant homology to a mammalian cysteine protease, interleukin-1β converting enzyme (ICE), with novel specificity for aspartic acid; no mammalian homologues for *ced4* have been identified. The observation of homology between the amino acid sequence of *ced3* and ICE was followed by the demonstration that over-expression of ICE in cells triggered apoptosis (Miura *et al.*, 1993). *Ced-9* was found to have an antagonistic activity to *ced-3* and *ced-4*. *Ced-9* loss of function mutations result in excess cell death. Loss of either *ced-3* or *ced-4* genes appears to compensate for the loss of the *ced-9* function. The mammalian inhibitor of cell death, *bcl*-2, was found to be highly homologous to the *C.elegans ced-9* gene (Hengartner and Horvitz, 1994). The role of *bcl-2* family members in the regulation of apoptosis will be addressed later (Section 1.7.1). The other seven *ced* genes so far identified appear to encode proteins involved in disposal of the dead cell's corpse. Another cell death effector gene, *nuc-1*, encodes an endonuclease involved in digestion of the dead cell's DNA. Other players in the apoptotic pathway include *p53*, c-*myc* and other oncogenes are discussed in more detail in Section 1.7.

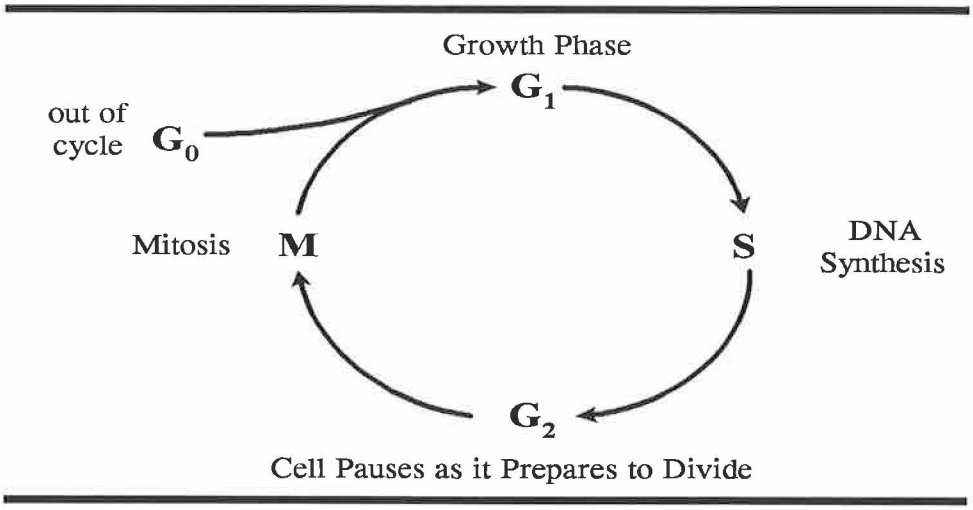
The importance of apoptosis in development, tissue homeostasis, defence against viral infection and oncogenesis is becoming increasingly evident. Developmental cell death shapes the digits in foetal hands by the deletion of interdigital cells and removes the superfluous neurons seen in the foetus as compared to the adult. Apoptosis of virus-infected cells interrupts viral replication, thus preventing the spread of infection to other cells in the organism. Apoptosis is used by the immune system to remove potentially autoreactive lymphocytes or those that fail to form a functional antigen receptor. DNA-damaged cells are also known to undergo apoptosis induced by p53, thus preventing the establishment of a mutated cell in the population. Apoptosis is also physiologically involved in the hormone-response of many tissues such as involution of the mammary glands in late lactation. The anti-tumour cytokines, tumour necrosis factor -α and -β, mediate their effect by inducing apoptosis; a molecule essential in the regulation of

apoptosis is the cell surface receptor CD95 (Fas/APO-1), a member of the tumour necrosis factor receptor family (Nagata and Golstein, 1995). More than 100 different stimuli, including many chemotherapeutic drugs and toxins, have been shown to induce apoptosis in experimental systems.

1.3.2 Apoptosis and Chemotherapeutic Drugs

Several chemotherapeutic agents commonly used in the treatment of both solid and haematological tumours have been shown to induce apoptosis in a number of cell lines *in vitro*. These include adriamycin (Ling *et al.*, 1993; Skladanowski and Konopa, 1993), cisplatin (Demarcq *et al.*, 1994), the epipodophyllotoxins (Holm *et al.*, 1994), macromolecular synthesis inhibitors (Gorczya *et al.*, 1993; Okamoto *et al.*, 1996), DNA alkylating agents (O'Connor *et al.*, 1991; Gorczya *et al.*, 1993), and topoisomerase inhibitors (Solary *et al.*, 1994; Dubrez *et al.*, 1995). One hypothesis used to explain the apoptotic response of chemotherapeutic drugs is that of a non-specific defense response to a perceived threat (Vaux and Haecker, 1995), thus explaining why so many agents of differing pharmacological action provoke the same response. Chemotherapeutic drugs act primarily by causing DNA damage, which in turn stimulates the p53 protein. Stimulation of p53 leads to cell cycle arrest in G_1 or G_2 (Figure 1.3.2.1) and results either in the repair of DNA damage and cell recovery or in the induction of apoptosis. p53 has been shown to affect apoptosis directly by down-regulating expression of the Bcl-2 protein and up-regulating Bax and Bcl-x_L expression (Miyashita *et al.*, 1994; Miyashita and Reed, 1995; Merchant *et al.*, 1996). Recently it has been shown that chemotherapeutic drugs mediate an apoptotic pathway by inducing the expression of the CD95 (Fas/APO-1) and its ligand (Micheau *et al.*, 1997). The up-regulation of CD95 expression may be at the "crossroad" of DNA damage, p53 accumulation, and the apoptosis response pathway (Debatin, 1997).

Figure 1.3.2.1 Cell Cycle



1.4 Multidrug resistance (MDR)

Failure to cure cancer by chemotherapy is caused primarily by the clinical development of chemotherapeutic drug resistance whereby tumours continue to proliferate in the presence of a cytotoxic agent designed to prevent proliferation; resistance can be either intrinsic (to the untreated tumour) or acquired (by an initially sensitive tumour after exposure to a chemotherapeutic agent). Although resistance of a tumour to one chemotherapeutic drug prevents the recovery of the patient, combination chemotherapy approaches could, in theory, alleviate this problem. The ultimate failure of cancer chemotherapy is seen in the development of Multidrug Resistance (MDR) - a phenomenon whereby a particular tumour is resistant not only to one drug but rather a variety of structurally unrelated and mechanistically varied natural product chemotherapeutic drugs such as the anthracyclines, the vinca alkaloids, epipodophyllotoxins, colchicine, actinomycin D and Taxol. The sensitivity of the tumour to alkylating agents, antimetabolites or platinum compounds appears unaltered (Clynes, 1993). Tumour types vary in their response to anti-cancer drugs - many tumours are inherently resistant to chemotherapy, such as colon carcinoma; others are sensitive to chemotherapy, such as non-Hodgkin's lymphomas, Wilm's tumour; but other tumour types, such as breast and ovarian cancers, which are initially responsive to chemotherapy, relapse and present with drug-resistant tumours (Spiers, 1994).

The underlying cause of progressive drug resistance is spontaneous genetic mutations which occur in all living cells. In any given cell population mutants that are resistant to a given drug occur at a frequency of somewhere between 1 in 10^5 and 1 in 10^8 cells. In a tumour of detectable size (1cm) there is at least 10^8 cells, some of which may be resistant, and by treatment with a drug, these potentially lethal cells are selected from a mostly drug-sensitive population (Kartner and Ling, 1989). The first insight into the MDR-phenotype was revealed in the 1960s when cross-resistance to completely unrelated drugs was found in tumours. The cause of this phenomenon *in vivo* is quite complex, however, and properties such as tumour size, vascularisation and specific cellular mechanisms such as increased drug efflux from cells, increased drug detoxification, DNA repair and others, may all be involved in the survival of a resistant tumour when under attack from a chemotherapeutic regimen.

The MDR phenomenon was first described *in vitro* in 1970 by Biedler and Riehm in studies of Chinese hamster lung cells and P388 leukaemia cells and since then much

research has been carried out on cellular models of multidrug resistance which act as "magnifiers" of the cellular response to commonly used chemotherapeutic agents. These studies have identified the main players involved in *in vitro* drug resistance and include efflux pumps such as P-glycoprotein, MRP and LRP. Topoisomerases have also been found to be involved in MDR.

1.4.1 P-glycoprotein and its role in Multidrug Resistance

Several genes have been found to be over-expressed in multidrug resistant human cancer cell lines and the product of the *mdr1* gene, P-glycoprotein, has been extensively studied in many *in vitro* model systems. This protein acts as an energy-dependent efflux pump located in the plasma membrane.

P-glycoprotein is present at high levels in normal human kidney and adrenal glands; at intermediate levels in lung, liver and colon; and at low levels in most other tissues (Tannock and Hill, 1992). Those organs which constitutively express P-glycoprotein often give rise to tumours that are innately drug resistant; other tumours, such as breast, which are initially responsive to chemotherapy (and express *mdr1* infrequently) often relapse and present with P-glycoprotein positive tumours (Nooter and Herweijer, 1991).

The results of a series of experiments raise the possibility that expression of the *mdr1* gene is regulated by the genes that drive tumour progression (Chin *et al.*, 1992). It was found that the *mdr1* gene promoter is a potential target of *ras* and *p53* and that the *mdr1* gene was turned on as a result of activation of *ras* genes (Section 1.6.2) and inactivation of the *p53* tumour suppressor gene, common events which occur during tumourigenesis. A study by Cornwell and Smith (1993) revealed that stimulation of signal transduction through the *c-Raf* kinase pathway (Section 1.6.2.2) also increased the activity of the *mdr1* promoter.

1.4.2 Other Mechanisms involved in Drug Resistance

The Multidrug Resistance-associated Protein, MRP, is another cellular pump which effluxes drug from resistant cell lines. The gene has been identified in non-P-glycoprotein multidrug cell lines derived from small cell lung, leukaemias, fibrosarcomas, breast and other carcinomas. Its expression appears to precede that of P-glycoprotein as a series of drug-resistant cell lines were found to over-express MRP in low-level resistant cells, followed by P-glycoprotein over-expression in the same cells at higher levels of resistance,

and, may for this reason be a more clinically relevant marker of resistance (Loe *et al.*, 1996).

A lung-resistance related protein (LRP), identified originally in a non-P-glycoprotein MDR lung cell line (Scheper *et al.*, 1993), is also believed to play a role in low-level MDR cells. Studies in different cancers are underway to determine if LRP expression in tumours is predictive of response to chemotherapy and prognosis (Izquierdo *et al.*, 1996).

Topoisomerases, proteins that facilitate the unwinding of DNA during DNA replication and transcription, have also been shown to be important in multidrug resistant cell lines (Beck, 1989). Many chemotherapeutic agents e.g. adriamycin, etoposide and others, act by targetting the topoisomerase enzymes, binding to the complex formed with DNA and preventing rejoining of the DNA strands (Chan *et al.*, 1993a). Low level Topoisomerase activity has been observed in many drug-resistant cell lines and resulted in relative insensitivity to Topoisomerase II targetting drugs (Long *et al.*, 1991). *Topoisomerase IIα* expression has been found co-amplified with c-*erb*B-2 (a poor prognostic marker in breast cancer, Section 1.6.1.3) and those patients that over-expressed c-*erb*B-2 were found to be sensitive to Topoisomerase II inhibitors (Isaacs *et al.*, 1995).

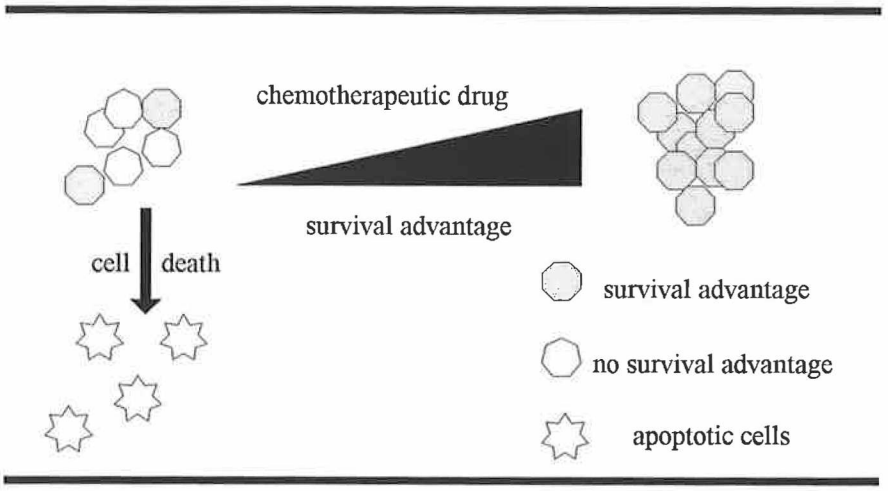
Resistance to cisplatin and other platinum compounds is usually associated with DNA repair activity. Cisplatin reacts readily with compounds containing a thiol group and resistance in some cases has been found to correlate with glutathione content (Pratt *et al.*, 1994). Another thiol-containing modifier of drug response is the metal-binding protein metallothionein. The metallothionein is a small 6-7kDa protein, 30% of which comprises of cysteine residues. High levels of metallothioneins are often seen in cisplatin-resistant cell lines (Kelley *et al.*, 1988).

The enzyme dihydrofolate reductase (DHFR) is responsible for the conversion of folate to a variety of coenzymes that are required for the synthesis of thymidylate, purines, methionine and glycine. Methotrexate blocks this process and in so doing results in inhibition of DNA, RNA and protein synthesis. Methotrexate resistant cells, therefore, frequently over-express the DHFR enzyme although other mechanisms of resistance are also known (Pratt *et al.*, 1994). Normal conversion of dUMP to dTMP is mediated by the thymidylate synthase (TS) enzyme and is inhibited by the fluoropyrimidines. Over-expression of TS is, therefore, seen in 5-fluorouracil resistant cell lines.

1.4.3 Control of Cell Death and Proliferation as a means of Multidrug Resistance

As control of cell number is intrinsic to normal cellular growth, and apoptosis has such an important role to play in normal cellular regulation, disturbance of these tightly controlled pathways could have alarming consequences. It has been shown that a disordered apoptotic pathway is responsible for many pathogenic diseases involving a hyper-active (virus-induced lymphocyte depletion (AIDS), Alzheimer's disease, Parkinson's disease, aplastic anaemia, myelodysplastic syndrome (reviewed by Thompson, 1995)) or a hypo-active (neoplasia and some autoimmune diseases such as systemic lupus erythematosis (for review see Uren and Vaux, 1996)) death response. The interest for a role of apoptosis in cancer is two-fold. Deficient apoptosis promotes tumorigenesis by allowing the accumulation of dividing cells and by not removing damaged variants with enhanced malignant potential. Secondly, awareness of the apoptotic pathway is vital in understanding the treatment of cancer by chemotherapy. Cells which are given a survival advantage due to the over-expression of anti-apoptotic or proliferation genes or down-regulation of pro-apoptotic genes are less likely to die when exposed to an apoptotic stimulus such as chemotherapeutic drugs (Figure 1.3.1). Clinical evidence supports this theory as it has been shown that the common solid tumours that frequently develop resistance to chemotherapeutic drugs have a low apoptotic index (Staunton and Gaffney, 1995). These cells survive attack of an apoptotic agent and subsequently develop "resistance" to that agent.

Figure 1.3.1 Emergence of drug resistant cells when treated with chemotherapeutic drug



The inherent problem with drug resistant cells is that they proliferate when they ought not,

the fundamental basis of the malignant process. The emergence of MDR is frequently, but not always, associated with tumour progression (Kellen, 1994) which is the result of oncogene activation. Over-expression of almost all classes of oncogenes has been shown to affect the cell's response to attack from chemotherapeutic agents. The epidermal growth factor receptor and its homologue, c-*erb*B-2, increase the resistance profile of some cell lines (Shin *et al.*, 1991; Meyers *et al.*, 1993; Muss *et al.*, 1994; Sabbatini *et al.*, 1994); signal transducers such as the Ras and Raf proteins which play a fundamental role in cellular proliferation affect resistance levels in *in vitro* systems as well (Isonishi *et al.*, 1991; Sabbatini *et al.*, 1994). Over-expression of nuclear oncogenes which act as transcription factors has increased resistance in cell lines (Scanlon *et al.*, 1991; Funato *et al.*, 1992; Zhao *et al.*, 1995); the role of the bcl-2 family in drug resistance is well documented (Kitada *et al.*, 1994; Teixeira *et al.*, 1995; Wagener *et al.*, 1996) as are other apoptosis effecting genes such as p53 (reviewed in Kellen, 1994) and c-myc (Kashani-Sabet *et* al., 1990; Sklar and Prochownik, 1991; Mizutani *et al.*, 1994). The anti-apoptotic oncogene Bcr-abl has been definitively shown to block cell death in neutrophils caused by anticancer drugs and other cellular stresses (McGahon *et al.*, 1994). Treatment of cells with drug has also demonstrated an inductive effect of certain oncogenes (Kashani-Sabet *et al.*, 1990; Kim and Beck; 1994; Sinha *et al.*, 1995; Tu *et al.*, 1996). The common factor uniting all of these genes in the regulation of drug resistance is the fact that they all promote cellular proliferation, suggesting that the MDR phenotype is fundamentally a result of inappropriate proliferation.

1.5 Oncogenes in the Study of Multidrug Resistance

Oncogenes are dominant mutated forms of normal cellular proteins involved in signal transduction cascades, expression of which results in inappropriate proliferation. There are three main methods whereby the normal cellular protein (proto-oncogene) is mutated to its oncogenic counterpart. The methods involved include :-

(a) deletion or point mutation in coding sequence of gene resulting in a hyperactive protein made in normal amounts;

(b) gene amplification leading to the over-production of a normal protein;

(c) chromosomal rearrangement whereby a gene is under transcriptional regulation of an actively transcribed gene. (Alberts *et al.*, 1994).

As the fundamental theory of chemotherapy demonstrates, chemotherapeutic drugs are more toxic to malignant cells than normal cells suggesting a role for the transformation from normal to cancerous cells in determining the response of a cell to the drug. A causative role for oncogenes in drug-resistance has received much attention in the last few years. This is not surprising as most chemotherapeutic drugs act by inducing apoptosis which is controlled by many signal transduction pathways involving oncogenes (Skladanowski and Konopa, 1993; Hickman, 1996). Drug-resistant cells proliferate in the presence of anti-proliferative drugs, therefore a mechanism is present which results in the dominant proliferation and survival of cells in the presence of a chemotherapeutic drug. A number of oncogenes are currently under investigation in the broad discipline of chemotherapy, such as targetting oncogene-expressing cells with antibody-linked chemotherapeutic drug (Suzuki *et al.*, 1995), and especially in the area of drug resistance. When treated with chemotherapeutic drugs, increased expression of genes which promote proliferation, either by increasing cell division or blocking cell death, would be expected to give cells a survival advantage and therefore result in the emergence of clinical drug resistance.

It is possible to group oncogenes into two main categories - those that increase the rate of cell division and those that influence cell death (both groups often overlap); this latter group can be sub-divided into genes that suppress or induce apoptosis. The balance of these genes in the cell determines the cells ability to survive an attack, for example, by a chemotherapeutic agent and emerge as a resistant population. Therefore, the consequence of inhibiting cells from undergoing apoptosis is another method whereby cells can develop resistance to chemotherapy.

1.6 Oncogenes and their role in Cell Proliferation

Oncogenes were discovered due to their characteristic capacity of increasing cellular proliferation, thereby resulting in the growth of a tumour. Oncogenes fall into many cellular categories, including growth factor receptors, cytosolic proteins and transcription factors, and are part of a cascade of proteins leading from the plasma membrane through the cytoplasm and into the nucleus (for review see Bishop, 1991). Molecular co-operation between different classes of these oncoproteins results in the development of a proliferating mass of undisciplined cells.

Even though over one hundred oncogenes have been identified to date only three biochemical mechanisms are known whereby the genes exert their transforming capacities; these mechanisms involve (a) phosphorylation of proteins on serine, threonine or tyrosine residues, (b) transmission of signals by GTPases, or (c) transcription from DNA (Bishop, 1991). Protein phosphorylation, regulated by protein kinases and phosphatases, controls a wide range of cellular events, such as cell division, cell signalling, differentiation and metabolism. Intrinsic to the transforming ability of oncogenes is a phosphorylation cascade whereby phosphorylation of a membrane-associated protein transduces a signal through the cytosol to the nucleus.

1.6.1 Oncogenesis at the Plasma Membrane

A number of oncogenes encode mutant forms of cell surface growth factor receptors whereby the mutated form gives rise to a cell with enhanced propensity for growth despite the presence of normal regulatory messages. Many of these oncogenes are derived from receptor protein-tyrosine kinase genes which encode either receptors for known growth factors (e.g. EGF receptor, PDGF receptor, IGF receptor) or receptor-like proteins with unknown ligands (e.g. neu / c-*erb*B-2). Other non-receptor tyrosine kinase proteins exist which include the cytoplasmic Src family, of which Lck is a member, and the nuclear Abl protein. Normal cells rarely phosphorylate tyrosine residues unless stimulated by growth factors.

Receptor protein tyrosine kinases have an extracellular ligand-binding domain, a single transmembrane domain and a cytosolic domain. When activated by ligand binding, the cytosolic domains of receptor dimers cross-phosphorylate each other on tyrosine residues which causes a conformational change that enhances the kinase activity of the receptor

towards other substrates. Oncogenic variants of normal receptors include deletion of large portions of the extracellular domain, single amino acid substitutions in the trans-membrane domain or mutations in the cytosolic domain (for review see Cantley *et al.*, 1991). The effect of these mutations is the activation of the receptor without the need for ligand binding.

The prototype of the cytosolic non-receptor protein tyrosine kinase is the c-Src protein. All protein-tyrosine kinases show sequence homology over a stretch of 300 amino acids known to encode the kinase domain. The Src subfamily has additional regions of homology not found in the receptor family which includes a short amino-terminal sequence required for the addition of myristic acid (necessary for membrane localisation) and two additional domains named Src homology 2 and 3 (SH-2 and SH-3), required for protein interactions (Cantley *et al.*, 1991). Most members of the src family are highly regulated and exhibit little activity in normal cells in the absence of an activating sequence. This tight regulation is mediated via tyrosine phosphorylation in the carboxy-terminal tail which results in a conformational change which blocks the interaction of effector proteins with the SH-2 and SH-3 domains and also inactivates the enzymatic ability of the kinase (Cooper and Howell, 1993). Inhibiting the phosphorylation of the carboxy domain will therefore remove the inhibition of the Src protein and maintain the kinase in an active form capable of interacting with other proteins. Stimulation of Src occurs via interaction with the receptor tyrosine kinases (reviewed by Parsons and Parsons, 1997).

Studies have shown that up-regulation of the epidermal growth factor receptor confers a poor prognosis and increased invasive ability on tumour cells (Hölting *et al.*, 1995; Jones *et al.*, 1996; Nagane *et al.*, 1996). Previous studies have suggested a role for the up-regulation of the EGF receptor in multidrug resistant cell lines (Meyers *et al.*, 1993 and Shin *et al.*, 1991), although Reeve and co-workers (1990) demonstrated a reduced expression of the EGF receptor in a human large cell lung cancer MDR cell line. More recently, evidence in the literature suggests that the homologous c-*erb*B-2 protein affects chemosensitivity levels of tumour cells *in vivo* and *in vitro* (see Table 1.6.1.2.1). c-*erb*B-2 is well established as a marker of poor prognosis in many tumours, especially breast (DePotter and Schelfhout, 1995).

1.6.1.1 The *c-erb*B-2 Oncogene

EGFR (c-*erb*B1) and c-*erb*B-2 / neu belong to the transmembrane type I receptor tyrosine kinase (RTK) family and the expression of these genes is associated with an invasive/metastatic phenotype and relative insensitivity to conventional treatments (Eccles *et al.*, 1995). Evidence is gathering that these family members may co-operate with each other in their transforming capacity (Bacus *et al.*, 1994). The c-*erb*B-2 gene, localised on the long arm of chromosome 17, encodes a protein of 185 kDa and is identified either as c-*erb*B-2, neu or HER-2. Transfection experiments whereby the gene was introduced into NIH 3T3 cells led to a malignant phenotype with an increased growth potential (DePotter and Schelfhout, 1995). An interesting observation was noted by Imyanitov and co-workers (1993) where amplification of *erb*B-2 was combined with 17p deletions (chromosomal location of p53, the tumour suppressor). The promoter region of the c-*erb*B-2 gene contains sequences that respond to several environmental signals including EGF, TPA, cAMP and retinoic acid (Hudson *et al.*, 1990). EGF stimulates phosphorylation of c-*erb*B-2 and its effect on promoter activity provides a second mechanism whereby EGF may increase c-*erb*B-2 activity. A specific transcription factor, OB2.1, has been found in breast cancer cell lines that over-express the c-*erb*B-2 gene (Hollywood and Hurst, 1993).

The close similarity of c-*erb*B-2 to EGFR has suggested a role for this protein as a growth factor receptor, although evidence exists that its ligand may be a "non-classical" growth factor as expression of c-*erb*B-2 has been found in non-proliferating cells (Bargmann, 1988). The ligand for this receptor kinase remains elusive, although some molecules have been identified which specifically induce phosphorylation of the c-*erb*B-2 protein e.g. Heregulin (the human homologue of NDF (neu differentiation factor) (DePotter and Schelfhout, 1995). Activation of c-*erb*B-2 by Heregulin is cell type-specific (Wallasch *et al.*, 1995) It appears that the interaction of c-*erb*B-2 with NDF involves heterodimerization of the former with another member of the EGFR family e.g. HER3 (Bacus *et al.*, 1994; Wallasach *et al.*, 1995). Disruption of the tightly co-ordinated interplay between members of the type I RTKs may have severe consequences on the cell, a fact borne out by the frequent involvement of such genes in human cancers. NDF induces morphologic changes in cells overexpressing c-*erb*B-2 and correlates with up-regulation of intracellular adhesion molecule 1 (ICAM-1) which plays a role in inflammation and cancer in mammary cells.

Other than its role as a stimulator of mitogenesis in cells, c-*erb*B-2 has also been reported to play a role in the process of active cell death and is believed to act in a manner similar to that of the "dual role" of c-myc (see Section 1.7.1.2) (Harris *et al.*, 1995). Harris *et al.* (1995) reported that over-expression of c-*erb*B-2 (by transfecting with a mutated *neu* gene) resulted in a marked apoptotic response when serum levels were reduced. This was the first report whereby a link was made between receptor tyrosine kinases and apoptosis in epithelial cells. The relevance of this is yet to be fully realised but its importance in breast tissue is noted as apoptosis naturally occurs during involution of the lactating rodent mammary gland following weaning and in the human breast epithelium during the menstrual cycle but no specific mechanisms that induce or inhibit apoptosis in the normal mammary gland have yet been identified.

To add to this "dual role" hypothesis, Dolnikov *et al.* (1995) found that v-*erb*B (the viral counter-part of the human homologue c-*erb*B-2) expression prevents programmed cell death. Increased resistance to chemotherapeutic drugs due to the over-expression of c-*erb*B-2 may be associated with this gene's role in apoptosis.

1.6.1.2 c-*erb*B-2 in Human Tumours and Effect on Response to Chemotherapy

Alteration to c-*erb*B-2 expression has been seen in a wide variety of human tumours. The neu gene was first isolated from a rat neuroblastoma and since then the human homologue has been associated with cancers of the breast (Slamon *et al.*, 1987; van de Vijver *et al.*, 1988; DePotter *et al.*, 1995; Keshgegian *et al.*, 1995; Scorilas *et al.*, 1995; Slamon *et al.*, 1989; Quénel *et al.*, 1995), ovaries (Slamon *et al.*, 1989; Katsaros *et al.*, 1995; Simpson *et al.*, 1995; Wong *et al.*, 1995), stomach (Orita *et al.*, 1997), lung (Tateishi *et al.*, 1991; Rachwal *et al.*, 1995; Diez *et al.*, 1997), bladder and many other adenocarcinomas (Bargmann, 1988). Amplification of c-*erb*B-2 takes place in a different set of tumours to that of the EGF receptor (Bargmann, 1988).

Expression of the protein in many of these tumours correlates with increased metastatic capacity and poor prognosis (Eccles *et al.*, 1995; Tan *et al.*, 1997). Liver metastases and haematogenous metastases are frequently found in c-*erb*B-2-overexpressing patients (DePotter and Schelfhout, 1995). Adding further to the role of c-*erb*B-2 as a poor prognostic marker is the fact that over-expression of c-*erb*B-2 in some human tumours affects resistance to chemotherapy treatment. *In vitro* studies also propose a role for c-*erb*B-2 in drug resistance in tumour cell lines (results of these studies are summarised in

Table 1.6.1.2.1 and discussed in more detail in Section 4.1.1).

Table 1.6.1.2.1 Effect of c-erbB-2 expression on Chemoresistance

Tissue Type	Drug	Reference
breast[T]	CMF	Bacus *et al.*, 1994
breast[T]	CMF	Bitran *et al.*, 1996
breast[C]	cisplatin/5-fluorouracil	Dickson *et al.*, 1990
breast[T]	adriamycin	Muss *et al.*, 1994
breast[C]	adriamycin	Sabbatini *et al.*, 1994
breast[C]	taxol	Yu *et al.*, 1996
non-small cell lung[C]	cisplatin/doxorubicin/etoposide	Zhang and Hung, 1996

[C] *in vitro* studies on human cell lines; [T] *in vivo* studies on human tumour specimens; CMF = cyclophosphamide:methotrexate:5-fluorouracil.

1.6.1.3 c-*erb*B-*2* and its Predictive Role in the Outcome of Breast Cancer

Size of primary tumour, stage of disease at diagnosis, hormonal receptor status and number of axillary lymph nodes involved in the disease are all important prognostic factors in the prognosis of breast cancer. In previous studies it was found that c-*erb*B-2 was a useful prognostic marker coming second to the classical indicators such as number of invaded axillary lymph nodes, receptor status etc. (DePotter and Schelfhout, 1995) and correlated well with many of these classical disease parameters (Slamon *et al.*, 1987). Hormones have been shown to play a role in c-*erb*B-2 regulation in breast cancer as it has been found that oestrogen receptor positive tumours are well differentiated and less invasive whereas c-*erb*B-2 positive tumours are less differentiated and more aggressive (DePotter and Schelfhout, 1995). Antoniotti *et al.* (1994) demonstrated that oestrogen represses the transcription of the c-*erb*B-2 gene and also decreases the level of protein present in the cell. Over-expression of c-*erb*B-2 tyrosine kinase receptor is a common feature of human breast cancer and appears to be an early event in progression of the disease and follows through to metastatic spread. Amplification and overexpression of the c-*erb*B-2 gene occurs in approximately 30% of breast cancers and correlation with earlier relapses and shorter overall survival times has suggested its use as a prognostic marker in breast cancer patients.

1.6.2 Transmission of Membrane Signals

Molecular signals at the plasma membrane are transmitted through to the nucleus via kinases in the cytoplasm as part of a signal transduction cascade. The primary cascade involved in signal transduction initiates signalling through the family of small nucleotide binding proteins, namely small G-proteins, to effector proteins that eventually transmit the signal to the nucleus. The initiating protein in this event is the *ras* protein whose importance was high-lighted when it was discovered that the first transforming genes of human cancers to be isolated were activated cellular counterparts of the normal cellular homologues of viral *ras* oncogenes (Lacal Tronick, 1988).

1.6.2.1 G-proteins

There are two main families of G-proteins, namely the heterotrimeric proteins and the monomeric proteins. Trimeric G-proteins were discovered in the late 1970s as crucial mediaries in signal transduction (Linder and Gilman, 1992). The proteins belong to a large superfamily of proteins that are regulated by Guanine nucleotides and are in their inactive state when bound to GDP but are quickly activated if cellular signals result in the replacement of the GDP molecule with GTP. These proteins are arranged on the cytoplasmic side of the plasma membrane and receive a signalling message from a specific receptor in the plasma membrane and transduce this message to an effector molecule such as adenylyl cyclase which in turn releases a second messenger (e.g. cyclic AMP (c-AMP)) resulting in the biochemical effect expected of the initial signalling message. The G-protein consists of three subunits, namely α, β and γ with the guanine nucleotides binding to the α-subunit. After activation by binding of GTP to the α-subunit, the α-subunit dissociates from the rest of the molecule and diffuses along the plasma membrane and binds to an effector molecule which is then activated. The GTP is then hydrolysed to GDP and the α-subunit reassociates with the rest of the G-protein complex.

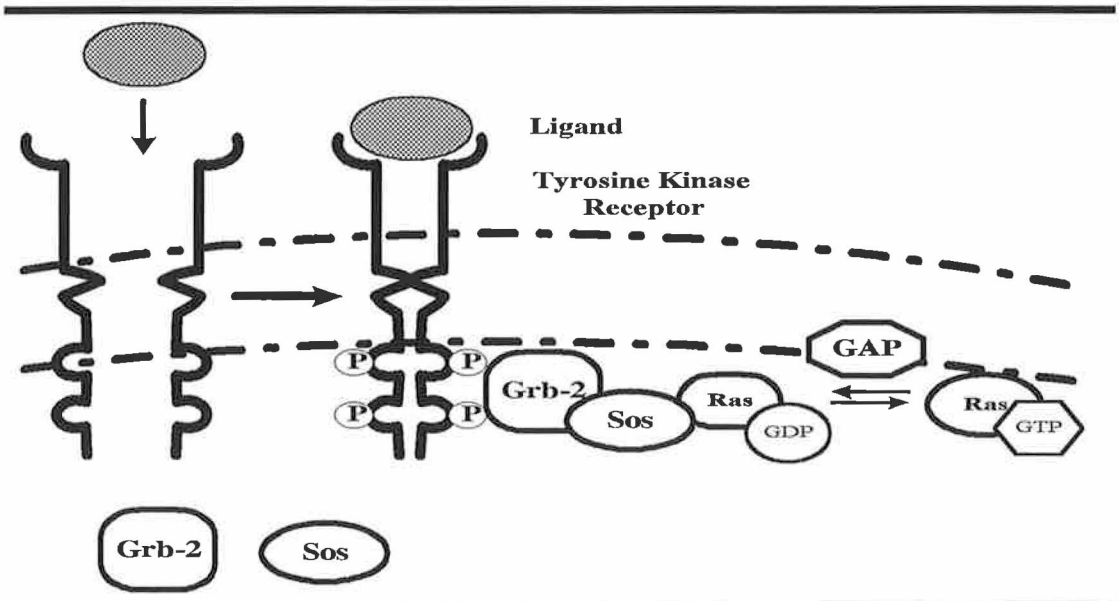
The *ras* family belong to the group of monomeric G-proteins, otherwise known as small G-proteins (also included in this group are the *rho* and *rab* families). The small G-proteins are most homologous to the α-subunit (Lacal Tronick, 1988) of the heterotrimeric proteins being active when bound with GTP and inactive when bound with GDP with the capacity to hydrolyse GTP to GDP. The molecular weight of all family members is 21-25kDa.

The *ras* oncogenes were first reported in 1964 as the Harvey sarcoma virus (H-*ras*) and since then other members have been discovered including Ki-ras and N-ras. In the case of these 21kDa proteins it has been shown that point mutations quite readily convert normal genes to proliferative oncogenes and these point mutations act by either reducing the inherent GTPase activity of the protein or decrease the affinity of the protein for GDP - both result in the continual activation of the protein by increasing the time the protein is bound to GTP. Many proteins have been identified which interact with the small G-proteins including GAP (GTPase Activating Protein), GIP (GTPase Inhibiting Protein) and GDI (GDP Inhibition Protein), among others (Lacal and McCormick, 1993). The normal function of ras in cells is believed to be in the regulation of growth and differentiation.

1.6.2.2 Transduction of Cellular Signal from Ras

The activation of Ras is stimulated via growth factor, such as platelet-derived growth factor or epidermal growth factor, binding to its receptor in the plasma membrane. This results in a conformational change, via phosphorylation, in the growth factor receptor and subsequent activation of the ras protein. Transfer of message from the growth factor receptor to the small G-protein is mediated via adaptor molecules (see Figure 1.6.2.2.1) (for review see Krontiris, 1995). Recruitment of Grb-2 (the growth factor receptor binding protein 2), an adaptor molecule, from the cytosol to the receptor following ligand binding, enlists the assistance of the Sos (son of sevenless) protein. Sos is a nucleotide exchange factor that engages the Ras-GDP protein and helps it exchange GDP for the active GTP-bound protein (reviewed by Krontiris, 1995). Ras GTPase activating protein (GAP) controls the lifetime of signalling by the ras protein by increasing the GTPase activity inherent in ras (it has been suggested that this GAP protein is the neurofibromatosis tumour suppressor gene (NF-1) (Xu *et al.*, 1990)). Other G-protein regulatory proteins have also been identified which down-regulate the activity of the protein, for review see Koelle (1997). As many oncogenes are truncated versions of growth factor receptors which no longer need a stimulus to become active and inhibitors of ras activation are absent in some tumours (e.g. NF-1), hyper-activity of ras is expected in many tumour cells.

Figure 1.6.2.2.1 Stimulation of Ras activity by Growth Factor Binding to Receptor



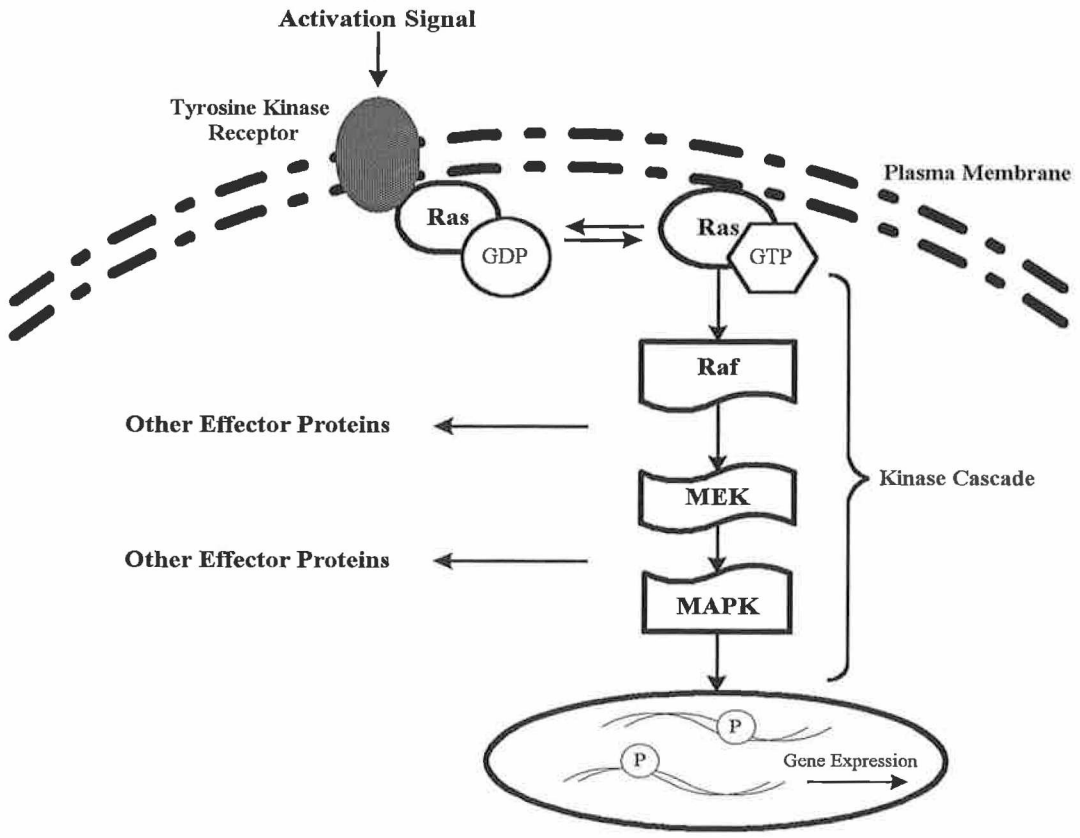
Activation of the tyrosine kinase receptor by ligand binding recruits the cytoplasmic proteins Grb-2 and Sos to the plasma membrane where they induce the Ras protein to exchange its bound GDP for GTP. GAP exerts its GTPase activity and converts the active GTP-bound Ras to its inactive GDP-bound form.

Growth factor stimulated Ras proteins interact on the cytosolic side of the plasma membrane yet it is in the nucleus that growth factors induce alterations in gene expression. To forward cell membrane messages, Ras begins a cytoplasmic signalling to the serine/threonine kinase protein, Raf. Three distinct members of the Raf family exists, namely, c-Raf-1, A-Raf and B-Raf which share three highly conserved regions (CR1-3) (Daum *et al.*, 1994). The role of Ras in the activation of c-Raf-1 is in the translocation of c-Raf-1 from the cytosol to the plasma membrane (Leevers, 1994). Translocation to the plasma membrane is not sufficient to activate c-Raf-1 and it has been found that phosphorylation on tyrosine residues is essential in this process. This phosphorylation can be carried out by the Src protein, among others (for review see de Vries *et al.*, 1996 and Morrison and Cutler, 1997). Recruitment of c-Raf-1 to the plasma membrane activates a number of kinases that eventually activate the transcription of a number of genes and phosphorylation of many proteins involved in cell proliferation. The substrate of the activated c-Raf-1 protein is the threonine/tyrosine protein kinase MAPK/ERK Kinase 1/2 (MEK1/2) that is activated by serine phosphorylation due to the kinase activity of c-Raf-1 (MEK kinase (MEKK)). MEK1 in turn activates the cytosolic serine/threonine Mitogen Activated Protein Kinases (MAPKs) by phosphorylation on threonine and tyrosine residues. De-phosphorylation of MAPKs is carried out by dual-specificity phosphatases

(DSPs) (reviewed in Neel and Tonks, 1997), such as MKP-1 (MAPK phosphatase 1). Mitogen activated protein kinases are constitutively activated in ras-transformed cell lines (Woodgett, 1992). MAPKs phosphorylate multiple proteins, including Sos and c-Raf-1, suggesting the existence of a negative feed-back loop (Cherniack *et al.*, 1994; Davis, 1993). Sustained activation of MAPKs induces translocation of the cytosolic kinases to the nucleus (Marshall, 1995) where nuclear transcription target proteins such as c-Myc, c-Jun, c-Fos and p53 reside (see Figure 1.6.2.2.2).

Cross-talk between the MAPK pathway and other signalling pathways occurs with integration occuring at many levels. A likely benefit of this cross-talk is an increase in the number of targets for positive and negative regulation of many cellular signals. The oncogenic pathway leading from ras therefore floods the cell with growth stimulatory signals and induces proliferation.

Figure 1.6.2.2.2 Signalling Cascade of Ras Protein



MAPK = Mitogen Activated Protein Kinase; P = Phosphorylation; MEK = MAPK/ERK Kinase

1.6.2.3 The Role of *Ras* in Survival Signalling and Proliferation

Wyllie *et al.* (1987) suggested a role for c-H-*ras* in survival signalling when the first studies of the effects of oncogenes on apoptosis were carried out; in these experiments it was surmised that spontaneous apoptosis was suppressed by the expression of c-H-*ras*. More recent evidence supporting a role for *ras* in survival signalling comes from experimental evidence by Kinoshita *et al.* (1995) which showed that *ras* rapidly up-regulates the survival factors *bcl-*x_L and *bcl-2* (see Section 1.7.1) but not the antagonistic homologue *bax* in haematopoietic cells exposed to interleukin-3 and GM-CSF. Chen and Faller (1995) found that activated Ras was a potent inducer of apoptosis when combined with down-regulation of protein kinase C (conflicting role in apoptosis process (Lucas and Sánchez-Margalet, 1995)) activity but could be abolished by co-expression of bcl-2.
Ras activity is also thought to be necessary in cell cycle progression, including G_0 to G_1 and G_2 to M transitions possibly via c-Myc activation. Another interesting observation in the path leading to oncogenesis is the recent finding (G.Breier, pers.comm.) where v-H-*ras* was found to increase the expression of the VEGF (vascular endothelial growth factor) gene thereby showing v-H-*ras* not only to have a direct effect on proliferation but also to play an indirect role by stimulating tumour angiogenesis as a result of up-regulation of VEGF mRNA.

1.6.2.4 Ras Expression in Human Tumours and Effect on Prognosis

Ras has demanded much attention as it has been found expressed in 30% of all human tumours. It is believed to be important in the initial steps leading to carcinogenesis (often detected in benign disease e.g. colon, thyroid and prostate (Bushman *et al.*, 1995) and results in malignancy when co-expressed with other oncogenes such as c-myc (Hunter, 1991). Mutations in K-ras are seen in 40% of colon tumours while H-*ras* mutations are associated with poor prognosis in cervical cancer. Elevated ras expression is associated with the pre-malignant "polyp" stage of colon cancer. Increased ras expression has also been seen in the progression of stomach, lung (K-ras mutations may be associated with the appearance of the malignant phenotype) (Sugio *et al.*, 1994) and breast cancer and correlates with disease recurrence in the latter (Field and Spandidos, 1990). Overexpression of members of the ras family occurs frequently (59%) in squamous cell carcinoma of the head and neck and McDonald *et al.* (1994) found this expression to play

an important part in the latter stages of tumourigenesis.

The role of Ras family members in clinical drug resistance has not been addressed. A number of *in vitro* studies have been carried out on H-*ras* and drug resistance in cancer cell lines, however (see Table 1.6.2.4.1; results are discussed in Section 4.1.2).

Table 1.6.2.4.1 Effect of H-ras Expression on Chemoresistance

Cell Type	Drug	Reference
rat liver	adriamycin/vinblastine	Burt *et al.*, 1988
NIH3T3 fibroblasts	cisplatin	Isonishi *et al.*, 1991
NIH3T3 fibroblasts	alkylating agents	Niimi *et al.*, 1991
human breast	adriamycin	Sabbatini *et al.*, 1994
NIH3T3 fibroblasts	cisplatin	Sklar, 1988

1.6.3 Orchestration of Cellular Signals at the Nucleus

To change the growth rate of cells, stable changes in cellular gene expression must occur and, therefore, gene transcription can be considered an ultimate target of oncogene activity. Those genes that control the expression of genes involved in proliferation bind DNA and are likely to function as transcription factors. Many oncogenic transcription factors appear to be constitutively activated forms of their normal cellular counterparts (Hunter, 1991).

The transcription factor AP-1 (HeLa cell activator protein 1) binds to a short sequence of DNA (namely TGACTCA) found in many human gene promoters to activate (or sometimes repress) expression of these genes. AP-1 is a known target of MAP kinase signalling pathways (reviewed by Whitmarsh and Davis, 1996). The complex consists of a variety of polypeptides including the Fos, Jun and activating transcription factor 2 (ATF2) proteins. The complex can act either as Jun (v-Jun, c-Jun, JunB, JunD) or ATF (ATF3, B-ATF) homo-dimers or Jun/ATF/Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) heterodimers but not as Fos homo-dimers. These genes are "B-Zip" proteins containing a basic DNA-binding region and a leucine Zipper region that allows both proteins to interact with each other. Both genes are known as "immediate early genes" and are induced by a wide variety of mitogens in different cell types. Much interest in the AP-1 complex and its relevance to drug resistance comes from the realisation that the hall-mark protein of drug resistance, P-glycoprotein, contains an AP-1 transcription site in its gene promoter (Teeter *et al.*, 1991).

1.6.3.1 c-Fos as Proliferation Inducer

c-Fos was originally recognised as a viral oncogene (Finkel-Biskis-Jinkins murine osteosaroma virus; FBJ-MuSV) that induced osteogenic sarcoma *in vivo* and transformed fibroblasts *in vitro*. The cellular homologue of FBJ-MuSV was identified in human cells and the human c-*fos* gene was found to encode a protein of 380 amino acids (van Straaten *et al.*, 1983).

The basal level of c-fos expression is very low in most cells, however, treatment with a variety of agents, including serum and chemotherapeutic drugs, leads to dramatic, but transient, induction of the gene as it acts to convert short-term signals occurring within a few minutes of the primary stimulus to long-term cellular responses that occur over hours

and days (Curran, 1988). In the search to find a common function for c-Fos a number of roles have been proposed for the gene in cell-cycle regulation, cellular proliferation and differentiation (Curran, 1988). Greenberg and Ziff (1984) found that stimulation of BALB/c-3T3 cells with serum, PDGF or TPA resulted in a transient increase in the transcription of c-*fos* during the transition from G_0 to G_1; a 15-fold increase was noted very soon after stimulation with a return to basal levels within 30min; increased transcripts are apparent as early as five minutes after serum addition, peak after ten minutes and begin to decrease by fifteen minutes. The authors concluded that continuous expression of c-fos might prevent a cell from ever entering a quiescent state resulting in a population of continually dividing cells. Anti-sense studies revealed that a reduction in c-fos mRNA levels resulted in an increase in doubling time of 3T3 cells and that the c-fos gene product appeared to have a required role in normal cell division (Holt *et al.*, 1986). Balsalobre and Jolicoeur (1995) found that c-Fos proteins can act as negative regulators of cell growth in some cell types, however, independently of the c-fos transforming pathway. It has been found that c-Fos is expressed immediately prior to apoptosis (and together with c-Jun may represent an important early event in the activation of the genetic cascade leading to cell death) (Colotta *et al.*, 1992) and induces apoptosis when continuously expressed (Smeyne *et al.*, 1993). It is for this reason that c-fos has been granted the title "harbinger of death". Continuous expression of Fos begins hours or days before cell death and is a hallmark of terminal differentiation and death in skin, hair follicle and bone. The part Fos has to play in cell death is as yet hypothetical but (i) it may have a primary role in the process of cell death; (ii) it may be necessary for apoptosis in only some circumstances; or (iii) it may be that a signal leading to cell death co-incidentally induces c-*fos*.

1.6.3.2 The c-Jun Oncogene

The v-*jun* oncogene was isolated from avian sarcoma virus 17 (ASV-17) which induces fibrosarcomas in chickens and can transform chick embryo fibroblasts. Analysis revealed the C-terminus to be similar to the DNA-binding domain of yeast transcriptional activator GCN4. The half life of the *jun* mRNA is very short (perhaps explaining the absence of introns in this gene) thus a signal leading to increased *jun* transcription can result in an increase in AP-1 activity rapidly and the low stability of the mRNA could be responsible for the correct termination of such a signal (Hattori *et al.*, 1988). c-Jun is also an

"immediate early gene" that responds to many cellular stimuli and therefore has an important role to play in mitogenesis. The induction of *fos* and *jun* is not always co-ordinated and usually the c-*jun* response is more persistent.

c-Jun is capable of transforming immortalized rat fibroblasts in co-operation with activated c-H-Ras (Binétruy *et al.*, 1991) independent of c-*fos* activity. Both oncogenes work in a common pathway whereby the upstream Ras stimulates c-Jun (see Figure 1.4.3.5.1) by inducing its phosphorylation through the activation of JNK. UV, a potent tumour promoting agent, is a very effective inducer of c-*jun* (via JNK) transcription but only modestly induces c-*fos*; this is one way in which c-*jun* can act as a mediator of tumour promotion by activating genes involved in proliferation (*jun* as transcription factor) in response to DNA damage such as UV light. JunB, a member of the Jun family, antagonizes the transforming properties of c-Jun (Schütte *et al.*, 1989).

Evidence has been gathering in recent years that c-Jun has a role to play in the apoptotic pathway. c-Jun expression was induced in cultured neurons undergoing β-Amyloid apoptosis and its expression was not induced in cells resistant to this type of apoptosis (Anderson, 1995); a possible role for JNK in gamma-irradiation induced apoptosis has also been proposed (Chen *et al.*, 1996).

1.6.3.3 AP-1 Target Genes

Binding of the AP-1 complex to many gene promoters activates transcription of that gene. Collagenase, human metallothionein IIA and transin are all activated by binding of the AP-1 complex to its promoter recognition sequence; the osteocalcin gene is, however, repressed by such binding. An AP-1 site has been identified in the *mdr1* promoter region and has been shown to be necessary for correct functioning of this gene (Teeter *et al.*, 1991). Topoisomerase I has also been found to contain an AP-1 binding site (Kunze *et al.*, 1990) in its promoter and a putative AP-1 site lies upstream of the translational initiation site of the dTMP synthase gene (Takeishi *et al.*, 1989).

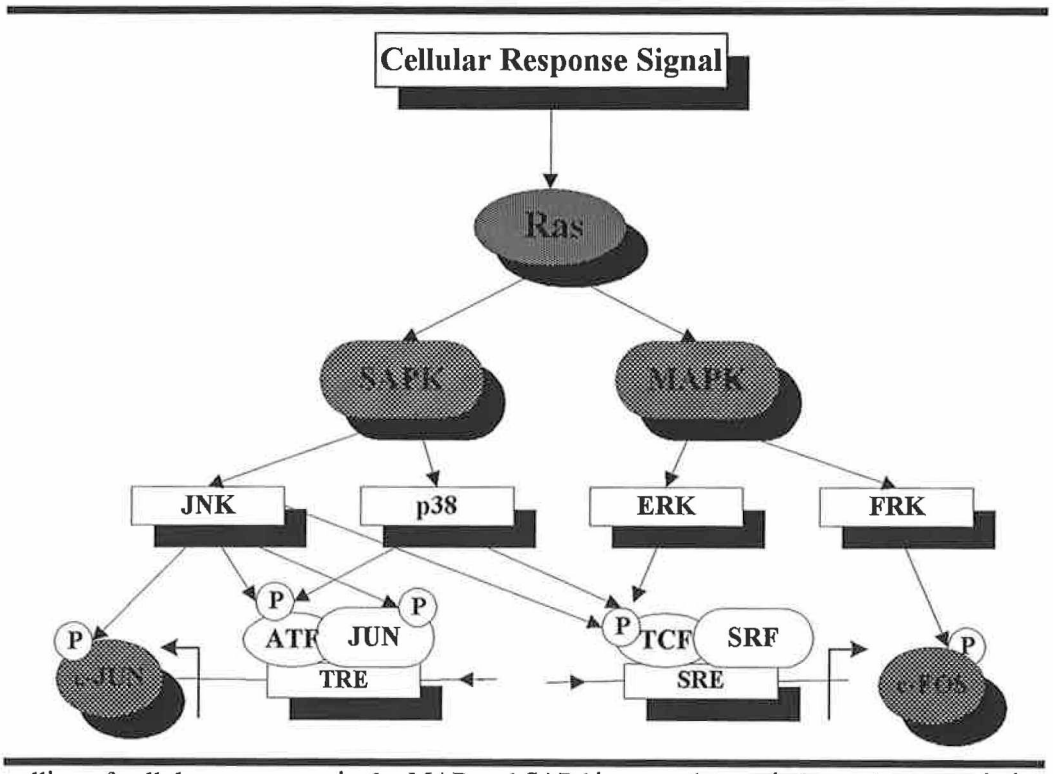
1.6.3.4 AP-1 as a Cellular Response Element

The c-*fos* promoter is activated by a variety of signals (e.g. growth factors, cytokines, UV-light and other DNA damaging agents) via signal transduction pathways involving the Ras family through MAP-K (mitogen activated protein kinase) and eventually activation of

the promoter at the serum response element (SRE) (Janknecht, 1995). c-Jun is activated by many cellular stimuli as well but regulation of its transcription unit is not as complex as that of c-fos. This kinase cascade is a common pathway shared by cell proliferation and stress-response signalling.

At least four different MAP-K proteins have been identified which activate the AP-1 complex, each with their own substrate specificities (Deng and Karin, 1994; for review see Karin, 1995). ERK (extracellular signal regulated protein kinase) is activated by growth factors, such as EGF and PDGF, and phorbol esters; FRK (Fos regulated kinase) is activated by growth factors alone; and JNK (c-Jun N-terminal kinase) and p38 are activated by UV, TNF and very weakly by growth factors and other cellular shocks. In response to DNA damaging agents, such as ionising radiation or cisplatin, JNK MAP kinases are activated by a pathway dependent on the non-receptor tyrosine kinase, c-Abl (Kharbanda *et al.*, 1995). These kinases act by phosphorylating either the c-Fos or c-Jun proteins or proteins in the transcription unit of either gene (see Figure 1.6.3.4.1).

Figure 1.6.3.4.1 Cellular Response Signalling Cascade of AP-1



Signalling of cellular messages via the MAP and SAP kinase pathways induces the transcription of the AP-1 complex proteins, c-Fos and c-Jun, and activates subsequent transcription of AP-1 target genes. SAPK = Stress Activated Protein Kinase; MAPK = Mitogen Activated Protein Kinase; JNK = c-Jun N-terminal Kinase; ERK = Extracellular Signal Regulated Protein Kinase; FRK = Fos Regulated Kinase; ATF = Activating Transcription Factor; TRE = TPA-Response Element; TCF = Ternary Complex Factor; SRF = Serum Response Factor; SRE = Serum Response Element:

1.6.3.5 AP-1 Expression in Human Tumours as a Marker of Poor Prognosis

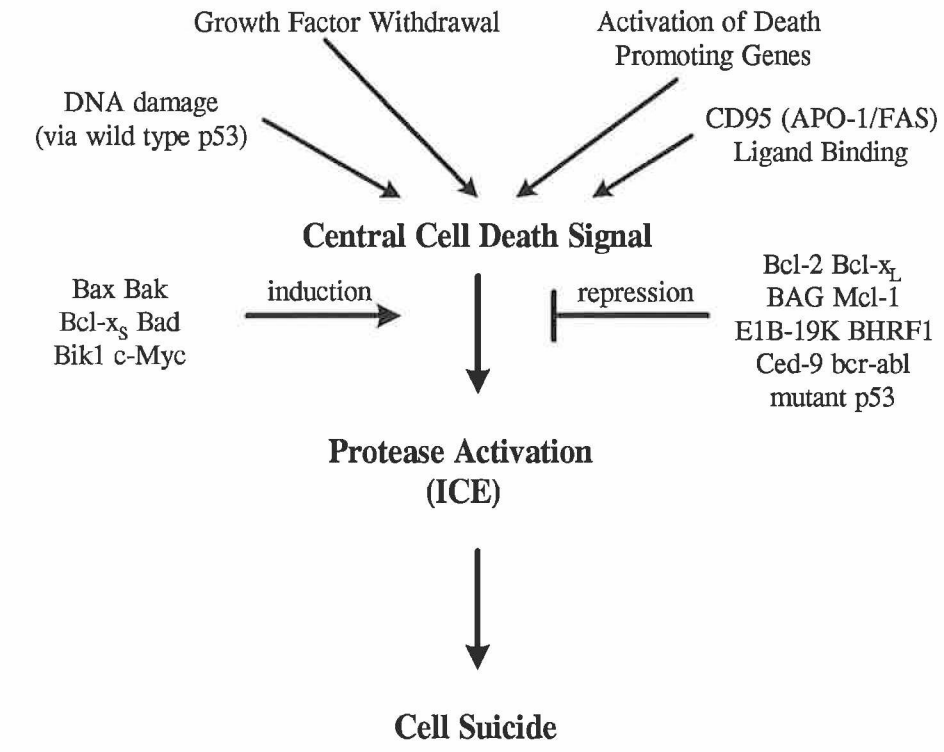
Both c-*fos* and c-*jun* were identified in osteosarcomas originally (Curran, 1988). Expression of both proteins has also been identified in breast cancer (Tiniakos *et al.*, 1994; Walker and Cowl, 1991) where Tiniakos *et al.* (1994) found 90% of all breast carcinomas studied to have c-Jun-specific nuclear staining. Down-regulation of these genes was hypothesised to be involved in the pathogenesis of lung cancer in a study by Levin *et al.* (1995). A significant relationship was seen between P-glycoprotein expression in human lung carcinomas and c-Fos and c-Jun expression (Volm, 1993). Volm and co-workers (1993) also found a significant relationship between c-*jun* expression in primary human small lung cancer and incidence of metastases, no correlation with c-*fos* expression was seen, however. Magrisso and co-workers (1993) found that c-Jun and c-Fos were important parameters in colorectal tumorigenesis.

A role for c-*jun* and c-*fos* in chemoresistance, especially to cisplatin, has been shown *in vivo* and *in vitro*. Zhao and co-workers (1995) demonstrated the up-regulation of c-*jun* by cisplatin in sensitive melanoma cell lines but less so in resistant variants. *In vivo* studies in head-neck tumours agreed with this finding (Los *et al.*, 1995). Up-regulation of the c-Fos protein is associated with cisplatin resistance in some cell lines (Funato *et al.*, 1992; Scanlon *et al.*, 1991) and human tumours (Kashani-Sabet *et al.*, 1990). The role c-Fos and c-Jun in drug resistance is discussed in greater detail in Section 4.1.3.

1.7 Suicide Genes and their role in Apoptosis

Apoptosis is regulated by many genes in normal tissues, some of which are normal cellular counterparts of genes influential in the transformation of normal to malignant cells. Human homologues of the *Caenorhabditis elegans ced* genes have been discovered and have added a wealth of knowledge to our understanding of cell suicide. The main players in the apoptotic pathway are the Bcl-2 family of genes, p53, ICE, CD95 (APO-1 /Fas), Bcr-abl and c-Myc (see Figure 1.7.1). Many oncogenes, described previously, have also been found to influence the cell's capacity to undergo apoptosis. These proteins can influence cell viability either by promoting or inhibiting cell death and it is the balance or interplay of this myriad of proteins which influences a cell's susceptibility to death. Over-expression of apoptosis promoting proteins will tip the balance in favour of cell death whereas over-expression of apoptosis suppressing proteins will favour survival.

Figure 1.7.1 Molecular Regulation of the Apoptotic Pathway



1.7.1 Bcl-2 Gene Family

The study of the Bcl-2 family as genes that influence cell viability and death independent of cell division represents a vast area of research in the whole field of cancer biology. Tsujimoto and Croce (1986) discovered the *bcl-2* (B-cell leukemia/lymphoma 2) gene through studies of chromosomal rearrangements frequently observed in the great majority of human hematopoietic malignancies. In most cases of follicular lymphoma a t(14;18)(q32;q21) translocation was observed and the *bcl-2* gene was discovered adjacent to this break-point. This head-to-tail juxtaposition of *bcl-2* (from chromosome 18q21) to the immunoglobulin heavy chain locus (at chromosome 14q32) is associated with an increase in expression of *bcl-2* believed to be due to the presence of enhancers in the immunoglobulin heavy (IgH) gene (Craig, 1995; Tsujimoto and Croce, 1986). In most follicular lymphomas studied the chromosome 18 break-points occur at the unusually long (5.4kb) untranslated 3' end of the *bcl-2* gene or are 3' of the involved *bcl-2* gene thereby preventing any interruption to the coding region of the bcl-2 gene.

Analysis by Tsujimoto and Croce (1986) indicated that the *bcl-2* gene consists of at least two exons that produce three transcripts of 8.5kb, 5.5kb and 3.5kb long mRNAs. The *bcl-2* cDNA sequence was analysed, from which two protein products were postulated, namely, *bcl-2*α (239a.a.) and *bcl-2*β (205a.a.), that differ only at their carboxyl terminus. The *bcl-2*α mRNA encodes a 26kDa protein that is homologous to the hypothetical Epstein-Barr virus protein BHFR1 (Cleary *et al.*, 1986). Most of the *bcl-2* ORF is encoded within the 5' exon. Structural analysis yielded no apparent transmembrane hydrophobic segment.

Studies of *bcl-2* knock-out mice highlight the problems associated with a dysfunctional apoptotic pathway and the role Bcl-2 has to play in it. Mice lacking Bcl-2 complete embryonic development and appear normal during the first week after birth. Subsequently, growth is retarded and small ears and immature facial features are observed. Premature death occurred and severe apoptosis in the kidney, thymus and spleen was seen with an almost complete loss of lymphocytes (Veis *et al.*, 1993).

1.7.1.1 Cellular Localisation and Function of Bcl-2

bcl-2 has a unique gene sequence without substantial homology to other proto-oncogene products. It lacks an N-terminal hydrophobic signal sequence, or obvious signal motifs,

that would target it to the endoplasmic reticulum or plasma membrane. *bcl-2* does possess a C-terminal hydrophobic 19 amino acid stretch similar to a membrane spanning segment, however, (Hockenbery *et al.*, 1990) and experiments showed that the protein localised to the inner mitochondrial membrane, a novel location for proteins with an oncogenic role. The localisation of Bcl-2 to the inner mitochondrial membrane where metabolic functions such as oxidative phosphorylation and electron and metabolite functions are carried out could mean a role for these processes in the cell's survival mechanism with a prominent role for Bcl-2 in a B-cell survival pathway. Susin and co-workers (1996) proposed that Bcl-2 functions as a repressor of apoptosis by favouring the retention of an apoptogenic protease, apoptosis-inducing factor (AIF), in the mitochondria. However, controversy over the subcellular localisation of Bcl-2 arose when the protein was discovered in other cellular membranes; indeed, a study by Jacobson *et al.* (1993) using a human fibroblast cell line completely depleted of mitochondrial DNA was still protected from apoptosis (induced by serum withdrawal) by the overexpression of Bcl-2, suggesting that neither apoptosis nor the protective effect of Bcl-2 depends on mitochondrial respiration. This study revealed the presence of the Bcl-2 protein bound to the continuous nuclear and endoplasmic reticulum membrane system. The Bcl-2 protein has also been found in the outer mitochondrial membrane, oriented so that the amino terminus faces the cytoplasm (Nakai *et al.*, 1993; Nguyen *et al.*, 1993). Cell-free systems studied by Janiak *et al.* (1994) suggest that Bcl-2 localises to the cytoplasmic side of multiple membranes, both *in vitro* and in human leukemic cells.

Although the Bcl-2 protein is found in different compartments the actual site(s) at which it exerts its function remains unknown. Results found by Nguyen *et al.* (1994) whereby deletion of the signal anchor sequence rendered Bcl-2 cytosolic and impaired its ability to prevent apoptosis in KB cells is consistent with a model in which the transmembrane segment contributes to the function of Bcl-2 by targeting and anchoring the protein to strategic membrane locations in the cell. Concentration of Bcl-2 at these sites may contribute to its proposed role as a regulator or component of an anti-oxidant pathway.
The idea that Bcl-2 is involved in an anti-oxidant pathway (perhaps to counter apoptotic death caused by cellular accumultion of reactive oxygen species) is consistent with the multiple membrane localistions of the protein because reactive oxygens are produced in mitochondria and at the nuclear envelope and endoplasmic reticulum (Akao *et al.*, 1994).
The fact that Bcl-2 can interfere with a diverse set of apoptotic stimuli implies that it

functions at a downstream step of what is ultimately a convergent and common mechanism of cell death (Nguyen *et al.*, 1994).

A role for Bcl-2 in cell survival was first proposed by Vaux *et al.* (1988) when it was noted that stable transfer of *bcl-2* expression vectors into immature pre-B-cells resulted in prolonged cell survival in the absence of IL-3 (known to maintain haematopoietic cell survival *in vitro* by preventing apoptosis), but without cell proliferation. The death suppressor activity of Bcl-2 has been extensively studied in neuronal cell death by Bredesen (1995). The role of Bcl-2 in Programmed Cell Death has been reviewed by Reed (1994); there is also some evidence to suggest that Bcl-2 can block necrosis (passive cell death) as well (Kane *et al.*, 1993). Evidence has been found to suggest that Bcl-2 functions as an apoptosis repressing gene primarily by inhibiting the cell death function of pro-apoptotic proteins such as Bax (Hunter and Parslow, 1996). The expression of Bcl-2 is not protective in all situations of cell death induction such as cell death induced, in some situations, by binding to the CD95 (APO-1/Fas) ligand (Nagata and Suda, 1995), implying that Bcl-2 dependent and independent mechanisms of apoptosis exist.

It has been suggested that Bcl-2 inhibits apoptosis by altering Ca^{2+} fluxes through intracellular organelles, a logical assumption as mitochondria and the endoplasmic reticulum are both sites of calcium storage, and also a calcium-dependent nuclease may be involved in the DNA fragmentation seen in apoptosis. As calcium can function as a second messenger in intracellular signalling this may be how Bcl-2 exerts its effect on cell death signal transduction pathways (Craig, 1995). It has been shown by Kinoshita and co-workers (1995) that activation of the Ras pathway induces the expression of Bcl-2 and Bcl-x_L but not Bax. Further work (Blagosklonny *et al.*, 1996) demonstrated that, following taxol treatment, c-Raf1 induced the phosphorylation of Bcl-2 and the subsequent induction of apoptosis. Taxol affects the integrity of microtubules (see Section 1.2.3) and analysis of other drugs with similar mode of action was also shown to phosphorylate Bcl-2 (in G_2-M) (Haldar *et al.*, 1997) suggesting that the normal physiological role of Bcl-2 is as a "guardian" of microtubule integrity. It has also been shown that Bcl-2 interacts with the G-protein, R-Ras, and induces apoptosis (Fernandez-Sarabla *et al.*, 1993). Cell cycle studies of the effect of over-expression of Bcl-2 suggest that expression of the protein is consistently associated with a retardation of cell proliferation due to a prolongation of the G_1 phase (Borner, 1996; Mazel *et al.*, 1996) and that Bcl-2 expression might exert its inhibition of apoptosis by regulating the kinetics of cell cycle progression at a critical

control point.

The Bcl-2 protein may also play a role in transport across membranes or it may interfere with the function or activation of proteins involved in the delivery of cell death signals i.e. Bcl-2 can inhibit p53 mediated apoptosis (for review see Núñez *et al.*, 1994).

1.7.1.2 The Bcl-2 Family of Genes

A family of Bcl-2 related proteins have been discovered which share three highly conserved stretches in their carboxyl regions. These conserved regions have been named the bcl homology domain 1 (BH1), bcl homology domain 2 (BH2) (Yin *et al.*, 1994) and bcl homology domain 3 (BH3) (Chittenden *et al.*, 1995) have been shown to play a role in the interaction of members of the Bcl-2 family.

A number of different genes have been discovered recently as members of this gene group, namely, Bcl-x (Boise *et al.*, 1993), Mcl-1 (Kozopas *et al.*, 1993), Bax (Oltvai *et al.*, 1993), Bak (Chittenden *et al.*, 1995), BAG-1 (Takayama *et al.*, 1995), Bad (Yang *et al.*, 1995), Bik (Boyd *et* al., 1995), Bfl-1 (Choi *et al.*, 1995) and A-1 (Karsan *et al.*, 1996). These proteins can homo- or hetero-dimerise and the balance of the pro- or anti-apoptotic effect determines whether a cell should live or die (for review see Farrow and Brown, 1996; Sedlak *et al.*, 1995).

Bcl-2 which represses cell death has been found to associate *in vivo* with a Bcl-2-associated X protein, namely Bax (Oltvai *et al.*, 1993). Bax was identified as a 21kDa protein with extreme amino acid homology with Bcl-2, and which forms homodimers or heterodimers with Bcl-2 *in vivo*. When Bax predominates apoptosis is increased and the repressive activity of Bcl-2 is countered. The gene encoding Bax consists of six exons all within a 4.5kb region and all six exons contribute information for protein coding. Three alternative transcripts of the gene have been identified - baxα, β and γ - baxα codes for the 21kDa protein product of the gene. RNA splicing may act as a differential regulator of Bax activity and localisation with baxβ providing an additional level of regulation; no function for baxγ has been identified.

Bax accelerates apoptotic cell death only following a death signal, this implies that the inherent ratio of Bax to Bcl-2 determines susceptibility to death following an apoptotic stimulus. The association between Bax and Bcl-2 has been described as "hand-to-hand combat" (Korsmeyer,1995) with Bax promoting cell death while Bcl-2 opposes it. Bcl-2

and Bax are associated in cells even before a death signal is received. When Bcl-2 is in excess the cell is protected from death (Bcl-2 competes for normal state of Bax homodimers forming Bcl-2/Bax heterodimers), however, when Bax is in excess the cell is susceptible to apoptosis (Bax homodimers predominate). The Bcl-2 to Bax ratio therefore acts as a "cell-autonomous rheostat" that predetermines a cell's life or death response to an apoptotic stimulus. In mutagenesis studies carried out by Yin *et al.* (1994) it was found that the same amino acids in the BH1 and 2 domains required for Bcl-2 function were also necessary for heterodimerization with Bax. Selective mutations enabled Bcl-2 to homodimerize but these homodimers were insufficient to protect cells from death, concluding that Bcl-2 must bind Bax to act as a death repressor. A "suicide" peptide sequence in *bax* was inserted into the *bcl-2* gene and it was found that the Bcl-2 inhibitor was converted to an activator of cell death (Hunter and Parslow, 1996). These results demonstrate that Bax is an active inducer of the cell death pathway and that the function of Bcl-2 in repressing the apoptotic pathway may be primarily to suppress the activity of Bax.

The *bax* promoter has four motifs with homology to consensus p53-binding sites. The wild type but not the mutant p53 transactivates the *bax* gene promoter. Bax has been described as a p53 primary response gene involved in a p53 regulated pathway for induction of apoposis (Miyashita and Reed, 1995). Experiments described by Miyashita and Reed (1995) showed that increasing p53 levels resulted in an increased *bax* mRNA and protein levels and a simultaneous decrease in the steady state levels of *bcl-2* mRNA and protein. p53 mediated elevations in Bax protein levels would render cells more susceptible to apoptotic cell death as lowering the Bcl-2:Bax ratio renders cells relatively more sensitive to induction of cell death by cytotoxic anticancer drugs. The Bax promoter region has also been identified as having binding sites for Myc, Mad, Max and Mxi-1.

Boise *et al.* (1993) isolated the bcl-x gene from gene libraries by hybridization with *a bcl-2* probe. Two distinct *bcl-x* mRNAs can form as a result of alternative splicing - the long form *bcl-*x_L (233 amino acids) and the short form *bcl-*x_S (170 amino acids) - the shorter form does not contain BH1 and BH2 domains.

Bcl-x can function as a Bcl-2-independent regulator of apoptosis but both transcripts work in a reciprocal fashion to one another i.e. X_L confers resistance to apoptosis whereas X_S, in a dominant manner, prevents increased levels of Bcl-2 from inducing resistance to apoptosis by acting in one of two proposed ways :

(i) X_S can form an inactive heteromeric complex with Bcl-2

(ii) X_S or X_L may bind directly to the same downstream regulators of apoptosis as Bcl-2.

There are close similarities between Bcl-x_L and the functional form of Bcl-2 (Bcl-2α) and Bcl-x_L functions in preventing apoptotic cell death at least as well, and under some circumstances better, than Bcl-2. Bcl-x_L knock-out mice exhibit massive cell death in the nervous and haematopoietic system (Motoyama *et al.*, 1995) unlike Bcl-2 knock-outs that die from polycystic kidney disease (Veis *et al.*, 1993) implying different roles for the two anti-apoptotic genes. The residues required for Bcl-x_L function are not identical to those required for Bcl-2 function. It has been suggested that Bcl-x_L also requires Bax (which is up-regulated by p53) to exert its repressive function, however, a study by Cheng and co-workers (1996) demonstrated that a Bax-independent inhibition of apoptosis by Bcl-x_L does exist. Co-operation of Bcl-x_L over-expression and loss of p53 was found to overcome a cell cycle check-point induced by mitotic spindle damage and result in genomic instability by allowing the accumulation of tetraploid cells (Minn *et al.*, 1996).

X-ray crystallography and NMR studies of the human Bcl-x_L protein revealed a strong similarity to the membrane translocation domain of bacterial toxins such as diphtheria toxin and the colcicins (Muchmore *et al.*, 1996). The diphtheria-toxin translocation domain is thought to dimerise and form a pH-dependent membrane pore, suggesting that the Bcl-2 proteins may also form pores in the cytoplasmic membranes where they localise. Minn and co-workers (1997) have now shown that Bcl-x_L does indeed form an ion-conducting pore in either synthetic lipid vesicles or planar lipid bilayers. The channel is pH sensitive and becomes cation-selective at physiological pH implying that Bcl-x_L may maintain cell survival by regulating the permeability of the intracellular membranes in which it is located.

Both Bcl-x_L and Bcl-x_S can form heterodimers and/or multimers with Bcl-2 (Sato *et al.*, 1994). The ratio of Bcl-x_L to Bcl-x_S in specific tissues is very important especially in hormone-dependent tissues (Krajewski *et al.*, 1994a). A study by Heermeier and co-workers (1996) on apoptosis in involuting mammary epithelial cells demonstrated that Bcl-x_S could facilitate apoptosis even when Bcl-x_L was present in excess.

1.7.1.3 Expression of Bcl-2 Family Members in Normal and Malignant Tissue

It is known that apoptosis regulates cell populations during embryonic growth and occurs widely in normal tissues to maintain a stable equilibrium with cell proliferation. A report has recently been published (Staunton and Gaffney, 1995) which assesses the extent of apoptosis in a wide range of different human tumour types. Results found suggest that each tumour type has a characteristic apoptotic index that reflects innate tumour cell susceptibility to undergo apoptosis. A high apoptotic index was found in rapidly growing tumours known to respond to chemotherapy (small cell carcinoma, Burkitt's lymphoma) and low apoptotic index in more aggressive neoplasms (follicular lymphoma).

To decipher the role Bcl-2 plays in tumourigenesis and apoptosis *in vivo*, many studies have been carried out to determine the distribution of Bcl-2 in human tissues. During differentiation, lymphoid cells undergo selection with only a small fraction of maturing cells surviving, the majority undergo apoptosis prior to terminal differentiation. These mature cells were found to be abundant in Bcl-2 protein (Hockenbery *et al.*, 1991; Núñez *et al.*, 1994). The role of Bcl-2 in normal differentiation, therefore, relates to the promotion of cell viability and it is to be expected then that this protein should be expressed in a variety of human lymphomas. The *bcl-2* gene was first discovered in human follicular lymphoma (Tsujimoto and Croce,1986), one of the most common human hematopoietic malignancies. The gene is also expressed in Hodgkin's disease (Lorenzen *et al.*, 1992; Corbally *et al.*, 1994).

Bcl-2's distribution profile is not limited to hematopoietic cells or tumours, indeed, the protein (or gene product) has been detected in a wide variety of tissues including breast, lung and neuroblastomas, among others (Hockenbery *et al.*, 1991).
Bcl-2 is expressed in normal breast epithelium, therefore, a tumour derived from normal breast epithelial cells might also be expected to express bcl-2 (Hockenbery *et al.*, 1991). A study by Leek *et al.* (1994) found a strong correlation between Bcl-2 expression and oestrogen receptor status in human breast carcinomas. The study showed 80% (70/88) Bcl-2 positive tumours to be oestrogen receptor (ER) positive compared with only 23% (7/30) of Bcl-2 negative tumours being ER positive. The converse relationship was found with Bcl-2 expression and epidermal growth factor receptor status, prompting the authors to suggest that Bcl-2 is an ER-regulated gene. Further evidence to support the association

between ER status and Bcl-2 in human breast cancer was proposed by Johnston *et al.* (1994). This study demonstrated Bcl-2 expression in 32% of invasive breast cancers and in 65% of tumours treated with the anti-oestrogen, tamoxifen. A subset of tumours lose expression of Bcl-2 at a later stage in their progression and this latter group has been associated with established markers of poor prognosis (e.g. EGFR, c-*erb*B-2, p53 positivity and ER negativity). Bcl-2's role in breast cancer progression may differ substantially from that seen in lymphoma, in which activation by translocation occurs (this translocation has never been described in breast carcinomas). Similar results were found by Doglioni *et al.* (1994) who found that the prevalence of Bcl-2 immunoreactivity is significantly higher in lobular carcinomas, in better differentiated and low-cycling tumours and in tumours lacking EGFR and p53 immunostaining; Bcl-2 positive immunostaining might indicate a less aggressive subset of breast carcinomas, a finding previously shown in squamous cell carcinomas of the lung (Pezzella *et al.*, 1993). In agreement with these results, Hellemans *et al.* (1995) concluded that the absence of Bcl-2 expression in axillary node-positive breast cancer was an independent marker of poor prognosis and that the absence of Bcl-2 expression in invasive ductal breast carcinoma possibly reflected the down-regulation of the Bcl-2 gene by mutant p53 suggesting an important role for Bcl-2 as a modulator of response to adjuvant therapy in breast cancer. Hurlimann *et al.* (1995) also found that Bcl-2 positivity conferred better prognosis as judged by overall survival at five years. The Bcl-2 protein seemed only to be an important prognostic factor in women over 54 years of age. Although the numbers studied in this report was small the authors stress the importance of studying both bcl-2 and p53 together. Expression of Bcl-2 protein was found in most (54/60) paraffin-embedded small cell lung carcinoma samples studied by Jiang *et al.* (1995). A number of studies have also been carried out on Bcl-2 expression in non-small cell lung carcinomas and findings were similar to those found for breast cancer i.e. an inverse relationship was seen with p53 expression and a survival advantage was seen in those patients expressing Bcl-2 (Fontanini *et al.*, 1995; Pezzella *et al.*, 1993).

Bcl-2 expression was found in tumours from patients with neuroblastomas who presented with poor stage disease (stage III/IV), unfavourable histology and N-myc amplification (Castle *et al.*, 1993). Kanitakis *et al.* (1995) showed that benign nevi and malignant melanomas were Bcl-2 positive whereas metastatic melanomas had decreased positivity compared with primary tumours suggesting a role for the Bcl-2 oncogene in metastatic progression of melanoma. Studies have also been carried out which report the detection of Bcl-2 in cancers of thymic origin (Brocheriou *et al.*, 1995), in hepatocellular carcinomas

(Zhao *et al.*, 1994), in prostate cancers (Bubendorf *et al.*, 1996; Colombel *et al.*, 1993; McDonnell *et al.*, 1992), in ovarian tumours (Henriksen *et al.*, 1995), in human gliomas (Alderson *et al.*, 1995), in cancers originating from the follicular epithelium of the thyroid gland (Pilotti *et al.*, 1994), in renal cell tumours (Paraf *et al.*, 1995) and in colorectal carcinoma (Öfner *et al.*, 1995). Many of the above studies found that the expression of Bcl-2 in tumours unexpectedly resulted in a good prognosis.

Expression of Bcl-2 conferring good prognosis is not the expected scenario as tumours positive for Bcl-2 would be expected to avoid apoptosis, live longer and therefore confer a worse prognosis on the tumour.

The Bcl-2 story is not complete when one looks only at the expression of this member of the gene family, however, as a study by Bargou *et al.* (1995) showed. These authors found no difference in expression of Bcl-2 and Bcl-x_L between normal breast epithelium and tumour tissue, in contrast, however, bax-α was found to be expressed in high amounts in normal breast tissue but only weakly, if at all, in malignant tissue. Lower Bax expression was also seen in studies on breast tumours by other groups (Krajewski *et al.*, 1995; Wagener *et al.*, 1996). The prognostic significance of Bcl-2 expression in breast carcinoma was enhanced in a study by Krajewski and co-workers (1997) when Bax and p53 expression was included. In this study a significant correlation was found between the poor prognostic c-*erb*B-2 marker and Bax expression. A strong correlation was also seen between Bax expression and c-*erb*B-2 and an inverse correlation with oestrogen receptor status (Binder *et al.*, 1996). The importance of the Bax:Bcl-2 ratio was shown in a study on gastric carcinoma (Koshida *et al.*, 1996) where the ratio was correlated with cellular proliferation. Neuroendocrine lung tumours which had a Bcl-2:Bax ratio of greater than 1 showed a lower apoptotic index and survival rate (Brambilla *et al.*, 1996). High Bax expression was observed in testicular cancers in a study by Chresta and co-workers (1996) which may explain the inherent sensitivity of these tumours to chemotherapy. Thus, the unexpected results of Hurlimann's (1995) and Hellemans' (1995) groups and others of Bcl-2 as a predictor of good response may be biased as only expression of the Bcl-2 gene was analysed without taking the other family members into consideration.

A study of the expression of Bcl-X in human and mouse tissues (Krajewski *et al.*, 1994a) revealed a different pattern to that reported for Bcl-2, suggesting that Bcl-X and Bcl-2 regulate cell life and death at different stages of cell differentiation through tissue-specific

control of their expression. In this study Bcl-X immunostaining was prominent in (a) neuronal populations in the brain; (b) cortical thymocytes, activated lymphocytes; (c) bone marrow cells; (d) reproductive tissue; (e) variety of epithelial cells. A further study by the same group (Krajewski *et al.*, 1994) found the expression of bax in mouse to be more wide-spread than that of bcl-2; bax staining was found in the hepatocytes of liver, the exocrine pancreas and the renal tubule epithelial cells whereas Bcl-2 was absent from these tissues. Krajewska and co-workers (1996) found an increase in expression of Bcl-x in undifferentiated primary colorectal cancer and a concomitant decrease in Bcl-2 and Mcl-1 expression. The authors concluded that in progression of colorectal cancer, expression of Bcl-2 and Mcl-1 is lost and Bcl-x takes over. This hypothesis was also suggested by Pezzella's group (1993) who found that Bcl-2 expression was high in a less aggressive subset of breast carcinomas. Lipponen and co-workers (1995) also found expression of Bcl-2 in well differentiated breast tumours resulting in a good prognosis.

The most recently discovered member of the Bcl-2 family, Bfl-1, was found abundantly expressed in the bone marrow and in some other tissues including the stomach (Choi *et al.*, 1995), and a correlation was seen between the expression of Bfl-1 and the development of stomach cancer.

1.7.1.4 Role of Bcl-2 Family Members and Chemoresistance

As Bcl-2 family members play a pivotal role in the apoptotic pathway in cells, it is not surprising that much research in recent years has concentrated on the role Bcl-2 family members play in the response of tumours to chemotherapeutic drug treatment. The fact that gene transfer studies with elevated levels of Bcl-2 (Miyashita and Reed,1992; Walton *et al.*, 1993; Dole *et al.*, 1994) and Bcl-x_L (Dole *et al.*, 1995; Minn *et al.*, 1995; Kühl *et al.*, 1997) protect cells from apoptotic death induced by chemotherapeutic drugs and antisense-mediated reductions in Bcl-2 (Kitada *et al.*, 1994) or over-expression of apoptosis-inducing family members sensitises cells to chemotherapeutic drugs (Wagener *et al.*, 1996; Sumantran *et al.*, 1995) has prompted the study of Bcl-2 family members in drug resistant tumours and cell lines and offers new targets in the treatment of cancer. Further evidence to suggest that Bcl-2 family members are involved in response to drug treatment comes from the fact that p53 down-regulates expression of Bcl-2 and up-regulates expression of the apoptosis promoting agent, Bax. A partial list of studies on Bcl-2 family members and chemoresistance is shown in Table 1.7.1.4.1.; results are

discussed in more detail in Section 4.1.5.

Table 1.7.1.4.1 Bcl-2 Family Members and Chemoresistance

Cell Type	Drug	Reference
neuroblastoma	cisplatin/VP-16	Dole *et al.*, 1994
ovarian	cisplatin	Eliopoulos *et al.*, 1995
lymphoma	methotrexate	Kitada *et al.*, 1994
ovarian	cisplatin	Perego *et al.*, 1996
breast	VP-16/taxol	Sumantran *et al.*, 1995
leukaemia	taxol	Tang *et al.*, 1994
breast	adriamycin	Teixeira *et al.*, 1995
myeloma	adriamycin/VP-16	Tu *et al.*, 1996
breast	epirubicin	Wagener *et al.*, 1996
glioma	cisplatin	Weller *et al.*, 1995

1.7.2 The c-Myc Oncogene

The myc gene was originally isolated as a transforming sequence of a subclass of avian leukaemia retroviruses (MC29) (Erisman and Astrin, 1988). Myc is a member of a multi-gene family consisting of at least three members, c-Myc, L-Myc (identified in small cell lung cancer) and N-Myc (expressed in neuroblastomas) (Erisman and Astrin, 1988); other related proteins have also been identified (Ingvarsson *et al.*, 1988; Sugiyama *et al.*, 1989). It is one of the immediate early growth response genes and is rapidly, but transiently, induced in quiescent cells upon mitogenic stimulation; mRNA and protein levels are sustained throughout the cell cycle in proliferating cells (Evan *et al.*, 1992). The gene is composed of three exons and is transcribed from two initiation sites. The gene is frequently seen translocated to the heavy chain locus of the immunoglobulin gene in Burkitt's lymphoma suggesting that c-*myc* has a primary role in transformation of some human haematopoietic cells (Watt *et al.*, 1983). Indeed in tumour cells elevated or deregulated expression of c-Myc is so widespread as to suggest a critical role for c-Myc gene activation in carcinogenesis.

c-Myc is a member of the nuclear oncoproteins and acts as a transcription factor (as a heterodimeric partner to Max) to promote cell proliferation and has an elusive role to play in control of the cell cycle (Pardee, 1989). Max is essential for Myc transformation activity. The Myc-Max heterodimer can transactivate several promoters containing CACGTG boxes, including p53 and ornithine decarboxylase. This partnership with Max allows cells to enter G_1 and begin cycling (D'Amico and McKenna, 1994). Ornithine decarboxylase is necessary for G_1 progression (Mihich and Schimke, 1994) and also cooperates with Ras in the transformation of NIH3T3 cells (Hibshoosh *et al.*, 1991). It remains to be established if the above mentioned genes are true downstream effectors of Myc. It has also been reported that Myc / Max binds to the 5' flanking region of the DHFR gene involved in DNA replication and induces amplification of this gene (Mai, 1994); the author concludes that c-Myc's contribution to cellular transformation may be based on the accumulation of genomic instabilities such as gene amplification. Recently, scientists at Cold Spring Harbor Laboratories (1996) discovered that the cdc25A phosphatase is a target of the c-Myc gene, thus confirming a role for c-Myc in cell cycle progression.

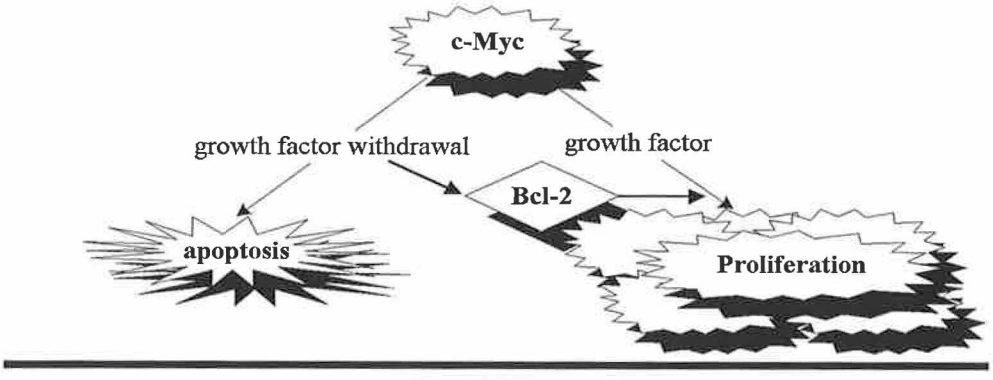
Myc usually stimulates proliferation, but if growth is inhibited by other factors, Myc-

induced apoptosis will occur. Myc therefore has a dual role to play in the cell: myc stimulates growth in the presence of survival factors and induces apoptosis in the presence of growth inhibiting factors (Mihich and Schimke, 1994).

1.7.2.1 c-Myc and its Dual Role

c-Myc, or the "Janus" (Roman God of two faces) gene, has a dual role to play in cellular control. c-Myc has been described as a "switch" directing cells towards proliferation or death with Bcl-2 determining the direction of the switch (Figure 1.7.2.1.1). The opposing roles of c-Myc in cell proliferation and cell death (first shown by Shi *et al.*, 1992) requires that this sort of molecular co-operation exists. It has been shown, for example, that c-Myc induces apoptosis in serum-starved fibroblasts. This can be overcome if Bcl-2 is increased in these cells leading to a block in apoptosis and cell proliferation (Bissonnette *et al.*, 1992).

Fig. 1.7.2.1.1 The Dual role of c-Myc in Response to Growth Factor

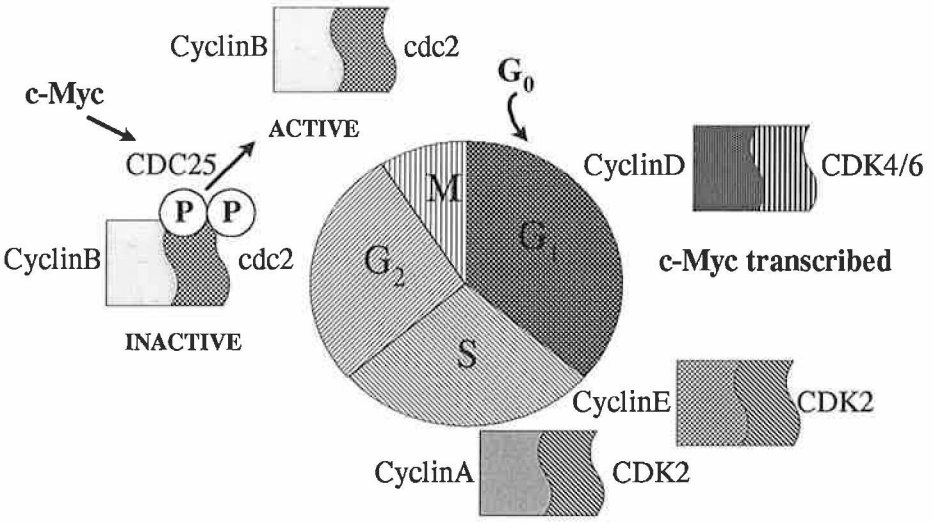


Bcl-2 can block the apoptotic role of c-Myc (due to the removal of growth factors) but it has no effect on the mitogenic function of the oncogene, therefore the combination of the block to apoptosis (by Bcl-2) and the induction of the mitogenic signal by c-Myc would allow a cell to proliferate. This is a novel mechanism of oncogene co-operation. Constitutive expression of c-Myc induces apoptosis only when combined with a negative growth signal e.g. serum withdrawal (cells proliferate to S-phase and then undergo apoptosis); growth arrest does not occur.

In order for cells to replicate they must enter the cell cycle consisting of four different phases; G_1 (growth phase), S (DNA replication), G_2 (cell pauses as it prepares to divide)

and M (mitosis). The four phases are controlled by the actions of specific serine/threonine kinases, the cdc-2 related proteins. cdc-2 related proteins (or cyclin-dependent kinase proteins (CDK)) bind to cyclin proteins and form an active complex which is regulated throughout the cell cycle. c-Myc is involved in the priming of quiescent cells in G_0 into the cell cycle at G_1 (Erisman and Astrin, 1988). TGF-β1, which arrests cell growth, specifically targets c-*myc* and reduces its mRNA levels and consequently leads to G_1 arrest (Alexandrow and Moses, 1995). PDGF stimulates c-*myc* levels and it has been shown that over-expression of c-*myc* eliminates PDGF requirement in cells for cell cycle progression (Armelin *et al.*, 1984). It is believed that the E2F transcription factor, which is active in the G_1 phase of the cell cycle (due to the removal of the inhibitory Retinoblastoma protein as a result of phosphorylation by cyclinD-cdk4/6 complex), regulates the transcription of the c-*myc* gene which then exerts its effect on the cell cycle by targetting the cdc25A phosphatase which converts the M-phase cyclinB/cdc2 complex from its inactive phosphorylated state to its active de-phosphorylated state promoting progression through mitosis (Figure 1.7.2.1.2).

Figure 1.7.2.1.2 Regulation of the Cell Cycle



When cells become quiescent c-Myc is down-regulated and growth arrest occurs; equally c-Myc has the power to convert quiescent cells to proliferating cells. This is a dangerous balance to maintain as any mutation deregulating c-Myc could result in tumour progression; as c-Myc is also involved in programmed cell death by controlling genes

involved in apoptosis (Evan *et al.*, 1992) these cells will also be primed for cell death and therefore removal of a possible tumour-forming mutation achieved. It has been suggested that c-Myc induces apoptosis (the normal intrinsic function of c-Myc) through sequence specific DNA binding, presumably by modulation of apoptotic and/or anti-apoptotic genes (Evan *et al.*, 1992; Amati *et al.*, 1993), complete overlap of structural sequence for transformation, dimerization and cell death functions is seen. Hermeking and Eick (1994) have shown that induction of p53 is part of the cellular response to activation of c-Myc in quiescent cells; c-Myc is dominant over p53 mediated growth arrest and can drive cells from G_1 into S-phase even in the presence of large amounts of p53. p53-induced apoptosis may be a common tumour suppressive mechanism to eliminate cells (that express certain oncogenes) that inappropriately by-pass the G_1-S check-point; failure to do this, due to loss of wild-type p53, may lead to carcinogenesis.

1.7.2.2 c-Myc Expression in Human Tumours and Effect on Chemoresistance

Expression of c-*myc* gene occurs in most cell types and is detectable in almost all proliferating cells and is vital for normal development. Not surprisingly then deregulated c-*myc* is found in a wide variety of malignancies. Over-expression of c-*myc* was shown to co-operate with activated c-H-*ras* and transform primary fibroblasts (Land *et al.*, 1983).
c-Myc can become oncogenic in a number of ways including, chromosomal translocation (Burkitt's lymphomas) and gene amplifications (colon, gastric, breast and other solid tumours) (Erisman and Astrin, 1988). Amplification of c-*myc* is infrequently found in primary tumours and it is therefore highly unlikely to be an initiating event in oncogenesis. Amplification may occur during tumour progression and may be an indication of advanced disease and a poor prognosis.
c-Myc has been found to be over-expressed or de-regulated in many clinical breast tumours (Escot *et al.*, 1986; Mariani-Costantini *et al.*, 1988; Berns *et al.*, 1992; Kreipe *et al.*, 1993) and amplification of the gene was found to be associated with early relapse and poor prognosis (Kreipe *et al.*, 1993; Martiani-Costantini *et al.*, 1988). c-Myc over-expression in carcinoma of the uterine cervix was found to correlate with a risk of relapse (Riou *et al.*, 1987). N-myc (a poor prognostic marker for small cell lung carcinoma, retinoblastoma and neuroblastoma (Knudson, 1985; Mizukami *et al.*, 1995)) expression in breast cancer has also been implicated in poor prognosis of breast carcinoma (Mizukami *et al.*, 1995) A number of *in vitro* studies have shown that elevated levels of c-*myc* result in

increased drug resistance to a variety of chemotherapeutic drugs in tumour cell lines (Table 1.7.2.2.1). Results found are described in Section 4.1.3.

Table 1.7.2.2.1 c-Myc Over-expression and Chemoresistance

Cell Type	Drug	Reference
colon	ciplatin/5-fluorouracil	Kashani-Sabet *et al.*, 1990
CHO9	methotrexate	Mai, 1994
murine Friend Erythroleukaemia	cisplatin	Mizutani *et al.*, 1994
NIH3T3 fibroblasts	cisplatin/adriamycin/cyclophosphamide	Niimi *et al.*, 1991
prostate	VP-16/cisplatin	Sinha *et al.*, 1995
small cell lung carcinoma	cisplatin	Sklar and Prochownik, 1991
bladder	cisplatin	VanWaardenburg *et al.*, 1996

1.8 Aims of Thesis

Observations in the literature suggest that proliferation promoting genes, such as c-*erb*B-2, c-H-Ras and the AP-1 complex, and genes involved in the process of apoptosis, Bcl-2 and c-Myc, have the capacity to affect the response of tumour cells to chemotherapeutic drugs. Over-expression of the above oncogenes in drug-resistant cell lines has previously been seen and genetic modulation of these genes sensitise the resistant cell line to drug. Few studies, however, compared the expression of oncogenes involved in a signal transduction pathway and apoptosis-influencing genes in the same cell lines. In this thesis, it was proposed to study the expression of a wide variety of oncogenes, known to influence proliferation and cell death, in drug resistant variants of the human lung carcinoma line, DLKP, developed by continuous exposure to commonly used chemotherapeutic drugs known to induce apoptosis, and in adriamycin-selected resistant variants of the human ovarian line, OAW42.

Many *in vitro* cellular models of drug resistant lines in common use have been developed by exposure to non-physiologically relevant levels of chemotherapeutic drug and it is not known if results from such studies accurately reflect the *in vivo* situation. To determine if oncogene over-expression may be a physiological response to clinically attainable levels of drug, a model of low-level MDR resistance was to be developed by pulse exposure to clinically attainable levels of drug over a short period of time. It was proposed for this work to develop low-level resistant variants of clonal populations of the DLKP cell line. Little evidence has been reported in the literature defining the initial steps in the development of drug resistance. In this thesis, the effect of certain oncogenes in the establishment of the MDR phenotype was to be addressed. Pharmacological inhibition of signalling cascades was also to be attempted in the low-level pulse-selected resistant cell line.

Finally, the problem of predicting the development of drug resistance *in vivo* was to be undertaken by analysis of MDR related gene and oncogene levels in breast cancer patients.

2. *Materials and Methods*

2.1 Preparation for Cell Culture

2.1.1 Water

Water used in the preparation of media and solutions was purified by passing it through a Millipore milli-RO Plus system with an Elga Elgastat UHP. A pre-treatment step involving activated carbon, pre-filtration and anti-scaling, was carried out followed by a reverse osmosis step. Organic adsorption, ion exchange, ultra-microfiltration, photo-oxidation and ultra-filtration completed the process. The quality of water was monitored on-line and a measure of 16MΩ/cm at 25°C was considered acceptable.

2.1.2 Glassware

All glassware and bottle-caps used were soaked, for 1-2 hours in a 2%(v/v) solution of RBS (AGB Scientific; RBS-25) in warm water. The bottles were then scrubbed and both bottles and caps were rinsed in warm water and machine washed using Neodiser detergent, followed by rinsing twice in double-distilled water and once in ultra-pure water. The bottles were then prepared for autoclaving. Waste bottles containing spent medium from cells were autoclaved, rinsed in tap water and treated as above.

2.1.3 Sterilisation

Water, glassware and solutions containing thermostable compounds were sterilised by autoclaving at 120°C for 20min. at 15 p.s.i. pressure. Temperature labile compounds were filtered through a 0.22μm sterile filter (Millipore; millex-gv).

2.1.4 Medium Preparation

Growth media for cell culture was prepared as indicated in Table 2.1.4.1. The pH was adjusted to 7.45 - 7.55 by the addition of sterile 1.5M NaOH, and the volume adjusted to 5 litres and filtered through a sterile 0.22μm bell filter (Gelman; G.1423S) into sterile 500ml bottles. Sterility checks were performed on each bottle by placing :

(a) 3ml in a sterile universal to check for turbidity

(b) 1ml streaked onto a Columbia (Oxoid; CM331) blood agar plate

(c) 1ml in a 5ml sample of sterile Sabauraud (Oxoid; CM421) dextrose

(d) 1ml in a 5ml sample of sterile Thioglycollate (Oxoid; CM173) broth.

Sterility checks were incubated at 37°C and 4°C for 1 month and checked every 24hrs. Blood agar plates were kept for 7 days. The media bottles were labelled, dated and stored at 4°C until required.

Table 2.1.4.1 Preparation of Growth Media.

Components	DMEM (Gibco;042-02501M)	Hams F12 (Gibco;074-01700N)	RPMI 1640 (Gibco;31870-025)	MEM (Gibco;21430-020)
10XMedium	500ml	Powder	1XRPMI	500ml
Ultra-pure H_2O	4300ml	4700ml	.....	4300ml
1MHepes (pH7.5)*	100ml	100ml	.....	100ml
7.5% $NaHCO_3$	45ml	45ml	.....	100ml

* The weight equivalent of 1M N-(2-Hydroxyethyl)piperazine -N'-(2-ethanesulfonic acid) (Hepes) was dissolved in an 80% volume of ultra-pure water and autoclaved. The pH was then adjusted to 7.5 with 5M NaOH.

2.2 Routine Management of Cells in Culture

All routine management of cells in culture, including cell feeding, sub-culturing, freezing and thawing, were performed aseptically in a down-flow re-circulating laminar flow cabinet (Holton or Gelman Cytoguard) which had been swabbed with 70% IMS (industrial methylated spirits). To maintain a sterile atmosphere inside of the laminar flow all articles entering the cabinet were also swabbed with 70% IMS. Gloves were worn at all times during these procedures.

2.2.1 Cell lines

All cell lines used throughout this thesis are outlined in Table 2.2.1.1. All cell lines are anchorage dependent except the leukaemia cell line, HL60 and HL60/ADR, which are suspension cells.

Cells were routinely grown in 25, 75 or 175cm^2 flasks (Costar; 3050, 3075: Nunc; 1-56502A, respectively). Cells were grown at 37°C and fed every 2-3 days or when a medium pH change was observed (colour change in medium due to the presence of a phenol red indicator). Waste media was removed from the cells at this stage and replaced

with fresh media, as indicated in Table 2.2.1.1. Separate waste and medium-containing bottles were kept exclusive to each cell line to prevent cross-contamination. When feeding more than one cell line a minimum of 15min should be left before introducing a new cell line in to the laminar flow to further ensure against cross-contamination.

Table 2.2.1.1 Cell lines used throughout the course of this Thesis.

Cell Line	Growth Medium	Cell Type	Source
A549	ATCC[1]	Human lung cancer	ATCC
BT-20	MEM[2]	Human breast cancer	ATCC
DLKP	ATCC[1]	Human lung squamous cell carcinoma	NCTCC
DLKP-A	ATCC[1]	Adriamycin-selected MDR variant of DLKP	NCTCC
DLKP-A2B	ATCC[1]	Clonal population of DLKP-A	NCTCC
DLKP-A5F	ATCC[1]	Clonal population of DLKP-A	NCTCC
DLKP-C14	ATCC[1]	Carboplatin-selected MDR variant of DLKP	NCTCC
DLKP-I	ATCC[1]	Clonal population of DLKP	NCTCC
DLKP-I*mdr1*Rz2D4	ATCC[1]	*mdr1* ribozyme DLKP-I transfectant	NCTCC
DLKP-SQ	ATCC[1]	Clonal population of DLKP	NCTCC
DLKP/VP-3	ATCC[1]	VP-16-selected MDR variant of DLKP	NCTCC
DLKP/VP-8	ATCC[1]	VP-16-selected MDR variant of DLKP	NCTCC
HL60	RPMI[3]	Human leukaemia cell line	M.Center
HL60/ADR	RPMI[3]	Adriamycin-selected MDR variant of HL60/S	M.Center
MCF-7	ATCC[4]	Human breast cancer	ATCC
OAW42-A	ATCC[5]	Adriamycin-selected MDR variant of OAW42-SR	NCTCC
OAW42-A1	ATCC[5]	Adriamycin-selected MDR variant of OAW42-SR	NCTCC
OAW42-S	ATCC[5]	Human Ovarian Carcinoma - sensitive clone	NCTCC
OAW42-SR	ATCC[5]	Spontaneously resistant population of OAW42	ECACC
PA1	MEM[2]	Human ovarian cancer	ATCC

[1] 50:50 mixture of Hams F12 medium and Dulbecco's Modified Eagle's medium supplemented with 5% foetal calf serum and 2mM L-Glutamine (Gibco; 25030-024) prior to use. [2] MEM medium supplemented with 10% foetal calf serum, 2mM L-Glutamine, 1% MEM non-essential amino acids (Gibco; 11140-035) and 1% Na-pyruvate (Gibco; 11360-039). [3] RPMI medium supplemented with 10% foetal calf serum, 2mM L-Glutamine and 5%CO_2 [4] 50:50 mixture of Hams F12 medium and Dulbecco's Modified Eagle's medium supplemented with 10% foetal calf serum, 2mM L-Glutamine and 10μg/ml Insulin (Sigma; I-1882) [5] 50:50 mixture of Hams F12 medium and Dulbecco's Modified Eagle's medium supplemented with 10% foetal calf serum, 2mM L-Glutamine and 1% Na-pyruvate.

2.2.2 Sub-Culture of Cell Lines

Monolayer cells grow attached to the bottom of flasks and upon reaching confluency (or when required for further studies) the cells were enzymatically detached from the flask base and sub-cultured. This involved removing waste medium from the flask of cells, rinsing the cells with 1ml of trypsin/EDTA (0.25% trypsin (Gibco; 043-05090), 0.01% EDTA (Sigma; EDS) solution in PBS (Oxoid; BR14a)) and then incubating with a further 4ml of the trypsin/EDTA solution for 5-10min (or until a single cell suspension had been obtained) at 37°C. An equal volume of complete medium was then added to the flask and the total cell suspension was transferred to a 30ml sterile universal (Sterilin; 128a) and centrifuged at 120g. for 5min. The medium was poured off the cell pellet which was then resupended in an appropriate volume of complete medium and re-seeded into fresh flasks at the cell density required (estimated by a cell count ; see section 2.2.3).
Suspension cells were sub-cultured simply by removing the cell suspension from the flask and pelleting the cells by centrifugation as above. Re-seeding and counting was carried out as for adherent cells.

2.2.3 Cell Counting

A sample of a single cell suspension was mixed in a ratio of 4:1 with trypan blue (Gibco; 525) and incubated for 2min. after which 10µl of the cell mixture was applied to a haemocytometer in the area under the cover-slip. Cells in the 16 squares of the four outer corner grids were counted, and the average of the four squares was multiplied by 10^4 and the initial dilution factor to determine the number of cells per ml of cell suspension. Cells which stained blue were considered non-viable while those unstained were accepted as viable cells.

2.2.4 Large-Scale Cell Culture

Cells required in large numbers were cultivated in roller bottles. Approximately 100ml of growth medium was allowed to equilibrate in a roller bottle at 37°C after which a single cell suspension of approximately $2x10^7$ cells was added. The roller bottle was incubated at 0.25rpm overnight and then the rotor speed was increased to 0.50rpm. The cells were allowed to grow to 80% confluency and were fed when determined necessary.

2.2.5 Freezing Cells in Culture

Stocks of all cells used in this study were frozen to allow their long-term storage and adequate supply within a given passage number range.

A single-cell pellet suspension was prepared (Section 2.2.2) from a sub-confluent large-scale culture of cells (Section 2.2.4). The cell pellet was resuspended in foetal calf serum (FCS) and an equal volume of 10% (v/v) DMSO (Sigma; D5879) in FCS was added drop-wise, with constant agitation, to result in a final concentration of 10^7 (viable) cells/ml. 1.5ml aliquots of the resulting cell suspension were placed in cryovials (Greiner; 122 278) (labelled with the cell line, passage number, date and operators initials) and stored in the vapour phase of liquid nitrogen for 2.5h. and then stored in the liquid phase until required. A vial of cells was thawed 2-5 days after freezing to determine the sterility and viability of the stock.

2.2.6 Cell Thawing

The required vial of cells was removed from its liquid nitrogen store and thawed in a 37°C water-bath. The thawed suspension was quickly transferred to a universal containing 5ml of medium and was centrifuged at 120g for 5min. The medium was poured off and the cell pellet was resuspended in 5ml of complete medium, transferred to a $25cm^2$ flask and incubated at 37°C. Following cell attachment, the cells were re-fed with fresh medium.

2.2.7 *Mycoplasma* Detection

All cell lines used in this study were routinely checked to ensure that *Mycoplasma* contamination had not occurred. These procedures were performed in isolation from the routine cell culture designated areas (by Dr. Mary Heenan and Mr. William Nugent) to avoid possible contamination of clean cell stocks. Two methods were used during analysis, namely the Hoechst 33258 indirect staining method and *Mycoplasma* culture methods.

The cell lines to be tested were grown in drug-free medium for a minimum of three passages following thawing. A 5ml aliquot of conditioned medium i.e. medium in which near-confluent cells had been grown for 2-3 days, was removed and analysed for the presence of *Mycoplasma*.

2.2.7.1 Hoechst 33258 Indirect Staining

Indicator cells (NRK) were grown ($2x10^3$) overnight on sterile coverslips in 1ml DMEM medium supplemented with 5% FCS and 2mM L-Glutamine, in individual 35mm sterile petri-dishes. 1ml aliquots of the conditioned media (Section 2.2.7), from each cell line to be tested, were added to duplicate cover-slips of NRK cells and incubated for 5 days (to approximately 50% confluency). The cover-slips were then washed of media twice with PBS, once with a 1:1 solution of ice-cold PBS:Carnoy's fixative (a freshly prepared 1:3 solution of glacial acetic acid (Sigma; A0808) with methanol (BDH; 101584W) which had been stored at -20°C for 30min prior to use), and fixed for 10min in Carnoy's fixative. The cover-slips were then allowed to air dry. 2ml of Hoechst 33258 stain (Sigma; B2883), at a concentration of 50ng/ml in PBS, was added to each cover-slip and incubated in darkness for 10min. The coverslips were then washed in water and mounted on a glass slide using 50% glycerol (BDH; 101184K) in 0.1M citric acid (Sigma; C2916), 0.2M disodium phosphate (Sigma; S9390), pH 5.5 as the mounting solution. The slides were examined for *Mycoplasma* contamination under oil immersion using a mercury fluorescent lamp. Hoechst 33258 stains nucleic acids and therefore staining in the NRK cell nuclei was observed, any extra-nuclear staining was an indication of the presence of *Mycoplasma* contamination of the cell line under analysis. Both positive (a sample of medium known to be contaminated) and negative (medium not exposed to cells) controls were included in this procedure.

2.2.7.2 *Mycoplasma* Culture Method

The substrate used for the *Mycoplasma* culture method of detection consisted of 90ml of *Mycoplasma* agar (Oxoid; CM401) and *Mycoplasma* broth (Oxoid; CM403) bases, which were supplemented with 16.33% FCS, 0.002% DNA (BDH; 42026), 2μg/ml fungizone (Gibco; 05290), $2x10^3$ U penicillin (Sigma; Pen-3) and 10ml of a 25% (w/v) yeast extract solution (which had been boiled for 10min. and filtered through a 0.2μm filter). A 0.5ml aliquot of sample medium from the cell line being tested was incubated with 3ml of the broth for 48h. at 37°C in a 5% CO_2 environment. An aliquot of the broth was then streaked onto a 10ml agar plate which was incubated for up to 3 weeks at 37°C in 5% CO_2, and frequently monitored microscopically for colony formation. The presence of "fried egg"-type colonies were indicative of *Mycoplasma* contamination of the cell line.

2.2.8 Serum Batch Testing

One of the main problems associated with the use of FCS in cell culture is its batch to batch variation. In extreme cases this variation may result in a lack of cell growth, whereas in more moderate cases growth may be retarded. To avoid the effects of the above variation, a range of FCS batches were screened for growth of each cell line. A suitable FCS was then purchased in bulk for a block of work with each particular cell line in use.

Screening involved growing cells in 96 well plates and growth was recorded as a percentage of growth of a serum with known acceptable growth rate.

Logarithmically growing cells were seeded into a 96 well plate (Costar; 3599) from a single cell suspension at a density of 10^3 cells/well in 100µl of medium without FCS. 100µl volumes of medium containing 10%, 20% or higher (if required by the particular cell line under analysis) (v/v) FCS was added to respective wells on the 96 well plate, resulting in final dilutions of the FCS to 5% and 10%, respectively. The first column of each plate was maintained as a control where FCS resulting in a known acceptable growth rate was used. Plates were placed at 37°C in 5% CO_2, for 5 days, after which growth was assessed by a crystal violet dye elution method or acid phosphatase (Martin and Clynes, 1991). Crystal dye elution method involved removing the media from the wells and rinsing twice in PBS. The wells were then fixed in 10% formalin (Sigma; F1635) for 10min, after which the formalin was removed and the plates allowed to dry. When the plates had dried 100µl of 0.25% crystal violet dye (Sigma; C3886) was added to each well of the plate for 10min; the dye was then removed and the plates were washed under running tap water 4 or 5 times and allowed to dry. The dye was eluted with a 33% solution of glacial acetic acid (Sigma; A6283) 100µl/well. The plates were then read in a dual beam plate reader at 570nm (reference wavelength 620nm) (Titertek; Multiskan). When growth was assessed by the acid phosphatase method, the plates were washed twice in PBS and incubated with 100µl of acid phosphatase buffer (consisting of 10mM p-nitrophenyl phosphate (Sigma; C104) in 0.1M Na-acetate, pH5.5 and 0.1% Triton X-100 (Sigma; X-100) for 2 hours at 37°C. Following incubation, 50µl of 1.0M NaOH was added to the buffer and the plates read in a dual beam plate reader at 405nm (reference wavelength 620nm) (Titertek; Multiskan).

2.2.9 Growth of Cell Lines in Serum-Free Media

For many studies analysis of cell lines grown in the absence of serum is required. A serum-free media which supports the growth of the DLKP cell line was developed in this laboratory (P.Meleady, 1997), based on that described by Mendiaz *et al.* (1986). Basal Hams F12 media is supplemented with the following components :-

Table 2.2.9.1 Supplements for Serum-Free Media to Support the Growth of DLKP*

Medium Supplements**	Concentration
Insulin	10μg/ml
Transferrin	5μg/ml
$FeSO_4$	5.0μM
Na_2SeO_3	$3.0x10^{-2}$μM
$CaCl_2$	$6.0x10^{-2}$μM
Linoleic acid	$3.0x10^{-1}$μM
L-glutamine	$1.0x10^{-2}$μM
Non-essential Amino Acids	$1.0x10^{-2}$μM
Trace Elements :	
$CuSO_4.5H_2O$	$1.0x10^{-3}$μM
$MnSO_4.5H_2O$	$1.0x10^{-3}$μM
$ZnSO_4.7H_2O$	$5.0x10^{-2}$μM
$SnCl_2.2H_2O$	$5.0x10^{-4}$μM
$NiCl_2.6H_2O$	$5.0x10^{-4}$μM
NH_4VO_3	$5.0x10^{-3}$μM
Na_2SiO_3	$5.0x10^{-2}$μM
$(NH_4)_6Mo_7O_{24}.H_2O$	$1.0x10^{-3}$μM

*The growth of some DLKP variants required further supplementation with fibronectin (Sigma; F2006) at a concentration of 5μg/ml. ** All medium supplements were purchased from Sigma, unless stated otherwise.

2.3 Miniaturised Toxicity Assay

Logarithmically growing cells were used in all miniaturised toxicity assays. The day prior to setting up the assay the cells were fed with fresh, complete medium. On the first day of the assay the cells were sub-cultured and a single cell suspension was obtained (as described in Section 2.2.2).

2.3.1 Toxicity Assay - 96 well plate

Cells were seeded, from a single cell suspension, into a 96 well plate (Costar; 3599) at a cell density of $1x10^3$cells/well in 100µl medium; the first column of the plate was not seeded with cell suspension and was used as a control containing only medium. The cells were allowed to attach overnight at 37°C in a 5% CO_2 environment. Drug concentrations used in each assay ranged from concentrations which would result in no kill (i.e. no drug) to approximately 100% kill. The required drug dilutions range was prepared (at twice the final concentration to be assayed) and 100µl of each drug dilution was added to each well, in replicas of eight. The plates were covered in aluminium foil (most chemotherapeutic drugs are light sensitive) and incubated at 37°C in a 5% CO_2 environment for a further 6 days or until control wells (where no drug was added) reached 80-90% confluency. Drug toxicity was then determined by crystal violet dye elution method or acid phosphatase method (see Section 2.2.8).

2.3.2 Toxicity Assay - 25cm² flask

25cm² flasks were seeded at a density of $2x10^5$cells/flask 2-3 days prior to drug addition. When cells had reached 50% confluency, a 1X concentration of drug was added to each flask in a 5ml volume. The drug was removed after a certain length of time (e.g. 4h). The flasks were rinsed twice in fresh medium, re-fed with 5ml of complete medium without drug and incubated at 37°C for a further 6 days. After this time the media was removed from the flasks which were then washed twice with PBS, fixed for 10min in 3ml 10% formalin, this fixative was then removed and the flasks were allowed to dry. When the flasks had dried, 3ml of crystal violet dye was added for 10min, removed, and the flasks were washed 4-5 times under running tap water. When dry, 1-5ml of 33% glacial acetic acid was added to the flask and the dye eluted. Eight replicas of 100µl volumes of the

eluted dye was placed in a 96 well plate for each drug concentration used. The toxicity of the drug was analysed as described previously (see Section 2.2.8). A variation of the acid phosphatase assay was also used to analyse growth in 25cm^2 flasks where 5ml of acid phosphatase was added to the flask and incubated at 37°C for 2 hours. Growth was analysed as described previously (Section 2.2.8).

2.4 Safe Handling of Drugs

There are many potential safety risks when using cytotoxic drugs and in order to minimise such dangers extreme care was exercised in handling and disposing of cytotoxic agents. All work with such drugs was performed in a Gelman "Cytoguard" laminar air flow cabinet (CG Series), face masks and double gloves were worn when dealing with concentrated stocks and all drug waste (pure or diluted in medium or in contaminated plastics) was disposed of as recommended by the manufacturers (outlined in Table 2.4.1).

Table 2.4.1 Storage and Disposal of Cytotoxic Drugs

Cytotoxic Agent	Stock concentration	Storage	Disposal
Adriamycin[1] (Doxoxrubicin)	2 mg/ml	4°C in darkness	Inactivate with 1% hyperchlorite Autoclave
Vincristine[2]	1 mg/ml	4°C in darkness	Autoclave Dispose with excess water
VP-16[3] (Etoposide)	20mg/ml	R.T. in dark	Incinerate
Carboplatin[2]	10mg/ml	R.T. in dark	Incinerate
Methotrexate[4]	5mg/ml	-20°C in dark	Autoclave Dispose with excess water
5-Fluorouracil[2]	25mg/ml	R.T. in dark	Neutralise with 5M NaOH Incinerate
Cyclophosphamide[5]	2mg/ml	4°C in darkness	Autoclave Dispose with excess water
Emodin[6]	0.1M	-20°C in dark	Incinerate
Genistein[7]	0.1M	-20°C in dark	Incinerate
Cyclosporin A[8]	50µg/ml	-20°C in dark	Incinerate

Cytotoxic drugs used were supplied by [1] Farmatalia; [2] David Bull Laboratories, Ltd.; [3] Bristol Myers Pharmaceuticals; [4] Lederle Lab., Gosport, Hampshire, England; [5] the cyclohexylamine salt, mafosfamide (D-17272) was a generous gift from Dr.J.Pohl, ASTA Medica, Frankfurt; [6] Sigma (E7881); [7] Alexis Corporation (LC-G-6055-M025); [8] Sandoz: (R.T. = room temperature)

2.5 Selection of MDR Resistant Variant from a Sensitive Parental Cell Line

Throughout the course of this study a number of low-level MDR-variant cell lines were developed by selection of the sensitive parental cell line, DLKP-SQ, with Adriamycin.

2.5.1 Continuous selection of DLKP-SQ with Adriamycin

DLKP-SQ were grown to 50% confluency in 25cm^2 flasks. The cells were then exposed to an initial selecting concentration of 25ng/ml Adriamycin which was equivalent to 80% kill when calculated using a 96 well plate miniature toxicity assay (see Section 2.3.2). The cells were re-fed with this drug concentration every 3-4 days and when the cells had regained a state of near 50% confluency, the concentration of drug to which they were exposed was increased approximately two-fold. This was repeated until the cells were exposed to a final drug concentration of 250ng/ml. When the cells were seen to grow in this concentration of drug, toxicity assays were carried out to monitor any changes in drug sensitivity when compared with the untreated parental population.

2.5.2 Pulse selection of Clonal Populations with Adriamycin

Cells were grown to 50% confluency in 25cm^2 flasks. The cells were then exposed to 250ng/ml Adriamycin for 4h. after which time the drug was removed and the cells rinsed twice with fresh media; the cells were then re-fed with 5ml of complete media without drug and incubated at 37°C for 6 days (re-feeding with fresh media every 2-3 days). This was repeated for at least 4 "pulses" after which time sensitivity to the selecting drug was monitored using a miniaturised toxicity assay (see Section 2.3.2).

2.6 Dilution Cloning

To propagate a clonal population from a mixed parent population, individual cells were plated into wells of a 96 well plate and allowed to grow as an individual clonal sub-population of the parental line. To achieve this a single cell suspension (see Section 2.2.2) was prepared at a density of approximately 1 cell per 300µl of media. The cell suspension was then plated out by placing 100µl into each well of a 96 well plate. The plates were then incubated at 37°C and 5% CO_2 and monitored after 2 days for cell attachment; wells

that were seen to have only one cell adhered after 2 days were chosen for expansion as clonal populations. When each individual well of the 96 well plate containing a clonal population had reached 80% confluency the cells were sub-cultured in to a well of a 24 well plate (Greiner; 662160) and grown again to confluency after which time they were transferred to a 25cm^2 flask. Frozen stocks of all clonal populations were made (Section 2.2.5) as soon as possible after propagation.

2.7 Subcellular Distribution of Adriamycin

The subcellular localisation of adriamycin in a number of cell lines was investigated by fluorescence microscopy.

Microscope glass coverslips (Chance Propper LTD) were washed with 70% alcohol and flamed with a bunsen burner. The sterile cover slips were placed in 35mm petri dishes (Greiner 627160). The cells used in the studies were grown until approximately 80% confluent and trypsinised while in exponential growth. The cells were pelleted by centrifugation and a single cell suspension of $1x10^5$ cells per ml was prepared. 1ml of the cell suspension was added to each of the petri dishes which were then incubated overnight at 37°C in 5% CO_2 to allow attachment of the cells to the sterile coverslips. The following day the medium was carefully removed from the petri dishes and the cells incubated with 1ml of medium containing 10μM adriamycin for 4.5 hours. After this time the drug containing medium was decanted and the cells were washed twice with ice-cold PBS. The coverslips were inverted onto clean glass slides and the edges sealed with silicon grease to protect the cells against dehydration. The cells were viewed for fluorescence under ultraviolet illumination using a Nikon microscope equipped with a mercury lamp. The ultraviolet illumination induced an orange fluorescence at the site of adriamycin accumulation. The cells were viewed immediately after mounting, as fading of the fluorescence occurred very rapidly.

2.8 Time-lapse Video Microscopy

Time-lapse video microscopy was carried out on a Nikon diaphot inverted microscope (Micron Optical, Bray, Ireland) equipped with phase contrast optics, linked to a Mitsubishi CCD-100 colour video camera. Images were recorded in S-VHS onto a Mitsubishi HS-S5600 video recorder with time-lapse capabilities. All time-lapse video-equipment was obtained from Laboratory Instruments (Ashbourne, Ireland). Recording speed was set at 3.22sec/field (480 hour mode), which at normal playback speed resulted in an acceleration factor of 160.

The temperature of the culture vessel was controlled by a Linkam C0102 warm stage controller. This controller was adjusted to keep the culture medium inside the vessel at 37°C, as measured using a TB3301 probe.

2.9 Preparation for RNA Analysis

Due to the labile nature of RNA and the high abundance of RNase enzymes in the environment a number of precautionary steps were followed when analysing RNA throughout the course of these studies.

- General laboratory glassware and plasticware are often contaminated by RNases. To reduce this risk, glassware used in these studies was baked at 180°C (autoclaving at 121°C does not destroy RNase enzymes) for at least 8h. Sterile, disposable plasticware is essentially free of RNases and was therefore used for the preparation and storage of RNA without pre-treatment. Polyallomer ultracentrifuge tubes, eppendorf tubes, pipette tips, etc. were all autoclaved prior to use. All spatulas which came in contact with any of the solution components were baked, chemicals were weighed out onto baked aluminium-foil and a stock of chemicals for "RNA analysis only" was kept separate from all other laboratory reagents.

- All solutions (which could be autoclaved) that came into contact with RNA were all prepared from sterile ultra-pure water and treated with 0.1% diethyl pyrocarbonate* (DEPC) (Sigma; D5758) before autoclaving (autoclaving inactivates DEPC), with the exception of Tris-containing solutions (DEPC reacts with amines and so is inactivated by Tris). The Tris-containing solutions were made with previously DEPC-treated ultra-pure water.

- Disposable gloves were worn at all times to protect both the operator and the experiment (hands are an abundant source of RNase enzymes). This prevented the introduction of RNases and foreign RNA/DNA into the reactions. Gloves were changed frequently.

- All procedures were carried out under sterile conditions when feasible.

* DEPC is a strong, but not absolute inhibitor of RNases. It is also a suspected carcinogen.

2.10 Total RNA Isolation, Preparation and Analysis by RT-PCR

Total RNA was extracted from cultured cell lines and human tumour specimens throughout the course of these studies and analysed by the reverse transcriptase polymerase chain reaction.

2.10.1 Total RNA Extraction from Cultured Cell Lines

The following procedure was carried out in a laminar flow cabinet to maintain sterile conditions. Adherent cells were grown in 175cm^2 tissue culture flasks until approximately 80% confluent. The medium was removed and the cells in two replica flasks were rinsed twice with DEPC-treated PBS. Non-adherent cells were pelleted then resuspended and pelleted twice in DEPC-PBS. Cells prepared by either method were then lysed directly in a 4M guanidium thiocyanate (GnSCN) solution (Appendix K) (25ml per cell sample). The pooled cell lysate was centrifuged at 120g. for 5min and layered on 5.5ml of a 5.7M caesium chloride (Appendix K) cushion in a polyallomer ultracentrifuge tube. The mixture was spun at 100,000g. at 15°C for 21-24h in a swinging bucket centrifuge. This resulted in the separation of protein (at the top of the GnSCN layer) and DNA (at the GnSCN:CsCl interface) from the RNA pellet (at the bottom of the tube). Care was taken to prevent disturbing the RNA pellet or contaminating the RNA with DNA. The GnSCN solution and the "jelly-like" layer below the GnSCN:CsCl interface was removed by aspiration (using a pasteur pipette), until approximately 1ml of CsCl remained. The bottom of the tube (containing the RNA pellet and 1ml of CsCl) was cut from the rest of the tube using a heated scalpel blade. The tube bottom was inverted and the pellet rinsed with 95% ethanol at room temperature and resuspended in 200µl of DEPC-treated water by gently pipetting up and down whilst keeping on ice. The resuspended pellet was transferred to an eppendorf tube and the suspension remains were rinsed into the eppendorf with a further 200µl of DEPC-treated water. The RNA was precipitated out of solution by the addition of 3M sodium acetate, pH 5.2, (to result in a final volume of 0.3M) and 2 volumes of ice-cold absolute ethanol, overnight at -20°C. The RNA was pelleted by spinning at 4°C, at maximum speed in a microfuge. The pellet was washed with 70% ethanol, the supernatant removed and the pellet was briefly air-dried*. The pellet was resuspended in 100µl of DEPC-treated water and stored at -80°C.

* The pellet was not allowed to dry completely as this greatly decreases its solubility. The solubility of RNA can be improved by heating to 55-60°C with intermittent vortexing or by passing through a pipette tip.

2.10.2 Total RNA Extraction from Paraffin-Embedded Tissue

100μm of formalin-fixed paraffin embedded sections were cut into a sterile eppendorf. The paraffin wax was removed from the sections by incubating in 400μl octane (BDH; 294 075A) for 10min at 60°C. The eppendorf was then spun at maximum speed in a microfuge at 4°C for 10min. and the wax layer on top was removed from the pelleted tissue using a sterile pipette tip. A second wash in octane was then carried out followed by two washes in 70% ethanol and spinning in between each wash at maximum speed in a microfuge at 4°C for 10min. The tissue pellet was then air-dried and resuspended in 400μl of digestion buffer (R&D Systems; MBK-001-25) to which 0.1-2.0μg/ml Proteinase K (Promega; V3021) had been added immediately prior to use. The tissue was incubated at 50°C with constant agitation for 4-18h after which time the tissue had fully digested. 600μl of water-saturated phenol (pH 4) was then added to the digested tissue and vortexed vigorously for 10min. after which 120μl of chloroform:isoamyl alcohol (24:1) was added to the digest/phenol, mixed and centrifuged for 10min. at maximum speed in a microfuge at 4°C. The upper aqueous phase was then removed and washed with 600μl chloroform:isoamyl alcohol (24:1) and spun as above. The aqueous phase (containing the extracted total RNA) was precipitated out of solution by the addition of 2M sodium acetate, pH 4.0, (to result in a final volume of 0.2M) and 2 volumes of ice-cold absolute ethanol, overnight at -20°C. The RNA was pelleted by spinning at 4°C, at maximum speed in a microfuge. The pellet was washed with 70% ethanol, the supernatant removed and the pellet was briefly air-dried. The pellet was resuspended in 20μl of DEPC-treated water and stored at -80°C.

2.10.3 Total RNA Extraction from Fresh Tissue

Fresh tissue was transported from St.Vincent's Hospital, Dublin 4, in complete media and reached the laboratory within 1-4 hours of operation. On arrival the tissue was cut into small pieces and snap frozen in liquid nitrogen. The sample was then either stored at -80°C or used directly. A sample was taken for RNA extraction. The specimen was digested in 400μl of digestion buffer (R&D Systems; MBK-001-25) to which 0.1-2.0μg/ml Proteinase K (Promega; V3021) had been added immediately prior to use. The tissue was incubated at 50°C with constant agitation for 4-18h after which time the tissue

had fully digested. The digest was then stored at -80°C for RNA extraction or extracted directly as described in Section 2.10.2.

2.10.4 RNA Quantitation

RNA was quantified spectrophotometrically at 260nm and 280nm. An optical density of 1 at 260nm is equivalent to 40mg/ml RNA. An A_{260}/A_{280} ratio of 2 is indicative of pure RNA. 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). In these studies 200μgRNA/175cm^2 flask was retrieved.

2.10.5 RNA Quality

RNA quality was checked by running a quantity of RNA on a 1% formaldehyde/agarose gel. For a 100ml, 1% gel, 1g of agarose was combined with 73.4ml sterile distilled water and dissolved by heating in microwave. In a fume hood 10ml of 10X MOPS buffer (0.25M MOPS (Sigma; M8899), 0.05M Na-acetate, 0.01M EDTA, pH 7.0) and 16.6ml formaldehyde (BDH; 15513) were added to the molten agarose. 2.5μl of ethidium bromide (Sigma; E8751) (10mg/ml) was added and the gel was then poured. 1.0μl formaldehyde, 5.0μl formamide (BDH; 33272), 0.5μl loading buffer (50% glycerol (Sigma; G5576), 1mg/ml xylene cyanol FF (BDH; 44306), 1mg/ml bromophenol blue (Sigma; B5525), 1mM EDTA) was added to 1.75μl of RNA, and incubated at 65°C for 15 minutes, placed on ice and then loaded onto the gel. The gel was run in 1X MOPS buffer. Intact RNA is detected as two ribosomal (28S and 18S) RNA bands.

2.10.6 Micropipette Accuracy Tests

Accuracy and precision tests were carried out routinely on all micropipettes used in all steps of the RT-PCR reactions. The accuracy and precision of the pipettes was determined by standard methods involving repeatedly pipetting specific volumes of water and weighing them on an analytical balance. The specifications for these tests were supplied by Gilson.

2.10.7 Reverse Transcription of total RNA to form cDNA

cDNA was formed on mRNA templates extracted from either cell lines (see Section 2.10.1), paraffin-embedded (see Section 2.10.2) or fresh tissue (see Section 2.10.3) using the following procedures. All reactions were incubated on ice under sterile conditions.

2.10.7.1 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/µl) (Promega; C1101), 1µl of total RNA (1µg/µl), and 3µl of DEPC-H_2O were mixed together and heated at 70°C for 10min and then chilled on ice to remove any RNA secondary structure formation and allow the 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 $MgCl_2$), 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) (40,000U/µl) (Gibco; 5108025 SA) was then added to the heat-denatured RNA complex and the mixture was incubated at 37°C for 1h 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 2min. The cDNA was used immediately in the PCR reaction or stored at -20°C until required for analysis.

2.10.7.2 Reverse Transcription of RNA isolated from Paraffin Sections

The basic procedure used for the reverse transcription of mRNA isolated from paraffin sections was similar to that used for mRNA from cell lines (see Section 2.9.7.1). Due to the degraded nature of such mRNA, however, alterations had to be made to the priming of this mRNA during reverse transcription (see Section 3.13.1).

2.10.7.3 Reverse Transcription of RNA isolated from Fresh Tissue

RNA extracted from fresh tissue was reverse transcribed as described in Section 2.10.7.1.

2.10.8 Polymerase Chain Reaction (PCR) Analysis of cDNA

The cDNA formed in the above reaction was used for subsequent analysis by PCR for the expression of specific mRNAs.

2.10.8.1 PCR Analysis of cDNA formed from mRNA isolated from cell lines

Typical PCR reactions were set up as 50µl volumes using 5µl of cDNA formed during the RT reaction (see Section 2.9.7.1). cDNA was amplified for varying cycle numbers but where possible amplification was carried out in the exponential phase of amplification.
Each PCR reaction tube contained 26.5µl of water, 5µl 10Xbuffer (100mM Tris-HCl, pH 9.0, 50mM KCl, 1% Triton X-100), 2µl 25mM $MgCl_2$, 1µl of first strand target primer* (250ηg/µl), 1µl of second strand target primer* (250ηg/µl), 0.5µl of first strand endogenous control primer* (250ηg/µl), and 0.5µl of second strand endogenous control primer* (250ηg/µl). 5µl of cDNA (pre-heated to 95°C for 3min. 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 5min (reduces non-specific binding of primers to template). 8µl of 1.25mM dNTP and 0.5µl of Taq DNA Polymerase enzyme (Promega; N1862) was then added to the above and a drop of liquid paraffin (BDH; 29436) was added to each reaction tube. The cDNA was then amplified by PCR (Techne; PHC-3) using the following program:

- 94°C for 1.5min (denature double stranded DNA);
- 15-35 cycles 94°C for 1.5min (denature double stranded DNA);
  54 or 60°C for 1min (anneal primers to cDNA);
  72°C for 3min (extension);
- 72°C for 7min (extension).

* All oligonucleotide primers used throughout the course of this thesis were made to order on an "Applied BioSystems 394 DNA/RNA Synthesiser" by R&D Systems Ltd., 4-10 The Quadrant, Barton Lane, Oxon, England. Sequences of primers used are shown in Table 3.1.1 and Appendix C

All reaction tubes were then kept at 4°C until analysed by gel electrophoresis followed by densitometry.

A 10µl aliquot of tracking buffer, consisting of 0.25% bromophenol blue (Sigma; B5525)

and 30% glycerol in water, was added to each tube of amplified cDNA products. 10μl of cDNA products from each tube were then separated by electrophoresis overnight at 30mV through a 4% agarose (Promega; V3122) gel containing ethidium bromide (Sigma; E8751), using TBE (22.5mM Tris-HCl, 22.5mM boric acid (Sigma; B7901), 0.5mM EDTA) as running buffer. Molecular weight markers "ϕ-X174" Hae III digest (Promega; G1761) were run, simultaneously, as size reference.
The resulting product bands were visualised as pink bands (due to the intercalation of the cDNA with the ethidium bromide) when the gels were placed on a transilluminator (UVP Transilluminator). The gels were photographed and the negatives produced were analysed by densitometry (Imaging Densitometer, Bio-Rad. Model GS-670).

2.10.8.1.1 Densitometric Analysis

Densitometric analysis was carried using the MS Windows 3.1 compatible Molecular Analyst software/PC image analysis software available for use on the 670 Imaging Densitometer (BioRad, CA) Version 1.3.
Negatives of PCR gels were scanned using transmission light and the image transferred to the computer. The amount of light blocked by the DNA band is in direct proportion to the intensity of the DNA present. A standard area is set which is scanned and a value is taken for the OD of each individual pixel on the screen. The average value of this OD (within a set area) is normalised for background of an identical set area. This normalised reading is taken as the densitometric value used in analysis.

2.10.8.2 PCR Analysis of cDNA formed from mRNA isolated from Paraffin Sections

PCR analysis of cDNA from paraffin sections was carried out as in Section 2.10.8.1 but amplification was carried out for all target genes for at least 30 cycles - no analysis on exponential phase was undertaken.

2.10.8.3 PCR Analysis of cDNA formed from mRNA isolated from fresh tissue

PCR analysis of cDNA from paraffin sections was carried out as in Section 2.10.8.1. Amplification was carried out for all target genes for at least 30 cycles of PCR.

2.10.8.4 Restriction Digestion of RT-PCR Products

Products formed after RT-PCR amplification were digested with an appropriate enzyme as follows. A 10µl aliquot of the particular RT-PCR product, 10 Units of the relevant endonuclease restriction enzyme (Table 2.10.8.4.1), 5µl of 10X buffer (supplied with each restriction enzyme) and the appropriate volume of water to result in a total volume of 50µl, were mixed in an eppendorf tube and incubated at 37°C for 1h. A 20µl aliquot of the resulting products was analysed by electrophoresis through a 4% agarose gel (see Section 2.10.8.1).

Table 2.10.8.4.1 Restriction Enzymes used to Identify RT-PCR Products

Amplified Product	Restriction Enzyme	Supplier	Catalogue Number
bcl-2α	BamHI	Promega	R602A
bcl-2β	BamHI	Promega	R602A
bcl-xL	Bsp12861	Promega	R674A
bcl-xs	KpnI	Promega	R634A
bax-α/β	PvuII	Boehringer Mannheim	642 690
bax-β	BamHI	Promega	R602A
c-fos	HinfI	Boehringer Mannheim	779 652
c-jun	HincII	Promega	R603A
c-myc	HaeIII	Promega	R6171
c-Ha-ras-1	FokI	Promega	R6781
c-erbB-2	PvuII	Boehringer Mannheim	642 690

2.11 RNA Analysis by *In Situ* Hybridisation

Non-isotopic *in situ* hybridisation (NISH) was carried out on formalin-fixed paraffin-embedded sections using a digoxigenin(DIG)-labelled *mdr1* riboprobe and detected using an alkaline phosphatase-conjugated antibody.

2.11.1 Digoxigenin Labelling of *mdr1* Riboprobe

pHDR5A, consisting of a 1.4kb insert of *mdr1* in transcription vector pGEM4, was a generous gift from Dr.M.M.Gottesmann and was used as a template to create a digoxigenin-labelled riboprobe of *mdr1* for use in *in situ* hybridisation.

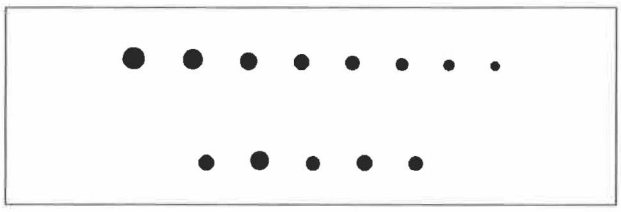
To generate the *mdr1* antisense probe, the plasmid was linearised with BamHI (Promega; R602A) and transcribed using Sp6 polymerase; the sense probe was generated by linearising the plasmid with NheI (Promega; R6502) and transcribed using T7 polymerase. A transcription kit (Boehringer-Mannheim; 1-175-025) was used to digoxigenen(DIG)-label the riboprobes according to the manufacturer's instructions. Incorporation of labelled nucleotides was measured using a dot-blot assay (see Section 2.11.1.1) and showed yields of ~5μg of RNA per μg of template cDNA. Sp6 labelling efficiency was increased greatly when an incubation temperature of 40°C was used in preference to the recommended 37°C.

A poly-dT probe, used to assess RNA preservation, was purchased as part of a kit from R&D Systems (BBS8). A β-actin probe (also used to assess RNA preservation in tissue) was purchased pre-labelled with digoxigenin (Boehringer-Mannheim; 1-498-045). An *In Situ* Workstation kit (R&D Systems; BBS1) was used for optimisation of digestion and post-hybridisation steps.

2.11.1.1 Dot-blot assay to estimate digoxigenin-labelled probe

1μl of each probe was dotted onto a nylon membrane (Amersham; Hybond N, RPN.3050N); standard controls of known labelling intensity were dotted in decreasing concentration on the top line, probes of unknown labelling concentration were placed on a lower line (Figure 2.11.1.1.1).

Figure 2.11.1.1.1 Dot-Blot Analysis to quantify DIG-labelled RNA



The detection procedure is a variation of that used by Boehringer-Mannheim (described in Boehringer-Mannheim; 1-175-041) and was carried out at room temperature.

The membrane was washed in 20ml 0.1M Tris-Cl, 0.15M NaCl, pH7.5 and 0.2% Triton X-100 for 1min followed by a 30min wash in 20ml 0.1M Tris-Cl, 0.15M NaCl, pH7.5 with 2% sheep serum. The membrane was then incubated for 30min in 20ml of a 1:5000 antibody conjugate in 0.1M Tris-Cl, 0.15M NaCl, pH7.5 and 1% sheep serum. The membranes were washed twice for 15min. in 0.1M Tris-Cl, 0.15M NaCl, pH7.5 and then equilibrated for 2min in 0.1M Tris-Cl, 0.1M NaCl, 0.05M $MgCl_2$, pH9.5. To assess probe intensity, the membranes were incubated in the dark in colour solution consisting of 0.1M Tris-Cl, 0.1M NaCl, 0.05M $MgCl_2$, pH9.5, 35µl BCIP (Boehringer Mannheim; 1-383-221) and 33.75µl NBT (Boehringer Mannheim; 1-383-213). When the colour had developed, the membranes were washed in 10mM Tris-Cl, 1mM EDTA, pH 8.0 and under running tap-water to stop colour development. The labelled probe concentration was estimated visually by comparing the probes of interest with the known standard concentrations.

2.11.2 Optimisation of *In Situ* Hybridisation Protocol for detection of *mdr1* mRNA

Many conditions were optimised in order to achieve successful specific hybridisation of probe to the tissue; these steps included -

- identifying a suitable digestion enzyme and concentration for the tissue
- optimising hybridisation conditions
- successful removal of unbound labelled probe subsequent to hybridisation
- sufficient blocking of slides prior to detection steps.

Proteinase K and pepsin are common digestion enzymes used for *in situ* hybridisation. To determine which enzyme was appropriate in this study, an *In Situ* Instructor kit (R & D

Systems; BBS1) was utilised to assess the effect of different digestion conditions on hybridisation of a labelled insulin probe to rat pancreas sections. It was found that Proteinase K, up to a concentration of 30μg/ml, allowed sufficient detection of the hybridised probe. The concentration of pepsin used (2mg/ml) was found to be sufficient to allow hybridisation of rat insulin probe and a small poly-dT probe to sections. This latter digestion was used thoughout the course of this work since it has generally been found to be effective on a wider range of tissues than Proteinase K.

Hybridisation conditions are the most critical steps to optimise for *in situ* hybridisation. Hybridisation with a buffer containing excess blocking agents or inappropriate stringency conditions prevents binding of the probe to the section while insufficient blocking allows for non-specific binding of probe and the generation of false positives. Probe concentration was also found to play an important role, with excess probe leading to high background and insufficient probe quantities resulting in the loss of signal from some cells. Hybridisation temperature is also important and pre-heating of sections to 95°C for 15min. resulted in a reduction of optimal hybridisation time at 50°C from 18 hours to 2 hours.

Initially hybridisation buffers were used which contained formamide, salt solutions, dextran sulphate (effectively increases the concentration of probe in the hybridisation buffer by forming a matrix in the hybridisation mixture; including dextran sulphate in the buffer can increase the hybridisation reaction by a factor of 3) and a detergent such as Triton-X100 (Sigma; X-100), but no blocking agents. When blocking agents, such as herring sperm DNA and transfer RNA, were added to the buffer a dramatic decrease in non-specific staining was observed. High stringency washes in formamide with SSC were found to be sufficient to remove any unbound labelled probe. The blocking steps used in this protocol were also sufficient as no staining was detected in control sections hybridised with control probes such as the sense (T7) probe or hybridised with the positive anti-sense probe (Sp6) pre-treated with RNase.

Specific staining of *mdr1* expression in erythroid precursors (haemopoeitic cells) in the sinusoids of foetal liver was detected using this procedure on formalin-fixed paraffin-embedded sections (Dr.S.Kennedy pers. comm.).

2.11.3 Non-Isotopic *In Situ* Hybridisation of *mdr1* riboprobe to Paraffin Sections

5μm sections were mounted onto poly-L-lysine (Sigma; P1274) coated slides. Sections

were deparaffinized twice in Xylene (2X 10min) and rehydrated in 99% ethanol (2X 2min), 95% ethanol (2X 2min) and incubated in DEPC-H_2O at 37°C for 10min. Tissues were digested in Pepsin/HCl (immediately prior to use 5mg Pepsin (Sigma; P6687) was dissolved in 2.17ml DEPC-H_2O; 0.33ml 1.5M HCl was then added slowly to the pepsin solution) at 37°C for 20min and then rinsed in 1XPBS at 37°C. Control sections were treated with RNaseA (Sigma; R9009) (100µg/ml) for 30min at 37°C to destroy all RNA. Post-fixation in 0.4% paraformaldehyde (in 1XPBS (Appendix K)) was then carried out at 4°C for 20min. and the slides were rinsed in DEPC-H_2O. Slides were pre-hybridised in pre-hybridisation buffer (50% deionized formamide (BDH; 33272), 2XSSC (Appendix K), 5% dextran sulfate, 0.3% Triton X-100, 1X Denhardt's solution (100X consists of 2%(w/v) bovine serum albumin (Sigma; A2153), 2% (w/v) Ficoll 400 (Sigma; F4375), 2% (w/v) polyvinylpyrrollidone (Sigma; PVP40) in water), 150µg/ml herring sperm DNA (Promega; D181A), 150µg/ml tRNA (Sigma; R8508), in DEPC-H_2O) for 1h at 37°C in a humid chamber. The slides were covered in hybridisation buffer (as for pre-hybridisation buffer but supplemented with the specific probe required (0.1-2.5µg/ml)) and cover-slipped; hybridisation was carried out at 95°C for 15min followed by 50°C for 2hour. To remove any unhybridised probe the slides were washed twice in solutions containing 30% formamide and (a) 4XSSC, (b) 2XSSC and (c) 0.2XSSC for 5min each at 50°C.

To detect the hybridised probe an alkaline phosphatase anti-DIG antibody Fab fragments (Boehringer Mannheim; 1-093-274) was used. The slides were blocked first in 1XTBS (Appendix K)/0.1%BSA/0.1%Triton X-100 for 25min at room temperature with gentle shaking. The antibody was then added to the slides (1:500 in 1XTBS/0.1%BSA) for 35min at room temperature. To remove any unbound antibody the slides were then washed twice in 1XTBS/0.1%BSA for 5min each at room temperature with vigorously. The revealing step involved the formation of a purple precipitate at the site of hybridisation due to the enzymatic action of alkaline phosphatase with NBT/BCIP. The slides were washed 2-3 times in 100mM Tris-HCl, pH9.5, 100mM NaCl and 50mM $MgCl_2$ and then colour solution (100mM Tris-HCl, pH9.5, 100mM NaCl and 50mM $MgCl_2$, 17.5µl BCIP (Boehringer Mannheim; 1-383-221), 16.88µl NBT (Boehringer Mannheim;1-383-213) per 5ml and levamisole (Dako; X3021)) was added for 10min in the dark. When the colour developed the slides were washed under running tap water and allowed to dry. The sections were counter-stained with 3% methylene green for 15sec and the slides mounted with an aqueous mounting gel such as GLYCERGEL (Dako; C563).

Notes:-

- Parafilm was used as cover-slips during hybridisation
- All hybridisation steps were carried out in a humid atmosphere. This was achieved simply by lying tissue soaked in 2XSSC at the base of a sealed hybridisation chamber.
- The amount of solution required during hybridisation was reduced (thus lowering the overall cost of the procedure) by circling the area of the slide containing the section with an Immuno-pen (Dako; S2002) that forms a seal with the slide.
- For accurate analysis of mRNA expression by NISH, a number of controls must be set up with the positive slide, namely, hybridisation with (i) sense probe, (ii) control probe (gene not present in cells under investigation), (iii) oligo-dT or β-actin probe (to assess mRNA quality throughout the cell sample), (iv) pre-treatment with RNase and (v) hybridisation buffer without labelled probe.

2.12 Gene Induction by Adriamycin and Analysis by RT-PCR

To determine the effect Adriamycin has on gene expression, DLKP-SQ cells were exposed to varying concentrations of drug and after set time points total RNA was extracted from the cells and analysed by RT-PCR.

2.12.1 Extraction of total RNA for analysis of Gene Induction by Adriamycin

DLKP-SQ cells were grown to 50% confluency in 25cm^2 flasks. The cells were then exposed to adriamycin (at 25ng/ml continuously or 250ng/ml for 4h after which the drug was removed, the cells washed twice in complete media and re-fed in media without drug) and total RNA was extracted after 0, 8, 15, 30min and 1, 2, 4, 6, 9, 24, 48 and 72h. Control samples were also taken from cells, at the same time-points, which had been fed with fresh media without drug. Total RNA was extracted from the cells using the Ultraspec-II kit (Biotecx; BL-12200). Briefly, this involved lysing the cells with 1ml of lysis buffer (guanidine and phenol) and further chloroform extraction. Once the cells had been lysed directly in the flask the sample was stored at -80°C for further processing; this involved a chloroform extraction by adding 0.2ml of chloroform to the 1ml cell lysate, centrifuging and precipitating the total RNA in the aqueous layer out of solution by incubating overnight at -20°C in 2 volumes of absolute ethanol and sodium acetate, pH 4.0. The RNA was pelleted at 4°C and resuspended in 20µl of DEPC-H_2O.

2.12.2 RT-PCR Analysis of Gene Induction

Total RNA extracted to analyse gene induction by Adriamycin was amplified by RT-PCR as described in Sections 2.10.7.1 and 2.10.8.1

2.13 Protein Analysis

Protein analysis was carried out by Western blotting using whole cell extracts and purified nuclear extracts.

2.13.1 Whole Cell Extract Preparation

Cells were grown in 175cm^2 flasks until they reached 80-90% confluency. The cells were then trypsinised and centrifuged at 120g. for 5 min. The pellet was washed in PBS and re-pelleted twice. 1ml of lysis buffer (PBS, 1% NP-40 (Sigma; N-3516), 1X protease inhibitors and 0.2mg/ml PMSF(Sigma; P7626)) was added to the pellet and left on ice for 20 min. A 100X stock solution of protease inhibitors consisted of 400mM DTT (Sigma; D5545), 1mg/ml aprotonin (Sigma; A1153), 1mg/ml leupeptin (Sigma; L2884), 1mg/ml soybean trypsin inhibitor (Sigma; T9003), 1mg/ml pepstatin A (Sigma; P6425) and 1mg/ml benzamidine (Sigma; B6506). If cell lysis had not occurred after 20 min the cells were subjected to sonication. Whole cell extracts were aliquoted and stored at -80°C.

2.13.2 Nuclear Extract Preparation

Cells were grown in 175cm^2 flasks until they reached 80-90% confluency. The cells were trypsinised and centrifuged at 120g. for 5 min. The cell pellet was then washed in TE buffer and re-pelleted twice. The cells were re-suspended in 10mM Tris-HCl, pH7.5, 10mM NaCl, 3mM $MgCl_2$, 0.5% NP-40, 0.2mg/ml PMSF and 1X protease inhibitors at 1x10^{7}cells/ml on ice until cell lysis was observed, as determined by trypan blue staining. The lysate was centrifuged at 800g. for 10min to pellet the nuclear extract. The pellet was washed in 10mM Tris-HCl, pH7.5, 10mM NaCl, 3mM $MgCl_2$, 1X protease inhibitors, 0.2mg/ml PMSF and centrifuged. To lyse the nuclei, the nuclear pellet was incubated in 350mM NaCl, 5mM KH_2PO_4, pH7.0, 2mM $MgCl_2$, 0.1mM EDTA, 0.2mg/ml PMSF and 1X protease inhibitors on ice for 30 min at a cell density of 1x10^{8}cells/ml. The lysate was aliquoted and stored at -80°C.

2.13.3 Quantification of Protein

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad; 500-0006)

with a series of bovine serum albumin (BSA) (Sigma; A9543) as standards. The dye reagent was provided as 5-fold concentrate. The appropriate standards (0.02ml) and test samples (0.02ml) were placed in clean, dry test tubes. The diluted dye reagent (1ml) was added and the mixture vortexed. After a period of 5 min to 1h, the OD_{595} was measured, against a reagent blank. From the plot of the OD_{595} of BSA standards versus their concentrations, the concentration of protein in the test samples was determined.

2.13.4 Gel electrophoresis

The protein present in the cell preparations were separated on a size basis using SDS polyacrylamide gel electrophoresis (SDS-PAGE) :-

Table 2.13.4.1 SDS-PAGE Recipes for 2 x 0.75mm Thick Gels

	Resolving Gel			Stacking Gel
	7.5%	**12%**	**15%**	**5%**
Acrylamide Stock*	3.8ml	5.25ml	7.5ml	0.8ml
Distilled H_2O	8.0ml	6.45ml	4.3ml	3.6ml
1.875M Tris, pH 8.8	3.0ml	3.0ml	3.0ml	.....
1.25M Tris, pH 6.8	.....	.....	.....	0.5ml
10% SDS	150µl	150µl	150µl	50µl
10% NH_4-persulphate	60µl	50µl	50µl	17µl
TEMED	9.0µl	10µl	7.5µl	8µl

* Acrylamide stock solution consists of 29.1g acrylamide (Sigma; A8887) and 0.9g NN'-methylene bis-acrylamide (Sigma; 7256) dissolved in 60ml UHP water and made up to 100ml final volume. The solution was stored in the dark at 4°C for up to 1 month. All components were purchased from Sigma; SDS (L4509), NH_4-persulphate (A1433) and TEMED, N,N,N',N'-tetramethylethylenediamine (T8133).

The resolving gel was immediately poured into two clean 10cm x 8cm gel cassettes comprising of a glass and aluminium plate separated by two 0.75cm spacers on either outer edge. The gel was overlayed with a 10% SDS solution and allowed to set. Once set, the SDS solution was poured off and the stacking gel layered on top of the resolving gel. A comb of appropriate thickness and well size was immediately inserted and the gel allowed to set. When the wells had formed, the gel combs were removed and the gels transferred to a mini-electrophoresis apparatus. The gels were flooded with running buffer (1.9M glycine (Sigma; G6761), 0.25M Tris, 0.1% SDS, pH 8.3 without adjustment).

Protein samples were loaded into the wells, based on equal protein loading. The samples to be loaded were diluted 1:1 with loading buffer (50mM Tris-HCl, pH 6.8; 0.1% SDS; 5% 2-mercaptoethanol (Sigma; M6350); 5% glycerol and 0.1% bromophenol blue) and boiled for 2 min. They were then loaded onto the gel, as were the appropriate molecular weight markers (New England Biolabs; 77085). The gels were run for approximately 1.5 hours with voltage set at 250V and current set at 45mA.

2.13.5 Western Blotting Procedure

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris; 192mM glycine; pH 8.3 - 8.5 without adjustment). Nitrocellulose filter (Amersham; Hybond-ECL RPN2020D), which was cut to the same size as the gel, was soaked in transfer buffer for 5 min. Six stacked sheets of gel-size Whatman 3mm filter paper were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Excess air was removed from between the filters by sliding a pipette over and back on the filter paper. The nitrocellulose was placed over the filter paper, again ensuring no air bubbles became trapped. The acrylamide gel was placed on the nitrocellulose and the nitrocellulose was marked at the sites of the gel lanes and size markers. Six more sheets of pre-soaked filter paper was placed on top of the gel. The protein was transferred from the gel to the nitrocellulose at a current of 0.34mA/0.15V for 30 min. The nitrocellulose was then blocked in blocking buffer and exposed to specific antibodies. Negative blots were also performed whereby the primary antibody was replaced with antibody diluent.

2.13.5.1 P-glycoprotein

Total protein was separated on a 7.5% SDS polyacrylamide gel. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked for 3 hr at room temperature in TBS (500mM NaCl; 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed twice with TBS and was exposed to the primary antibody (1 in 2 dilution of supernatant (BRI)) at 4°C overnight. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary antibody (horse radish peroxidase-conjugated (HRP)) (1:1000 dilution of anti-mouse-HRP (Sigma; A6782) in TBS, 0.1% Tween-20) for 1.5 hours at room temperature. The nitrocellulose was again

washed three times in TBS and was developed as outlined in Section 2.13.6.

2.13.5.2 c-erbB-2

Total protein was separated on a 7.5% SDS polyacrylamide gel. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked for 2 hours at room temperature in TBS (500mM NaCl; 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed twice with TBS and was exposed to the primary antibody at a dilution of 1:500 (c-erbB-2, DAKO; A0485) at 4°C overnight. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary antibody (1:1000 dilution of anti-rabbit-HRP (Sigma; A4914) in TBS, 0.1% Tween-20) for 1 hour at room temperature. The nitrocellulose was again washed three times in TBS and was developed as outlined in Section 2.13.6.

2.13.5.3 c-Fos

Nuclear protein was separated on a 12% SDS polyacrylamide gel. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked overnight at 4°C in TBS (500mM NaCl; 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed twice with TBS and was exposed to the primary antibody (c-Fos(Ab-2), Calbiochem; PC05) at 2.5μg/ml at room temperature for 2 hours. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary (1:1000 dilution) anti-rabbit-HRP antibody (Sigma; A4914) in TBS, 0.1% Tween-20 for 1 hour at room temperature. The nitrocellulose was again washed three times in TBS and was developed as outlined in Section 2.13.6.

2.13.5.4 Bcl-x

Total protein was separated on a 15% SDS polyacrylamide gel. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked for 2 hours at room temperature or over-night at 4°C in TBS (500mM NaCl; 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed

twice with TBS and was exposed to the primary antibody (Bcl-x(Ab-1), Calbiochem;PC67), at 2.5μg/ml, for 2 hours at room temperature. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary antibody (1:1000 dilution of anti-rabbit-HRP (Sigma; A4914) in TBS, 0.1% Tween-20) for 1.5 hours at room temperature. The nitrocellulose was again washed three times in TBS and was developed as outlined in Section 2.13.6.

2.13.6 Development of Western Blots by ECL

Western blots were developed by a chemiluminescence method. An equal volume of ECL solutions 1 and 2 (Amersham; RPN2209) were mixed together (3ml/blot). The nitrocellulose was placed, protein side up, on a piece of flat cling-film and covered with the detection reagent for 1 min at room temperature. Excess detection reagent was poured off and the membranes were wrapped in cling-film, ensuring no air-pockets were created. A sheet of autoradiography film was placed on top of the membranes and exposure time varied. Film was developed and fixed using standard methods.

2.14 Transfection of Mammalian Cells with Exogenous DNA

Throughout the course of this thesis it was found necessary to introduce foreign DNA into host cells either to increase the level of expression of a particular gene (by transfecting with an expression plasmid) or decrease the level of expression of a particular gene (by transfecting with a plasmid containing a ribozyme).
Sufficient plasmid was produced by transforming JM109 with the plasmid required, growing up a large stock of these cells and isolating the plasmid from them; this isolated plasmid was then transfected into the chosen cell line.

2.14.1 Plasmids used

The c-*fos* ribozyme was cloned into the pMAM-*neo* inducible mammalian expression vector and was a generous gift from Dr. Kevin Scanlon. The plasmid is induced by adding dexamethasone to the growth medium. The *bcl-x*$_S$ cDNA was a generous gift from Dr. Gabriel Nuñez.

2.14.2 $MgCl_2$ / $CaCl_2$ Transformation of JM109 Cells

10ml of LB broth (Appendix K) was inoculated with a single colony of JM109 bacteria from an agar plate and incubated overnight at 37°C at 200r.p.m. The following day 500µl of this suspension was inoculated into 50ml of LB broth and grown to an OD_{600nm} of 0.3. The cells were then pelleted at 3000r.p.m. for 10min, the supernatant removed and the pellet was resuspended in 10ml of 100mM $MgCl_2$, on ice for 15min. The cells were again precipitated at 3000r.p.m. for 10min and the pellet was resuspended in 10ml of 100mM $CaCl_2$ on ice for a further 15min. The precipitation step was then repeated and the pellet was resuspended in 1-2ml of 100mM $CaCl_2$ and left on ice for at least 15min. The cells were now competent and ready for transformation with the foreign DNA required.
100µl of the competent cell suspension was mixed with 20ng DNA and placed on ice for 40min after which the mixture was heat-shocked at 42°C for 90sec and then placed on ice for 3min. 1ml of LB broth was added to the competent cell suspension and incubated at 37°C for 40min. 400µl of this suspension was spread on a selecting agar plate (Ampicillin/AMP (Boehringer Mannheim; 835 269)) and incubated overnight at 37°C. Single colonies which grew on these selecting plates were further colonised on another

selecting plate and allowed to grow overnight.

2.14.2.1 Isolation of Plasmid from JM109 cells

A single colony (from 2.14.2) was inoculated into 10ml of LB AMP 50μg/ml and grown overnight; 2ml of this suspension was added to 200ml of TB AMP 50μg/ml and left to grow overnight at 37°C for large scale isolation of plasmid from JM109 cells. The following day the cells were pelleted and pZ523 spin columns (5 Prime → 3 Prime Inc.; 5-523523) were used to isolate the plasmid according to the manufacturer's instructions. This procedure involved lysing the pellet in 20ml of an ice-cold solution containing 50mM glucose, 25mM Tris-Cl, 10mM EDTA, pH8.0 and 5mg/ml lysozyme (Sigma; L6876) at room temperature for 10-15min. 40ml of a 0.2N NaOH and 1.0% SDS solution was gently mixed with the lysate until the suspension became clear and incubated on ice for 10min. 30ml of 3M K-Acetate, pH5.2 was added to the above and mixed gently until a flocculent precipitate appeared at which stage the mixture was stored on ice for at least 10min. The sample was centrifuged at 35,000g. for 1h at 4°C after which the supernatant was recovered and added to 0.6 volume of 100% Isopropanol, mixed gently and left at room temperature for 20-30min. The suspension was then centrifuged at 35,000g. for 30min at 20°C after which the supernatant was discarded and the pellet washed in ice-cold 70% ethanol and resuspended in 5ml of TE, pH8.0. To remove any contaminating RNA the plasmid solution was treated with RNase Plus (5 Prime → 3 Prime Inc.; 5-461036) (to a final dilution of 1:250) for 30min at 37°C followed by phenol:chloroform:isoamyl alcohol extractions. 10M ammonium acetate was added to the aqueous phase to a final concentration of 2.0M and 0.6 volume of 100% Isopropanol was added to the sample, mixed and stored at room temperature for 20-30min. The sample was centrifuged at maximum speed in an epifuge and the DNA pellet was washed in 70% ethanol and resuspended in 3.6ml of 10mM Tris-Cl, 1mM EDTA, and 1.0M NaCl, pH8.0. 1.8ml of this sample was loaded into one of two pZ523 columns (following the manufacturer's instructions) and the column effluent was precipitated with 0.6 volume 100% Isopropanol, as described previously. The DNA was pelleted at maximum speed in an epifuge, washed in 70% ethanol and resuspended in TE. The DNA concentration was determined by measuring the OD_{260nm}.

2.14.3 $CaPO_4$ Transfection of Mammalian Cells

On the day prior to transfection the cells to be transfected with plasmid DNA were plated from a single cell suspension (Section 2.2.2) and seeded into a $75cm^2$ flask at $5x10^5$ cells per flask. The plasmid DNA was diluted to 1μg/μl in TE and 10μg DNA was stored overnight in 410μl H_2O at 4°C.

On the day of the transfection the diluted DNA was incubated at 37°C for 1h 60μl 2M $CaCl_2$ was added dropwise to the DNA with continual mixing. Immediately the DNA-$CaCl_2$ mixture was added dropwise into the 2XHBS (Appendix K) solution with continual mixing and left at room temperature for 30min to form a DNA-$CaPO_4$ mixture. The DNA-$CaPO_4$ mixture was added to the flask of cells (containing media) dropwise, swirling constantly to ensure even mixing. The cells were then incubated for 4h at 37°C after which time the cells were "shocked" with glycerol to aid the entry of the DNA into the cells. Glycerol-shocking was done by removing the media from the cells and adding 5ml of 10% glycerol in 1XHBS to the cells for 3min. The glycerol was then removed, the cells rinsed twice in 5ml serum-free media and then re-fed with 10ml fresh growth media and incubated for 2-3 days at 37°C.

2.14.3.1 Selection and Isolation of Colonies

In order to study the true effect of transfection studies, single colonies of stably transfected cells were selected and isolated. The selection process was carried out by feeding the "transfected" cells with media containing geneticin (Sigma; G9516) - the plasmids used had a geneticin-resistant gene, therefore, only those cells containing the plasmid will survive treatment with geneticin. 2 days after transfection the flask of cells was fed with 200μg/ml geneticin in complete media, when the cells grew readily in this concentration of selecting agent, the concentration was increased step-wise to a final concentration of 600μg/ml. At this stage the cells were plated at clonal density (see Section 2.6) and clonal populations were propagated, as described previously. Transfected cells were periodically challenged with geneticin to establish stability of transfectants.

2.14.4 Induction of c-Fos Ribozyme

To assess the effect of the c-fos ribozyme in cells, the plasmid was induced by feeding cells in 5μM dexamethasone (Sigma; D1756) 24 hour prior to drug addition.

3. Results

3.1 Design of Oligonucleotide Primers for RT-PCR Studies of Oncogene mRNAs

Primer pairs were designed to allow detailed analysis of the expression of a number of oncogenes, in multi-drug resistant cell lines and human tumours, in order to determine if any of these genes have a role to play in MDR *in vitro* or *in vivo*.

The genes chosen for study were those of the *bcl-2* family, including *bcl-2α*, *bcl-2β*, *baxα*, *baxβ*, *bcl-x_L* and *bcl-x_S*; also 3 nuclear oncogenes were studied, namely, c-*fos*, c-*myc*, c-*jun*; two other oncogenes, c-*erb*B-2 and c-*Ha-ras*1, were also included in this study. All other primers used for the study of MDR-related genes have been published previously (O'Driscoll *et al.*, 1993).

β-actin was used as a control in all reactions as it has previously been found to be most suitable for the study of mRNA expression in MDR-related studies (O'Driscoll *et al.*, 1993). Depending on the target length to be amplified, one of two pairs of PCR primers were used - a 383bp (previously published) or a 142bp *β-actin* template was amplified in all reactions with the gene of interest. Some primers were co-amplified initially with *β-2-microglobulin* (114bp product produced) as control.

All primers were chosen to conform to as many as possible of the guidelines discussed in Appendix A. The guideline that was adhered to most vigorously was that of sequence specificity with the uniqueness of each primer checked *via* the DNA data-base at EMBL. It was not possible to comply with all of the guidelines for each primer pair and the primers chosen were found to be the best compromise in each individual situation. All primers chosen maintain 100% homology with the gene in question. The primers chosen contain no common homologies with the internal control primers to amplify *β-actin*. The specific criteria of base composition, length, T_m and amplified target length for each primer pair can be seen in Table 3.1.1 (see also Appendix A).

3.1.1 Examples of chosen Primer Pairs

The primers chosen for the amplification of members of the *bcl-2* family of genes and the guidelines followed for their selection are discussed below.

(a) Bcl-2α and *bcl-2β*

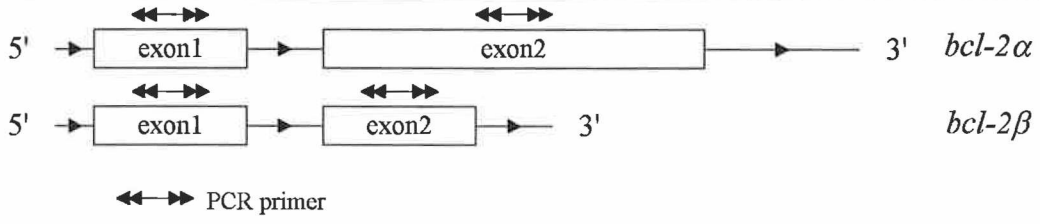
A common 5'primer for *bcl-2α* and *bcl-2β* but different 3' primers were chosen (to distinguish the *α*- from the *β*-form); this latter primer was chosen from different locations

on the template, thus allowing both forms to be differentiated by size. The α- and β-forms of bcl-2 are identical in exon1 but have different second exons where α has a very long 3'untranslated region and β does not.

The 5'primer was chosen from exon1 and the 3'primer of the α-form was chosen from exon2 (Figure 3.1.1.1). *bcl-2* commonly forms a hybrid with immunoglobulin heavy chain transcripts as a result of t(14;18 translocations). These hybrids consist of the 5' half of the *bcl-2* mRNA fused to a "decapitated" Ig heavy chain mRNA. All of the break-points identified thus far in the *bcl-2α* gene have been found at the 3' end of the second exon and care was taken not to include this region in the primers. Although these primers will not distinguish between an amplified sequence in a normal *bcl-2* mRNA or the hybrid mRNA, no other common homologies between the primers were identified.

The 3' primer of the β-form was chosen from its second exon and no homology to the α-form was apparent. The primers were chosen from the open reading frame.

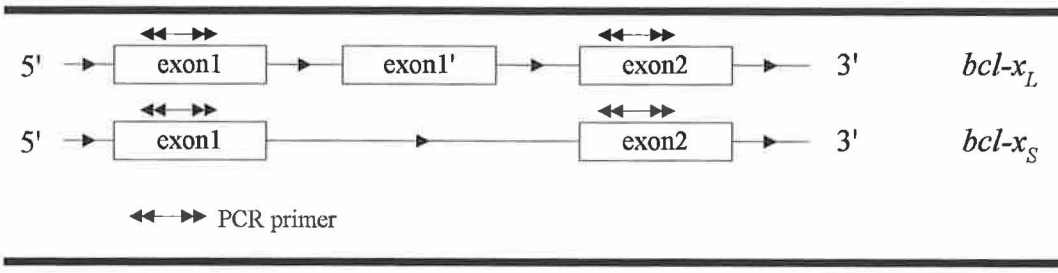
Figure 3.1.1.1 Gene structure of *bcl-2α* and *β* and location of primers chosen on template



(b) Bcl-x_L* and *bcl-x_S

The *bcl-x* gene is transcribed to give two alternatively spliced forms of the gene, a long form (*bcl-x_L*) and a short form (*bcl-x_S*). The sequences of both transcripts are identical except that the shorter form has a central exon spliced from the mRNA. The primers used were designed to flank this missing exon and therefore could be used to amplify both templates and differentiate between them by size.

Figure 3.1.1.2 Gene structure of *bcl-x_L* and *bcl-x_S* and location of primers chosen on template

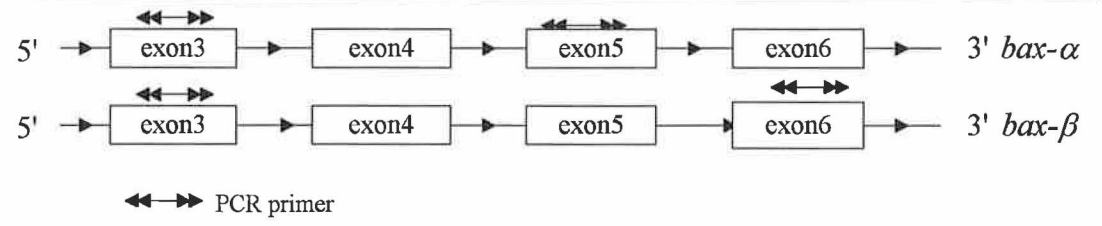


(c) Bax-α and *bax-β*

When designing primers for the bax genes the 3' region of the α-form was avoided as it was found to be highly homologous to other human sequences; to compensate for this a pair of common *α/β* primers were chosen and also a specific 3' primer to the *β*-form as the *α- and β*-forms differ only in their 3' regions, specifically in the sixth exon. The 3' primer of the *β*-form is very close to the 3' end of the gene and this is important to note if analysing degraded mRNA.

The 5' primer is located on exon3 and the 3'primer for *α /β* on exon 5; the 3' primer for the *β*-form was chosen from exon6.

Figure 3.1.1.3 Gene structure of *bax-α* and *bax-β* and location of primers chosen on template



3.1.2 Trial Amplification and Restriction Digestion of Amplified Oncogene Products

Trial amplification of all genes to be studied by RT-PCR was carried out to ensure that each primer pair chosen amplified a specific cDNA, producing a single band of the predicted size. PCR reactions were set up as described in Section 2.10.8.1 using cDNA formed on mRNA templates extracted from a number of cell lines included in this study, some of which were known to express the specific cDNA in question.

All reactions were set up in duplicate and an endogenous control (*β-actin*) was co-amplified with the required oncogene product. Primers to produce a short *β-actin* band of 142bp were included in the PCR reaction mix with primers for each of the following oncogenes; *bcl-x*, *bax*, *bcl-2*, c-*fos*, c-*jun*, c-*myc* and c-*erb*B-2. Co-amplification of c-Ha-*ras1* was carried out with *β-actin* primers to amplify a band of 383bp. Results are shown in Fig. 3.1.2.1. (In preliminary studies *β-2-microglobulin* (114bp band) was used as an internal control). Amplification of all products was successful, resulting in a single band of appropriate size (Figure 3.1.2.1).

An aliquot of each amplified product (after 30 cycles of PCR) was digested with an

appropriate restriction enzyme. An example of these results (from DLKP variant cDNA analysis) is illustrated in Figure 3.1.2.1(i). Co-amplification of a 383bp *β-actin* band is seen with *bcl-2* (Figure 3.1.2.1(i)); *β-2-microglobulin* internal controls (114bp) are included with the c-*fos* and c-*erbB-2* digests (Figures 3.1.2.1(i)).

All PCR products are run with ϕX174/HaeIII digested DNA marker comprising of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72bp fragments.

3.2 Establishing Exponential PCR Cycle Range

To allow for semi-quantitative analysis of PCR products formed during the course of these studies, the exponential PCR cycle range for each primer pair described in Table 3.1.1 was determined. cDNA formed on mRNA templates extracted from DLKP and DLKP-A cell lines, which were used as representative sensitive and drug resistant variants, respectively, were used in this analysis.

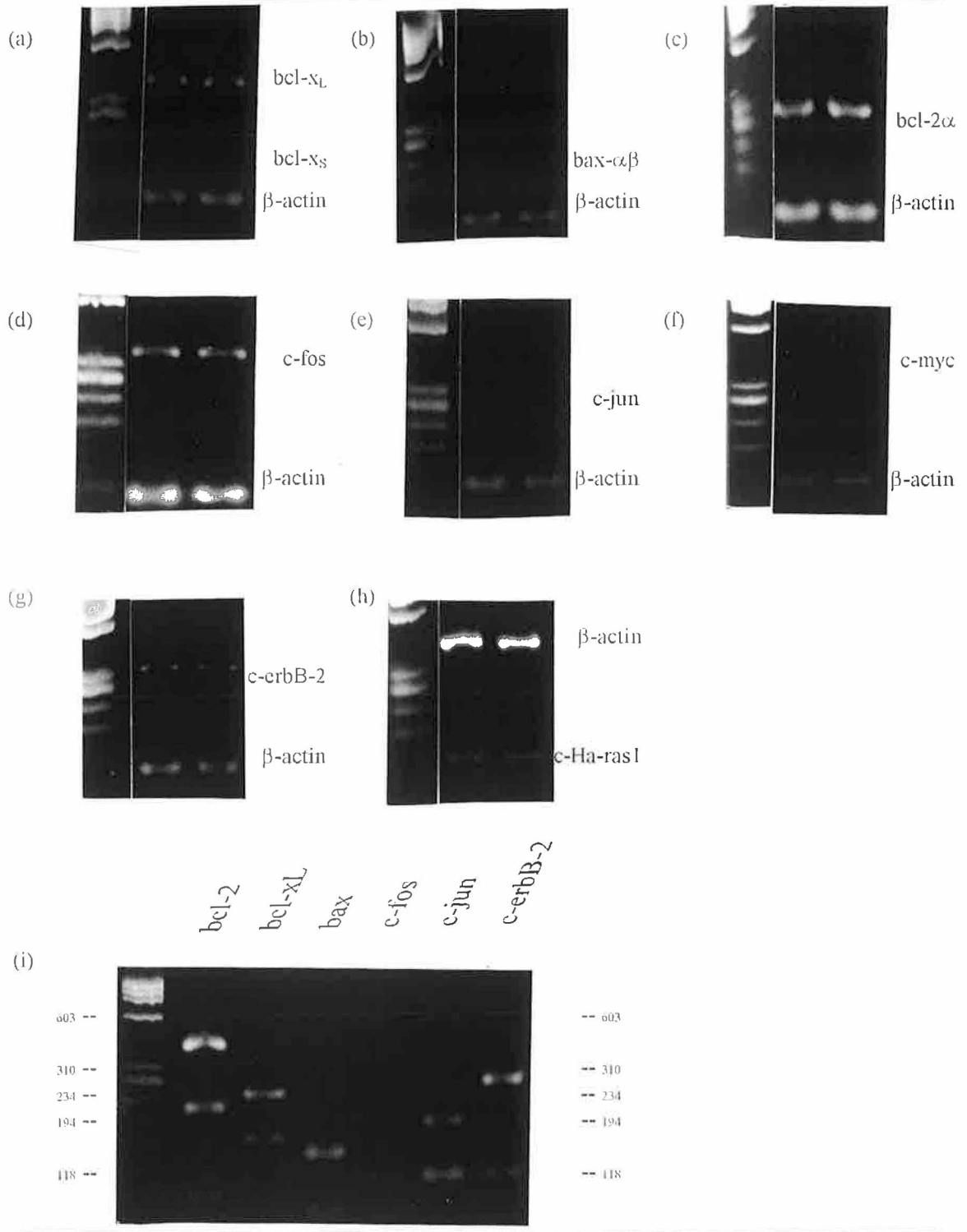
100μl reaction volumes were set up in a tube for each PCR reaction and 10μl volumes were removed at a number of time points, ranging from 15 to 35 cycles. Exponential range studies were carried out in duplicate, from two independent RNA extractions. All results were analysed by densitometry.

A representative study is illustrated in Figure 3.2.1 of the exponential range of *bax* expression in DLKP and DLKP-A.

Gene		Primer length (bases)	A+T:G+C	T_m	Amplified cDNA length (bases)	Diagnostic R.E.	Restriction product length (bases)	Amplified DNA sequence length (bases)	Location on template (accession #)
bcl-2α	(a)	22	11:11	66	306	BamHI	97+209	Not Known	1925-1946 (M13994)
	(b)	24	14:10	68					2207-2230
bcl-2β	(a)	22	11:11	66	145	BamHI	97+48	Not Known	613-634 (M13995)
	(b)	21	11:10	62					737-757
bcl-xL	(a)	20	9:11	62	396	Bsp1286I	235+161	Not Known	345-364 (L20121)
	(b)	19	8:11	60		KpnI	91+305		722-740
bcl-xs	(a)	20	9:11	62	207	KpnI	91 +116	Not Known	345-364 (L20122)
	(b)	19	8:11	60					533-551
bax-α/β	(a)	21	11:10	62	230	PvuII	88+142	Not Known	202-222 (L22473/4)
	(b)	20	9:11	62					412-431
bax-β	(a)	21	11:10	62	439	BamHI	251+188	Not Known	202-222 (L22474)
	(b)	21	12:9	60					619-639
c-fos	(a)	20	10:10	60	343	HinfI	84+259	457	2514-2533 (K00650)
	(b)	20	10:10	60					2951-2970
c-jun	(a)	20	10:10	60	292	HincII	178+114	292	234-253 (J04111)
	(b)	20	10:10	60					506-525
c-myc	(a)	18	7:11	58	336	HaeIII	74+262	1712	1154-1171 (V00568)
	(b)	21	10:11	64					1469-1489
c-Ha-ras-1	(a)	20	10:10	60	129	FokI	65+64	302	2177-2196 (V00574)
	(b)	19	8:11	60					2460-2478
c-erbB-2	(a)	20	10:10	60	332	PvuII	82+250	Not Known	2478-2497 (X03363)
	(b)	20	10:10	60					2790-2809
β-Actin	(a)	23	11:12	68	142	Fnu4HI	44+98	254	901-923 (X00351)
	(b)	22	11:11	66					1021-1042

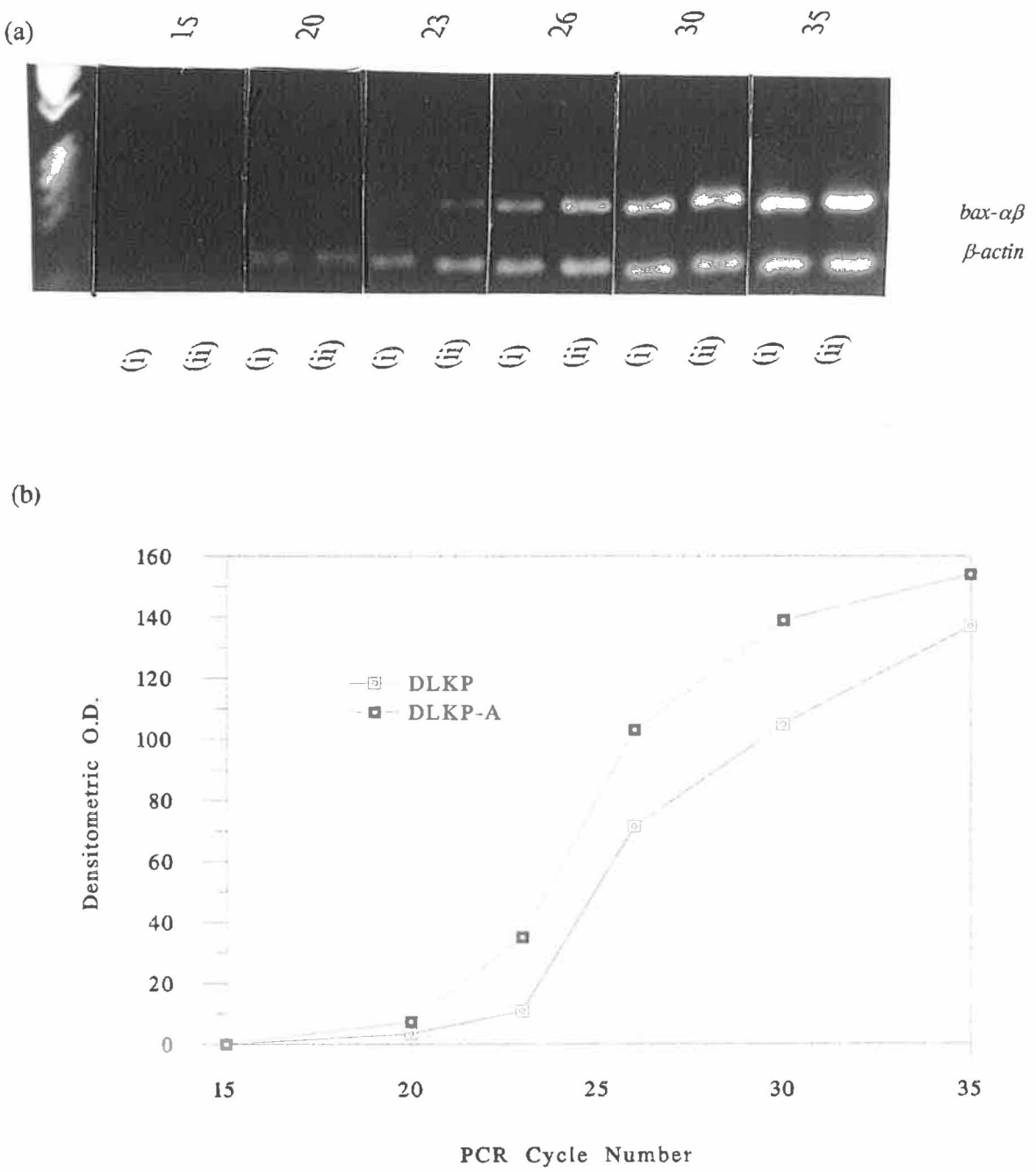
Table 3.1.1 Primers to amplify cDNA formed by reverse transcription on mRNA templates for oncogene expression analysis

Figure 3.1.2.1 Trial Amplification and Product Restriction of Oncogene Products



PCR Amplification for (a) bcl-xL (396bp) and bcl-xS (207bp); (b) bax (230bp); (c) bcl-2 (306bp); (d) c-fos (343bp); (e) c-jun (292bp); (f) c-myc (336bp); (g) c-erbB-2 (332bp) and (h) c-Ha-ras1 (129bp) co-amplified with a 142bp (a-g) or 383bp (h) β-actin product. Restriction digest of PCR products is shown in (i). All PCR products are run with ϕX174/HaeIII digested DNA marker comprising of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72bp fragments.

Figure 3.2.1 Exponential Range of PCR Amplification for *Bax* Expression in DLKP and DLKP-A



cDNA from DLKP(i) and DLKP-A(ii) was used as template to establish exponential phase for the amplification of the *bax* gene. 100μl reaction volumes were set up for PCR and 10μl aliquots were removed after 15, 20, 23, 26, 30 and 35 cycles. Equal volumes for each cycle were run on agarose gels (a) and analysed by densitometry (b). This representative study would suggest semi-quantitative analysis of *bax* gene levels in DLKP and DLKP-A be carried out between 23 and 26 cycles of PCR.

3.3 Analysis of mRNA Transcripts of Oncogene Products in Sensitive vs MDR Cell Line Variants of DLKP

Total RNA was extracted from the parental DLKP cell line and a number of well characterised resistant variants of that cell line, namely, DLKP-A (adriamycin selected variant), DLKP/VP-3, DLKP/VP-8 (VP-16 selected variant) and DLKP-C14 (carboplatin) cell lines (see Appendix D for fold resistance levels). This RNA was used as template for reverse transcription as described previously (Section 2.10.7.1). cDNA was analysed by PCR in the exponential phase for each of the following genes : *bcl-x, bax, bcl-2,* c-*fos,* c-*jun,* c-*myc,* c-*erbB*-2 and c-H-*ras1*.

The resulting bands were analysed to determine if any change in expression of these genes was evident between the sensitive parental cell line, DLKP, and DLKP-A, DLKP/VP-3 DLKP/VP-8 or DLKP-C14 (see Figures 3.3.1 and 3.3.2). No analysis on c-*jun* levels is shown as this gene is intronless and mRNA amplification and DNA contamination could not be differentiated between by PCR analysis.

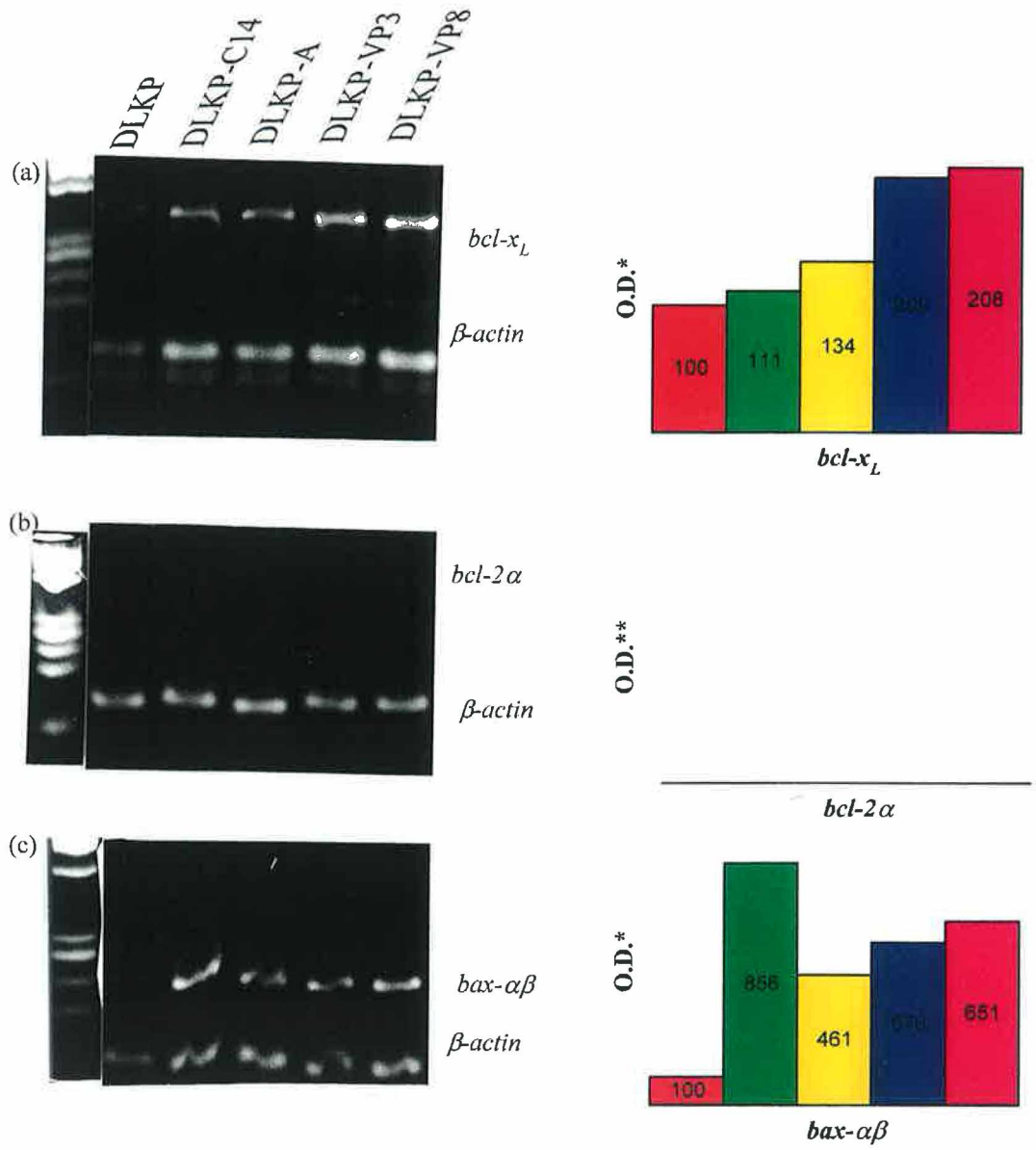
Initial observations revealed that *bcl-*x_L gene expression was increased in the resistant lines studied; *bax* levels were also increased in the variants, however no expression of *bcl-2* was detected even after 30 cycles of PCR (Figure 3.3.1). Increased levels of c-*erb*B-2 was detected in all the resistant lines studied compared to the sensitive DLKP line. c-*fos* expression was increased only in the adriamycin selected line and not in those cell lines selected with carboplatin or VP-16. c-*myc* levels were increased in the DLKP-C14 line but decreased in the adriamycin and VP-16 selected lines. Slight alteration in c-Ha-*ras1* levels was evident under these conditions (Figure 3.3.2).

3.3.1 Expression of Oncogene Products in Clonal Populations of DLKP-A

DLKP-A is a well characterised heterogeneous population of an MDR cell line. Clonal populations with differing resistance profiles have previously been isolated from this resistant line (Heenan *et al.*, 1997). In the current study total RNA isolated from the least resistant clonal population (DLKP-A2B) and the highest resistant population (DLKP-A5F) of DLKP-A was analysed by RT-PCR to determine if alterations in oncogene expression play a role in the development of sensitive and resistant populations within a single heterogeneous population.

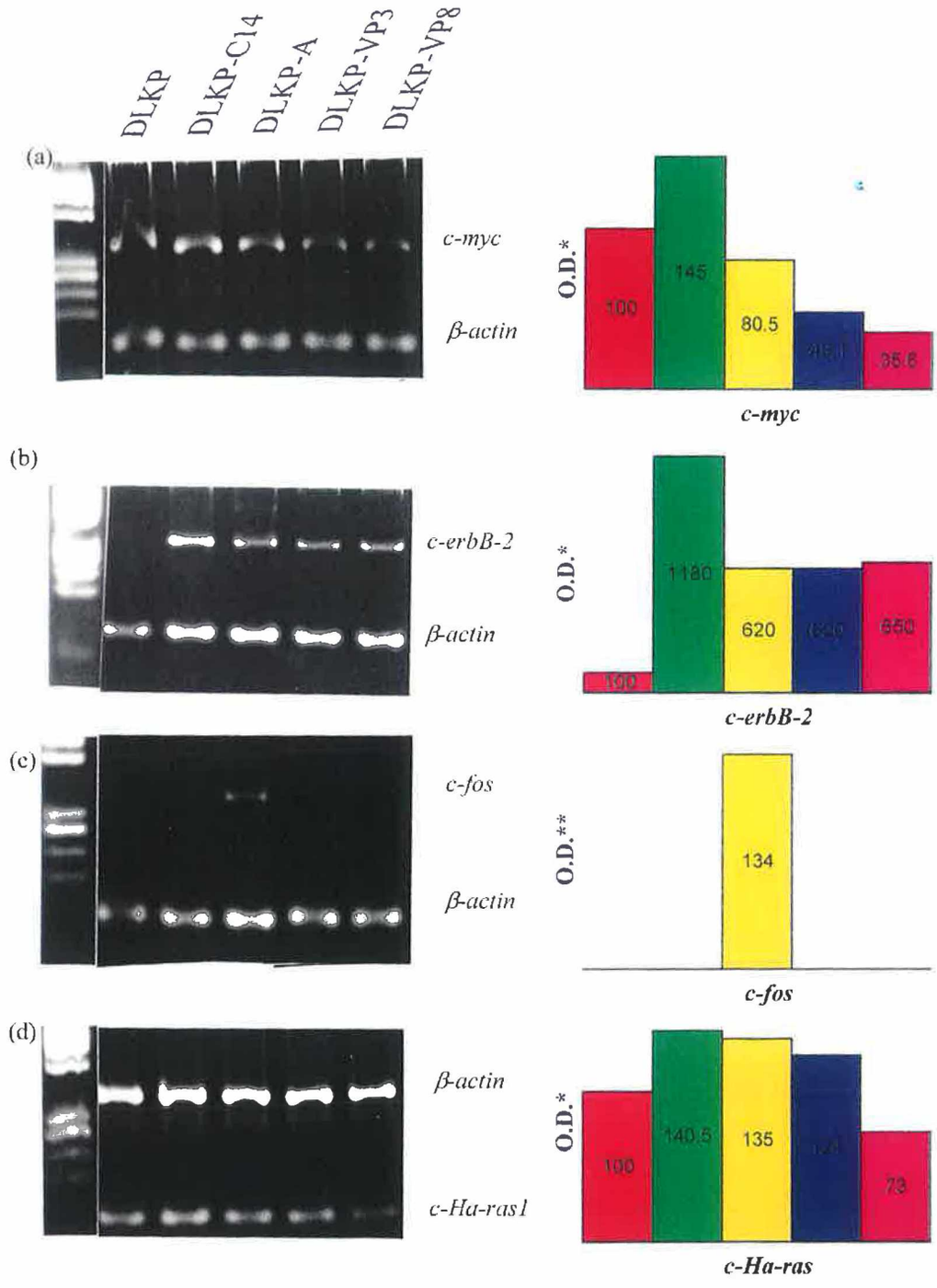
RT-PCR analysis after 30 cycles of PCR revealed differential expression of *bcl-2α* (referred to as *bcl-2* in further analysis) mRNA in DLKP-A and the clonal variants analysed. Increased levels of c-*fos* were observed in both the DLKP-A and DLKP-A5F variants but not in DLKP-A2B cells relative to the parental DLKP-A cell line (Figure 3.3.1.1). RT-PCR analysis revealed differential expression of *bcl-x_L* in the three resistant cell lines studied (Figure 3.3.1.2(a)). *bcl-x_L* levels were increased in DLKP-A5F cells relative to DLKP-A2B cells. Analysis of *bax-αβ* (referred to as *bax* in further analysis) levels also revealed differential expression among the variants with increased levels seen in DLKP-A2B relative to DLKP-A and DLKP-A5F (Figure 3.3.1.2(b)). From these studies it was seen that the most sensitive clone, DLKP-A2B, had a higher *bax:bcl-x_L* ratio than the most resistant DLKP-A5F clone. Higher levels of c-*erb*B-2 were detected in DLKP-A5F relative to DLKP-A2B cells, although the ratio of the clones to the parental DLKP-A cell line was seen to differ between reactions (Figure 3.3.1.3(a)). Elevated levels of c-Ha-*ras1* were observed in the DLKP-A5F clone as compared to DLKP-A and DLKP-A2B which had expressed similar amounts of c-Ha-*ras1* mRNA (Figure 3.3.1.3(b)).

Figure 3.3.1 RT-PCR Analysis of Oncogene Expression in Resistant Variants of DLKP



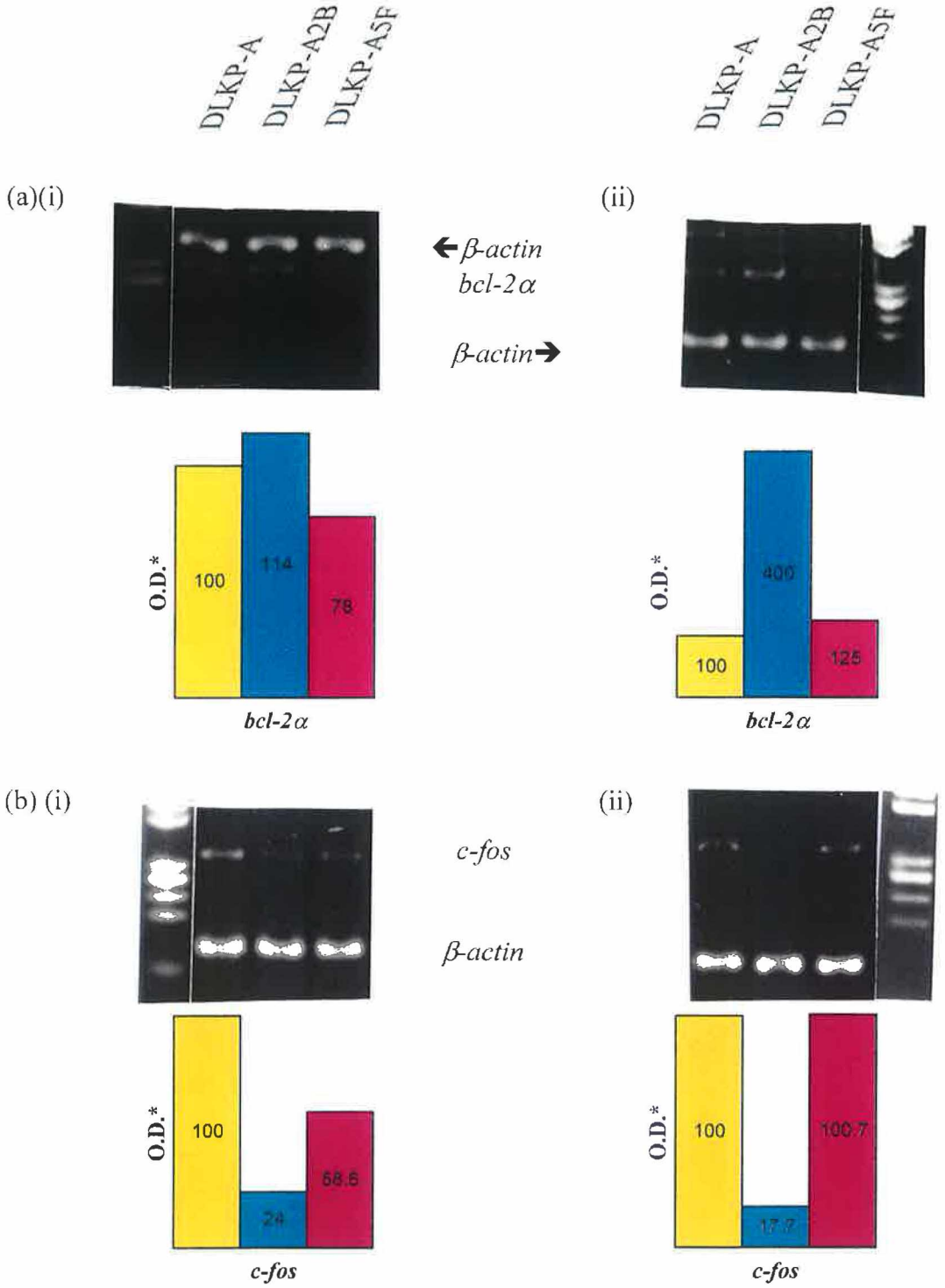
cDNA from DLKP (■), DLKP-C14 (■) (carboplatin-selected line), DLKP-A (■) (adriamycin-selected cell line), DLKP-VP3 (■) and DLKP-VP8 (■) (VP-16-selected cell lines) was amplified by PCR for the expression of (a) *bcl-xL* (23 cycles of PCR); (b) *bcl-2α* (30 cycles of PCR) and (c) *bax-αβ* (23 cycles of PCR). A 142bp β-actin product was co-amplified in all PCR reactions. Densitometric O.D.* is given as the ratio of the levels of the specific gene product to the internal *β-actin* control and is normalised to 100% for DLKP. No bands were detected for the expression of *bcl-2α***.

Figure 3.3.2 RT-PCR Analysis of Oncogene Expression in Resistant Variants of DLKP



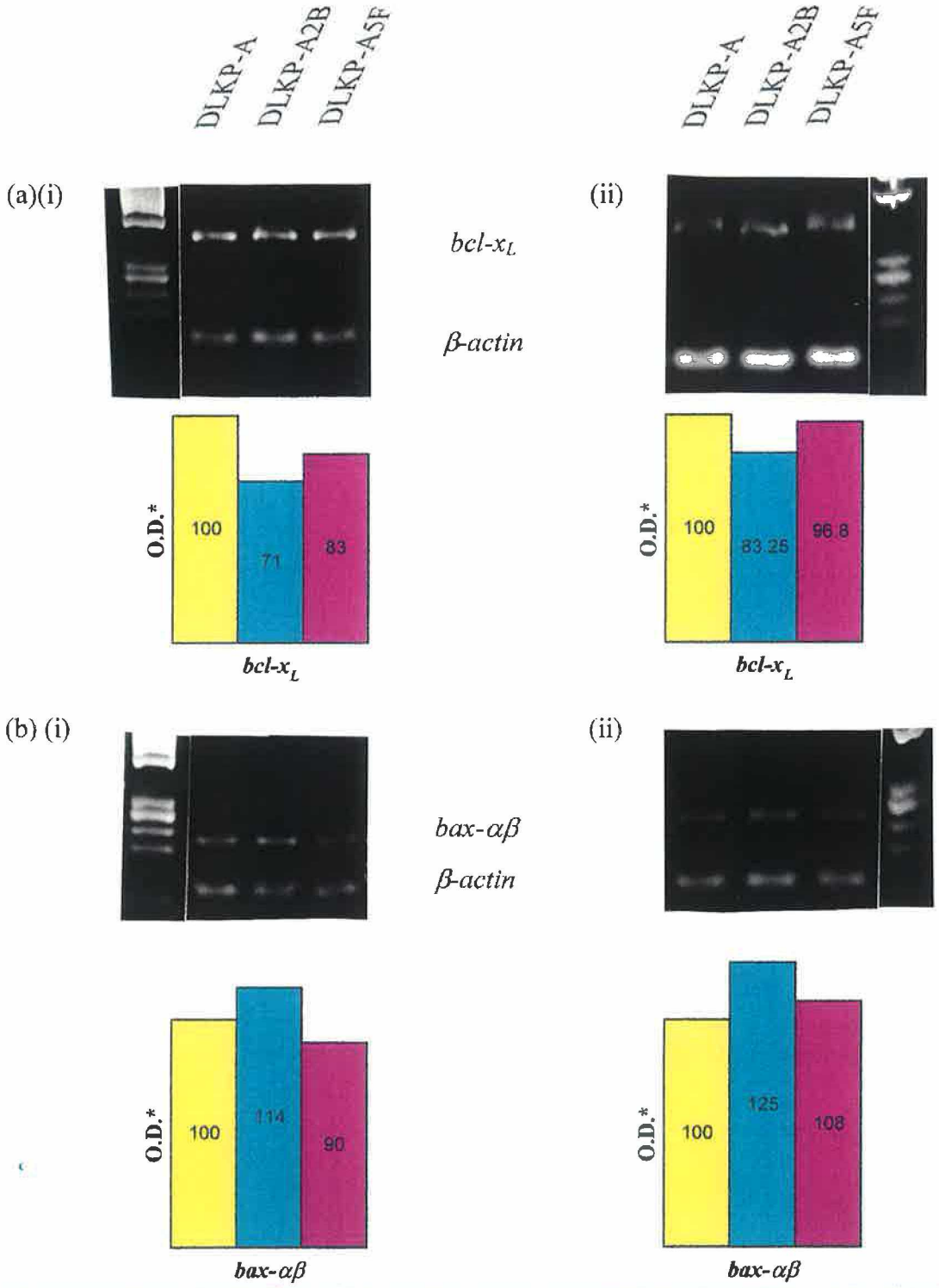
cDNA from DLKP (■), DLKP-C14 (■), DLKP-A (■), DLKP-VP3 (■) and DLKP-VP8 (■) was amplified by PCR for the expression of (a) *c-myc* (25 cycles); (b) *c-erbB-2* (24 cycles); (c) *c-fos* (30 cycles) and (d) *c-Ha-ras1* (30 cycles). A 142bp *β-actin* product is co-amplified with *c-myc*, *c-erbB-2* and *c-fos* products. A 383bp *β-actin* band is co-amplified with the *c-Ha-ras1* product. Densitometric O.D.* is presented as the ratio of the levels of specific gene product to the internal *β-actin* control and

Figure 3.3.1.1 RT-PCR Analysis of Oncogene Expression in DLKP-A and Clonal Variants



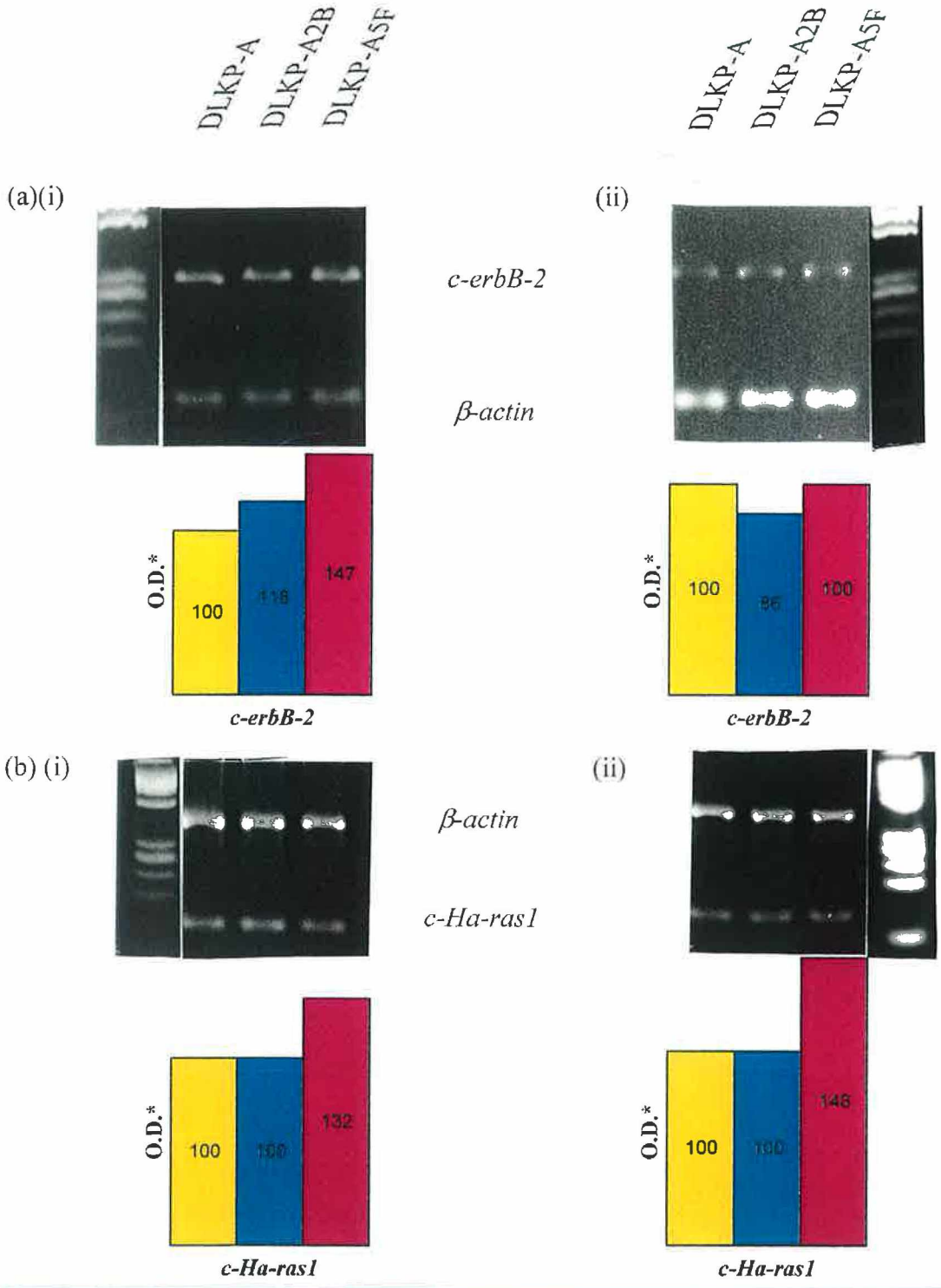
cDNA from DLKP-A (■), DLKP-A2B (■) and DLKP-A5F (■) was amplified by PCR for the expression of (a) *bcl-2α* (30cycles of PCR) and (b) *c-fos* (30 cycles of PCR) from two independent RNA extractions. A 142bp or 383bp *β-actin* product was co-amplified in all reactions. Densitometric O.D.* is presented as the ratio of the levels of *bcl-2* and *c-fos* gene product to the internal *β-actin* control and is normalised to 100% for DLKP-A.

Figure 3.3.1.2 RT-PCR Analysis of Oncogene Expression in DLKP-A and Clonal Variants



cDNA from DLKP-A, DLKP-A2B and DLKP-A5F was amplified by PCR for the expression of (a) *bcl-xL* (23 cycles of PCR) and (b) *bax-αβ* (23 cycles of PCR) from two independent RNA extractions. A 142bp *β-actin* product was co-amplified in all reactions. Densitometric O.D.* is presented as the ratio of the levels of *bcl-x* and *bax* gene product to the internal *β-actin* control and is normalised to 100% for DLKP-A.

Figure 3.3.1.3 RT-PCR Analysis of Oncogene Expression in DLKP-A and Clonal Variants



cDNA from DLKP-A (■), DLKP-A2B (■) and DLKP-A5F (■) was amplified by PCR for the expression of (a) *c-erbB-2* (25 cycles of PCR) and (b) *c-Ha-ras1* (30 cycles) from two independent RNA extractions. A 142bp or 383bp *β-actin* product was co-amplified in all reactions. Densitometric O.D.* is presented as the ratio of the levels of *c-erbB-2* or *c-Ha-ras1* gene product to the internal *β-actin* control and is normalised to 100% for DLKP-A.

3.3.2 Western Blot Analysis of Oncogene Expression in Resistant Variants

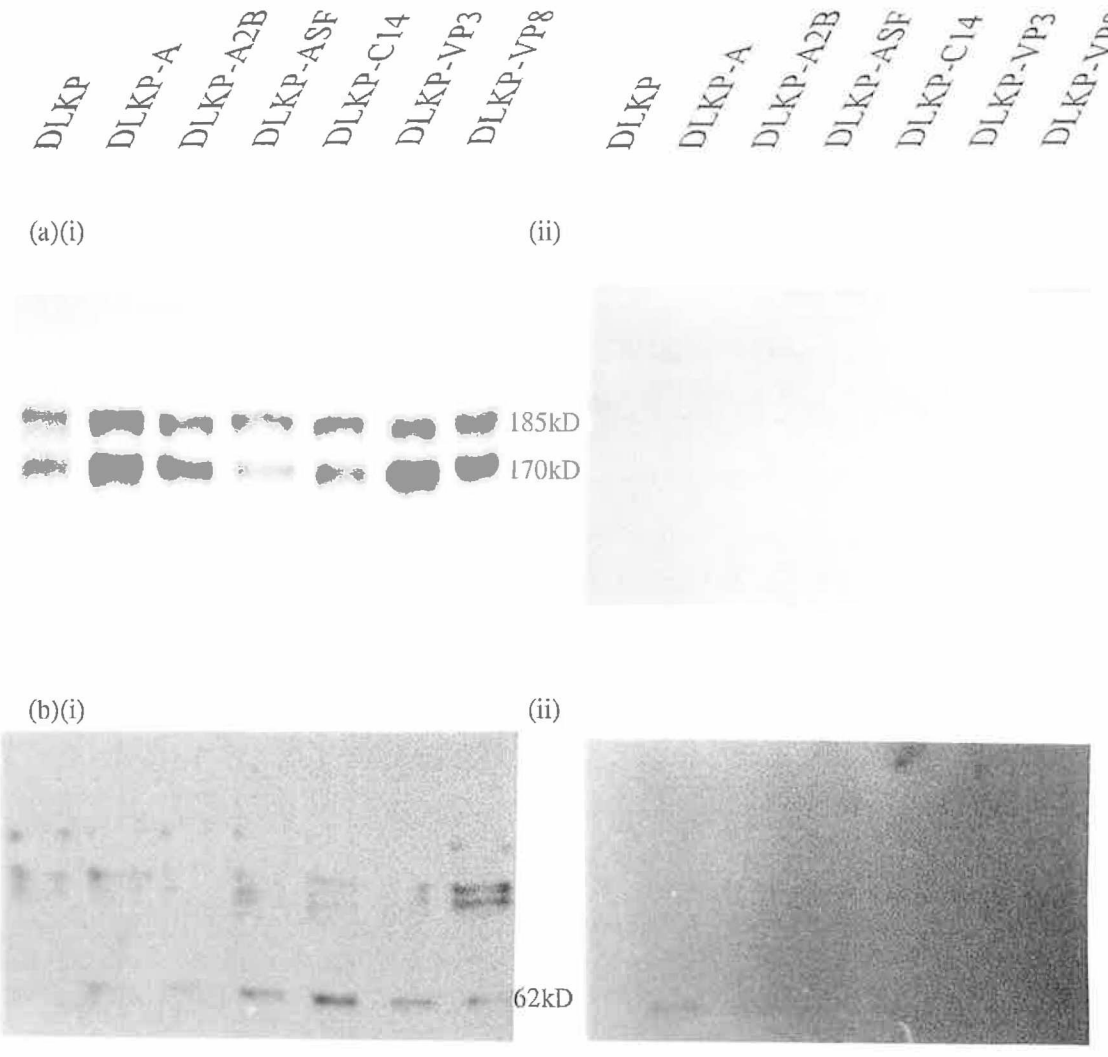
Analysis of c-*erb*B-2, c-Fos and Bcl-x protein expression was performed using Western blotting techniques. Successful analysis was achieved using the Dako c-*erb*B-2 polyclonal antibody. Two specific bands were detected for this antibody (p185 and p170), in agreement with previous results (Slamon *et al.*, 1989a). An increase in expression of c-*erb*B-2 protein was seen in DLKP-A, DLKP-A2B, DLKP-VP3 and DLKP-VP8 cell lines relative to the parental DLKP cell line (Figure 3.3.2.1(a)). c-*erb*B-2 protein levels were not found to be over-expressed in the DLKP-A5F cell line. Slightly elevated levels were seen in the DLKP-C14 cell line. Differential expression was seen between the two bands detected for the c-*erb*B-2 protein, the significance of this is unknown. The trend at the protein level is different to that previously seen at the mRNA level (Figure 3.3.2(b)). mRNA levels, as analysed by RT-PCR, of c-*erb*B-2 demonstrated overexpression of the gene in all the resistant variants of DLKP analysed with greatest overexpression observed in the DLKP-C14 cell line.

Nuclear protein extracts were used to analyse c-Fos levels in cells, limited success was achieved (Fig. 3.3.2.1(b)). A very faint band was seen for the 62kDa c-Fos protein in the DLKP cell line. Stronger bands were detected for the resistant variants. However, a band in the negative control was also seen at 62kDa for DLKP-A. Highest expression of c-Fos protein was seen in the DLKP-C14 cell line and to a lesser extent in the DLKP-A5F and VP-16-selected DLKP-VP3 and DLKP-VP8 variants. This was not the observed trend when mRNA levels were analysed (Figure 3.3.2(c) and Figure 3.3.1.1(b)) as c-*fos* mRNA was only detected in the DLKP-A and DLKP-A5F cell lines.

Total protein was analysed for the expression of Bcl-x protein by Western blotting without success (results not shown).

Analysis was carried out on one protein extraction from each cell line. Time constraints prevented further analysis.

Figure 3.3.2.1 Western Blot Analysis of c-*erb*B-2 and c-Fos Expression in DLKP Variants



(a) Total protein isolated from DLKP, DLKP-A, DLKP-A2B, DLKP-A5F, DLKP-C14, DLKP-VP3 and DLKP-VP8 was analysed for the expression of c-*erb*B-2 protein using a 1:500 dilution of an anti-c-*erb*B-2 antibody (Dako). (b) Nuclear protein isolated from DLKP, DLKP-A, DLKP-A2B, DLKP-A5F, DLKP-C14, DLKP-VP3 and DLKP-VP8 was analysed for the expression of c-Fos protein using an anti-c-Fos antibody at 2.5μg/ml (Calbiochem). Positive blots are shown in (a)(i) and (b)(i); negative controls are shown in (a)(ii) and (b)(ii).

3.4 c-*fos* Ribozyme Transfection and Effect on Toxicity in DLKP-A cells

A c-*fos*-specific ribozyme was transfected into DLKP-A. Expression of this ribozyme was induced by dexamethasone. The transfection technique involved calcium-phosphate precipitation of the ribozyme onto monolayer cultured cells followed by glycerol shocking as described previously (Section 2.14.3). Six individual colonies were maintained as individual cell lines after selection in 600μg/ml geneticin. The resulting cell lines were designated DLKP-A/*fos*RzB4, DLKP-A/*fos*RzB5, DLKP-A/*fos*RzB6, DLKP-A/*fos*RzE2, DLKP-A/*fos*RzC11 and DLKP-A/*fos*RzF3.

3.4.1 Adriamycin Toxicity Analysis of Transfected Clones

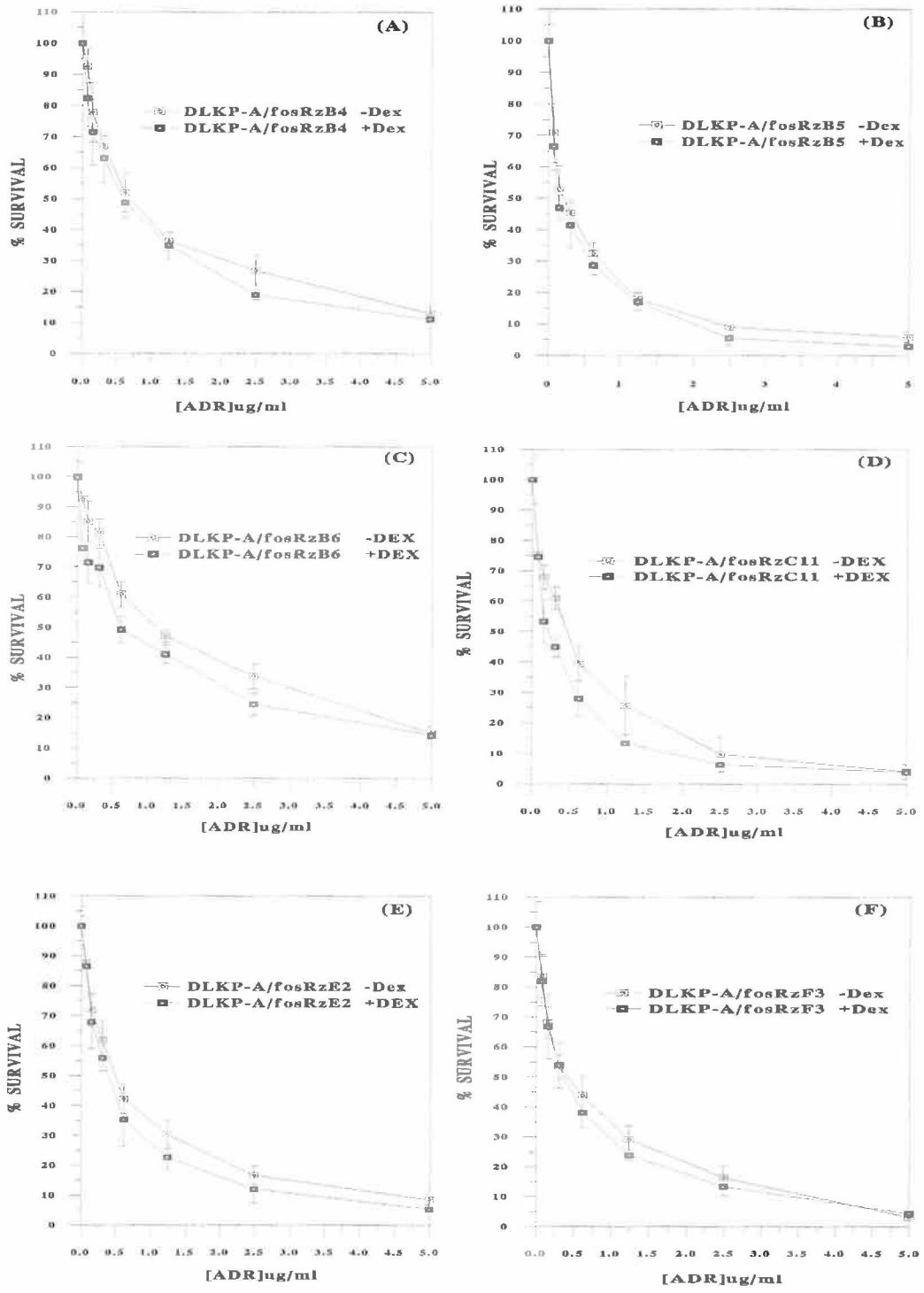
The toxicity profile of adriamycin in the c-*fos* ribozyme transfected cell lines revealed minimum effect of ribozyme induction on adriamycin toxicity in the selected cells. The ribozyme was induced with 5μM dexamethasone (used previously by Funato *et al.*,1992) 24 hours prior to addition of drug. The drug was added in dexamethasone supplemented medium. The IC_{50} of adriamycin in DLKP-A cells is in the order of 3.7μg/ml which is substantially higher than that seen in any of the *fos*-ribozyme transfected clones (see Figure 3.4.1.1). As DLKP-A is composed of a mixed population of varying resistance levels it is not known whether transfection has selected inherently sensitive clones. The least resistant DLKP-A clone, DLKP-A2B, has an IC_{50} value of 0.5μg/ml adriamycin whereas the most resistant clone, DLKP-A5F, has an adriamycin IC_{50} value of 4.8μg/ml. A two-fold decrease in adriamycin resistance, as determined by comparing IC_{50} values, was seen in clones B6 (Figure 3.4.1.1(C)) and C11 (Figure 3.4.1.1(D)) when cells were treated with 5μM dexamethasone (see Table 3.4.1.1); no decrease in toxicity was seen in the other clones isolated (Figure 3.4.1.1(A),(B),(E),(F)) when treated with dexamethasone.

Table 3.4.1.1 Adriamycin Toxicity Level in c-*fos* Ribozyme Transfected DLKP-A Cells

IC_{50} μg/ml**	ATCC (-Dex)	ATCC (+Dex)*
DLKP-A	3.7	.....
DLKP-A2B	0.5	.....
DLKP-A5F	4.8	.....
DLKP-A*fos*RzB6	1.2	0.625
DLKP-A*fos*RzC11	0.5	0.25

**IC_{50} values for the *fos* ribozyme transfected cells is significantly lower than the value for the parental DLKP-A cell line with or without dexamethasone treatment. Treatment with 5μM dexamethasone* for 24 hours reduced the IC_{50} values of the ribozyme-expressing cells two-fold. The parental DLKP-A cell lines were not analysed in the presence of dexamethasone.

Figure 3.4.1.1 Adriamycin Toxicity Profile of *c-fos* Ribozyme Transfected Clones



Adriamycin toxicity profile is shown for six clones of DLKP-A transfected with the c-fos ribozyme. Cells were pre-treated for 24 hours with 5μM dexamethasone (+Dex) to induce the ribozyme and then treated with adriamycin in dexamethasone-supplemented media. Control assays were set up without dexamethasone (-Dex).

3.4.1.1 Transfection of DLKP-A5F with c-*fos* Rz and effect on Adriamycin Toxicity

To determine if a clearer result could be found on the effect c-*fos* ribozyme expression has on adriamycin toxicity in the DLKP-A cell line, DLKP-A5F, a highly resistant clonal population of DLKP-A shown to over-express c-*fos* when compared to DLKP, was transfected with the c-*fos* ribozyme using those methods described in Section 2.14.3. Individual colonies were not isolated from the transfected population.

The resulting transfected population, DLKP-A5F*fos*Rz, was analysed, in the presence and absence of dexamethasone, for adriamycin toxicity levels. Results revealed no significant effect of c-*fos* ribozyme transfection on resistance levels in this cell line (Table 3.4.1.1.1).

Table 3.4.1.1.1 Adriamycin Toxicity (IC_{50}) in DLKP-A5F c-*fos* Ribozyme Transfected Cells

	DLKP-A5F	DLKP-A5F*fos*Rz (-Dex)	DLKP-A5F*fos*Rz (+Dex)
[ADR]μg/ml	1.9 ± 0.1	2.2 ± 0.4	1.7 ± 0.3

Adriamycin toxicity levels in DLKP-A5F cells and c-*fos* ribozyme transfected DLKP-A5F cells fed with (+Dex) or without (-Dex) 5μM dexamethasone as control.

3.4.2 RT-PCR Analysis of c-*fos* Ribozyme Variants

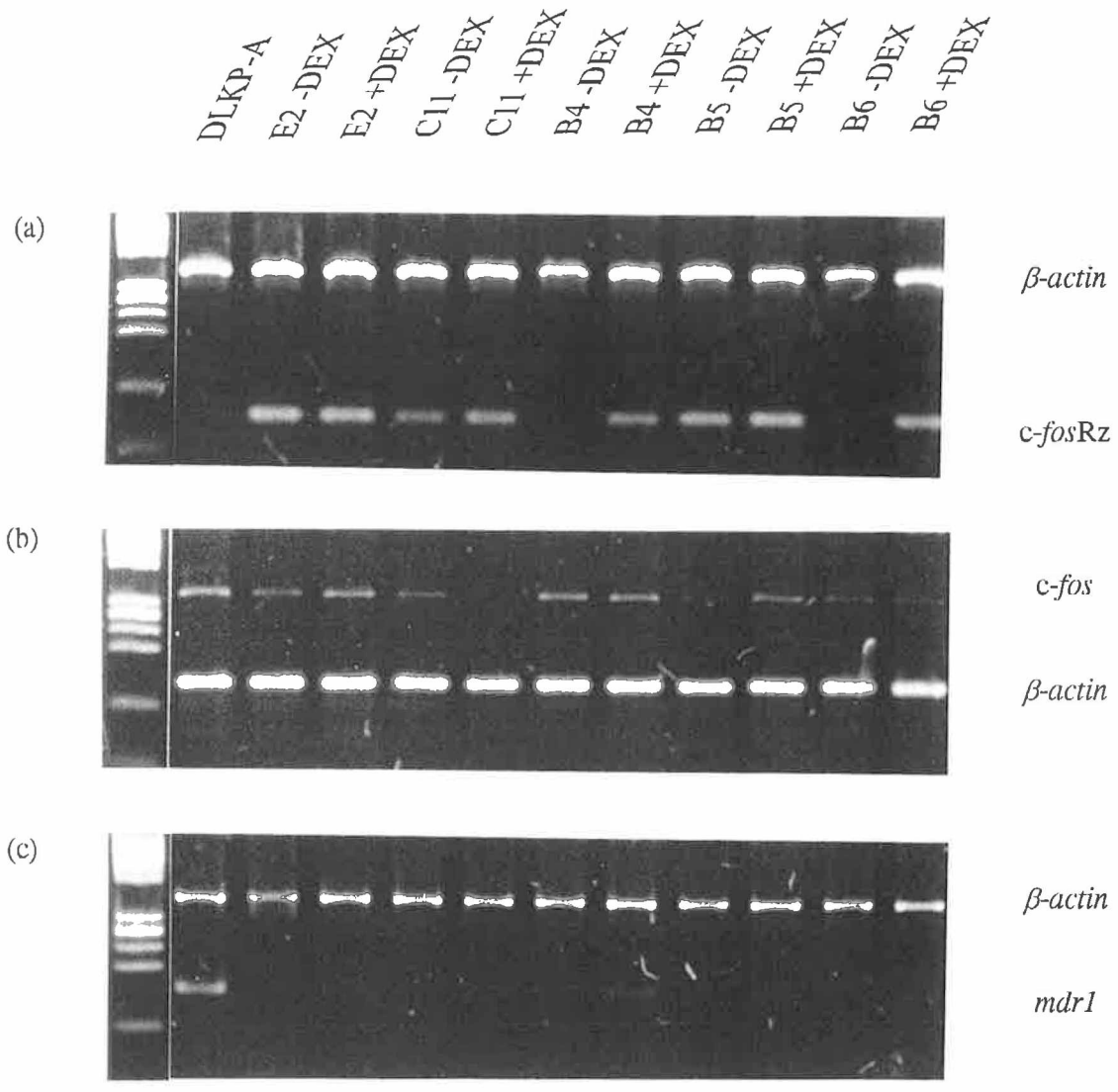
To determine if the c-*fos* ribozyme was induced by basal levels of dexamethasone in serum RNA was extracted from five of the ribozyme transfected clones which had not been treated with dexamethasone. RNA was also extracted from the cell lines following 24 hour incubation in 5μM dexamethasone. Results found show that the ribozyme is induced in some of the cell lines (E2, C11, B6) by basal levels of dexamethasone in serum (Figure 3.4.2.1(a)). All cell lines analysed (except for control DLKP-A) expressed the ribozyme following dexamethasone treatment.

c-*fos* mRNA levels were also analysed in these cell lines (Figure 3.4.2.1(b)). Analysis revealed expression of the c-*fos* mRNA in all cell lines. However, expression was not detected in the C11 clone following dexamethasone treatment. These analyses were not repeated.

To determine if c-*fos* down-regulation affects *mdr1* levels in these clones, RT-PCR was carried out on the same RNA extractions for the expression of the *mdr1* gene. A band for the *mdr1* gene was detected for DLKP-A (following 20 cycles of PCR) but not for the c-*fos* ribozyme-expressing cell lines (Figure 3.4.2.1(c)). It is not known if this down-

regulation in expression is due to inherently sensitive clonal sub-populations in the DLKP-A cell line or if it a result of the c-*fos* ribozyme expression.

Figure 3.4.2.1 RT-PCR Analysis in c-*fos* Ribozyme transfected DLKP-A cells



RT-PCR analysis, for the expression of (a) c-*fos* ribozyme (Rz), (b) c-*fos*, (c) *mdr1*, on DLKP-A and c-*fos* ribozyme transfected clonal sub-populations DLKP-A*fos*RzE2 (E2), DLKP-A*fos*RzC11 (C11), DLKP-A*fos*RzB4 (B4), DLKP-A*fos*RzB5 (B5), DLKP-A*fos*RzB6 (B6), in the presence (+DEX) and absence (-DEX) of 5μM dexamethasone for 24 hours.

3.4.3 Culturing of DLKP-A/*fosRz*B6 in serum free media

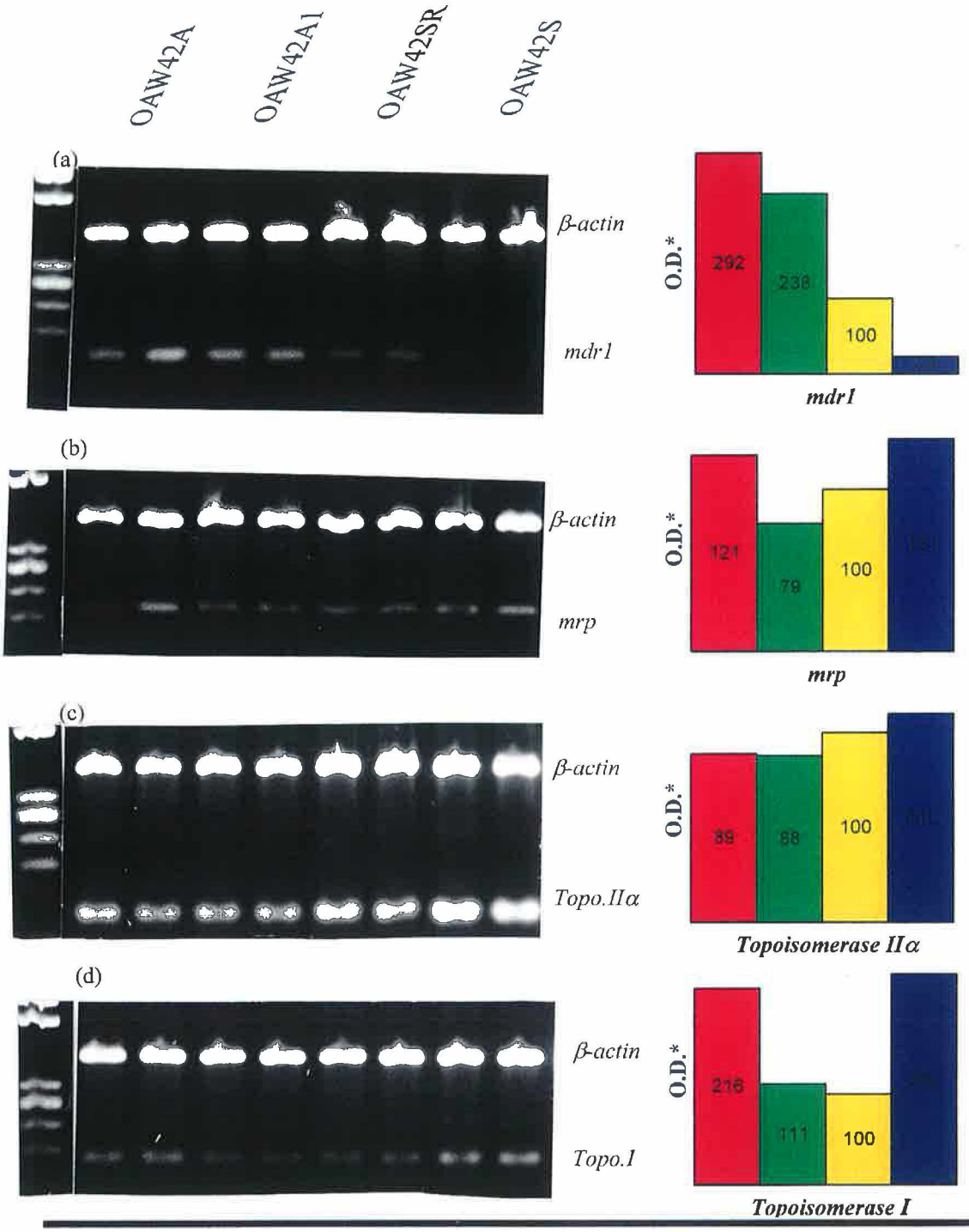
As dexamethasone is a component of serum and the cell lines analysed in Section 3.4.1 were grown in serum-supplemented media the true effect of the *fos* ribozyme may be masked, as it may already be induced in the cell lines without the need for supplemented dexamethasone (see Figure 3.4.2.1(a)). To reduce the effect of residual steroid levels in serum supplemented media, attempts were made to grow DLKP-A/*fos*RzB6 cells in a serum free media developed in this laboratory (P.Meleady, 1997). This serum-free medium (Section 2.2.9) was developed to support the growth of the parental DLKP-A cell line. The cell line DLKP-A/*fos*RzB6 did not grow in this serum-free medium, however, and analysis of the true effect of *fos* ribozyme induction could not be determined.

3.5 Analysis of Low-level MDR cell lines

3.5.1 Gene Expression Analysis by RT-PCR in Low-level Resistant Ovarian Cell Line

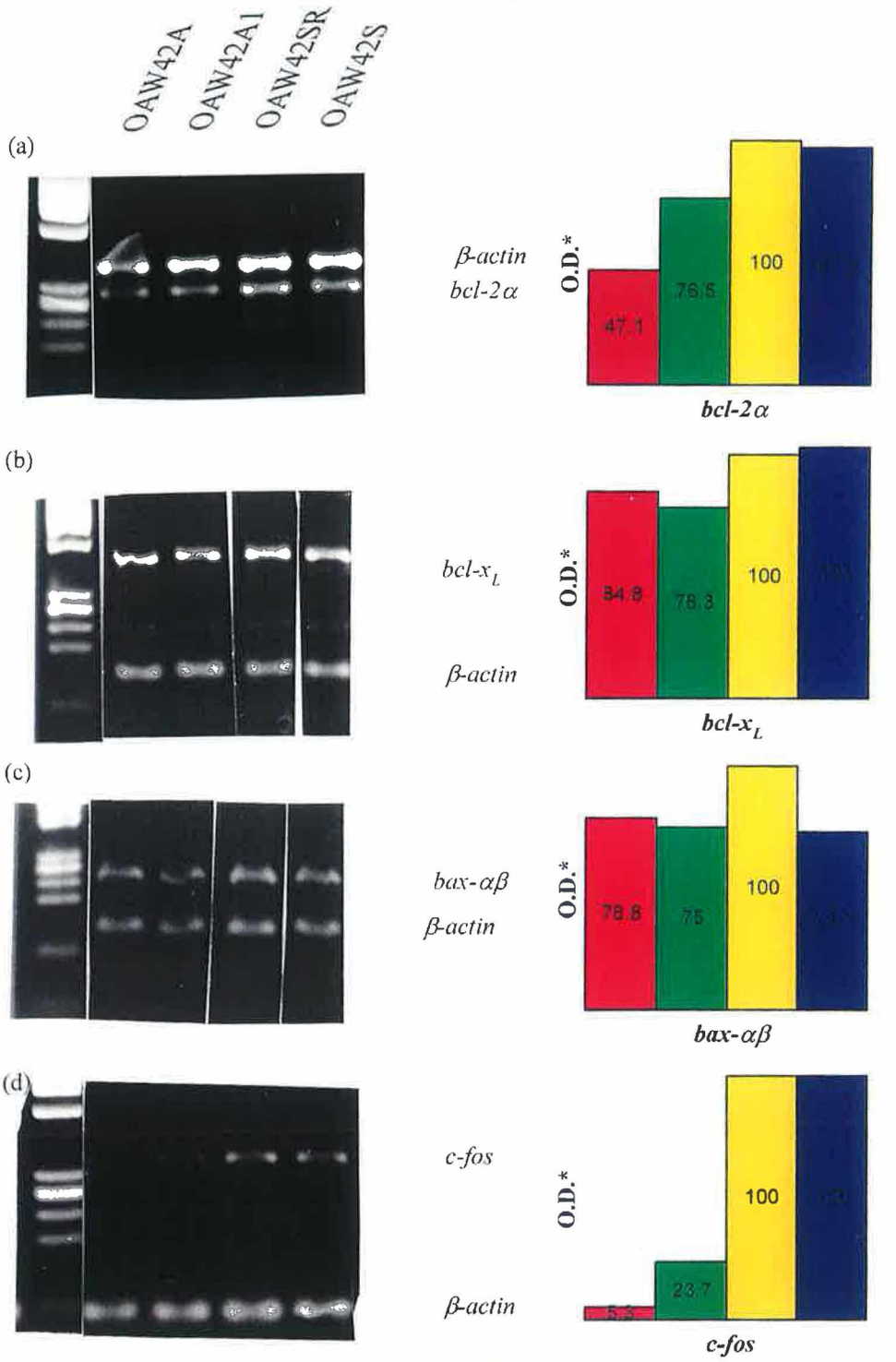
The cell lines analysed in Section 3.3 are highly resistant cell lines and do not accurately reflect resistance levels seen *in vivo*. In order to establish if RT-PCR can detect low-level changes in resistance at the mRNA level, well characterised low-level resistant variants of an ovarian cell line, OAW42, were analysed by RT-PCR for the expression of common MDR markers such as *mdr1*, *mrp*, *GST*π, *Topoisomerase I* and *Topoisomerase II*α (Appendix C). Duplicate PCR reactions were prepared for each resistant variant, OAW42A, OAW42A1 (adriamycin-selected resistant lines) and the parental population OAW42SR (spontaneously resistant variant of OAW42) and OAW42S (a sensitive clone previously isolated in this laboratory from the parental OAW42 population). The OAW42A cell line is the most resistant variant and was established by further exposure of the OAW42A1 cell line to adriamycin (for fold resistance levels see Appendix D). The results of this analysis are shown in Figure 3.5.1.1. Analysis of *mdr1* levels revealed expression of the gene in the most resistant variants (OAW42A and OAW42A1) and to a lesser extent in OAW42SR, but not in the sensitive OAW42S line (Figure 3.5.1.1(a)). Expression of *mrp* (analysis shown in Figure 3.5.1.1(b)) and *GST*π (results not shown) mRNA was observed in all cell lines analysed, however, no consistent differential expression was evident between the sensitive and resistant variants. Decreased mRNA levels of *Topoisomerase II*α (Figure 3.5.1.1(c)) were observed in the more resistant lines relative to the OAW42S cells. *Topoisomerase I* levels (Figure 3.5.1.1(d)) were decreased relative to the sensitive clone, OAW42S, but increased expression relative to OAW42SR was observed. Average densitometric values for each duplicate reaction is presented in Figure 3.5.1.1. Levels of *bcl-x*, *bax*, *bcl-2* and c-*fos* were also analysed in these cell lines (Figure 3.5.1.2). c-*fos* expression was detected in the sensitive OAW42SR and OAW42S cell lines with decreasing levels observed in the resistant variants (Figure 3.5.1.2(d)); this is in direct contrast to the situation observed in the DLKP variants where over-expression of the c-*fos* gene was seen in highly resistant adriamycin-selected cell lines (Figure 3.3.2(c) and Figure 3.3.1.1(b)) *bcl-2* mRNA levels were also reduced in the resistant variants relative to the sensitive OAW42SR and OAW42S cells (Figure 3.5.1.2(a)). Decreased levels of *bcl-*x_L and *bax* were seen in the resistant OAW42 variants (Figure 3.5.1.2(b),(c)) compared to the sensitive variants.

Figure 3.5.1.1 RT-PCR Analysis of MDR-related Gene Expression in OAW42SR Variants



cDNA from OAW42A (■) (adriamyin-selected variant of OAW42SR), OAW42A1 (■) (adriamyin-selected variant of OAW42SR), OAW42SR (■) and OAW42S (■) (sensitive clone from OAW42SR) was amplified by PCR for the presence of (a) *mdr1*, (b) *mrp*, (c) *Topoisomerase IIα* and (d) *Topoisomerase I*. A 383bp *β-actin* product was co-amplified in all reactions. Densitometric O.D.* is presented as the ratio of the levels of each specific gene product to the internal *β-actin* control and is normalised to 100% for OAW42SR.

Figure 3.5.1.2 RT-PCR Analysis of Oncogene Expression in OAW42SR and Resistant Variants



cDNA from OAW42A (■), OAW42A1 (■), OAW42SR (■) and OAW42S (■) was amplified by PCR for the presence of (a) *bcl-2α,* (b) *bcl-xL*, (c) *bax-αβ* and (d) c-*fos*. A 383bp *β-actin* product was co-amplified in (a) and a 142bp *β-actin* product was amplified in all other reactions. Densitometric O.D.* is presented as the ratio of the levels of each specific gene product to the internal *β-actin* control and is normalised to 100% for OAW42SR.

3.5.2 Selection of Low-level MDR Cell Lines from A Clonal Cell Population

Adriamycin-selected resistant variants of a sensitive clonal population, DLKP-SQ, were selected during the course of this work by two methods described below.

3.5.2.1 Selection of Low-level MDR Cell Line by Continuous Exposure to Adriamycin

The clonal population DLKP-SQ was made resistant by stepwise selection in increasing concentrations of adriamycin as described in Section 2.5.1. The cells were initially exposed to 25ng/ml adriamycin continuously until they readily adapted to growth, at this concentration. At this stage the concentration was increased in a step-wise manner to approximately two-fold concentration of drug. This selection process was continued until the cells grew in 250ng/ml adriamycin, and were then maintained in the absence of drug. This selected cell line was designated DLKP-SQ/A250c (c for continuous exposure to drug). Analysis of resistance stability was not addressed for this cell line.

3.5.2.2 Selection of Low-level MDR Cell Line by Pulse Exposure to Adriamycin

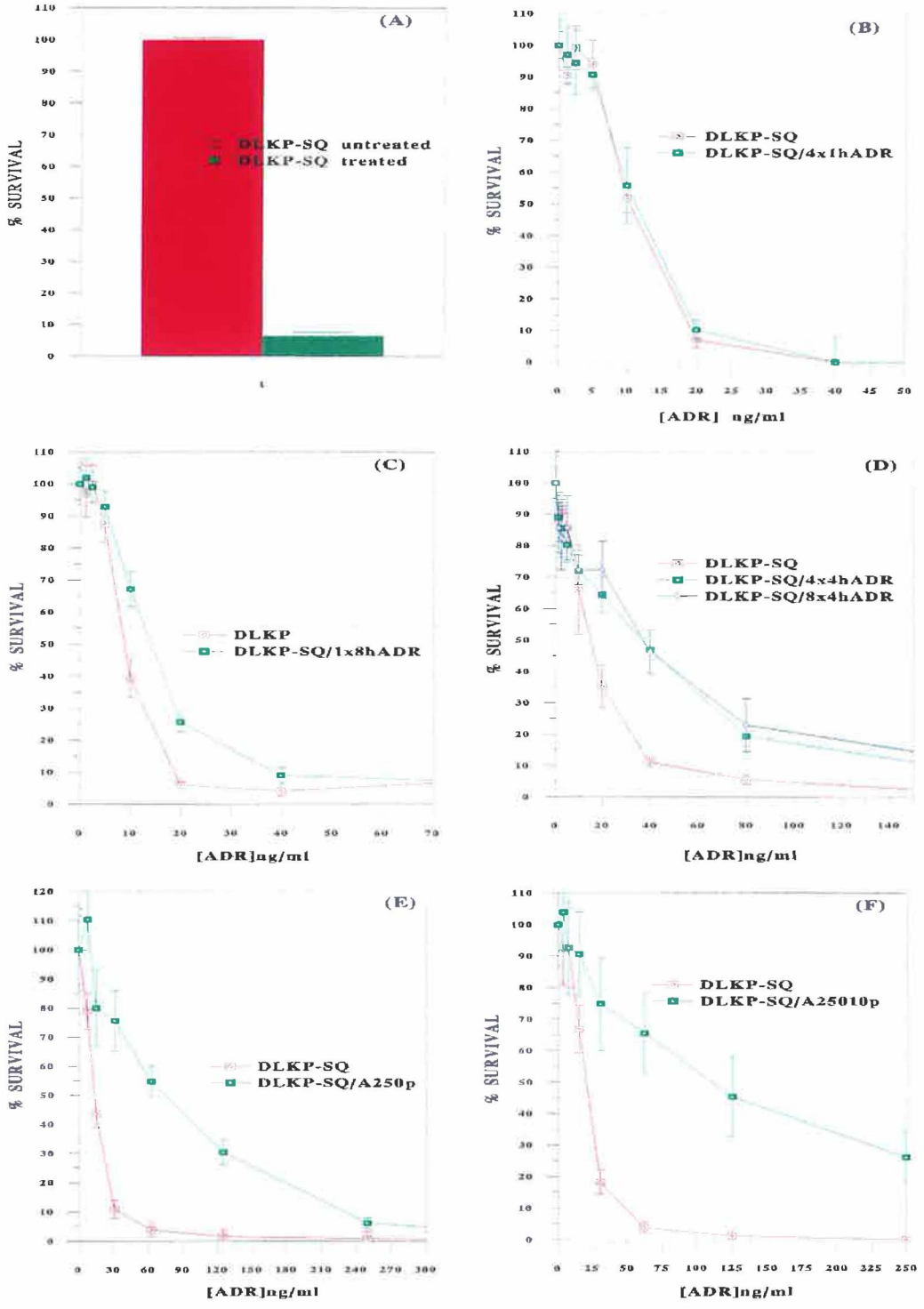
A concentration of adriamycin (250ng/ml) shown to be clinically attainable in blood (Piscitelli *et al.*,1993) was used to develop a resistant variant of DLKP-SQ by pulse exposure. A number of time schedules, with the selecting drug concentration, were attempted in the development of a selected line, including selection for 1, 4, 8, 24 and 48 hours. Pulses were repeated once every week. Following the first pulse only those cells which had been exposed to drug for 1 and 4 hours maintained some degree of viability; no cells survived following exposure to drug for 24 or 48 hours. Although an individual colony survived from the cells that were exposed to drug for 8 hours, further propagation in the absence of adriamycin did not result in increased resistance when compared to DLKP-SQ (this line was called DLKP-SQ/1x8h ADR) (Figure 3.5.2.2.1(C)). A slight increase in resistance was noted when DLKP-SQ/1x8h ADR was compared to the DLKP mixed population which is slightly more sensitive to drug than the DLKP-SQ cell line (Figure 3.5.2.2.1(C)). Cells exposed to drug for 1 and 4 hours were further treated with drug for at least 4 pulses after which time toxicity was assessed. DLKP-SQ/4x1h ADR (DLKP-SQ exposed to ADR for four 1 hour pulses) was not found to have increased

resistance compared to the parental population DLKP-SQ (Figure 3.5.2.2.1(B)). DLKP-SQ/4x4h ADR (DLKP-SQ exposed to ADR for four 4 hour pulses) was found to have 2 fold increased resistance over DLKP-SQ (see Table 3.5.2.2.1); further pulses with ADR did not result in any significant increase in resistance levels(DLKP-SQ/8x4h ADR) (Figure 3.5.2.2.1(D)).

Repeat exposure of DLKP-SQ to 250ng/ml adriamycin for four weekly pulses resulted in the development of a 4 fold resistant variant and this line was called DLKP-SQ/A250p (p for pulse exposure) (Figure 3.5.2.2.1(E)). Further pulsing of this variant with 250ng/ml adriamycin, until the cells grew in this concentration of drug, resulted in the development of the DLKP-SQ/A25010p variant (after 10 pulses); this variant was found to be more resistant than the A250p variant (Figure 3.5.2.2.1(F)). Repeat selection of DLKP-SQ with adriamycin resulted in a similarly resistant cell line named DLKP-SQ/A25010p^2 (Table 3.5.2.2.3). Selection of another clonal population of DLKP, namely DLKP-I, under the same conditions resulted in the development of a resistant variant, DLKP-I/A25010p (Table 3.5.2.2.3).

Selection of cells with adriamycin results in the establishment of a multiple drug resistance phenotype where cells are cross-resistant to adriamycin, the epipodophyllotoxins, the vinca alkaloids and taxol among others but are not cross-resistant to drugs such as 5-fluorouracil or the platinum compounds. The resistant variants of DLKP-SQ and DLKP-I established in these studies demonstrate this specific cross-resistance profile (Table 3.5.2.2.1 and 3.5.2.2.3). Although the lower resistant variant, DLKP-SQ/4x4hADR, showed similar levels of cross-resistance to adriamycin, vincristine and VP-16 (Table 3.5.2.2.1), the variants with greater resistance profiles (DLKP-SQ(I)/A25010p) showed alterations in cross-resistance levels with the greatest increase in resistance seen for vincristine, to a lesser extent for adriamycin, and lowest resistance to VP-16 (Table 3.5.2.2.4). A similar cross-resistance profile was evident in the resistant variants of DLKP and OAW42SR developed by continuous exposure to adriamycin (see Appendix D).

Figure 3.5.2.2.1 Pulse selection of DLKP-SQ with Adriamycin



(a) effect of one 4 hour exposure of 250ηg/ml adriamycin (DLKP-SQ treated) on survival of DLKP-SQ cells; adriamycin toxicity profile of (b) DLKP-SQ/4x1hADR; (c) DLKP-SQ/1x8hADR; (d) DLKP-SQ/4x4hADR and DLKP-SQ/8x4hADR; (e) DLKP-SQ/A250p and (f) DLKP-SQ/A25010p.

Table 3.5.2.2.1 Fold Resistance of DLKP-SQ/4x4hADR compared to DLKP-SQ

	[ADR]	[VP-16]	[VNC]	[CBP]	[5-FU]
DLKP-SQ/4x4hADR	2.23 ± 0.28	2.31 ± 0.31	2.53 ± 0.41	1.34 ± 0.58	1.0 ± 0

Fold resistance levels of DLKP-SQ/4x4hADR (adriamycin pulse-selected variant established after exposure of DLKP-SQ to four weekly four hour pulses of 250ng/ml adriamycin) for adriamycin (ADR), VP-16, vincristine (VNC), carboplatin (CBP) and 5-fluorouracil (5-FU) relative to DLKP-SQ.

Table 3.5.2.2.2 Adriamycin IC_{50} Values (ng/ml) for Low-level Resistant DLKP-SQ cell lines

	DLKP-SQ	DLKP-SQ/A250p	DLKP-SQ/A25010p	DLKP-A250c
IC_{50}	13.9 ± 3.5	73.6 ± 8.5	139.2 ± 58.8	323

Adriamycin toxicity levels of adriamycin-selected resistant variants of DLKP-SQ established after four (DLKP-SQ/A250p) and ten (DLKP-SQ/A25010p) weekly pulse exposures (of 250ng/ml adriamycin for 4 hours) and continuous selection (to a final concentration of 250ng/ml adriamycin).

Table 3.5.2.2.3 IC_{50} values of resistant variants of DLKP-SQ and DLKP-I

	[ADR]ng/ml	[VNC]ng/ml	[VP-16]ng/ml	[5-FU]μg/ml
DLKP-SQ	13.9 ± 3.5	1.1 ± 0.4	333.3 ± 100.0	0.7 ± 0.4
DLKP-SQ/A25010p	139.2 ± 58.8	47.2 ± 15.7	980.4 ± 454.3	1.4 ± 1.1
DLKP-SQ/A25010p^2	110.6 ± 60.2	33.1 ± 28.6	1058.5 ± 153.4	0.7 ± 0.1
DLKP-I	10.0 ± 2.4	2.7 ± 0.4	171.8 ± 71.4	0.9 ± 0.2
DLKP-I/A25010p	165.6 ± 119.8	161.5 ± 50.2	598.0 ± 164.0	0.7 ± 0.5

Toxicity profile of resistant variants of DLKP-SQ (DLKP-SQ/A25010p and DLKP-SQ/A25010p^2(repeat selection)) and DLKP-I (DLKP-I/A25010p) developed by ten weekly exposures to 250ng/ml adriamycin for 4 hours.

Table 3.5.2.2.4 Fold Resistance of DLKP-SQ and DLKP-I Resistant Variants.

	[ADR]	[VNC]	[VP-16]	[5-FU]
DLKP-SQ/A25010p*	10.0 ± 4.2	42.9 ± 14.3	2.9 ± 1.4	2.0 ± 1.6
DLKP-SQ/A25010p^2*	8.0 ± 4.3	30.1 ± 26.0	3.2 ± 0.5	1.0 ± 0.1
DLKP-I/A25010p**	16.6 ± 12.0	59.8 ± 18.6	3.4 ± 1.0	0.8 ± 0.6

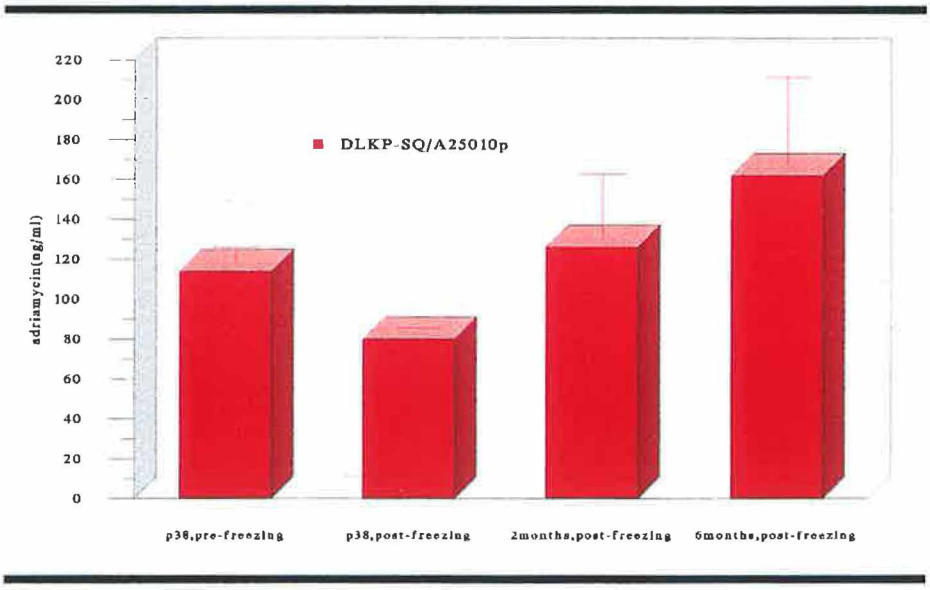
Fold resistance profile of resistant variants of DLKP-SQ/A25010p and DLKP-SQ/A25010p^2 (repeat selection), relative to DLKP-SQ*, and DLKP-I/A25010p, **relative to DLKP-I, developed by ten weekly exposures to 250ng/ml adriamycin for 4 hours.

3.5.2.2.1 Stability of the DLKP-SQ/A25010p Cell Line

To determine if the resistant phenotype of the DLKP-SQ/A25010p cell line was stable following sub-culturing and freezing of cells, adriamycin toxicity levels were analysed in the resistant variant over a range of five passages and six months following freezing. Initial toxicity analysis on this cell line prior to freezing produced an IC_{50} value of 113.8 ± 6.6ng/ml adriamycin at passage 38. Two months following freezing analysis was repeated on passage 38 and an IC_{50} value of 80ng/ml adriamycin was obtained. These cells were maintained in culture for four to six weeks and analysed by three consecutive toxicity assays, the average result of which can be seen in Figure 3.5.2.2.1.1 (value obtained in analysis in Table 3.5.2.2.3). The passage numbers used in this analysis ranged from passage 40 to 43. A stable adriamycin resistance profile was seen following two months of freezing. Further analysis of the latter passages of cells following six months storage in liquid nitrogen revealed a stable adriamycin resistant phenotype also. Therefore, this pulse selected cell line maintained stable resistance over six months storage in liquid nitrogen.

The stable resistance profile of DLKP-SQ/A25010p, maintained throughout the course of this work without further exposure to adriamycin, is also supported by similar resistance levels of clonal populations of the cell line (C.O'Loughlin, pers.comm.).

Figure 3.5.2.2.1.1 Stability of Adriamycin Resistance in DLKP-SQ/A25010p Cell Line



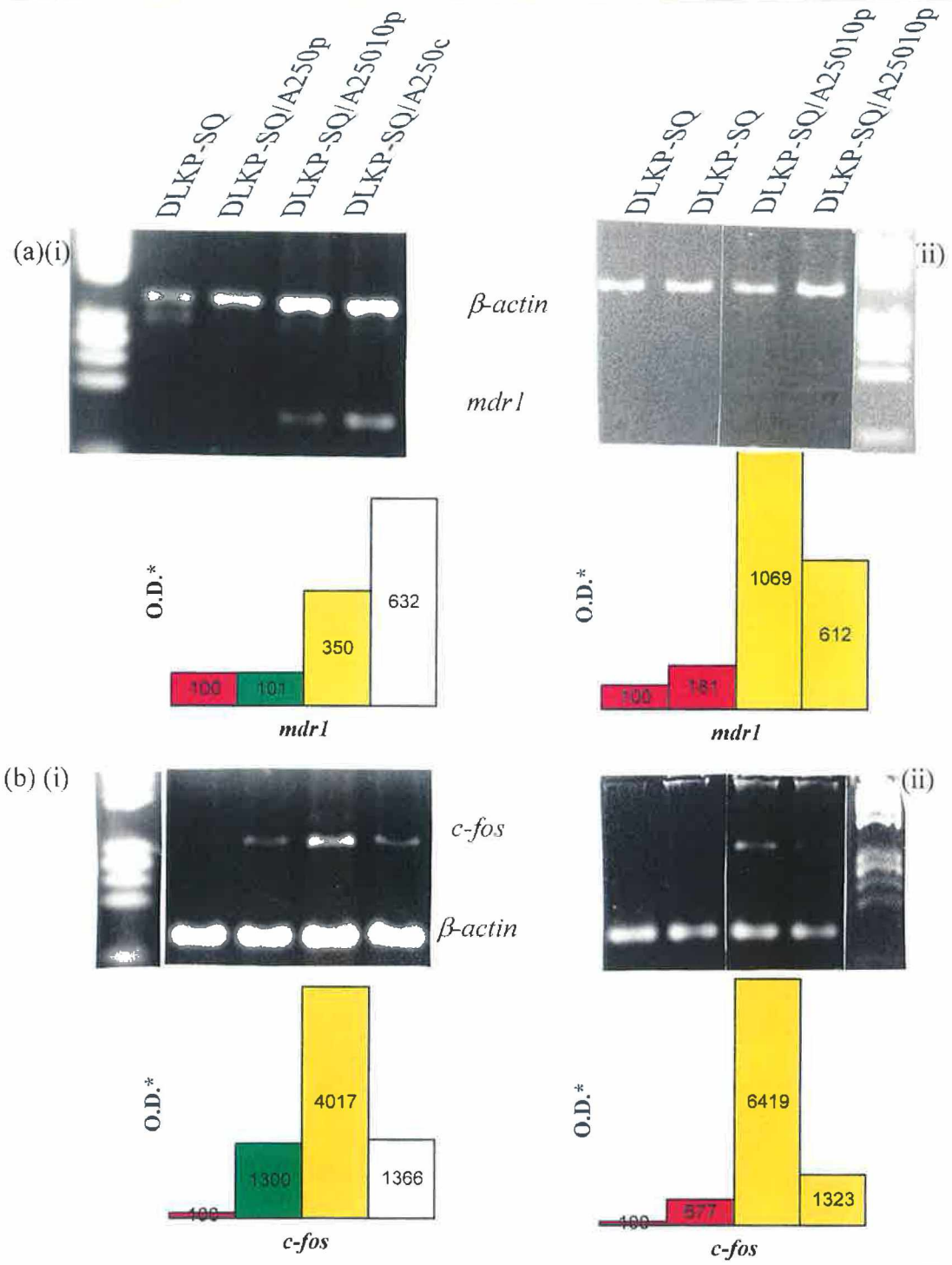
Initial analysis of adriamycin IC_{50} values in DLKP-SQ/A25010p prior to freezing (p38,pre-freezing) and two (2months,post-freezing) and six (6months,post-freezing) months post-freezing and maintained in culture without further exposure to adriamycin.

3.5.2.3 RT-PCR Analysis of Low-level DLKP-SQ Resistant Variants

RT-PCR analysis was carried out on mRNA extracted from the low-level resistant variants selected from the parental DLKP-SQ cell line. RNA extracted from DLKP-SQ/A250p, DLKP-SQ/A25010p and DLKP-SQ/A250c was analysed by RT-PCR for the expression of *mdr1*, c-*erb*B-2, *bcl-x*, *bax* and c-*fos* gene levels.

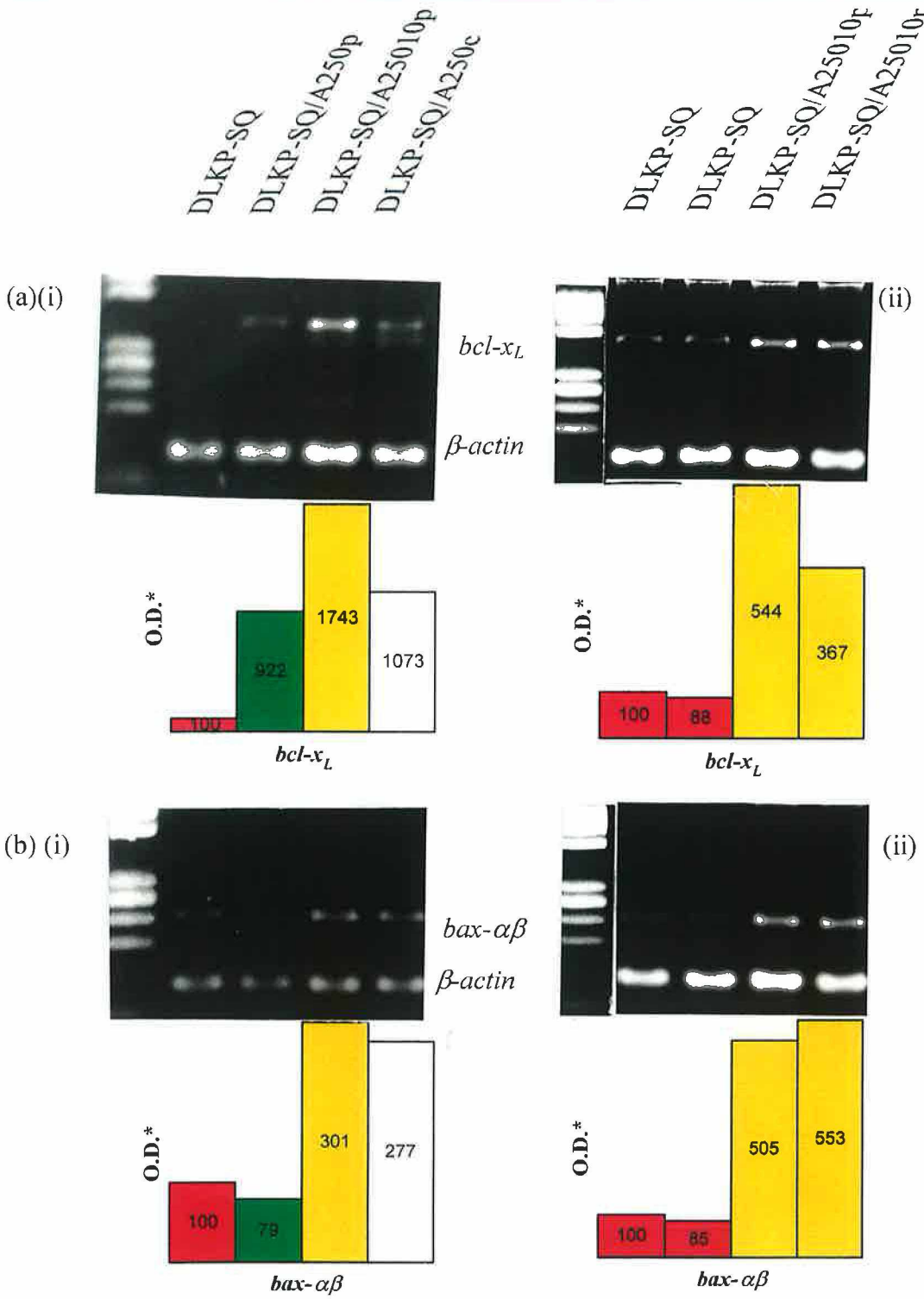
The results obtained indicate an increase in *mdr1* expression in DLKP-SQ/A250c and to a lesser extent in DLKP-SQ/A25010p (Figure 3.5.2.3.1(a)) relative to DLKP-SQ. No increase in *mdr1* levels was seen in the DLKP-SQ/A250p cell following 25 cycles of PCR (Figure 3.5.2.3.1(a)) although further amplification to 30 cycles resulted in a slight increase in *mdr1* mRNA levels (results not shown). Levels of c-*fos* were also found to be elevated in the resistant variants, but most obviously in DLKP-SQ/A25010p (Figure 3.5.2.3.1(b)). Previous results on the DLKP resistant variants have shown elevated levels of c-*fos* in the adriamycin-selected lines expressing the highest levels of *mdr1* mRNA (Figure 3.3.1.1(b) and Appendix E); the results on the DLKP-SQ variants suggest that this is not the case and that higher levels of c-*fos* are seen in the pulse-selected cell line when compared to the continuously-selected variant, although higher *mdr1* levels are seen in the latter cell line. Analysis of *bcl-*x_L and *bax* levels in these variants have shown greater expression in the resistant variants relative to the sensitive DLKP-SQ cell line (Figure 3.5.2.3.2(a),(b)); this was also seen for the resistant variants of DLKP (Figure 3.3.1). Initial studies revealed little alteration in c-*erb*B-2 levels between variants (Figure 3.5.2.3.3(ai)) however further extractions have shown increased c-*erb*B-2 expression in DLKP-SQ/A25010p relative to DLKP-SQ (Figure 3.5.2.3.3(aii)). The poor amplification of *β-actin* mRNA in the former reaction (Figure 3.5.2.3.3(ai)) may account for the absence of observed alteration of c-*erb*B-2 mRNA levels in the cell lines.

Figure 3.5.2.3.1 RT-PCR Analysis of Low-level DLKP-SQ Resistant Variants



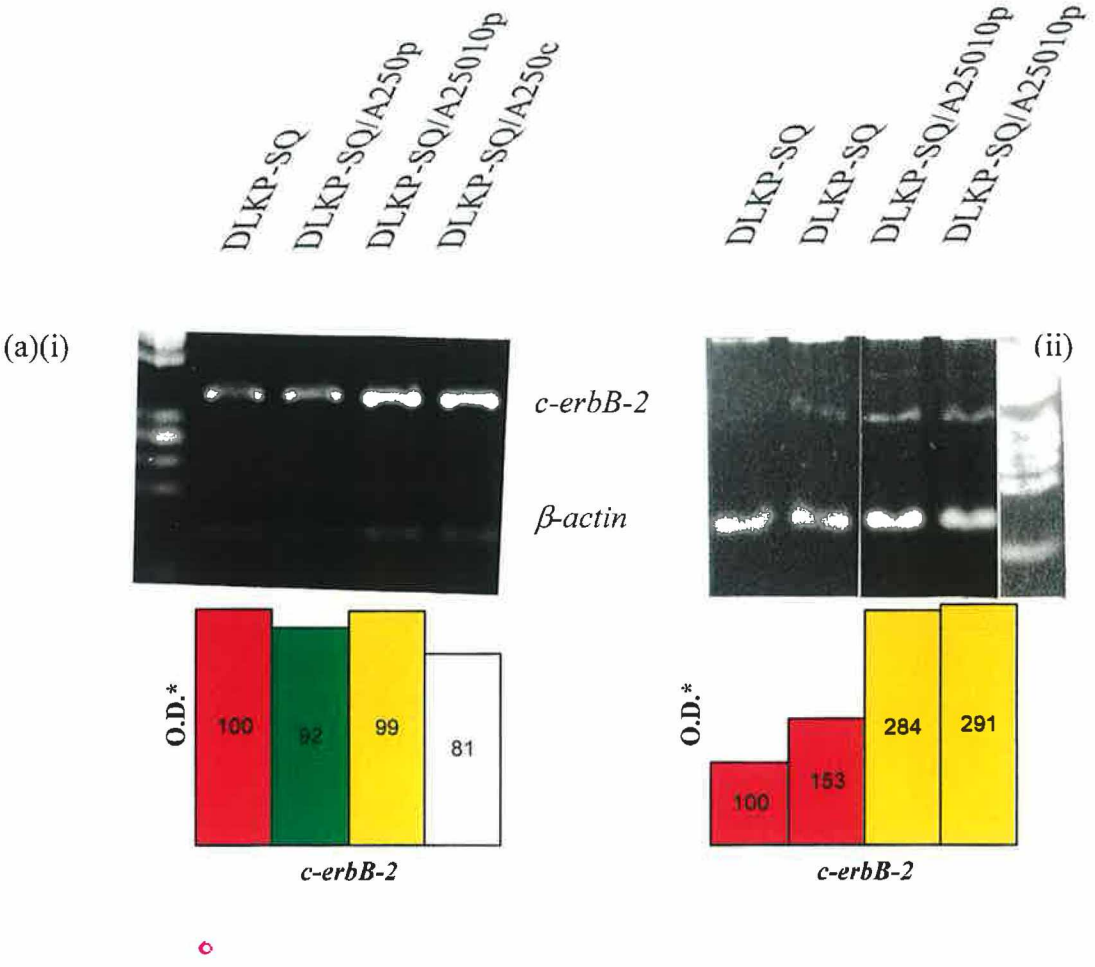
cDNA from DLKP-SQ (■), DLKP-SQ/A250p (■), DLKP-SQ/A25010p (■) and DLKP-SQ/A250c (□) was amplified by PCR for the expression of (ai) mdr1 (25 cycles of PCR) and (bi) c-fos (30 cycles of PCR). Further analysis on repeat extractions of DLKP-SQ and DLKP-SQ/A25010p was also carried out ((aii) and (bii)). Densitometric O.D.* is presented as *mdr1/c-fos:β-actin* and is normalised to 100% for DLKP-SQ.

Figure 3.5.2.3.2 RT-PCR Analysis of Low-level DLKP-SQ Resistant Variants



cDNA from DLKP-SQ (■), DLKP-SQ/A250p (■), DLKP-SQ/A25010p (■) and DLKP-SQ/A250c (□) was amplified by PCR for the expression of (ai) *bcl-x* (25 cycles of PCR) and (bi) *bax* (25 cycles of PCR). Further analysis on repeat extractions from DLKP-SQ and DLKP-SQ/A25010p was also carried out ((aii) and (bii)). Densitometric O.D.* is presented as *bcl-x/bax:β-actin* and is normalised to 100% for DLKP-SQ.

Figure 3.5.2.3.3 RT-PCR Analysis of Low-level DLKP-SQ Resistant Variants

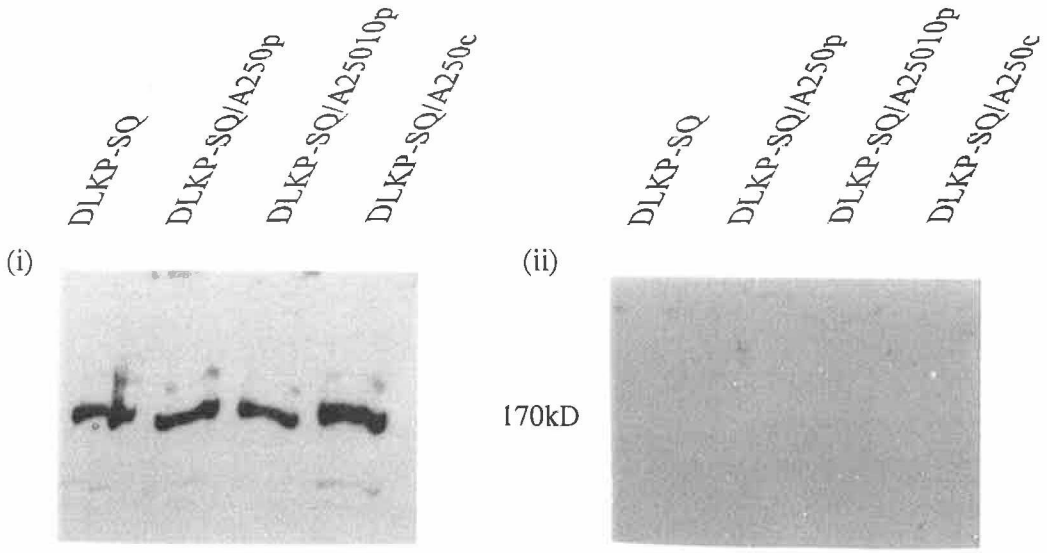


cDNA from DLKP-SQ (■), DLKP-SQ/A250p (■), DLKP-SQ/A25010p (■) and DLKP-SQ/A250c (□) was amplified by PCR for the expression of (ai) *c-erbB-2* (25 cycles of PCR). Further analysis on repeat extractions of DLKP-SQ and DLKP-SQ/A25010p was also carried out (aii). Densitometric O.D.* is presented as *c-erbB-2:β-actin* and is normalised to 100% for DLKP-SQ.

3.5.2.4 Analysis of P-glycoprotein levels in DLKP-SQ Variants

P-glycoprotein protein expression was analysed in DLKP-SQ, DLKP-SQ/A250p, DLKP-SQ/A25010p and DLKP-A250c. Protein expression was detected at a similar level in DLKP-SQ, DLKP-SQ/A250p and DLKP-SQ/A25010p. Greater expression was seen in the DLKP-SQ/A250c variant (Figure 3.5.2.4.1). DLKP-SQ/A250c also showed the greatest overexpression of *mdr1* mRNA (Figure 3.5.2.3.1(a)). Elevated *mdr1* mRNA was also observed in the DLKP-SQ/A25010p cell line (Figure 3.5.2.3.1(a)) although overexpression was not obvious at the protein level.

Figure 3.5.2.4.1 P-glycoprotein Expression in DLKP-SQ Resistant Variants

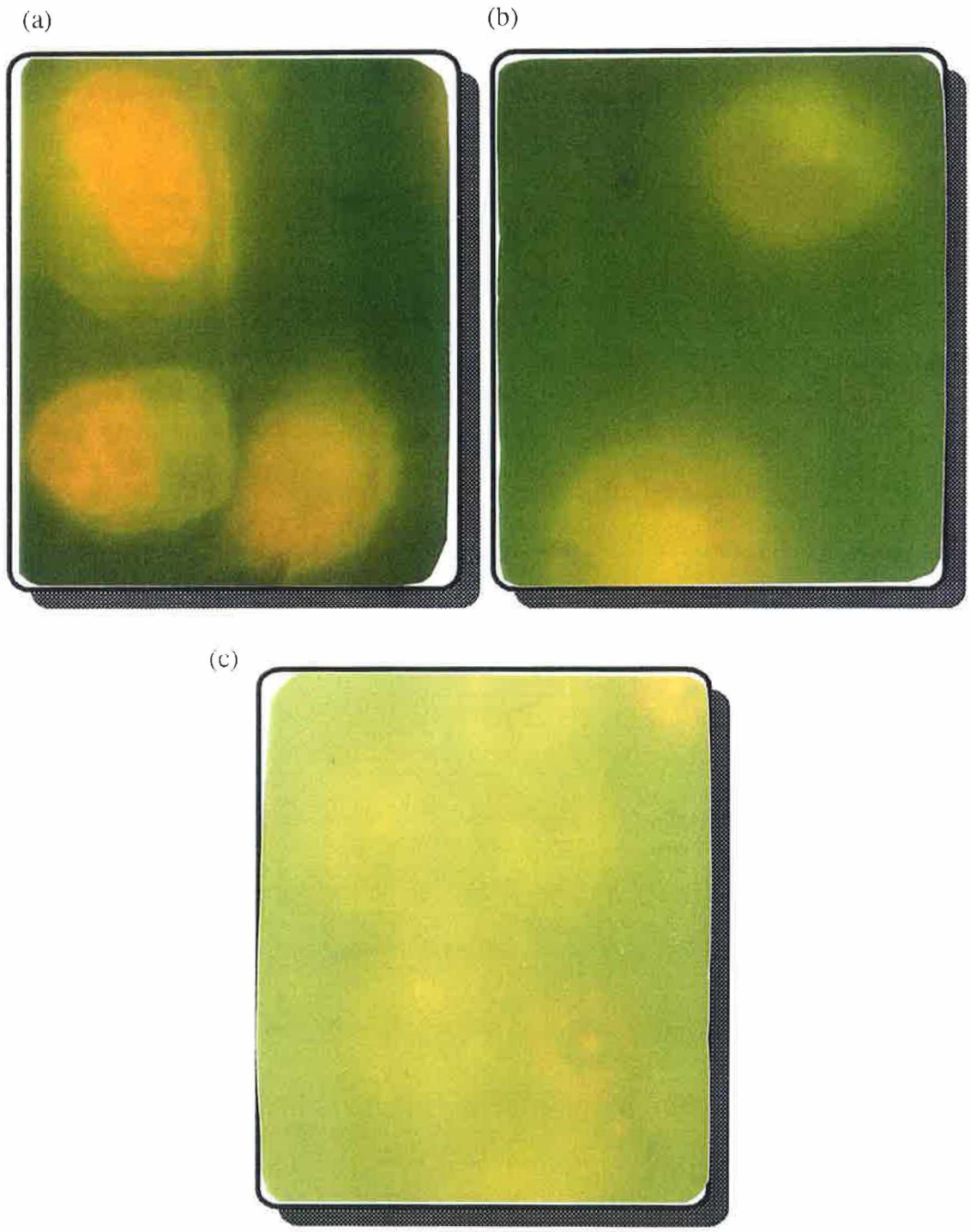


P-glycoprotein expression in DLKP-SQ and the adriamycin-selected variants DLKP-SQ/A250p, DLKP-SQ/A25010p and DLKP-SQ/A250c. Positive blot is shown in (i) and control blot (without primary antibody) is seen in (ii).

3.5.2.5 Adriamycin Uptake in DLKP-SQ Variants

Alterations in drug uptake and accumulation is a hallmark of drug resistance. To determine if alterations in drug accumulation were associated with the drug resistance profile seen in the DLKP-SQ resistant variants, adriamycin uptake was monitored in the resistant cell lines. DLKP-SQ, DLKP-SQ/A25010p and DLKP-SQ/A250c were incubated in 10μM adriamycin for 4.5 hours. Cells were then washed free of any residual drug. Cells were viewed under ultraviolet light; at this wavelength adriamycin appears as bright orange fluorescence. Sensitive cells accumulate adriamycin mainly in the nucleus whereas resistant variants accumulate less drug and it is concentrated in the cytoplasm; degree of drug accumulation is indirectly proportional to the level of cellular resistance. In the sensitive cell line DLKP-SQ, bright nuclear fluorescence was evident (Figure 3.5.2.5.1(a)). Minimal cytoplasmic fluorescence was visible, most likely due to the length of incubation in drug. The resistant variant, DLKP-SQ/A25010p, is clear of nuclear fluorescence and the adriamycin was seen to accumulate in the cytoplasm (Figure 3.5.2.5.1(b)). The more resistant cell line DLKP-SQ/A250c had no nuclear accumulation of adriamycin (Figure 3.5.2.5.1(c)) and less cytoplasmic accumulation of adriamycin than the pulse-selected variant, DLKP-SQ/A25010p.

Figure 3.5.2.5.1 Adriamycin Uptake in DLKP-SQ Variants



DLKP-SQ variants were exposed to 10μM adriamycin for 4.5 hours, drug was then removed and cells washed free of any residual drug. Adriamycin uptake is observed as orange fluorescence. Adriamycin accumulation is observed in the nucleus of DLKP-SQ (a) but is greatly reduced in the adriamycin-resistant pulse variant DLKP-SQ/A25010p (b) where cytoplasmic staining is observed. The more highly resistant cell line DLKP-SQ/A250c (c) shows no nuclear fluorescence and some cytoplasmic fluorescence.

3.5.2.6 Effect of Co-treatment on Adriamycin Toxicity in DLKP-SQ/A25010p Cells

DLKP-SQ and DLKP-SQ/A25010p were analysed for the effect cyclosporin A (an inhibitor of P-glycoprotein), emodin (shown previously to affect the activity of c-*erb*B-2) and genistein (a tyrosine kinase inhibitor) had on adriamycin toxicity in these cell lines. DLKP-SQ cells were treated with 15ng/ml adriamycin and DLKP-SQ/A25010p were treated with 150ng/ml adriamycin in a 7 day 96-well toxicity assay in triplicate (set up on the same day) (see Figures 3.5.2.6.1 and .2). The sensitive and resistant cell lines were co-treated with three different concentrations of cyclosporin A (2μg/ml; 1μg/ml or 0.5μg/ml), genistein (15μM, 7.5μM or 3.75μM) or emodin (25μM, 12.5μM or 6.25μM).

Increasing concentrations of cyclosporin A had a dramatic effect on the toxicity of adriamycin in the DLKP-SQ/A25010p cells, the highest concentration (2μg/ml) decreased cell survival from 90.9% (cyclosporin A alone) to only 4.9% (cyclosporin A and adriamycin) (Table 3.5.2.6.1), implying that P-glycoprotein plays a prominent role in the development of resistance in these cells. Cyclosporin A also affected survival in the DLKP-SQ cell line but the effect seen was less dramatic and less dependent on concentration of cyclosporin A (Table 3.5.2.6.1). The average of the results for three toxicity plates set up on the same day is given in Table 3.5.2.6.1

Table 3.5.2.6.1 Effect of CyclosporinA on Adriamycin Toxicity in DLKP-SQ variants.

% growth	DLKP-SQ	DLKP-SQ/A25010p
control	100*	100*
adriamycin	79.9 ± 4.1	87.3 ± 7.9
cyclosporin1	88.0 ± 2.8	98.1 ± 3.2
cyclosporin1 + adriamycin	63.1 ± 5.0	60.0 ± 1.2
cyclosporin2	90.5 ± 2.2	96.4 ± 4.0
cyclosporin2 + adriamycin	58.1 ± 1.2	20.5 ± 3.0
cyclosporin3	82.6 ± 5.4	90.9 ± 1.2
cyclosporin3 + adriamycin	46.7 ± 4.7	4.9 ± 1.4

DLKP-SQ and DLKP-SQ/A25010p were exposed to 15ng/ml and 150ng/ml adriamycin, respectively. Control survival is given as 100% growth (100*) in the absence of any drug for each plate analysed. Three concentrations of cyclosporin A - 0.5μg/ml (cyclosporin1); 1.0μg/ml (cyclosporin2) or 2.0μg/ml (cyclosporin3) - were used with both cell lines.

Increasing concentrations of genistein also increased the effect co-treatment of genistein and adriamycin had on survival in the DLKP-SQ/A25010p and DLKP-SQ cell line (Table 3.5.2.6.2) but to a lesser extent than that seen for cyclosporin A. At higher concentrations of genistein the effect was lost as genistein alone caused dramatic cell kill. Co-treatment of cells with emodin and adriamycin over seven days did not enhance the toxicity of adriamycin in DLKP-SQ or DLKP-SQ/A25010p cell lines (Table 3.5.2.6.3).

Table 3.5.2.6.2 Effect of Genistein on Adriamycin Toxicity in DLKP-SQ variants.

% growth	DLKP-SQ	DLKP-SQ/A25010p
control	100*	100*
adriamycin	80.5 ± 1.3	93.9 ± 2.4
genistein1	72.6 ± 0.7	84.7 ± 7.4
genistein1 + adriamycin	41.8 ± 8.3	57.1 ± 3.8
genistein2	36.4 ± 1.7	61.3 ± 5.1
genistein2 + adriamycin	24.1 ± 2.5	38.5 ± 5.4
genistein3	1.0 ± 1.0	22.1 ± 2.5
genistein3 + adriamycin	1.4 ± 1.0	14.4 ± 0.7

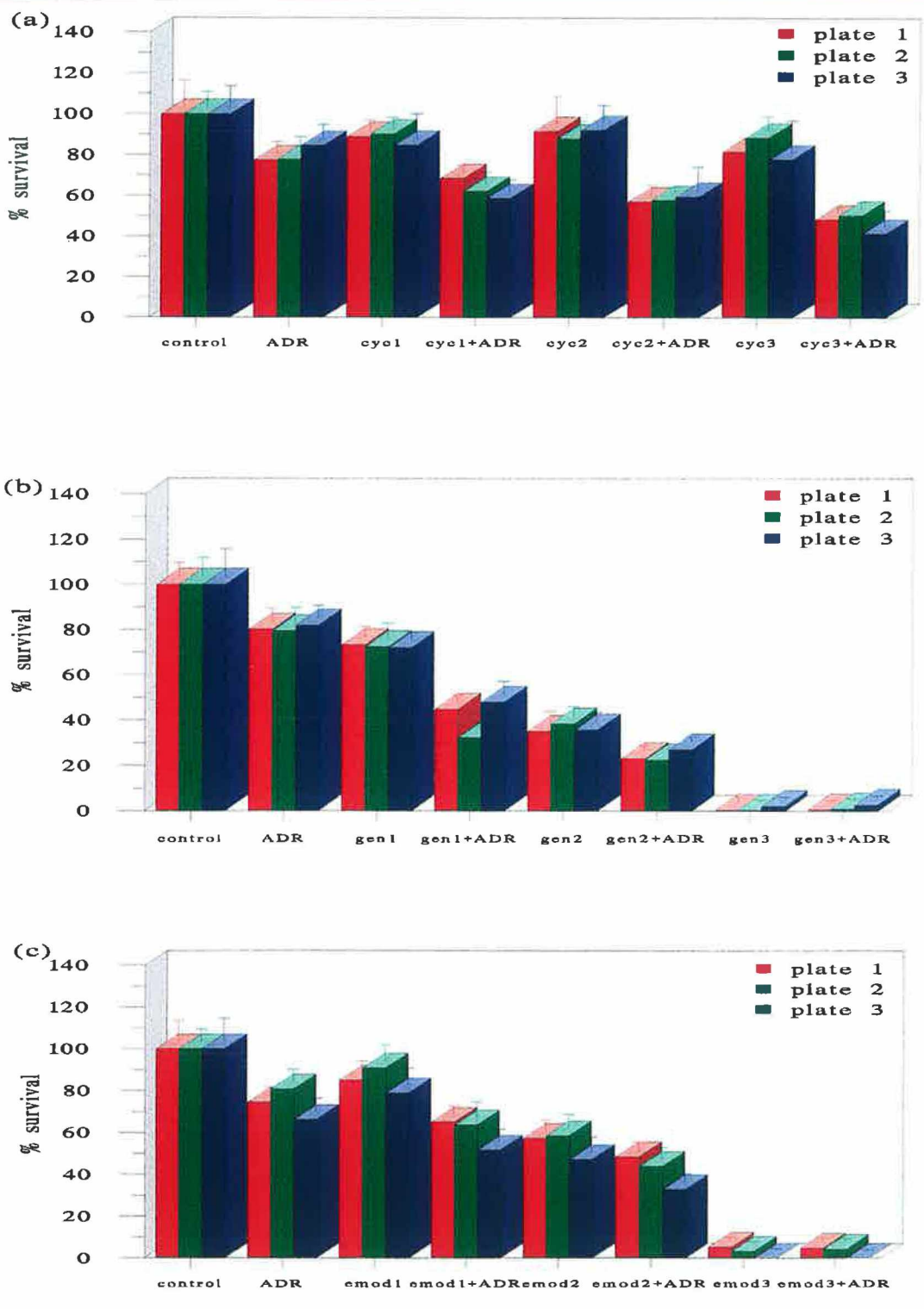
DLKP-SQ and DLKP-SQ/A25010p were grown in 15ng/ml and 150ng/ml adriamycin, respectively. Control survival is given as 100% growth (100*) in the absence of any drug for each plate analysed. Three concentrations of genistein - 3.75μM (genistein1); 5.7μM (genistein2) or 15μM (genistein3) - were used with both cell lines.

Table 3.5.2.6.3 Effect of Emodin on Adriamycin Toxicity in DLKP-SQ variants.

% growth	DLKP-SQ	DLKP-SQ/A25010p
control	100*	100*
adriamycin	73.8 ± 7.3	92.4 ± 3.0
emodin1	84.9 ± 6.0	90.7 ± 13.8
emodin1 + adriamycin	60.2 ± 7.4	89.9 ± 7.6
emodin2	54.2 ± 6.3	72.5 ± 5.2
emodin2 + adriamycin	41.6 ± 8.1	68.7 ± 6.3
emodin3	2.8 ± 2.6	3.5 ± 1.9
emodin3 + adriamycin	3.0 ± 2.6	5.4 ± 0.9

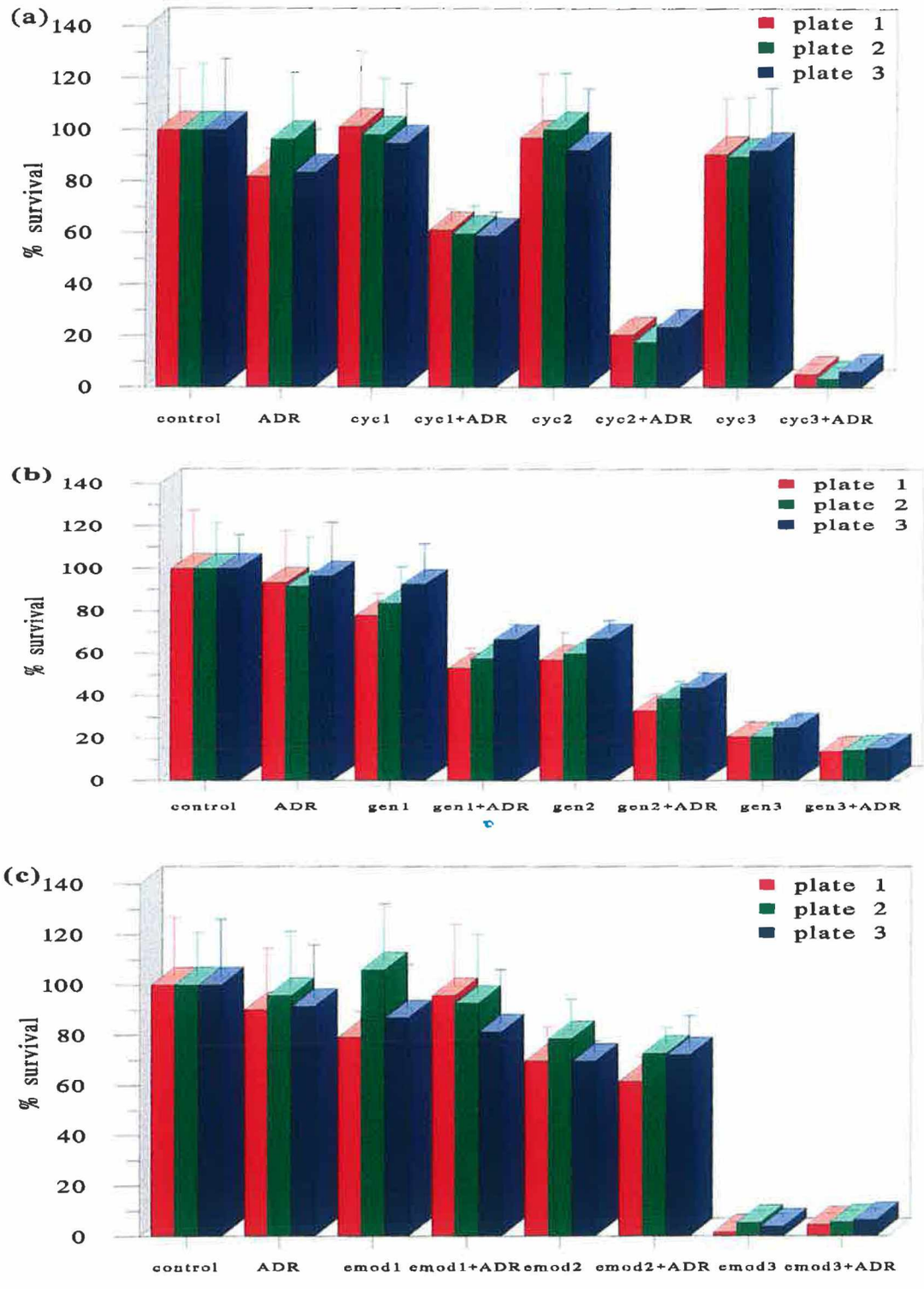
DLKP-SQ and DLKP-SQ/A25010p were exposed to 15ng/ml and 150ng/ml adriamycin, respectively. Control survival is given as 100% growth (100*) in the absence of any drug for each plate analysed. Three concentrations of emodin - 6.25μM (emodin1); 12.5μM (emodin2) or 25μM (emodin3) - were used with both cell lines.

Figure 3.5.2.6.1 Enhancement of Adriamycin Toxicity in DLKP-SQ



DLKP-SQ cells were analysed in a 96-well toxicity assay co-treated with 15ηg/ml adriamycin (ADR) and (a) 0.5μg/ml (cyc1), 1.0μg/ml (cyc2), 2.0μg/ml (cyc3) cyclosporinA; (b) 3.75μM (gen1), 7.5μM (gen2), 15μM (gen3) and (c) 6.25μM (emod1), 12.5μM (emod2), 25μM (emod3) emodin.

Figure 3.5.2.6.2 Effect of Co-treatment on Adriamycin Toxicity in DLKP-SQ/A25010p



DLKP-SQ/A25010p cells were analysed in a 96-well toxicity assay co-treated with 150ηg/ml adriamycin (ADR) and (a) 0.5μg/ml (cyc1), 1.0μg/ml (cyc2), 2.0μg/ml (cyc3) cyclosporinA; (b) 3.75μM (gen1), 7.5μM (gen2), 15μM (gen3) and (c) 6.25μM (emod1), 12.5μM (emod2), 25μM (emod3) emodin.

3.6 Transfection of *Bcl-x_S* cDNA and Effect on Toxicity in DLKP-SQ/A25010p cells

The *bcl-x_S* cDNA was transfected into DLKP-SQ/A25010p cells by the calcium phosphate method (Section 2.14.3). Five colonies were maintained as individual cell lines following selection in 600μg/ml geneticin. The resulting cell lines were designated DLKP-SQ/A25010p-*bclx$_S$*.1 (clone1), DLKP-SQ/A25010p-*bclx$_S$*.2 (clone 2), DLKP-SQ/A25010p-*bclx$_S$*.3 (clone 3), DLKP-SQ/A25010p-*bclx$_S$*.4 (clone 4) and DLKP-SQ/A25010p-*bclx$_S$*.5 (clone 5).

3.6.1 Toxicity Profile of *Bcl-x_S* Transfected Clones

Toxicity analysis of four of the *bcl-x_S* transfected clones was carried out for adriamycin, vincristine, VP-16 and 5-fluorouracil and the results shown in Table 3.6.1.1. Clones 1 and 4 showed a slight decrease in toxicity to adriamycin, vincristine and VP-16. Clone 2 showed a slight increase in resistance to adriamycin and VP-16 but a two-fold increase in vincristine resistance. Clone 3 showed a much greater sensitivity to the three multi-drug resistance drugs, vincristine, VP-16 and adriamycin, the decrease being most significant for adriamycin. No significant alteration was seen in sensitivity to 5-fluorouracil in any of the clones analysed.

3.6.2 RT-PCR Analysis of *Bcl-x_S* Transfected Clones

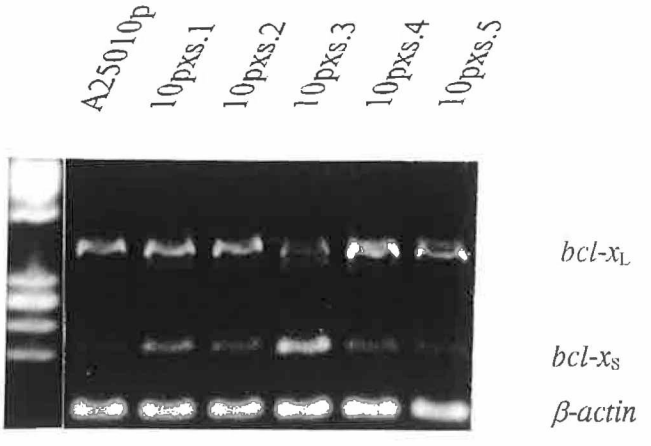
RT-PCR analysis for the expression of *bcl-x_S* was carried out on the five clones. Analysis revealed the presence of a 207bp *bcl-x_S* band in all five clones (Figure 3.6.2.1). The greatest expression was seen in clone 3 which showed the greatest reversion to sensitivity of the four clones analysed (Table 3.6.1.1). RT-PCR analysis of *mdr1* expression in the least resistant clone (DLKP-SQ/A25010p-*bclx$_S$*.3) did not reveal any alteration in *mdr1* levels when compared to the parental DLKP-SQ/A25010p (result not shown).

Table 3.6.1.1 Toxicity Profile of DLKP-SQ/A25010p-bclx$_S$ transfected clones

IC_{50}	[ADR]ng/ml	[VNC]ng/ml	[VP-16]ng/ml	[5'FU]μg/ml
DLKP-SQ	13.9 ± 3.5	1.1 ± 0.4	333.3 ± 100.0	0.7 ± 0.4
DLKP-SQ/A25010p	139.2 ± 58.8	47.2 ± 15.7	980.4 ± 454.3	1.4 ± 1.1
DLKP-SQ/A25010p-bclxs.1	100.0 ± 28.4	34.7 ± 2.9	736.2 ± 298.1	0.8 ± 0.5
DLKP-SQ/A25010p-bclxs.2	177.5 ± 94.3	101.2 ± 7.6	1269.3 ± 881.7	0.8 ± 0.4
DLKP-SQ/A25010p-bclxs.3	46.7 ± 10.7	28.9 ± 9.9	437.5 ± 212.6	0.8 ± 0.5
DLKP-SQ/A25010p-bclxs.4	100.9 ± 42.5	45.3 ± 18.8	851.3 ± 509.8	1.2 ± 1.0

Toxicity profile of DLKP-SQ/A25010p cells transfected with the *bcl-x$_S$* cDNA.

Figure 3.6.2.1 RT-PCR Analysis of *Bcl-x$_S$* transfected DLKP-SQ/A25010p clones



Expression of *bcl-x$_S$* in DLKP-SQ/A25010p cells transfected with the *bcl-x$_S$* cDNA. Parental cell line (DLKP-SQ/A25010p) is shown in the first lane. DLKP-SQ/A25010p-*bcl-x$_S$*.1, .2, .3, .4 and .5 are named as 10pxs.1, .2, .3, .4 and .5, respectively.

3.7 Adriamycin selection of an *mdr1* ribozyme expressing DLKP clone

A clonal population of DLKP, DLKP-I, was transfected previously with an *mdr1* ribozyme plasmid (S.McBride, 1995). No significant change in toxicity profile was seen in the transfected clone (Table 3.7.1). The clone which most strongly expressed the ribozyme, DLKP-I*mdr1*Rz2D4 (as determined by S.McBride, 1995) was used in this study to determine if the presence of *mdr1* is essential in the development of resistance in this cell line.

The parental line, DLKP-I, and the *mdr1* ribozyme expressing clone, DLKP-I*mdr1*Rz2D4, were exposed to ten weekly four-hour exposures of 250ng/ml adriamycin. Both cell lines developed resistance as shown in Table 3.7.1. Selection of DLKP-I with adriamycin resulted in the development of a 16 fold adriamycin-resistant variant, DLKP-I/A25010p. Selection of the *mdr1* ribozyme expressing clone produced a 10-fold adriamycin resistant cell line, DLKP-I*mdr1*Rz2D4/A25010p.

3.7.1 RT-PCR Analysis of DLKP-I Resistant Variants

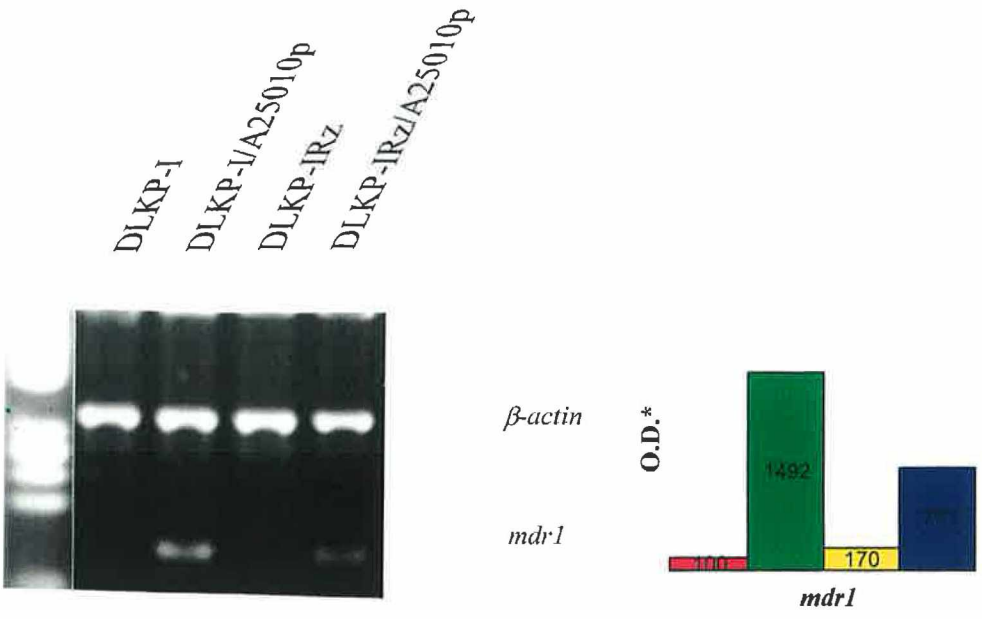
RT-PCR analysis was carried out on DLKP-I and DLKP-I*mdr1*Rz2D4 and their resistant variants. Levels of *mdr1* cDNA were faintly detected in the sensitive cell lines, after 30 cycles of PCR, and were increased in DLKP-I/A25010p and to a lesser extent in DLKP-I*mdr1*Rz2D4/A25010p (Figure 3.7.1.1). This change in *mdr1* levels in the resistant variants was not significantly expressed in toxicity levels of these cell lines to adriamycin or vincristine, two P-glycoprotein-associated drugs (Table 3.7.1). Technical difficulties prevented the detection of the *mdr1* ribozyme in these cell lines. However, the ribozyme-transfected cells did grow in the presence of geneticin.

Table 3.7.1 Toxicity Profile of DLKP-I Variants

IC_{50}	[ADR]ng/ml	[VNC]ng/ml	[VP-16]ng/ml	[5'FU]µg/ml
DLKP-I	10.0 ± 2.4	2.7 ± 0.4	171.8 ± 71.4	0.9 ± 0.2
DLKP-I/A25010p	165.6 ± 119.8	161.5 ± 50.2	598.0 ± 164.0	0.7 ± 0.5
DLKP-I*mdr1*Rz2D4	9.8 ± 5.6	2.7 ± 0.4	199.7 ± 45.6	1.6 ± 0.5
DLKP-I*mdr1*Rz2D4/A25010p	103.7 ± 54.3	189.0 ± 42.4	446.7 ± 47.1	2.1 ± 0.1

Toxicity profile of DLKP-I and resistant variant, DLKP-I/A25010p, and an *mdr1* ribozyme expressing clone, DLKP-I*mdr1*Rz2D4, and its resistant variant, DLKP-I*mdr1*Rz2D4/A25010p. Resistant variants were developed by pulse exposure of the sensitive parental cells to ten weekly four hour pulses of 250ng/ml adriamycin.

Figure 3.7.1.1 RT-PCR Analysis of *mdr1* Gene Expression in DLKP-I Variants



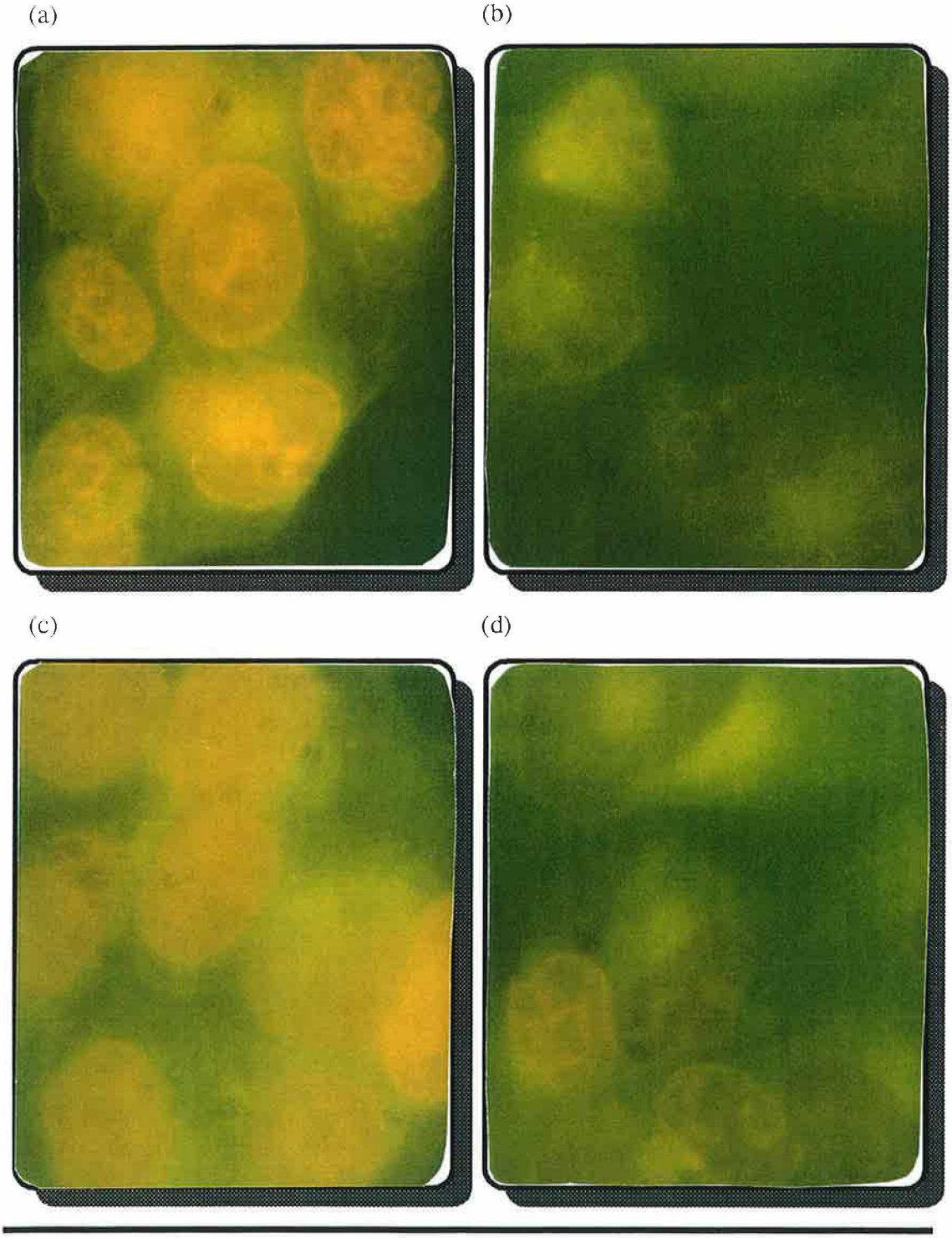
cDNA extracted from DLKP-I (■), DLKP-I/A25010p (■), DLKP-I*mdr1*Rz2D4 (■) (DLKP-IRz), and DLKP-I*mdr1*Rz2D4/A25010p (■) (DLKP-IRz/A25010p) was analysed for the expression of the *mdr1* gene. A 383bp *β-actin* product was co-amplified in all reactions. Densitometric O.D.* is presented as the ratio of the levels of each specific gene product to the internal *β-actin* control and is normalised to 100% for DLKP-I.

3.7.2 Adriamycin Uptake in DLKP-I Variants

To determine if the difference in *mdr1* expression seen in the resistant variants of DLKP-I and DLKP-I*mdr1*Rz2D4 affected drug accumulation as a means of resistance, adriamycin accumulation was analysed in the sensitive and resistant variants. Adriamycin uptake analysis in DLKP-I and DLKP-I/A25010p and DLKP-I*mdr1*Rz2D4 and DLKP-I*mdr1*Rz2D4/A25010p showed a dramatic decrease in nuclear accumulation of adriamycin in the resistant variants following incubation of all cells in 10μM adriamycin for 4.5 hours.

Nuclear accumulation of adriamycin was observed in DLKP-I with very little cytoplasmic accumulation of drug (Figure 3.7.2.1(a)). The resistant variant of DLKP-I, DLKP-I/A25010p, had adriamycin-free nuclei and some cytoplasmic accumulation (Figure 3.7.2.1(b)) but much less total cellular accumulation of drug. DLKP-I*mdr1*Rz2D4 cells appeared to have more accumulation of drug in their nucleus relative to the DLKP-I parental cell line (Figure 3.7.2.1(c)) suggesting a role for P-glycoprotein mediated drug accumulation in sensitive cells. The resistant variant DLKP-I*mdr1*Rz2D4/A25010p were seen to be a heterogenous population with some cells displaying nuclear fluorescence and others free of nuclear accumulation; this may indicate that the ribozyme is only expressed in some cells or that these cells have lost resistance. Most cells in this population, however, showed clear nuclei with cytoplasmic accumulation of adriamycin (Figure 3.7.2.1(d)), more so than the DLKP-I/A25010p variant (Figure 3.7.2.1(b)) suggesting that decreased *mdr1* mRNA levels may result in increased drug accumulation. This increased drug accumulation does not appear to affect toxicity levels of the cell line (Table 3.7.1).

Figure 3.7.2.1 Adriamycin Uptake in DLKP-I Variants



DLKP-I variants were exposed to 10μM adriamycin for 4.5 hours, drug was then removed and cells washed free of any residual drug. Adriamycin uptake is observed as orange fluorescence. Adriamycin accumulation is observed in the nucleus of the sensitive DLKP-I (a) but is greatly reduced in the adriamycin-resistant pulse variant DLKP-I/A25010p (b). The sensitive cell line DLKP-I*mdr1*Rz2D4 (c) appears to have much more intense nuclear staining than DLKP-I. DLKP-I*mdr1*Rz2D4/A25010p (d) (resistant variant) are a mixed population with less nuclear fluorescence than its sensitive parental line; greater cytoplasmic fluorescence is observed in these cells.

3.8 Adriamycin Selection from Five DLKP-SQ *Bcl-x_S*-expressing Clones

DLKP-SQ was transfected with the *bcl-x_S* cDNA using calcium-phosphate precipitation and glycerol shock method (Section 2.14.3). Five clones were maintained as individual cell lines following selection in 600μg/ml geneticin and included DLKP-SQ/*bclx$_S$*.1, DLKP-SQ/*bclx$_S$*.2, DLKP-SQ/*bclx$_S$*.3, DLKP-SQ/*bclx$_S$*.4 and DLKP-SQ/*bclx$_S$*.5.

The five isolated clones were exposed to weekly four-hour exposures of 250ηg/ml adriamycin. Four of the clones were found to grow readily in increasing pulses of adriamycin, further analysis was not carried out. One clone, DLKP-SQ/*bclx$_S$*.1, did not survive exposure to the first weekly pulse of 250ng/ml adriamycin. Repeat analysis confirmed this result.

3.8.1 Adriamycin Toxicity Analysis of DLKP-SQ/*bcl-x_S* clones

Adriamycin toxicity analysis was carried out for four of the original DLKP-SQ/*bcl-x_S* clones described in Section 3.8. Initial results indicated that the IC_{50} for the clones was quite similar to that of the parental line, DLKP-SQ. Further analysis of three clones, DLKP-SQ/*bcl-x_S*.1, DLKP-SQ/*bcl-x_S*.4 and DLKP-SQ/*bcl-x_S*.5, confirmed this result (Table 3.8.1.1).

3.8.2 RT-PCR Analysis of DLKP-SQ/*bcl-x_S* clones

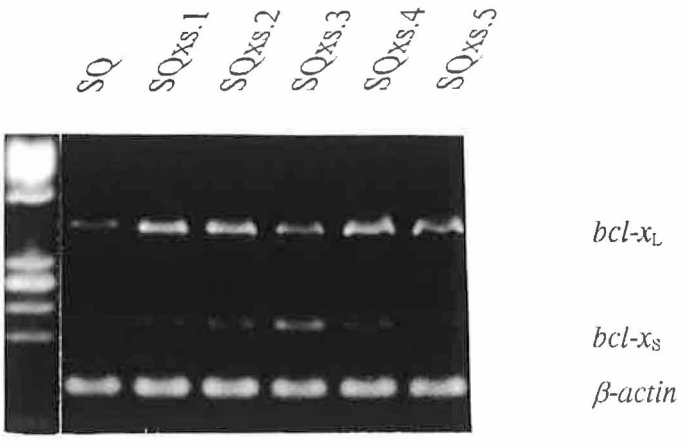
RT-PCR analysis was carried out on the five DLKP-SQ/*bcl-x_S* clones to show the presence of *bcl-x_S* cDNA. A 207bp *bcl-x_S* band was detected for all clones after 30 cycles of PCR. Highest levels of expression were seen in DLKP-SQ/*bcl-x_S.3* compared to the other clones (Figure 3.8.2.1); however, this was not reflected in increased sensitivity to adriamycin treatment. *mdr1* mRNA levels were not detected in the DLKP-SQ/*bcl-x_S* clones (result not shown).

Table 3.8.1.1 Adriamycin IC_{50} values of DLKP-SQ/*bcl-x$_S$* clones

	DLKP-SQ	DLKP-SQ/*bcl-x$_S$*.1	DLKP-SQ/*bcl-x$_S$*.4	DLKP-SQ/*bcl-x$_S$*.5
[ADR]ng/ml	13.9 ± 3.5	17.5 ± 6.6	15.5 ± 0.7	17.6 ± 0.9

Adriamycin toxicity in DLKP-SQ and clonal populations transfected with the *bcl-x$_S$* gene (DLKP-SQ/*bcl-x$_S$*.1, which did not survive exposure to adriamycin, and DLKP-SQ/*bcl-x$_S$*.4 and .5, which grew readily in repeated pulses of adriamycin).

Figure 3.8.2.1 RT-PCR Analysis of DLKP-SQ/*bcl-xs* clones



Expression of *bcl-x$_S$* in DLKP-SQ cells transfected with the *bcl-x$_S$* cDNA. Parental cell line (DLKP-SQ) is shown in the first lane (SQ). DLKP-SQ/*bcl-x$_S$*.1, .2, .3, .4 and .5 are named as SQxs.1, .2, .3, .4 and .5, respectively.

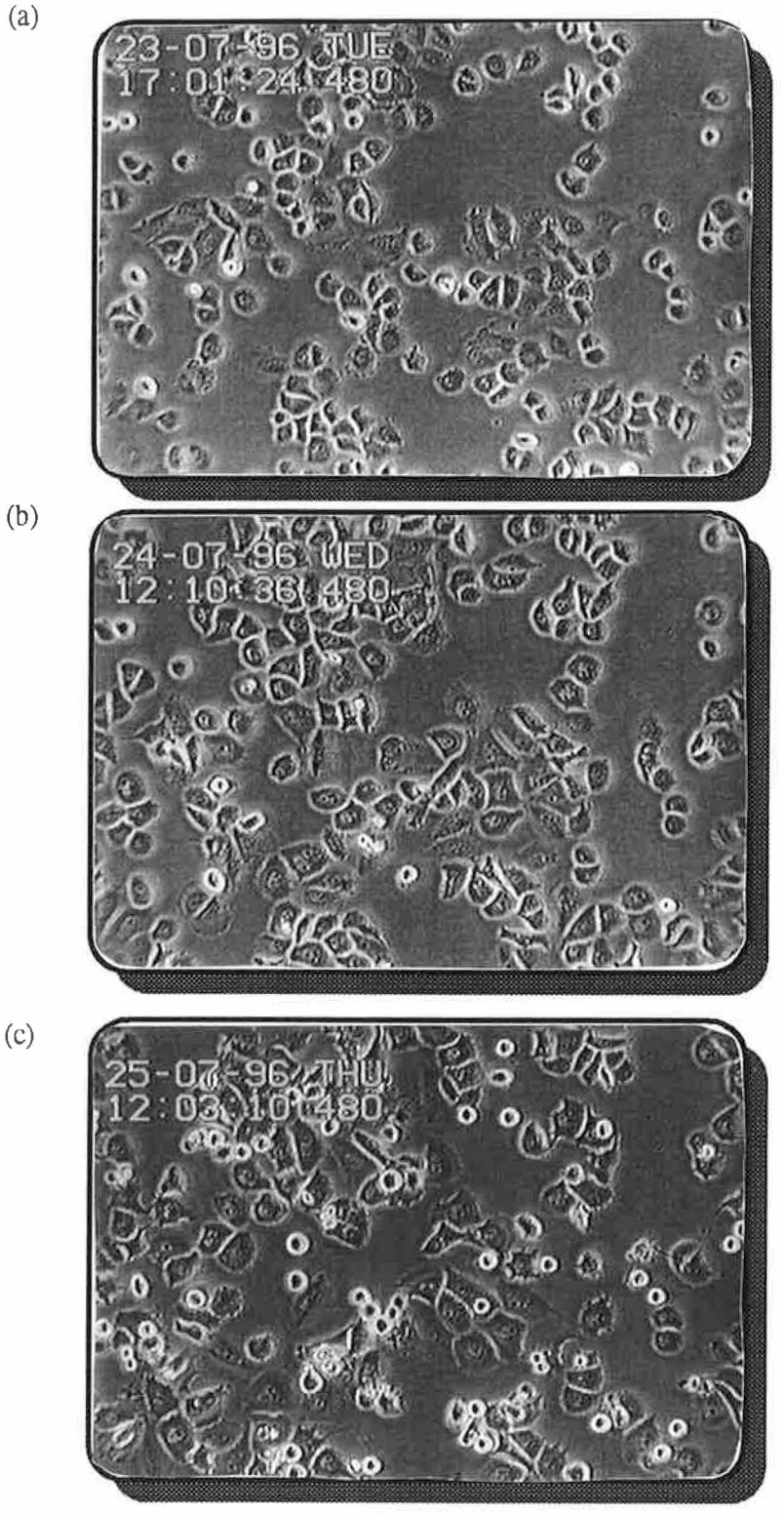
3.8.3 Time-lapse Analysis of DLKP-SQ/*bcl-x$_S$*.1 exposed to Adriamycin

DLKP-SQ and DLKP-SQ/*bcl-x$_S$*.1 cell growth was monitored by time-lapse video-microscopy for four days either in the absence of drug or following exposure to 250ng/ml adriamycin for 4 hours. Results are shown in Figures 3.8.3.1 and 3.8.3.2. Cells growing in 25cm^2 flasks were monitored at 10X magification by time-lapse video-microscopy equipment described in Section 2.8. To analyse cell death or mitotic events the recording was played back at accelerated speed. Groups of cells were chosen and the time at which either apoptotic or mitotic events was seen was recorded. Apoptotic cells round up and cell blebbing is then observed with destruction of the cell. Mitotic events also begin with rounding up of the cell and subsequent cell division.

Pulse exposure of DLKP-SQ cells with 250ng/ml adriamycin for 4 hours revealed cell kill by apoptosis after a lag phase of 0 - 25 hours (Figure 3.8.3.1 - cell blebbing and apoptotic events observed). Only five apoptotic events were seen up to 25 hours after addition of drug. 40 cells (80%) underwent apoptosis between 25 and 80 hours (Figure 3.8.3.3). Nine mitotic events were observed between 35 and 95 hours, six of which occurred between 35 and 47 hours (Figure 3.8.3.1(f) - mitotic event is marked with **M**). Mitotic events in untreated (without ADR) DLKP-SQ cells is shown for comparison (Figure 3.8.3.4).

The DLKP-SQ/*bcl-x$_S$*.1 cell line was also exposed to 250ng/ml adriamycin for 4 hours and monitored by time-lapse video-microscopy. Drug was removed after 4 hours and cells were maintained in adriamycin-free media. Again a lag-phase of up to 35 hours was seen for this cell line where only 6 apoptotic events were observed. After 35 hours, however, apoptotic events were more frequent and 50 (100%) cells observed had died by 100 hours (Figure 3.8.3.3). These cells were found to swell dramatically between 4 and 24 hours (prior to induction of increase in apoptotic events) when drug had been removed (see Figure 3.8.3.2). The rate of apoptosis was quite similar in both the DLKP-SQ and DLKP-SQ/*bclx$_S$*.1 cell line (see Figure 3.8.3.3). No mitotic events were seen in the DLKP-SQ/*bclx$_S$*.1 cell line, however (Figure 3.8.3.4).

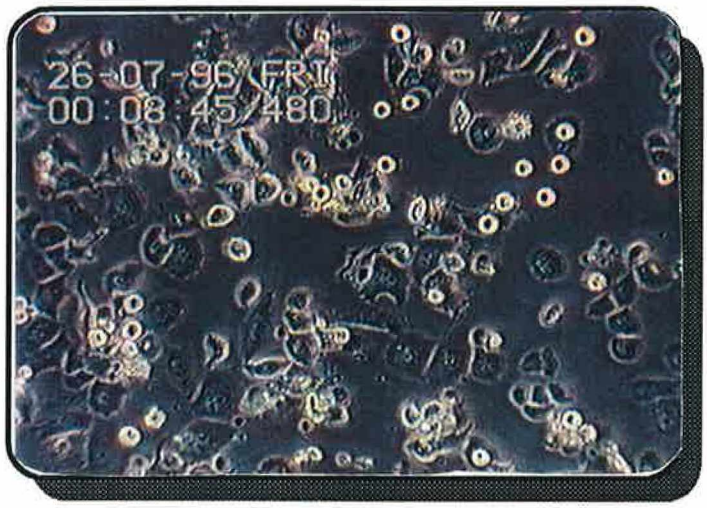
Figure 3.8.3.1 Time-lapse Video-microscopy of DLKP-SQ in the presence of 250ng/ml adriamycin



Adriamycin was added at time t=0 and removed at time t=4 (a). Cell survival was monitored by time-lapse video-microscopy for 4 days (t=24hour (b); t=48hour (c); t=60hour (d); t=72hour (e); t=80hour (f)). Mitotic event in (f) is marked as **M**.

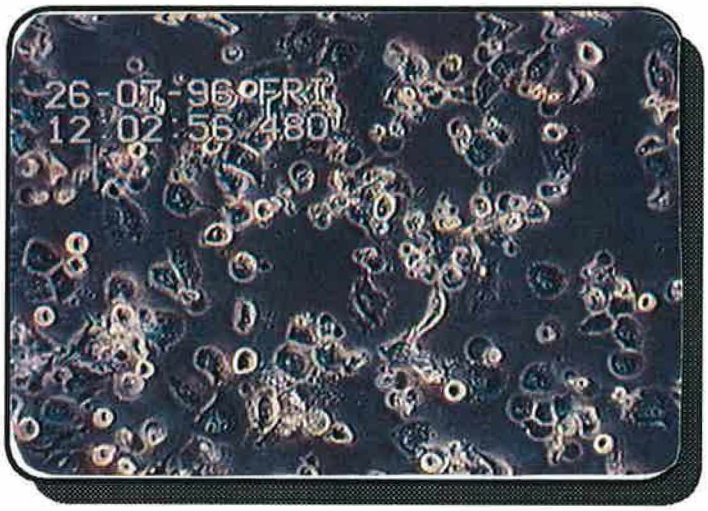
Figure 3.8.3.1 (cont.) Time-lapse Video-microscopy of DLKP-SQ in the presence of 250ng/ml adriamycin

(d)



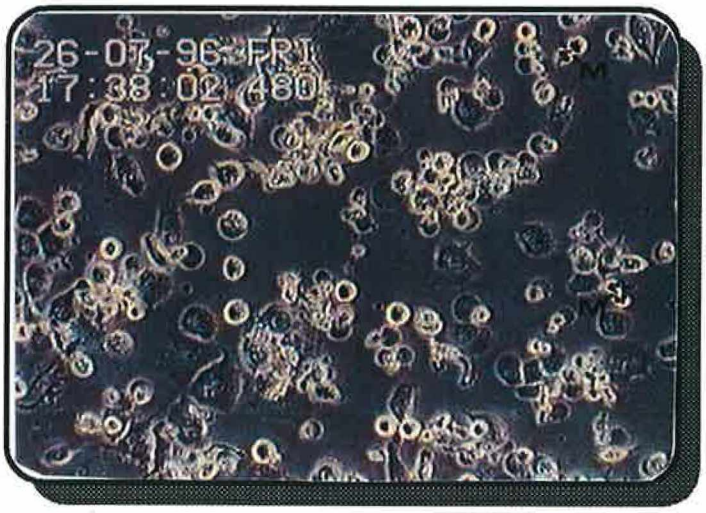
t = 60hour

(e)



t = 72hour

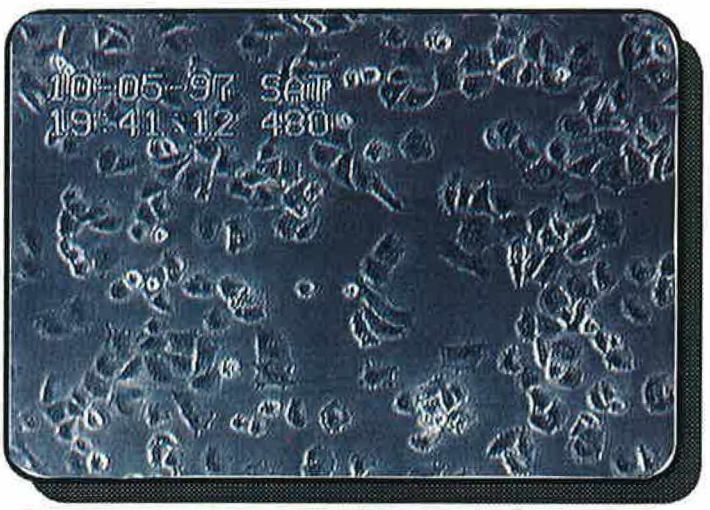
(f)



t = 80hour

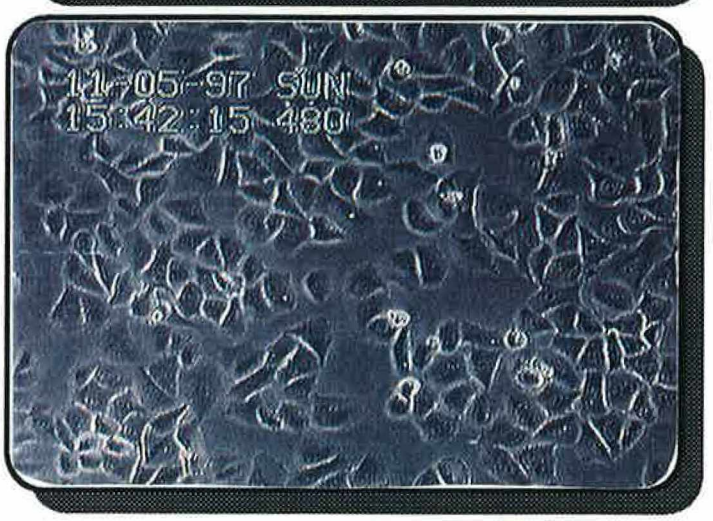
Figure 3.8.3.2 Time-lapse video-microscopy of DLKP-SQ/*bclx$_S$*.1 in the presence of 250ng/ml adriamycin

(a)



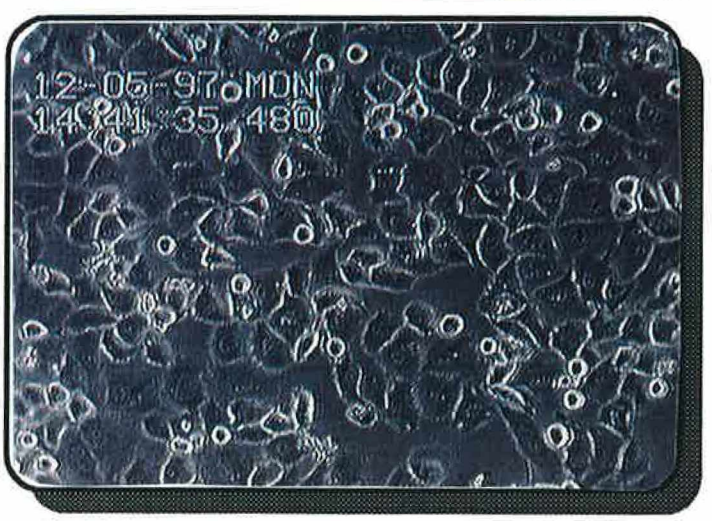
t = 4hour

(b)



t = 24hour

(c)

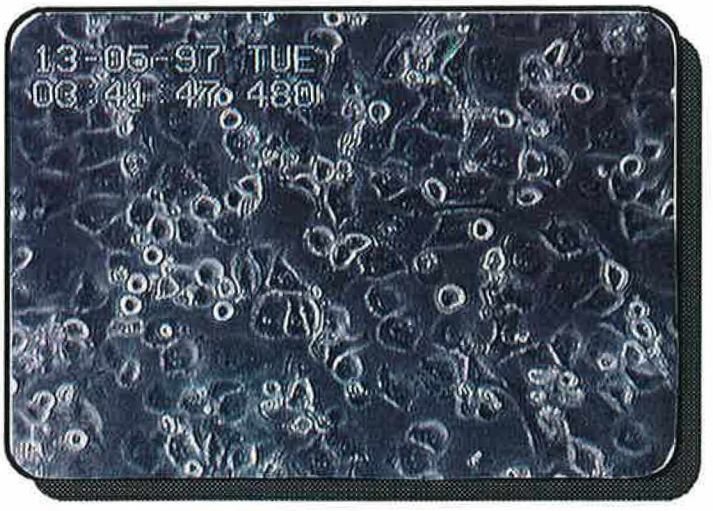


t = 48hour

Adriamycin was added at time t=0 and removed at time t=4 (a). Cell survival was monitored by time-lapse video-microscopy for 4 days (t=24hour (b); t=48hour (c); t=60hour (d); t=72hour (e); t=80hour (f)).

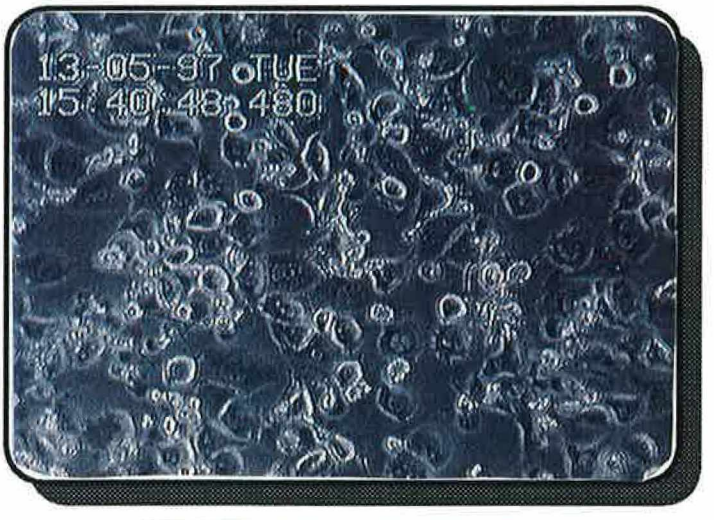
Figure 3.8.3.2 (cont.) Time-lapse Video-microscopy of DLKP-SQ/*bclx$_S$.1* in the presence of 250ng/ml adriamycin

(d)



t = 60hour

(e)

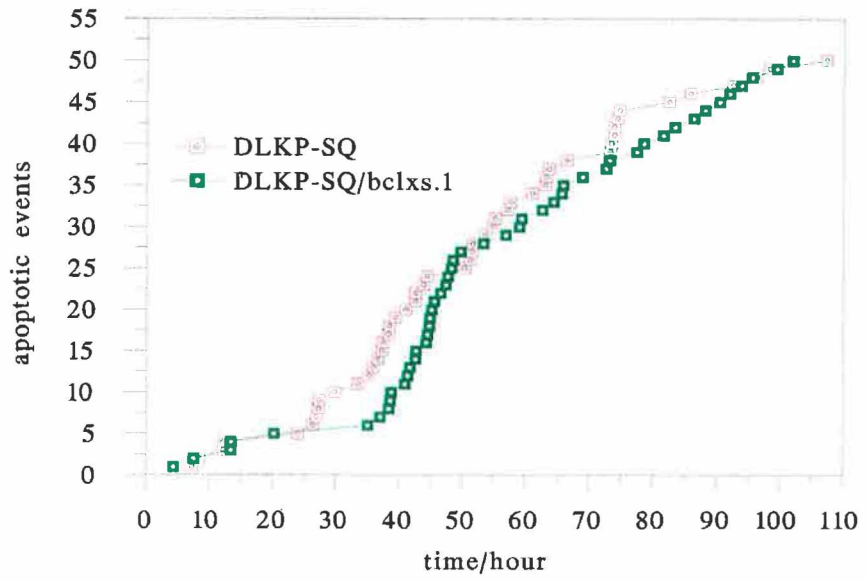


t = 72hour

(f)

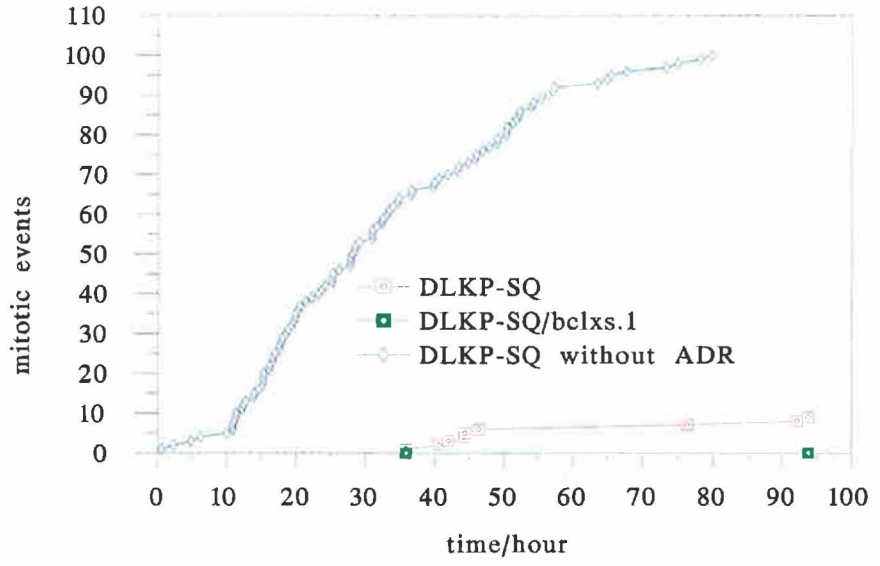
t = 80hour

Figure 3.8.3.3 Rate of Apoptotic events in DLKP-SQ and DLKP-SQ/*bclx*$_S$.1 cell lines



Apoptotic events were monitored by time-lapse video microscopy in the DLKP-SQ and DLKP-SQ/*bcl-x*$_S$.1 cell line following 4 hour treatment with 250ng/ml adriamycin. Drug was added at time t=0 and removed at t=4 when cells were fed in fresh media without adriamycin.

Figure 3.8.3.4 Mitotic Events in DLKP-SQ



Mitotic events were monitored by time-lapse video microscopy in the DLKP-SQ and DLKP-SQ/*bcl-x*$_S$.1 cell line following 4 hour treatment with 250ng/ml adriamycin and DLKP-SQ without adriamycin (ADR) treatment (DLKP-SQ without ADR). Drug was added (to DLKP-SQ and DLKP-SQ/*bclx*$_S$.1) at time t=0 and removed at t=4 when cells were fed in fresh media without adriamycin.

3.9 Adriamycin Selection of DLKP-SQ clones transfected with c-*fos* Ribozyme

DLKP-SQ cell line was transfected (Section 2.14.3) with an inducible c-*fos* ribozyme plasmid. Individual colonies were propagated in 600μg/ml geneticin and maintained as the following cell lines - DLKP-SQ*fos*Rz/E1, DLKP-SQ*fos*Rz/A6 and DLKP-SQ*fos*Rz/B10. The c-*fos* ribozyme was induced by 24 hour incubation in 5μM dexamethasone (used previously by Funato *et al.*, 1992).

Triplicate flasks of each cell line were pre-treated for 24 hours in 5μM dexamethasone and then exposed to 250ng/ml adriamycin (in dexamethasone containing medium) for 4 hours; control selections were set up without dexamethasone treatment. These treatments were carried out once weekly. Selection failed with each cell line tested, including those cells set up without dexamethasone, as all cells died following exposure to the first pulse with adriamycin.

3.9.1 Adriamycin Toxicity Profile of DLKP-SQ*fos*Rz clones

Adriamycin toxicity analysis of the DLKP-SQ c-*fos* ribozyme expressing clones was carried out on one representative clone, namely DLKP-SQ*fos*RzA6. No significant alteration to adriamycin sensitivity levels was seen between the parental DLKP-SQ cell line and the c-*fos* ribozyme-expressing cell line in serum-supplemented media (Table 3.9.1.1).

3.9.2 RT-PCR Analysis of c-*fos* Ribozyme transfected cells

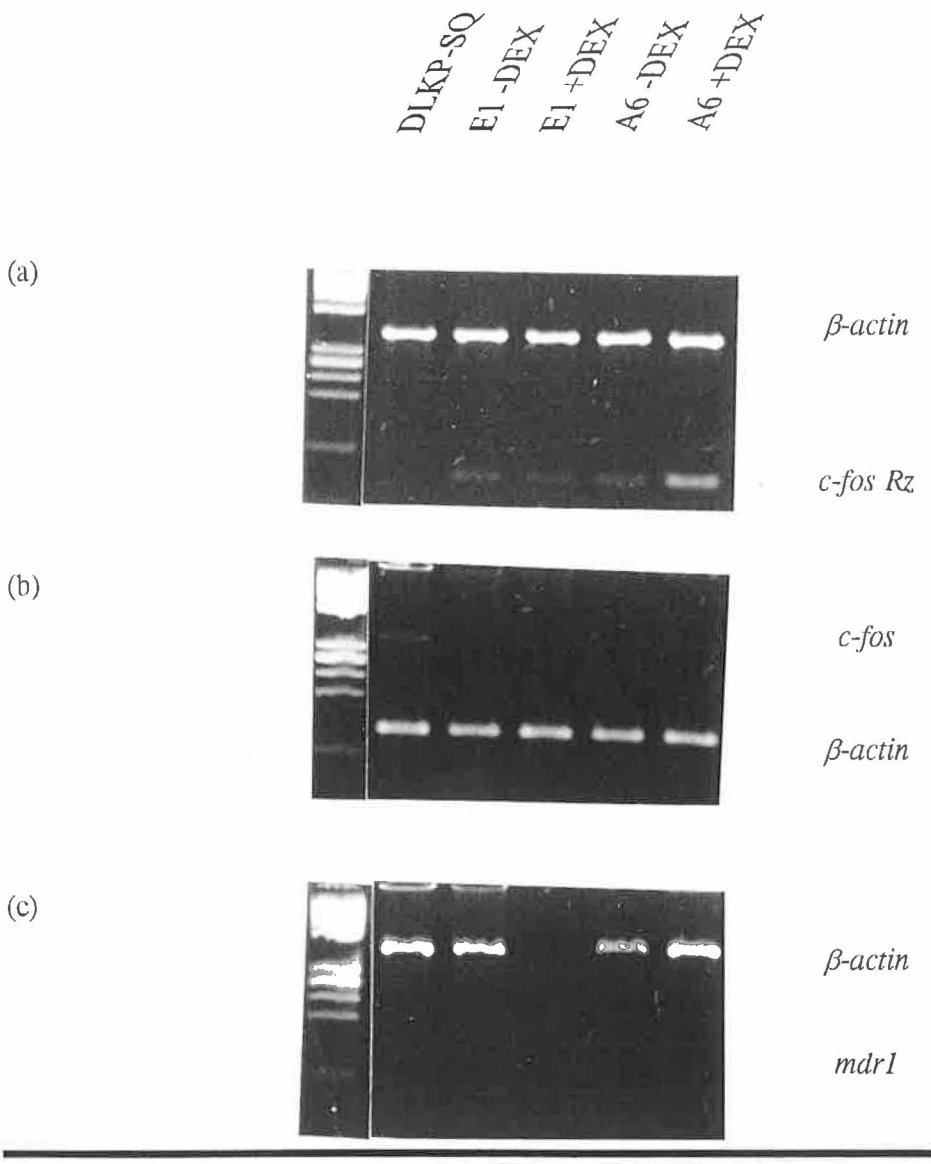
RT-PCR analysis was carried out on mRNA extracted from the c-*fos* ribozyme expressing clones either in the presence or absence of dexamethasone-supplemented medium. Analysis of c-*fos* ribozyme expression, c-*fos* mRNA levels and *mdr1* mRNA levels was undertaken. The c-*fos* ribozyme expression was detected in the two clones analysed (E1 and A6) in the presence and absence of dexamethasone (Figure 3.9.2.1(a)). c-*fos* mRNA was detected in the parental DLKP-SQ cell line but not in the ribozyme-transfected clones (Figure 3.9.2.1(b)). No *mdr1* mRNA was detected in the cell lines after 25 cycles of PCR (Figure 3.9.2.1(c)).

Table 3.9.1.1 Adriamycin IC_{50} values of DLKP-SQ/*fos*Rz clone

IC_{50}	DLKP-SQ	DLKP-SQ/*fosRz*A6
[ADR]ng/ml	13.9 ± 3.5	18.6 ± 12.5

Adriamycin toxicity in DLKP-SQ and a clonal population (DLKP-SQ/*fosRz*A6) transfected with the c-*fos* ribozyme cDNA.

Figure 3.9.2.1 RT-PCR Analysis in c-*fos* Ribozyme transfected DLKP-SQ cells



RT-PCR analysis, for the expression of (a) c-*fos* ribozyme, (b) c-*fos*, (c) *mdr1*, on DLKP-SQ and c-*fos* ribozyme transfected clonal sub-populations DLKP-SQ*fos*RzE1 (E1), DLKP-A*fos*RzA6 (A6), in the presence (+DEX) and absence (-DEX) of 5μM dexamethasone for 24 hours.

3.9.3 Culturing of DLKP-SQ*fos*Rz clones in serum-free media

As serum is known to contain certain levels of dexamethasone which could be enough to induce the expression of the c-*fos* ribozyme, attempts were made to grow these cell lines in serum-free media.

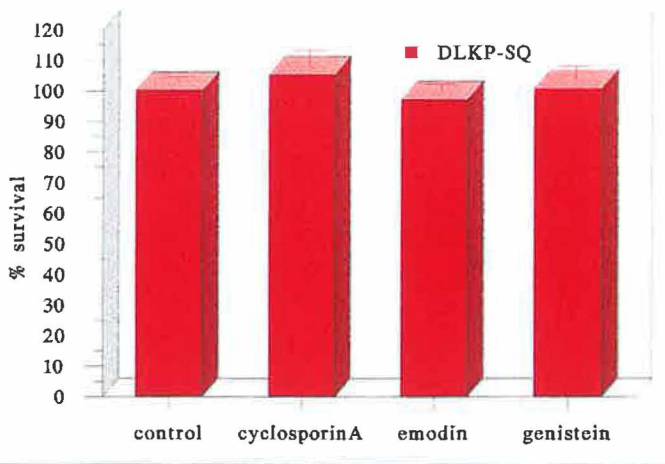
Initially cells were grown in a serum-free media designed to support the growth of the DLKP cell line (P.Meleady, 1997). This media was insufficient to support the growth of the c-*fos* ribozyme transfected cell lines. Media was then supplemented with fibronectin at a concentration of 5μg/ml which resulted in readily adherent cell lines but did not induce mitotic events. As growth in serum-free media proved unsuccessful attempts were made to grow cells in serum-free media supplemented with 1% serum. This media was insufficient to support the growth of the c-*fos* ribozyme transfected clones.

3.10 Effect of Potential Toxicity Enhancing Agents on Adriamycin Toxicity

Three drugs (cyclosporin A, emodin, genistein) were chosen as potential enhancing agents with adriamycin to determine if they could act as toxicity enhancers in these studies. Non-toxic levels of the three drugs were used (Figure 3.10.1), as determined by duplicate toxicity assays set up in 25cm^2 flasks (see Section 2.3.2). The pulse concentration of adriamycin used in this analysis was 250ng/ml for 4 hours and included the appropriate co-selecting agent where appropriate. The adriamycin was then removed from the cells which were then re-fed for 24 hours either in media without any drug or supplemented with the appropriate potential toxicity enhancing agent.

Each analysis shown (Figures 3.10.2 and .3) is the average of at least two 25cm^2 flasks set up on the same day.

Figure 3.10.1 Effect of 28 hour exposure to selecting agent on DLKP-SQ



DLKP-SQ cells were set up in 25cm^2 flasks until 50% confluent; cells (in triplicate flasks) were then fed in fresh media (control) or media supplemented with 2.0μg/ml Cyclosporin A (cyclosporin A), 25μM Emodin (emodin) or 15μM Genistein (genistein) for 28 hours. Media was then removed and cells were grown in control media until control flasks reached 90% confluency. Cell growth was then assessed as previously described (Section 2.3.2).

3.10.1 Co-selection of DLKP-SQ with Adriamycin and Cyclosporin A

DLKP-SQ cells were exposed to 250ng/ml adriamycin for 4 hours in medium containing 2.0μg/ml cyclosporin A, followed by removal of adriamycin and re-fed for 24 hours in 2.0μg/ml cyclosporin A. This process was carried out once a week for 2 weeks. Cell survival was analysed at the end of the second week and it was found that co-selection with the non-toxic level of cyclosporin A reduced cell survival from 100% (adriamycin alone) to 4.0% (Figure 3.10.2). Further analysis following 1 weekly pulse reduced survival to 50% and a second pulse, one week later, reduced survival to 13.2% (Figure 3.10.3).

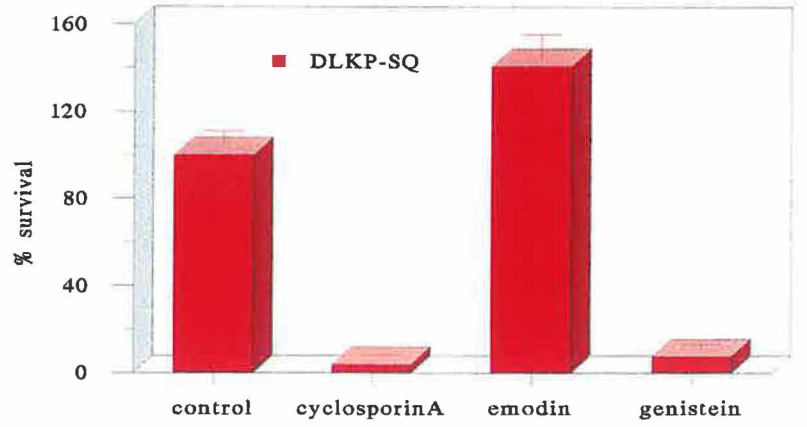
3.10.2 Co-selection of DLKP-SQ with Adriamycin and Emodin

DLKP-SQ cells were exposed to 250ng/ml adriamycin for 4 hours in media containing 25μM emodin, followed by removal of adriamycin and re-fed for 24 hours in 25μM emodin. This process was carried out once a week for 2 weeks. Cell survival was analysed at the end of the second week and it was found that co-selection with the non-toxic level of emodin increased cell survival from 100% (adriamycin alone) to 140% (Figure 3.10.2). Further analysis following 1 weekly pulse increased survival to 161% and a second pulse, one week later, increased overall survival, relative to adriamycin only treated cells, to 172% (Figure 3.10.3).

3.10.3 Co-selection of DLKP-SQ with Adriamycin and Genistein

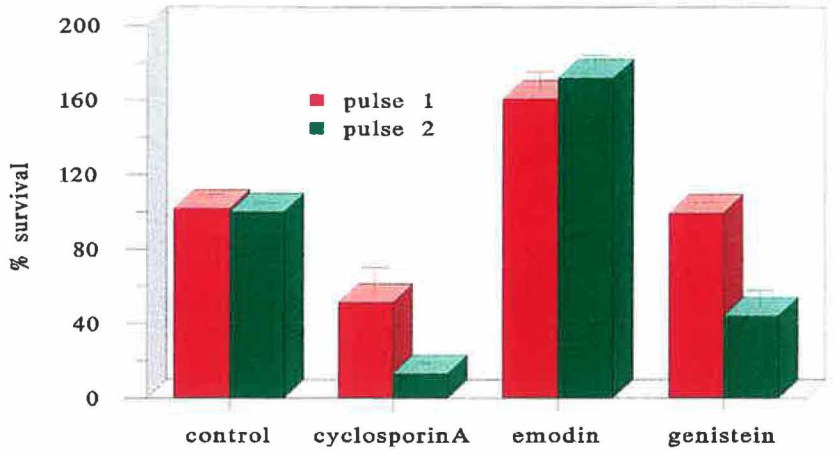
DLKP-SQ cells were exposed to 250ng/ml adriamycin for 4 hours in media containing 15μM genistein, followed by removal of adriamycin and the cells were then re-fed for 24 hours in 15μM genistein. This process was carried out once a week for 2 weeks. Cell survival was analysed at the end of the second week and it was found that co-selection with the non-toxic level of genistein decreased cell survival from 100% (adriamycin alone) to 7.6% (Figure 3.10.2). Further analysis following 1 weekly pulse did not affect cell survival, however, a second weekly pulse reduced survival to 44% (Figure 3.10.3).

Figure 3.10.2 Effect of Potential Toxicity Enhancing Agents on Adriamycin Toxicity



DLKP-SQ cells were set up in 25cm^2 flasks until they reached 50% confluency. Cells were then fed in medium supplemented with 250ng/ml adriamycin only (control) or adriamycin and 2.0μg/ml Cyclosporin A (cyclosporin A), 25μM Emodin (emodin) or 15μM Genistein (genistein) for 4 hours; after 4 hours adriamycin-supplemented medium was removed, cells rinsed in fresh medium and re-fed for a further 24 hours in control medium or medium supplemented with the appropriate selecting agent. All flasks were then re-fed in fresh medium. Cells were allowed to grow for a further 6 days and then the procedure was repeated. Following the second 6-day recovery phase growth was assessed as described previously (Section 2.3.2).

Figure 3.10.3 Effect of CyclosporinA, Emodin and Genistein on Adriamycin Toxicity



DLKP-SQ cells were set up in 25cm^2 flasks until they reached 50% confluency. Cells were then fed in media supplemented with 250ng/ml adriamycin only (control) or adriamycin and 2.0μg/ml Cyclosporin A (cyclosporin A), 25μM Emodin (emodin) or 15μM Genistein (genistein) for 4 hours; after 4 hours adriamycin-supplemented medium was removed, cells rinsed in fresh medium and re-fed for a further 24 hours in control mediumor medium supplemented with the appropriate selecting agent. All flasks were then re-fed in fresh medium. Cells were allowed to grow for a further 6 days after which time cell survival was assessed (pulse 1). The procedure was repeated. Following the second 6-day recovery phase growth was assessed (pulse 2) as described previously.

3.11 Gene Induction Studies with Adriamycin

Previous results have indicated a role for a number of oncogenes in the expression of the MDR phenotype. To determine if alterations in the expression of these genes occurs as an early response to the selecting drug DLKP-SQ was used as a model to follow gene induction. After exposure to the drug concentrations used in the development of resistant variants, e.g. 25ng/ml adriamycin continuously or 250ng/ml adriamycin for 4 hours, total RNA was extracted as previously described (Section 2.12.1) after a certain timeframe (from 0 minutes to 72 hours) had elapsed. RNA was subsequently reverse transcribed and studied by PCR for the expression of the *bcl-x*$_L$, c-*erb*B-2 and c-*fos* genes.

3.11.1 Effect of Adriamycin treatment on expression of *bcl-x* mRNA in DLKP-SQ

RT-PCR analysis of *bcl-x*$_L$ expression in RNA extracted from cells 0, 8, 15, 30min, 1, 2, 4, 6, 9 and 24 hours after addition of 25ng/ml adriamycin revealed a gradual induction in expression of this gene with time after 30min exposure to the drug (see Figure 3.11.1.1(a)). A repeat of this experiment did not display the same trend in expression (see Figure 3.11.1.1(d)).

Similar analysis of RNA extracted from cells that were treated with 250ng/ml adriamycin for 4hours also revealed an increase in *bcl-x*$_L$ levels with time, although the increase was not seen until further time had elapsed (see Figure 3.11.1.1(b)). Repeating this experiment did not reveal the same trend (Figure 3.11.1.1(e)).

Figure 3.11.1.1(c) is a control experiment where cells had not been exposed to adriamycin.

3.11.2 Effect of Adriamycin treatment on c-*erb*B-*2* mRNA expression in DLKP-SQ

Initial results following continuous treatment of DLKP-SQ with 25ng/ml adriamycin revealed an induction of c-*erb*B-*2* transcripts after 2hours exposure to adriamycin (Figure 3.11.2.1(a)). Repeat analysis of a second extraction showed increased expression after 4 hours in drug but this increase was not maintained at 6 hours (see Fig. 3.11.2.1(d)).

The trend when cells were treated with 250ng/ml adriamycin for 4 hours was similar to that of 25ng/ml adriamycin continuously with a steady increase evident, beginning at 4 hours (Figure 3.11.2.1(b)). Repeated analysis did not concur with the trend found in

initial experiments (Figure 3.11.2.1(e)).

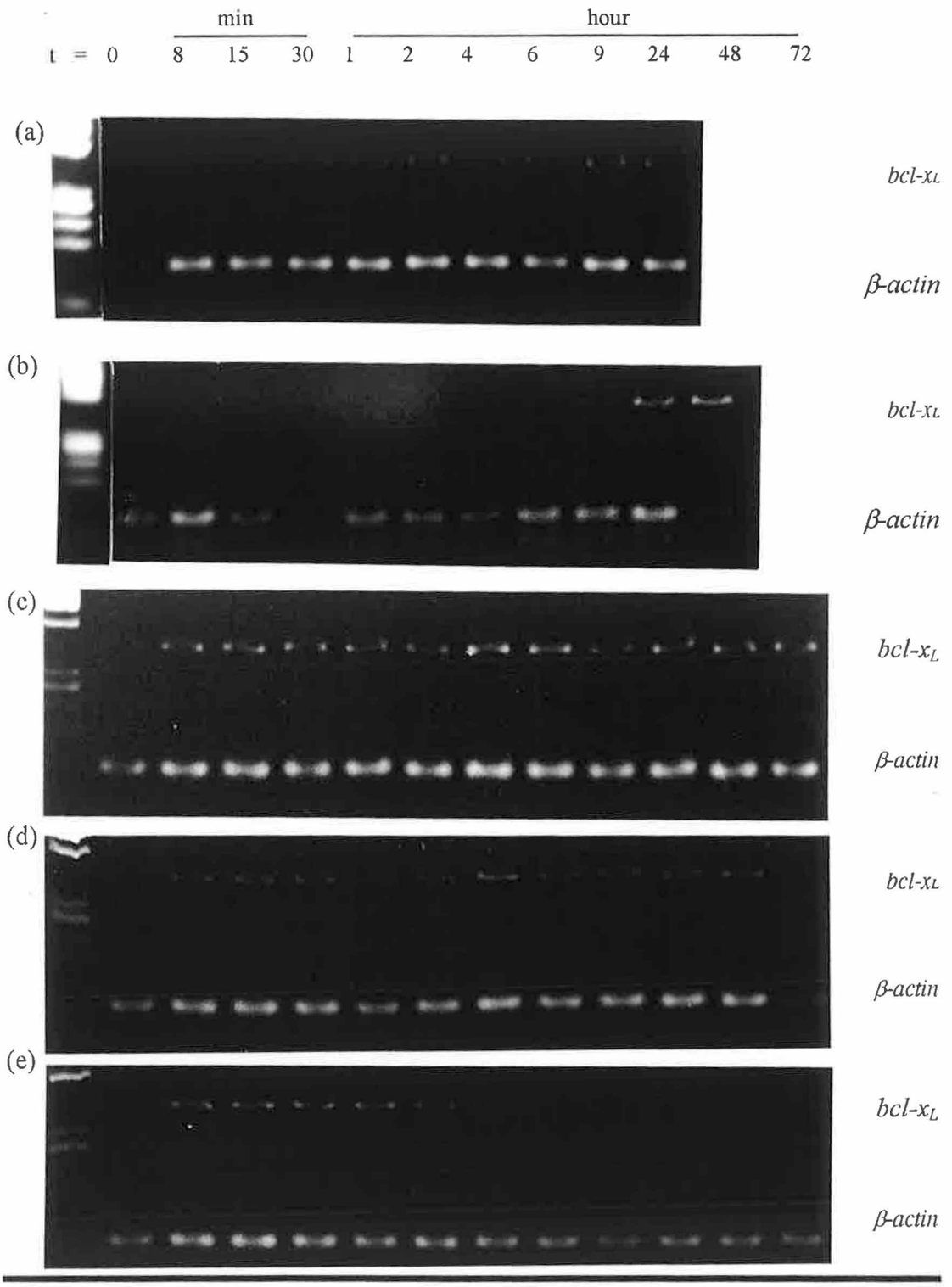
Figure 3.11.2.1(c) is a control experiment where cells had not been exposed to adriamycin.

3.11.3 Effect of Adriamycin treatment on expression of c-*fos* mRNA in DLKP-SQ

c-*fos* levels were increased in response to serum addition to cells and reached a maximum at 30 minutes (see Fig.3.11.3.1c); higher levels of expression were maintained for longer periods when cells were exposed to drug (see Fig. 3.11.3.1e). The effect of adriamycin on c-*fos* levels is not conclusive as repeat analysis did not reveal a similar trend.

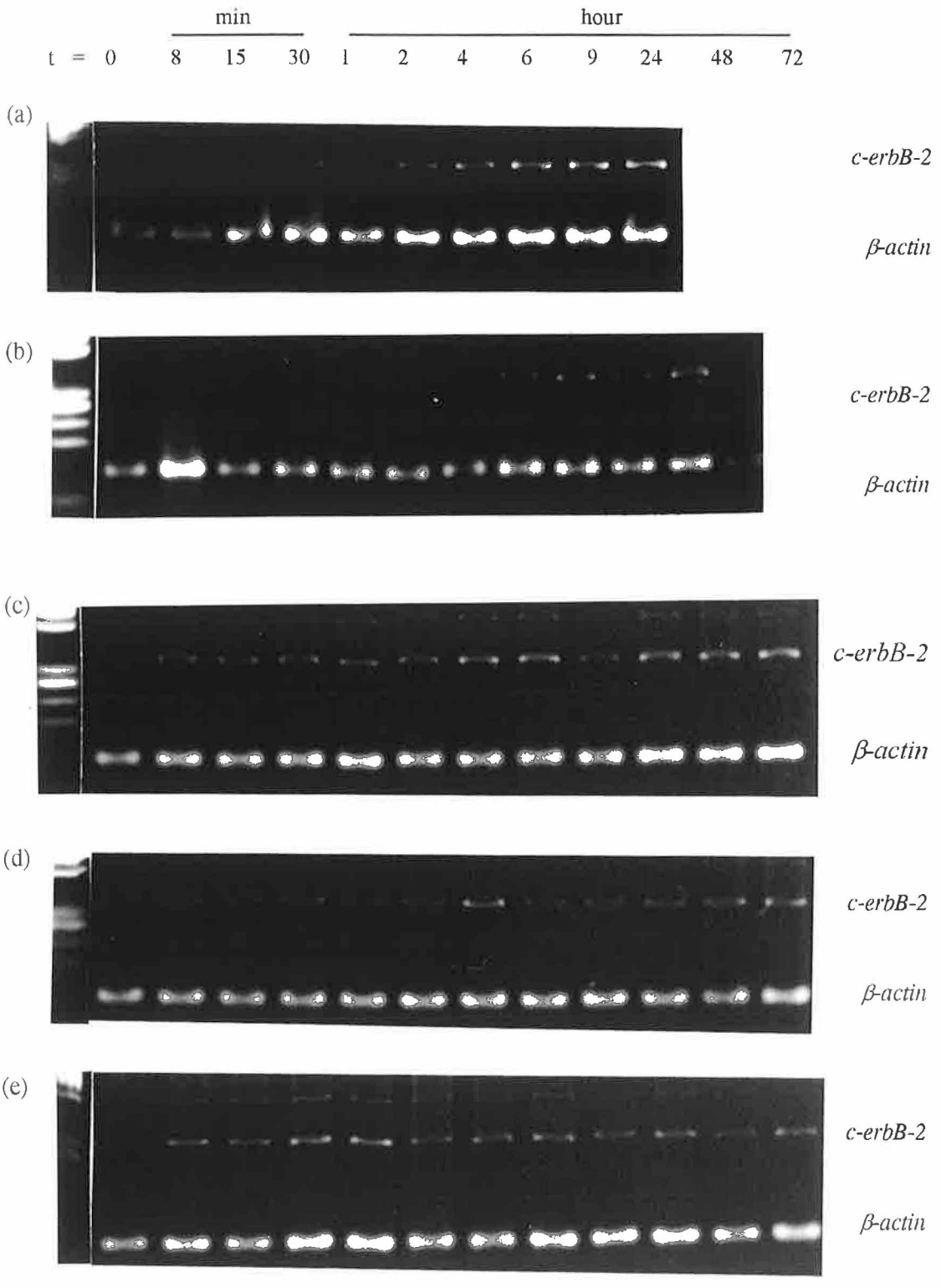
Figure 3.11.3.1(c) is a control experiment where cells had not been exposed to adriamycin.

Figure 3.11.1.1 Gene Induction Analysis of *bcl-x*$_L$ in DLKP-SQ following Exposure to Adriamycin



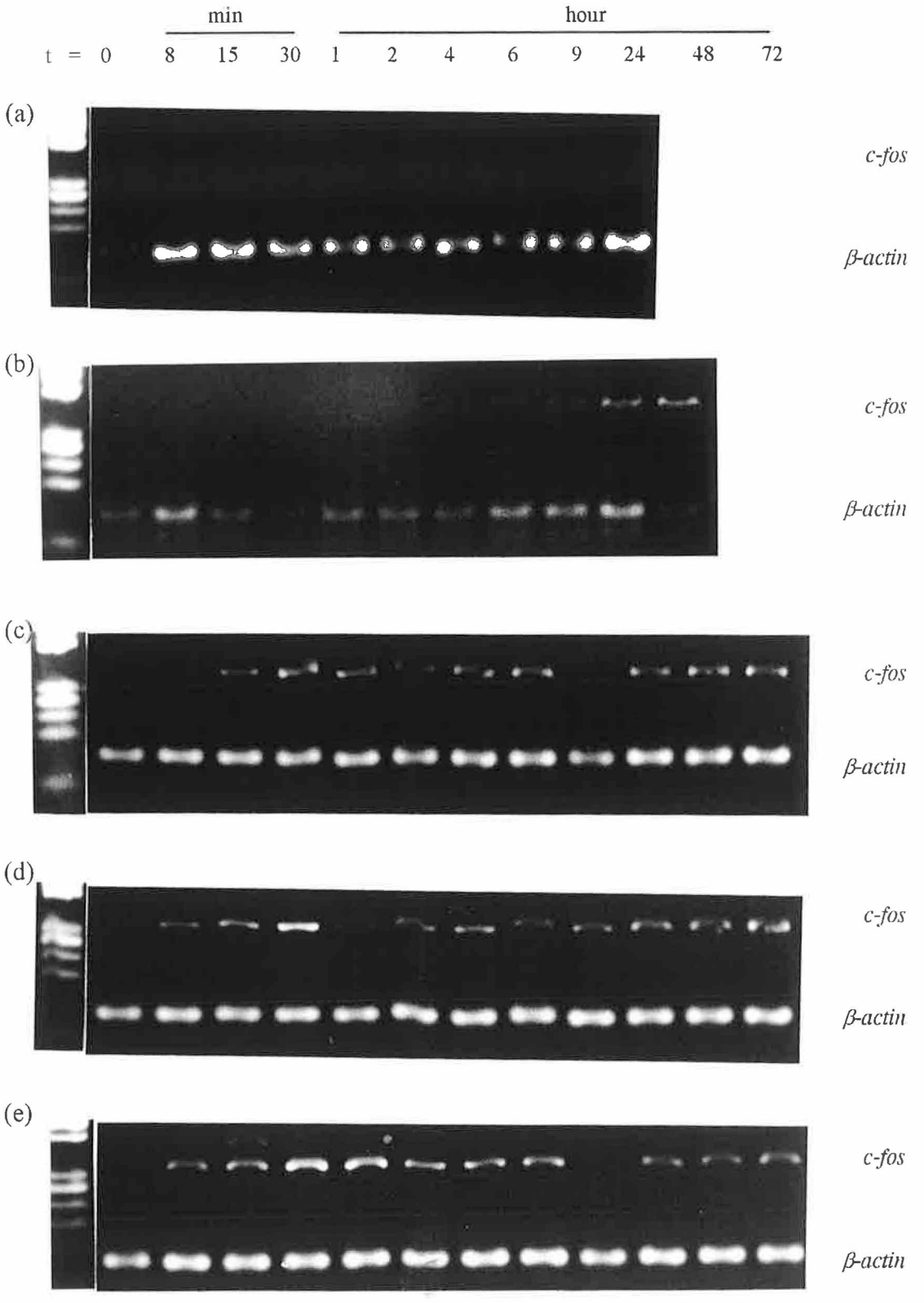
RT-PCR analysis (after 25 cycles) of *bcl-x*$_L$ on RNA extracted from DLKP-SQ exposed to adriamyin at (a) 25ng/ml continuously (1st extraction); (b) 250ng/ml for 4 hours (1st extraction); (c) 0ng/ml (2nd extraction); (d) 25ng/ml continuously (2nd extraction) and (e) 250ng/ml for 4 hours (2nd extraction).

Figure 3.11.2.1 Gene Induction Analysis of *c-erbB-2* in DLKP-SQ following Exposure to Adriamycin



RT-PCR analysis (after 25 cycles) of *c-erbB-2* on RNA extracted from DLKP-SQ exposed to adriamyin at (a) 25ng/ml continuously (1st extraction); (b) 250ng/ml for 4 hours (1st extraction); (c) 0ng/ml (2nd extraction); (d) 25ng/ml continuously (2nd extraction) and (e) 250ng/ml for 4 hours (2nd extraction).

Figure 3.11.3.1 Gene Induction Analysis of *c-fos* in DLKP-SQ following Exposure to Adriamycin



RT-PCR analysis (after 30 cycles) of *c-fos* on RNA extracted from DLKP-SQ exposed to adriamyin at (a) 25ng/ml continuously (1st extraction); (b) 250ng/ml for 4 hours (1st extraction); (c) 0ηg/ml (2nd extraction); (d) 25ng/ml continuously (2nd extraction) and (e) 250ng/ml for 4 hours (2nd extraction).

3.12 Development of a Resistant variant of BT-20

Attempts were made throughout the course of this work to develop a resistant variant of the breast cell line BT-20 (derived from a patient not previously treated with chemotherapy). Drug resistance profile of BT-20 is shown in Table 3.12.1. The selection process was initiated with 25ng/ml adriamycin which had little effect on the growth of these cells. Continuous selection was then attempted with higher adriamycin concentrations (e.g. 30 and 35ng/ml adriamycin); these higher concentrations caused dramatic cell kill and selection was not successful as the cells did not recover from exposure to adriamycin at these concentrations. Pulse exposure of a clonal population of BT-20, BT-20/B10, to 250ng/ml adriamycin for 4 hours also failed to select a resistant variant.

The ease with which DLKP-SQ could be selected with adriamycin compared to the difficulty in selecting BT-20 with this drug is surprising as toxicity levels of adriamycin in both cell lines are quite similar (see Table 3.5.2.2.2 (IC_{50} value of ADR in DLKP-SQ is 13.9 ± 3.5ng/ml) and Table 3.12.1). Observations made while maintaining the parental BT-20 population in culture revealed a strong dependence on high cell density for successful growth of this cell line under normal culturing conditions. This was not the case for DLKP-SQ which grew readily at all cell densities tested.

Attempts were also made to co-select the BT-20 cell line with cyclophosphamide :methotrexate:5-fluorouracil (CMF), a regimen frequently used in the treatment of breast cancer. Initial toxicity work (Table 3.12.1) revealed an extraordinarily high resistance in this cell line to methotrexate (IC_{50} of MCF-7 for methotrexate is only 0.04 ± 0.01μg/ml). This is quite surprising as the patient from whom the BT-20 cell line was derived had not been treated with chemotherapy.

Table 3.12.1 IC_{50} Values for BT-20

	[ADR] ng/ml	[VNC] ng/ml	[VP-16] ng/ml	[MTX] μg/ml	[CPM] μg/ml	[5-FU] μg/ml
IC_{50}	17.5 ± 1.9	1.0 ± 0	80*	27.9 ± 7.3	2.33 ± 1.53	0.43 ± 0.12

Toxicity profile of a sensitive breast cell line, BT-20. ADR = adriamycin; VNC = vincristine; MTX = methotrexate; CPM = mafosfamide; 5-FU = 5-fluorouracil: *analysis was only carried out once.

3.12.1 RT-PCR Analysis of Cell Lines with Varying Tendencies to Develop Resistance

Unpublished observations found in this laboratory previously, and throughout the course of this work, have shown that cell lines vary in their tendencies to develop resistance - some lines develop resistance readily (e.g. DLKP) whereas others do not (e.g. BT-20, DLKP-SQ/*bclx*$_S$.1 and DLKP-SQ/*fos*Rz clones) (see Table 3.12.1.1). A group of six cell lines was chosen in this study to examine the level of common MDR markers and oncogene expression in paired lung (DLKP and A549), breast (MCF-7 and BT-20) and ovarian (OAW42SR and PA-1) cell lines to determine if any trends could be seen among groups. The BT-20 and PA-1 cell lines did not grow well at low cell density, perhaps explaining in part the fact that they do not develop resistance. The A549 cell line does, however, grow well at low cell densities.

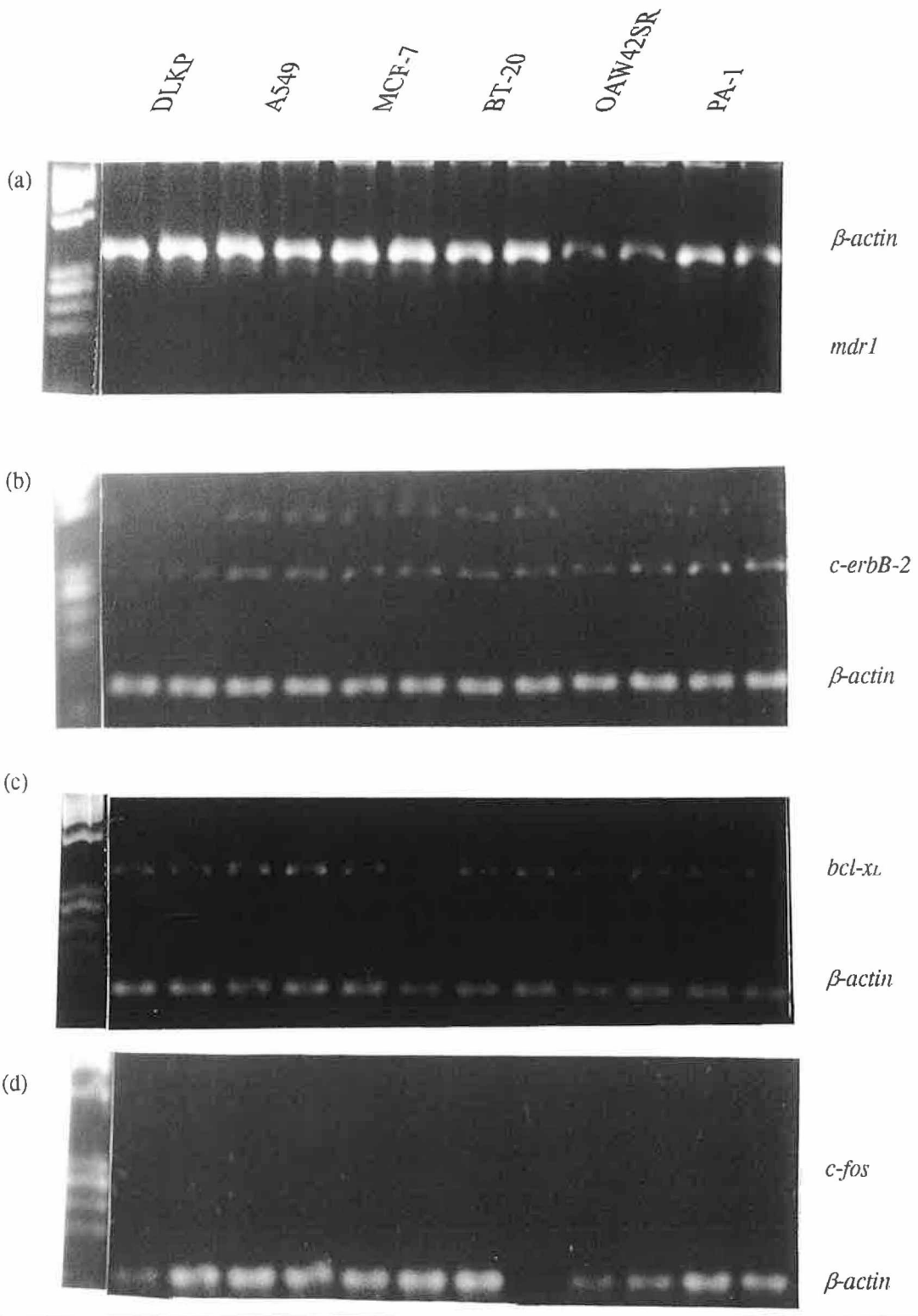
Table 3.12.1.1 Tendencies of Cell Lines to Develop Drug Resistance

Cell Line	DLKP	A549	MCF-7	BT-20	OAW42SR	PA-1
Resistance Development	√	**X**	√	**X**	√	**X**

Cell lines that do (√) and do not (**X**) develop resistance.

All of the genes previously described (e.g. *bcl-2, bcl-x, bax,* c-*fos,* c-*jun,* c-*myc,* c-*erb*B-*2,* c-*Ha-ras1*) and common MDR-related genes (*mdr1, mrp, Topoisomerases, GST*π) were analysed. Varied expression was evident for some genes in different cell lines e.g. *bcl-2* expression high in MCF-7 but not in BT-20 or the lung cell lines analysed. No consistent trend, at the mRNA level as detected by RT-PCR, was observed between the group of cell lines that do and those that do not develop resistance readily. RT-PCR analysis of *mdr1,* c-*erbB-2,* *bcl-x* and c-*fos* in these cell lines is shown in Figure 3.12.1.1

Figure 3.12.1.1 RT-PCR Analysis of Sensitive Cell Lines



RT-PCR analysis of paired lung (DLKP and A549), breast (MCF-7 and BT-20) and ovarian (OAW42SR and PA1) sensitive cell lines. Analysis was carried out for (a) *mdr1* for 25 cycles; (b) *c-erbB-2* for 24 cycles; (c) *bcl-x_L* for 25 cycles and (d) c-fos for 30 cycles of PCR amplification.

3.13 Clinical Studies of mRNA levels in Fresh and Archival Tissue

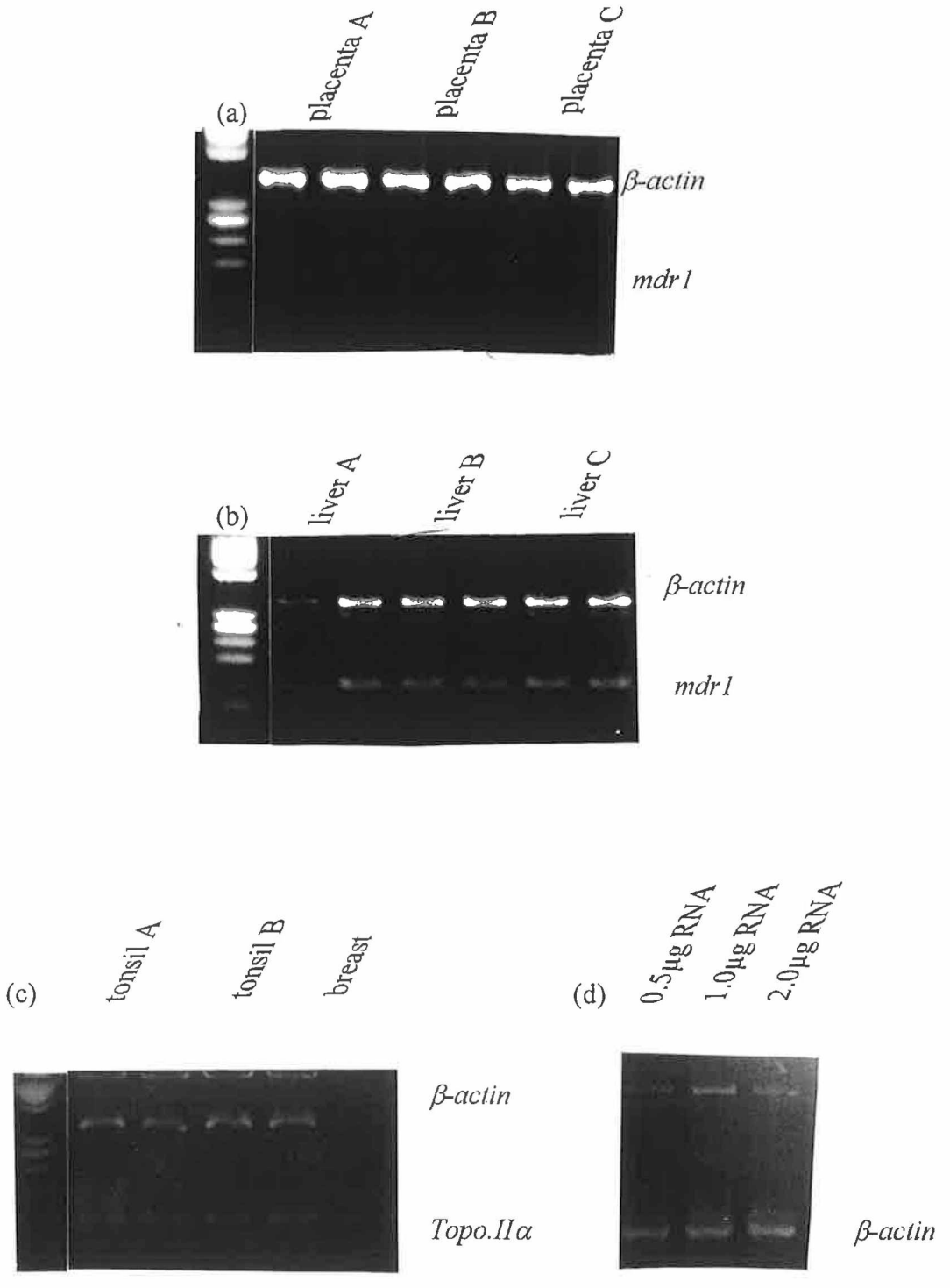
3.13.1 RT-PCR Analysis of mRNA Extracted from Archival Sections

Total RNA was extracted from formalin-fixed paraffin-embedded sections and mRNA levels analysed by RT-PCR. Successful extraction and amplification of mRNA from foetal liver, tonsil and placental tissue was achieved (Figure 3.13.1.1(a) to (c)) using a previously described protocol (O'Driscoll *et al.*, 1996).

When this protocol was applied to a broad range of 15 paired tumours from breast cancer patients (identified as pre- and post-chemotherapy paired samples) success was limited (results not shown). This protocol was found to be insufficient to digest many of these tumours. Increased de-waxing steps and longer digestion times (up to 72 hours) with increased enzyme concentrations did not significantly improve the success rate. RT-PCR analysis on 18 of these tumour sections revealed a very faint *β-actin* band in two sections and a faint *mdr1* band in another. No bands were seen for any other tissue sections (results not shown). RNA extracted from a section of normal breast was analysed for the expression of *Topoisomerase IIα* and *β-actin* and very faint bands, at the expected sizes, were evident (Figure 3.13.1.1(c)).

To address these problems tissue sections were digested in different digestion buffers. The buffer described in Section 2.10.2 was found to digest all tissues analysed to date. However, the RNA isolated from these tissues was not of sufficiently high quality to be reverse transcribed and used for PCR amplification by the method described previously (O'Driscoll *et al.*, 1996). To overcome this problem, steps were taken to optimise the reverse transcription step taking into account the degraded nature of the mRNA in these studies. As oligo-dT and random primers were not sufficient to act as primers to reverse transcribe degraded mRNA a mixture of the reverse primers used in the PCR reaction was prepared and used to prime the degraded RNA during reverse transcription. This cDNA was subsequently analysed by PCR for the expression of *β-actin* and c-*erb*B-*2* after 40 cycles of PCR. A band was evident at the expected size for *β-actin* although background bands were also present (Figure 3.13.1.1(d)), no band was seen at the expected site for c-*erb*B-*2* amplification. Further optimisation of this protocol is required.

Figure 3.13.1.1 RT-PCR Analysis of RNA Extracted from Archival Sections



RT-PCR analysis (30 PCR cycles) of RNA extracted from (a) 3 placenta samples; (b) 3 foetal liver sections; (c) 2 tonsil sections and 1 normal breast section and (d) RT-PCR analysis (40 PCR cycles) of degraded RNA using specific primers to reverse transcribe the RNA; amount of RNA used in the RT step was either 0.5, 1.0 or 2.0μg.

3.13.2 RT-PCR Analysis of mRNA Extracted from Fresh Breast Tissue

Analysis of mRNA extracted from fresh tumour samples was carried out using a similar procedure to that used for extraction from formalin-fixed paraffin-embedded sections (Section 2.10.3). The aim of this study was to compare mRNA extraction from tumour samples pre- and post-fixation and embedding procedures. In the space of 6 months 14 tumours were received from breast cancer patients attending St. Vincent's Hospital, Dublin 4. Total RNA was extracted from these tumours and analysed by RT-PCR for the expression of MDR-related genes and oncogene products. Successful amplification of *β-actin* was achieved for all tumours, however, amplification of a range of specific gene products was achieved for only four of these tumours. The gene expression detected in these four tumours is summarised in Table 3.13.2.1.

Table 3.13.2.1 Gene Expression in Fresh Tumour

	Patient Number			
	434992	434992a	435329	364788
mdr1	+	+	-	+
mrp	+/ -	+/ -	+/ -	+/ -
Topoisomerase IIα	+ +	+/ -	+/ -	+/ -
bcl-2	+ +	+ +	+	+ +
bcl-x_L	+	+	+	+
bax	+ +	+	+	+ +
c-*fos*	+	+	+	+
c-*myc*	+ +	+ +	+	+
c-*erb*B-2	+	+/ -	-	-

RNA isolated from axillary node (434992) and breast (434992a, 435329 and 364788) tissue was analysed for the expression of a range of genes by RT-PCR. Expression is indicated by (+), weak expression by (+/ -) and absence of expression by (-). All analysis was carried out following 30 cycles of PCR.

Patient history or prognosis is unknown. Time constraints prevented extraction or analysis of RNA from formalin-fixed and paraffin-embedded sections of the same tissue.

3.13.3 *mdr1* Analysis by Non-Isotopic *In Situ* Hybridisation of Archival Tissue

A protocol was developed throughout the course of this study to analyse *mdr1* gene expression in archival formalin-fixed paraffin-embedded tissue. Foetal liver was used to optimise hybridisation conditions as this tissue is known to express high levels of *mdr1* gene (van Kalken *et al.*, 1992). A digoxigenin-labelled riboprobe was used to identify the mRNA of interest.

Specific staining of *mdr1* expression in erythroid precursors (haemopoeitic cells) in the sinusoids was detected using this procedure on foetal liver sections (Dr.S.Kennedy pers. comm.) (Figure 3.13.3.1(a)). Immunohistochemical analysis of P-glycoprotein levels in the same tissue block was carried out by Ms. Annemarie Larkin and is included for comparison (Figure 3.13.3.1(b)).

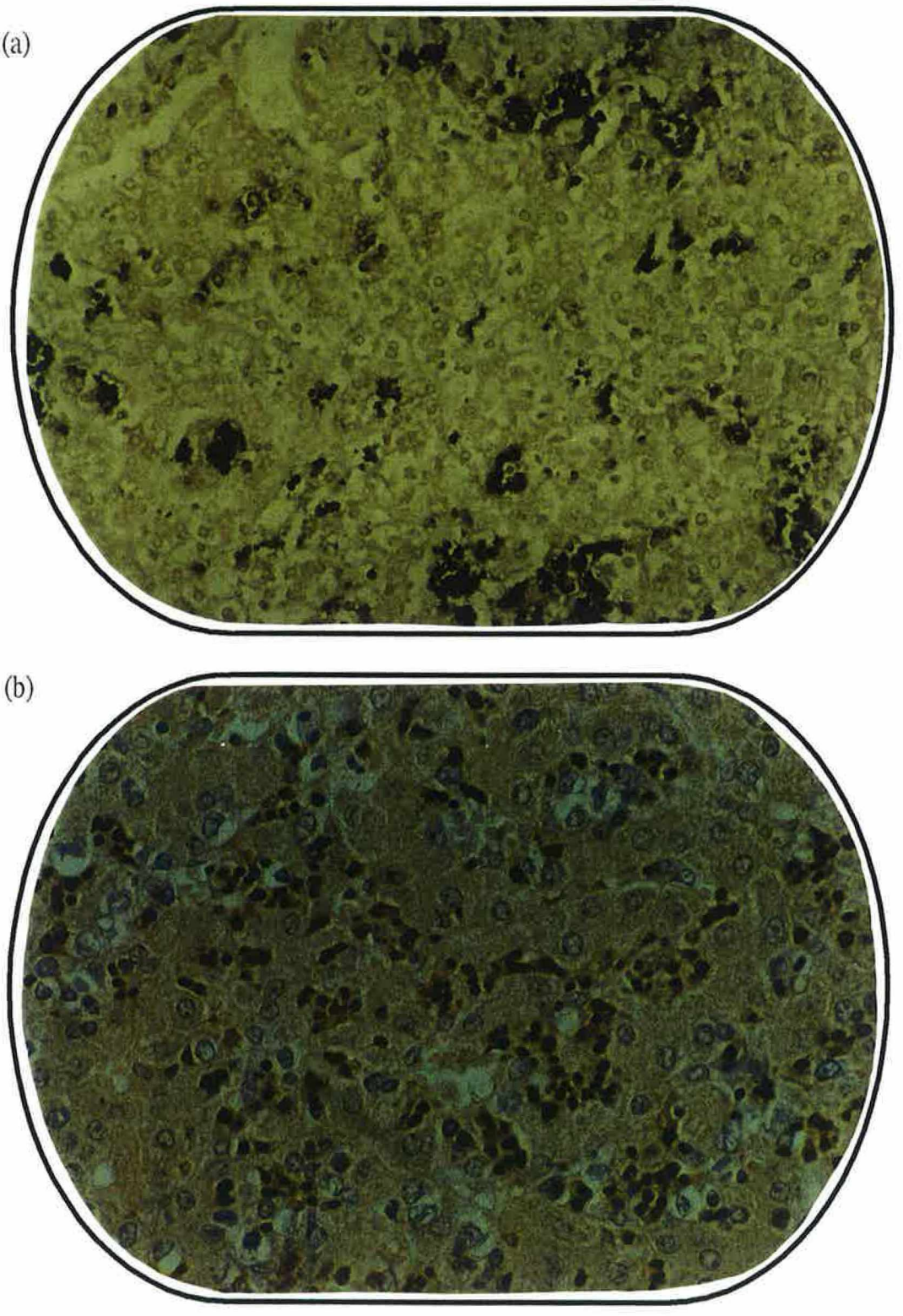
Four paired pre- and post-chemotherapy tumour blocks were chosen at random from the thirty blocks used in the archival RT-PCR studies. Successful staining for poly-A mRNA and *mdr1* were seen by *in situ* hybridisation in these studies. All patients had been treated with courses of CMF.

Table 3.13.3.1 Archival Breast Tumour Blocks Used in *In Situ* Hybridisation Studies

	Pre-chemotherapy		Post-chemotherapy	
Patient #	**Block #**	**Tissue Type**	**Block #**	**Tissue Type**
306246	1349/89	breast	5136/90	skin
335969	17210/89	breast	18131/90	node
301897	19705/88	breast	S6683/1/94	breast
229544	13536/87	breast	5843/92	node

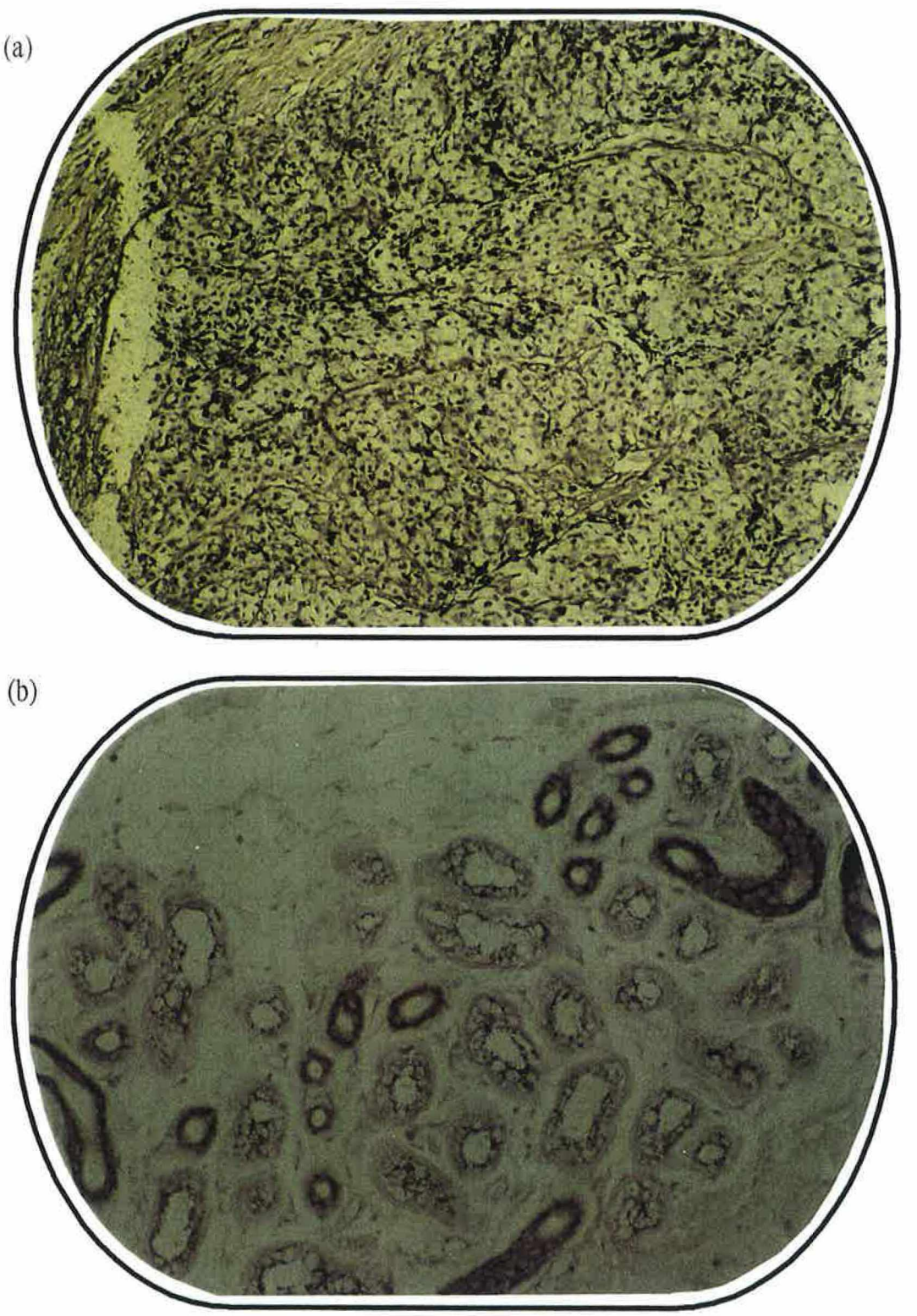
Analysis of *mdr1* mRNA expression in these sections was successful, although the quality of mRNA in some tumour blocks was not preserved throughout the entire specimen. Analysis of poly (A) staining with a poly (dT) digoxigenin labelled probe and specific *mdr1* staining is shown for block numbers 19705/88, S6683/1/94, 13536/87 and 5843/92 in Figures 3.13.3.2, .3, .4 and .5, respectively. Control hybridisations included RNase pre-treatment and hybridisation with labelled sense probe. All control hybridisations were negative (results not shown).

Figure 3.13.3.1 Expression of *mdr1* Gene and Protein Products *In Situ* on Foetal Liver Tissue



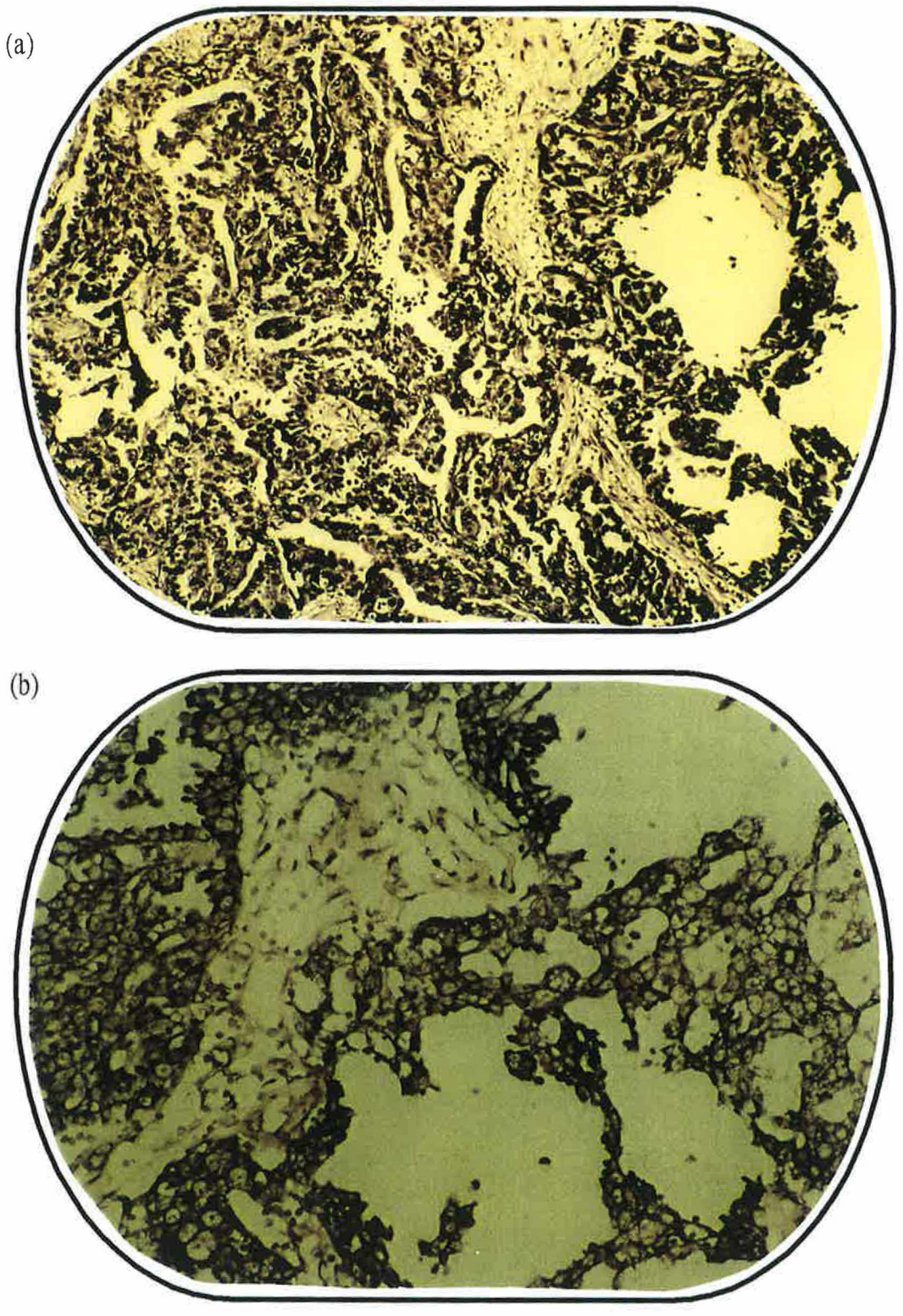
(a) hybridisation of the *mdr1* riboprobe to foetal liver tissue (20X); (b) staining of P-glycoprotein in foetal liver tissue (photograph courtesy of Ms.Annemarie Larkin).

Figure 3.13.3.2 *In Situ* hybridisation analysis of tumour block # 19705/88



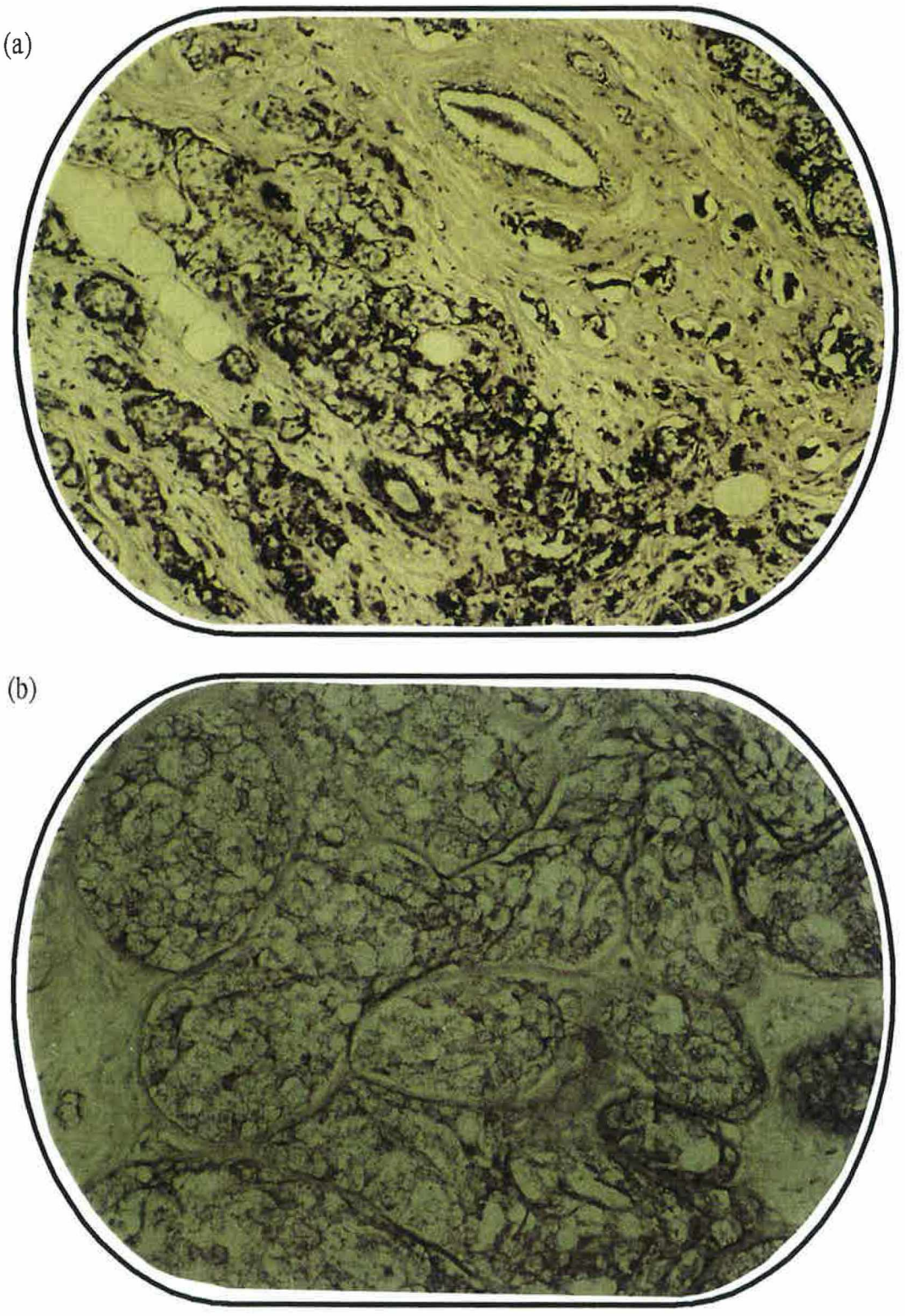
(a) hybridisation of poly (dT) probe (10X); (b) hybridisation of the *mdr1* riboprobe (20X)

Figure 3.13.3.3 *In Situ* hybridisation analysis of tumour block # S6683/1/94



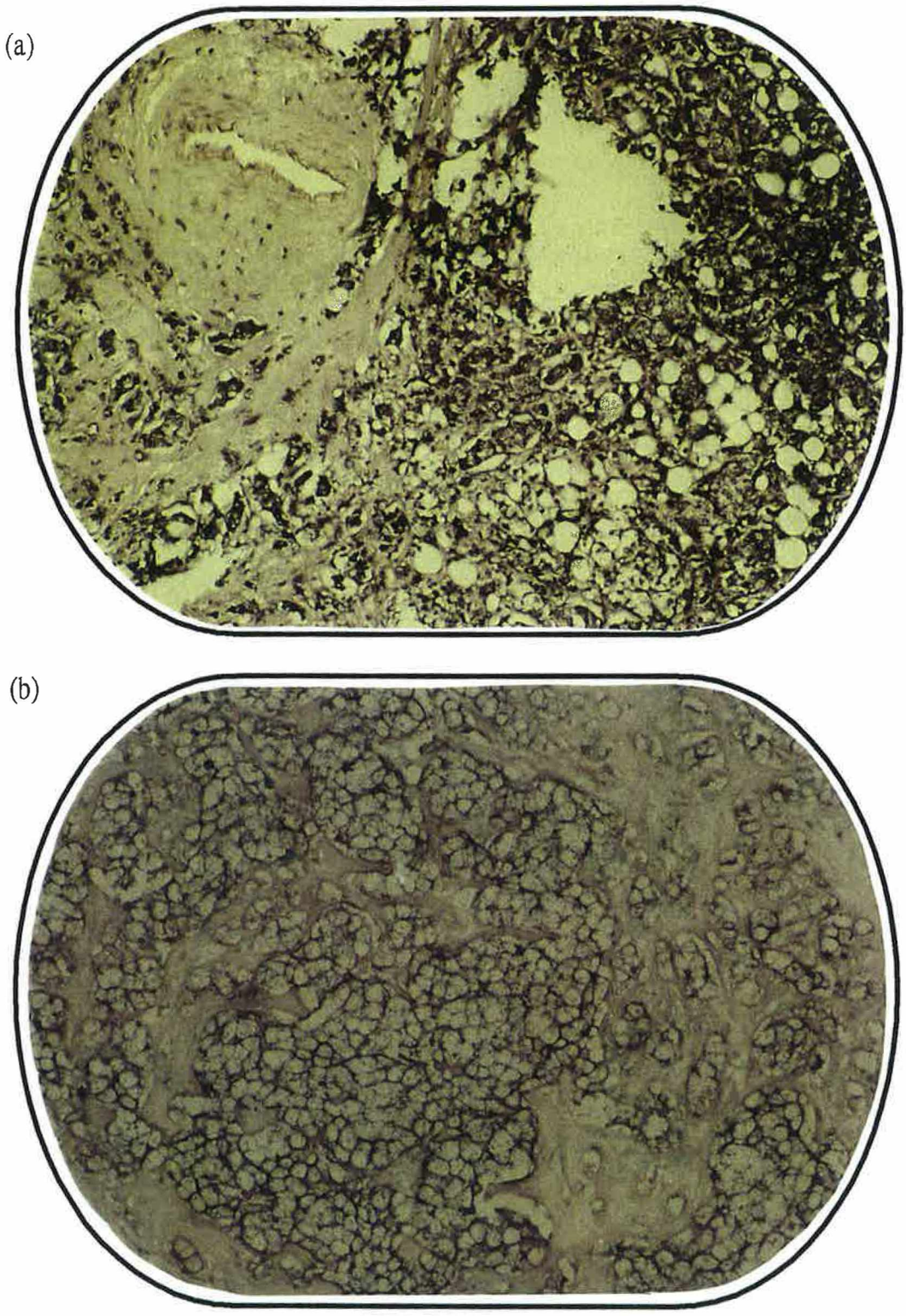
(a) hybridisation of poly (dT) probe (10X); (b) hybridisation of the *mdr1* riboprobe (20X)

Figure 3.13.3.4 *In Situ* hybridisation analysis of tumour block # 13536/87



(a) hybridisation of poly (dT) probe (10X) (b) hybridisation of the *mdr1* riboprobe (20X)

Figure 3.13.3.5 *In Situ* hybridisation analysis of tumour block # 5843/92



(a) hybridisation of poly (dT) probe (10X); (b) hybridisation of the *mdr1* riboprobe (20X)

4. Discussion

4.1 Analysis of Oncogene Expression in Multidrug Resistance

The observation that malignant cells commonly become resistant to chemotherapeutic drugs while normal cells rarely do suggests that molecular processes involved in malignancy, such as oncogene activation, may play a role in drug resistance (Sklar and Prochownik, 1991).

The focus of this project was to determine if alterations in oncogene expression could affect the MDR phenotype seen in highly resistant MDR variants of the DLKP cell line or clinically relevant resistant variants of a clonal population of DLKP. The oncogenes chosen in this study were representative of each class of known oncogenes involved in the signalling cascade from the plasma membrane to the nucleus. Expression of the genes chosen had previously been shown to result in poor prognosis in the clinic. Initial studies included analysis of c-*erb*B-2, c-Ha-*ras1*, c-*fos*, c-*jun*, c-*myc* and the *bcl-2* family of proteins.

4.1.1 c-*erb*B-2 and its role in MDR

Although some of the literature is conflicting, much evidence has been gathered which implies that c-*erb*B-2 may account for insensitivity to commonly used chemotherapeutic regimens in some human cancers, especially lung and breast and increased c-*erb*B-2 levels were seen in the tumours of ovarian carcinoma patients with recurrent or persistent disease after chemotherapy (van Dam, 1994).

Muss *et al.* (1994) showed that patients whose tumours over-expressed c-*erb*B-2 were relatively resistant to chemotherapy at conventional doses of the topoisomerase II inhibitor doxorubicin. Using higher dose doxorubicin improved survival in these patients. The authors concluded that over-expression of c-*erb*B-2 may be a useful marker in identifying patients who are most likely to benefit from high doses of adjuvant chemotherapy. However, a study by Bitran *et al.* (1996) on 25 patients with high-risk breast cancer found that those patients over-expressing c-*erb*B-2 were at risk for relapse, even when treated with high-dose chemotherapy.

Klijn *et al.* (1993) found that c-*erb*B-2 over-expression was a predictor of good response to cyclophosphamide:methotrexate:5-fluorouracil (CMF) chemotherapy in patients with metastatic disease. Other investigations, however, have shown that tumours over-expressing c-*erb*B-2 are less responsive to CMF regimens (Bacus *et al.*, 1994) whereas c-

*erb*B-2 over-expressing tumours were found to respond to high doses of cyclophosphamide:adriamycin:5-fluorouracil (CAF). Sensitivity of c-*erb*B-2 over-expressing breast carcinomas to doxorubicin was also shown by Paik (1992) where over-expression of c-*erb*B-2 was associated with resistance to several other cytotoxic agents. Bottini *et al.* (1996) described a study of breast cancer patients whose response to CMF treatment could not be correlated with c-*erb*B-2 expression.

Direct *in vivo* evidence of a role for c-*erb*B-2 in MDR came from studies by Schneider and co-workers (1994) in a sub-group of aggressive, locally advanced, inoperable carcinomas. This study showed a highly significant co-expression of P-glycoprotein and c-*erb*B-2. Volm *et al.* (1993) also demonstrated a significant relationship between the common MDR markers, P-glycoprotein, GST-π and Topoisomerase II, and c-*erb*B-2 in third or fourth passages from primary renal cell carcinomas in culture. Järvinen *et al.* (1996) also showed a significant correlation between c-*erbB-2* and Topoisomerase IIα expression in breast cancer patients.

In vitro studies on the effect of c-*erb*B-2 on MDR have also been undertaken and it has been shown by many groups that cell lines transfected with c-*erb*B-2 display varying degrees of resistance to chemotherapeutic drugs. Sabbatini *et al.* (1994) demonstrated that co-transfection of activated c-Ha-*ras* and c-*erb*B-2 into MCF-10A (human breast epithelial line, *mdr1* negative) resulted in the establishment of a *de novo mdr1*-expressing cell line with slight increase in resistance to adriamycin. Benz *et al.* (1992) showed that transfection of breast tumour cells with c-*erb*B-2 resulted in resistance to tamoxifen. Yu *et al.* (1996) demonstrated that over-expression of c-*erb*B-2 due to introduction of the human c-*erb*B-2 gene into the very low c-*erb*B-2 protein-expressing MDA-MB-435 (human breast cancer) cell line resulted in increased taxol resistance. No increase in P-glycoprotein levels was seen. c-*erb*B-2 over-expression and *in vitro* resistance to 5-fluourouracil and cisplatin (non-MDR drugs) has been reported by Dickson *et al.* (1990). Zhang and Hung (1996) described a study of non-small cell lung cancer cell lines that over-expressed the c-*erb*B-2 protein with concomitant increases in resistance to cisplatin, doxorubicin and etoposide. In this thesis resistant variants of the lung cell line, DLKP, developed by continuous exposure to carboplatin, adriamycin and VP-16 also displayed elevated levels of c-*erb*B-2 mRNA. Not all cell lines over-expressing c-*erb*B-2 resulted in resistant variants, however, which reflects work by Slamon (1992) where MCF-7 cells transfected with the c-*erb*B-2 cDNA were not found to have increased chemo-resistance.

4.1.2 c-Ha-*ras* and its role in MDR

c-Ha-*ras* is involved in signal transduction pathways governing many regulatory pathways in the cell and is expressed in 30% of all human cancers and is a marker of poor prognosis in many cancers. The role of c-Ha-*ras* and *in vivo* MDR has yet to be addressed, however, much *in vitro* work has been carried out and results found implicate a role for c-Ha-*ras* in the establishment of the MDR phenotype.

Burt *et al.* (1988) showed that transfection of rat liver epithelial cells with v-H-*ras* or v-*raf*, independently of chemical exposure, resulted in the establishment of an MDR phenotype. Increased expression of *mdr1* and *GST*π was observed in the resistant cell lines with concomitant increase in resistance to adriamycin, vinblastine and 2-acetylaminofluorene. As mentioned previously (Sabbatini *et al.*, 1994), co-transfection of activated c-Ha-*ras* and c-*erb*B-2 into MCF-10A cells also resulted in the establishment of a *de novo mdr1*-expressing cell line with slight increase in resistance to adriamycin. Direct evidence for the regulation of *mdr1* by the *ras* family of proteins came from studies by Chin *et al.* (1992) and Cornwell and Smith (1993) which showed that the human *mdr1* gene promoter is activated by Ras and p53, and c-Raf kinase, respectively. In this thesis elevated levels of c-Ha-*ras* mRNA were detected in the MDR cell lines DLKP-A (selected with adriamycin) and DLKP-VP3 but not DLKP-VP8 (selected with VP-16). Elevated levels of c-Ha-*ras* were seen in a 10-fold resistant variant of the human prostate PC3 cell line (Sinha *et al.*, 1995) which is resistant to a wide variety of anticancer agents including VP-16 and cisplatin.

A role for c-Ha-*ras* in cisplatin resistance has been demonstrated by many groups on a variety of cell lines. A *ras*-transformed variant of the non-malignant mammary epithelial cell line, HBL100, displayed a selective 2.7 fold increased resistance to cisplatin but not to other DNA interacting agents such as adriamycin (Levy *et al.*, 1994). Increased resistance to cisplatin was found to be specific for H-*ras* transformants and did not include K-*ras* transfected cell lines (Shinohara *et al.*, 1994); induction of cisplatin resistance was mainly due to a reduction of cisplatin accumulation and an impairment of Na^+,K^+-ATPase activity in the membrane fraction. NIH3T3 fibroblasts transfected with several types of cellular and viral *ras* oncogenes were shown to have increased resistance to the non-MDR drug, cisplatin (Sklar, 1988). The carboplatin resistant line used in this thesis, DLKP-C14, also expressed elevated levels of c-Ha-*ras* mRNA. Isonishi and co-workers (1991) also showed that over-expression of a mutant c-Ha-*ras* gene in murine NIH3T3 cells

conferred increased resistance (8.2 ± 1.3 fold) to cisplatin without altering cellular glutathione content or the activity of glutathione-S-transferase in the transfected cells. Studies by Niimi *et al.* (1991) disagreed with these results when they showed that NIH3T3 cells transfected with c-Ha-*ras* had increased resistance to alkylating agents (e.g. melphalan) but not to adriamycin, VP-16 or cisplatin.

4.1.3 Transcription Factors and their role in MDR

Transcription factors are known to be early responders to cellular stress from chemotherapeutic drugs. Evidence in the literature suggests that the three oncogenic transcription factors, c-*fos*, c-*jun* and c-*myc*, affect chemoresistance in cancer cell lines.
A study by Zhao *et al.* (1995) demonstrated that c-*jun* is induced by exposure to cisplatin in a sensitive melanoma cell line but less so in cisplatin-resistant variants of the human melanoma line, RPMI8322, and may be involved in a cisplatin-induced apoptosis pathway. A preliminary *in vivo* study described the induction of c-*jun* mRNA in head-neck tumour biopsies from patients responding to cisplatin-based chemotherapy, but not in samples from resistant tumours (Los *et al.*, 1995). Ribozyme-mediated reduction of c-*fos* levels has previously been shown to affect drug sensitivity in cisplatin resistant cell lines (Funato *et al.*, 1992; Scanlon *et al.*, 1991) and other multidrug resistant variants (Scanlon *et al.*, 1994). In these studies it was observed that c-*fos* mRNA was only over-expressed in the adriamycin-selected DLKP-A variant and not the carboplatin or VP-16 selected cell lines. A correlation has been shown between expression of P-glycoprotein and c-*fos* and c-*jun* products in human lung carcinomas (Volm *et al.*, 1993). In renal cell carcinomas a significant relationship also existed between P-glycoprotein, GST-π and Topoisomerase II and c-*fos* (Volm *et al.*, 1993). The AP-1 transcription factor (complex of Fos/Jun dimers - see Section 1.4.3) was previously shown to be crucial for full promoter activity of the Chinese hamster P-glycoprotein (Teeter *et al.*, 1991).

Cell lines derived from a patient presenting with adenocarcinoma of the colon and treated with cisplatin and 5-fluorouracil showed a two fold amplification in DNA copy number of the c-*myc* gene and a fifteen fold increase in c-*fos* amplification (and also c-Ha-*ras* (4 fold)) (Kashani-Sabet *et al.*, 1990). More direct evidence of a role for Myc family members in clinical drug resistance came from a study by Bordow and co-workers (1994) who showed that expression of the MRP gene correlated with amplification and over-

expression of the N-*myc* oncogene in childhood neuroblastoma; no correlation was found with P-glycoprotein expression.

In vitro studies carried out by Niimi *et al.* (1991) demonstrated that NIH3T3 cells transfected with c-*myc* had increased resistance to cisplatin, 4-hydroxycyclophosphamide, adriamycin, melphalan and CPT-11 and were the first to report a role for c-*myc* in drug resistance. Mai (1994) found that c-*myc* over-expression precedes amplification of the dihydrofolate reductase gene in CHO-9 cells which has been shown to affect resistance to methotrexate. Levels of c-*myc* and c-*jun* were increased in resistant variants of the human prostate PC3 cell line and treatment with VP-16 or cisplatin induced c-*myc* mRNA in sensitive PC3 cells (Sinha *et al.*, 1995). Walker and co-workers (1996) found that the rat c-*myc* gene displayed a sustained (analysed after 2 and 7 days) 2-fold elevation in expression following single-dose of 1mg/kg cisplatin chemotherapy. Antisense approaches targeted at the c-*myc* gene reduced cell growth rate and the authors concluded that antisense therapy directed at c-*myc* in combination with cisplatin may achieve therapeutic efficacies *in vivo*. Sklar and Prochownik (1991) also demonstrated a direct link between levels of c-*myc* expression in Friend murine erythroleukaemia cells and cisplatin resistance. The increased expression of c-*myc* in the carboplatin resistant cell line, DLKP-C14, is in agreement with these findings in the literature of increased c-*myc* expression in cisplatin resistant cell lines.

4.1.4 Oncogene Expression in Resistant Variants of DLKP

As has been discussed in previous sections, evidence is gathering in the literature to suggest that expression of several oncogenes affects chemosensitivity levels in cell culture systems. In this thesis analysis of expression of c-*erb*B-2, c-Ha-*ras1*, c-*fos*, c-*myc* and the *bcl-2* family members, *bcl-2*, *bcl-x* and *bax*, was carried out on multidrug resistant variants of the human lung cancer cell line, DLKP. These variants were previously established by continuous exposure to adriamycin, (DLKP-A; Clynes *et al.*, 1992), carboplatin (DLKP-C14; I.Cleary, 1995) and VP-16 (DLKP-VP3 and DLKP-VP8; M.Heenan, 1994). Analysis of oncogene expression in a variety of different multidrug resistant variants selected by exposure to different drugs has not previously been carried out on such a broad range of oncogenes. These cell lines display differing cross-resistance profiles, as can be seen in Table 4.1.4.1. As these resistant variants are composed of heterogenous populations, analysis of gene expression can be masked by different genetic

clonal populations co-existing in the parental line. To address this problem clonal populations of the adriamycin selected line, DLKP-A, were also included in this study. The least resistant clone, DLKP-A2B, and the most resistant clone, DLKP-A5F, (Heenan, 1997) were analysed for the expression of oncogene levels. Fold resistance of the clonal populations is also given in Table 4.1.4.1.

Table 4.1.4.1 Fold resistance of DLKP Variants

	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKP-A	254 ± 119	1.46 ± 0.42	1504 ± 707	61 ± 8	1.75 ± 0.74
DLKP-A2B	37 ± 16	0.57 ± 0.07	228 ± 71	18 ± 8	1.28 ± 0.77
DLKP-A5F	331 ± 69	1.39 ± 0.03	1275 ± 625	51 ± 8	0.98 ± 0.47
DLKP-VP3	90 ± 13	0.32 ± 0.17	1089 ± 161	58 ± 2	0.47 ± 0.38
DLKP-VP8	272 ± 38	0.22 ± 0.06	1737 ± 348	101 ± 7	0.90 ± 0.43
DLKP-C14	1.67	12.2	1.12	1.9	0.77

This analysis was carried out by Dr.M.Heenan and Dr.I.Cleary.

Signalling cascades of cellular messages begins at the plasma membrane. The first players in this cascade are proteins such as growth factor receptors. In this work multidrug resistant cell lines were shown to over-express the c-*erb*B-2 gene, a plasma membrane protein that is highly homologous to the epidermal growth factor receptor, relative to the sensitive parental DLKP line. c-*erbB-2* expression levels (by RT-PCR) were increased dramatically in the resistant lines selected by exposure to adriamycin, carboplatin and VP-16 studied. Levels of c-*erb*B-2 mRNA in the sensitive cell line, DLKP, were barely detectable by densitometry (Figure 3.3.2(b)). This result is in direct agreement with a previous study which showed increased resistance to adriamycin, cisplatin and VP-16 in a study of human non-small cell lung cancer cell lines (Zhang and Hung, 1996). Western blot analysis for the c-*erb*B-2 p185 protein also showed elevated levels in the DLKP-A and VP-16 selected, DLKP-VP3 and DLKP-VP8, resistant variants of DLKP.

The study of these highly resistant cell lines suffers from two major disadvantages. The first is the high levels of resistance seen in these cells (DLKP-A is 200 fold more resistant than DLKP to adriamycin) which may distort the effect of a single gene, or a group of genes, in these systems. The second is the heterogenous nature of these cell lines making it difficult to study the true effect of gene expression in individually selected cells. To overcome the latter problem clonal populations of the adriamycin-selected cell line,

DLKP-A, were analysed for oncogene expression by RT-PCR. From the resistant line, DLKP-A, clonal populations of varied resistance levels were isolated (Heenan *et al.*, 1997). The least resistant clone, DLKP-A2B, and the most resistant clone, DLKP-A5F, were used in this study. Analysis was carried out on two RNA extractions for each gene analysed. Analysis revealed higher c-*erb*B-2 mRNA expression in the more resistant DLKP-A5F cell line relative to DLKP-A2B. The over-expression of c-*erb*B-2, at the mRNA level, in all resistant variants analysed implies a general role for c-*erb*B-2 in response to a variety of mechanistically different chemotherapeutic drugs and supports some clinical studies that suggest that c-*erb*B-2 expressing tumours would benefit from more aggressive treatment. A few reports have suggested that c-*erb*B-2 over-expressing tumours maintain sensitivity to adriamycin (Bacus *et al.*, 1994; Paik, 1992). In this thesis, however, MDR lines developed by exposure to adriamycin also over-expressed c-*erb*B-2. Highest mRNA levels were seen in the DLKP-C14 variant, however, which suggest that a c-*erb*B-2 over-expressing tumour should not be treated with carboplatin containing regimens.

Previous results have shown that the signal transducer protein, c-Ha-*ras*, is important in the resistance of cell lines to cisplatin and VP-16 (Sinha *et al.*, 1995). However, Niimi *et al.* (1991) demonstrated conflicting results when they showed that NIH3T3 cells transfected with c-Ha-*ras* had increased resistance to alkylating agents but not to adriamycin, VP-16 or cisplatin. In this thesis, c-Ha-*ras* levels of expression were elevated in the carboplatin-selected line, DLKP-C14, the adriamycin-selected line, DLKP-A and the VP-16 selected variant, DLKP-VP3, but not in DLKP-VP8 (Figure 3.3.2(d)). Increased expression of the poor prognostic marker c-Ha-*ras1* was also seen in the DLKP-A5F cells relative to DLKP-A2B cells (Figure 3.3.1.3(b)). c-Ha-*ras* has previously been shown to regulate the transcription of c-*fos* which has been shown to control transcription of *mdr1* (responsible for resistance in DLKP-A and the VP-16 selected lines) and metallothionein (responsible for resistance in DLKP-C14), therefore, elevations in the levels of c-Ha-*ras* is not unexpected in the DLKP resistant variants. Why decreased expression is seen in the DLKP-VP8 line relative to DLKP-VP3 is unknown as DLKP-VP8 is more resistant than the DLKP-VP3 variant. However, P-glycoprotein levels (Appendix E) are not always over-expressed in the DLKP-VP8 cell line relative to DLKP-VP3 (M.Heenan, 1994).

Transcription factors are the ultimate target of cellular signalling and are known to be early responders to cellular stress from e.g. chemotherapeutic drugs. Expression of three transcription factors, c-*fos*, c-*jun* and c-*myc*, which are known oncogenes, was analysed in drug resistant variants of the DLKP cell line.

c-*myc* expression was decreased in the adriamycin and VP-16 selected cell lines, although higher levels were observed in the carboplatin-variant (Figure 3.3.2(a)). c-*myc* has been shown to induce apoptosis in the presence of growth inhibiting factors (Mihich and Schimke, 1994), therefore, reduced c-*myc* in cells may give cells a survival advantage. c-*myc* levels are reduced in DLKP-A, DLKP-VP3 and DLKP-VP8 cell lines. The increased resistance of c-*myc* in the carboplatin selected line, DLKP-C14, is in agreement with previous studies by other laboratories (Niimi *et al.*, 1991; Sklar and Prochownik, 1991; Walker *et al.*, 1996).

c-*fos* expression was not detected (or detected at very low levels) in the sensitive cell line, DLKP, nor was it detected in the carboplatin or VP-16 selected lines, increased expression was, however, seen in the adriamycin-selected variant (Figure 3.3.2(c)). Expression of c-*jun* was detected, by RT-PCR, in the cell lines analysed but as it is an intron-less gene and its mRNA produces the same sized fragment as its amplified DNA sequence, it was not included in any further studies, as RT-PCR could not be relied on for definitive analysis. Pre-treatment of the RNA with a DNase enzyme was performed (results not shown) to reduce contaminating DNA but could not be relied on to destroy all residual DNA.

DLKP-A, DLKP-VP3 and DLKP-VP8 all over-express the *mdr1* gene (see Appendix E) and it is surprising to find that the VP-16 selected cell lines do not over-express c-*fos*. The AP-1 complex can substitute its c-Fos counterpart for a c-Jun homo-dimer or can be substituted by another member of the c-Fos family of genes such as Fra1 (Kim and Beck, 1994). Therefore AP-1 activity may be increased in these latter resistant variants without presenting an increase in c-Fos expression. The protein found to be responsible for resistance in the DLKP-C14 cell line is the metallothionein protein (I.Cleary, 1995), which also has an AP-1 site in its promoter (Scanlon *et al.*, 1991).

Analysis of c-*fos* levels in the clonal populations revealed high levels of expression in DLKP-A and DLKP-A5F, with very low levels of expression seen in the DLKP-A2B clone (Figure 3.3.1.1b). This trend was also seen for *mdr1* expression in these cell lines (see Appendix E).

Resistant variants of an ovarian cell line, OAW42SR, were used as a model of well characterised low-level resistant cell lines in this study. The adriamycin-selected resistance

variants OAW42A and OAW42A1 are 14 and 26 fold more resistant to adriamycin respectively, than the sensitive OAW42S clone, respectively (Appendix D). Differential expression of common MDR-related genes such as *mdr1* and *Topoisomerase IIα* and *Topoisomerase I* could be detected in these OAW42SR variants, as shown in Figure 3.5.1.1. In these resistant cell lines, however, a dramatic reduction was seen in the expression of c-*fos* mRNA in the resistant OAW42A and OAW42A1 cell lines.

4.1.5 Bcl-2 family and their role in MDR

Evidence is accumulating over the past ten years to suggest that expression of *bcl-2*, the apoptosis suppressing gene, in malignancies results in a poor prognosis, with chemotherapy having minimal impact on the disease (Horning and Rosenberg, 1984). The resulting "drug-resistant" tumours would, therefore, be expected to express elevated levels of survival genes as these cells have survived an attack from an apoptosis-inducing agent and subsequently developed resistance to that agent. The balance of expression of members of the Bcl-2 family determines if a cell should live or die. Increased expression of the apoptosis-suppressing genes would favour survival while pre-dominance of apoptosis-promoting genes would result in cell death (Section 1.7).

Reports in the literature are often conflicting in attempts to determine the role Bcl-2 plays *in vivo* as a prognostic marker for response to chemotherapy. Maung *et al.* (1994) demonstrated that increased expression of Bcl-2 in blasts from patients with acute leukaemia is associated with a poor initial response to chemotherapy and that expression is higher in patients on relapse than on presentation. Expression of the Bcl-2 protein and resistance to doxorubicin was shown in a study of squamous cell carcinomas (Volm and Mattern, 1995) where a correlation was found in these tumours between expression of P-glycoprotein and glutathione S-transferase-π, the common MDR markers. These results indicate that Bcl-2 may be a good parameter by which to determine the likelihood of developing resistance. Ironically, many reports have suggested that Bcl-2 expression is an indicator of good prognosis in response to chemotherapy (Gasparini *et al.*, 1995; Hurlimann *et al.*, 1995) whereas a study on Bcl-2 expression in 231 colorectal adenocarcinomas treated with 5-fluorouracil showed no influence of gene expression on treatment outcome (Schneider *et al.*, 1997). Stoetzer *et al.* (1996) demonstrated a significant correlation between the ratio of Bcl-2 to Bax expression, clinical response and

failure to achieve complete remission in patients with a Bcl-2:Bax ratio >1.

The importance of expression of Bcl-2 family members in tumour types which readily respond to chemotherapy or frequently develop resistance has been addressed *in vitro*. Metastatic testicular cancers are curable (Frei, 1985) whereas most other solid tumours are not e.g. bladder cancers. Three testicular cancer cell lines were analysed and they were all found to contain high expression of Bax and did not express the Bcl-2 protein; the three bladder cell lines analysed expressed Bcl-2 but did not express the Bax protein thereby adding further support to the relevance of Bax:Bcl-2 ratio and response to chemotherapy (Chresta *et al.*, 1996) in human tumours. This finding adds further support to the work of Stoetzer *et al.* (1996) who demonstrated the importance of Bax:Bcl-2 ratio and response to chemotherapy *in vivo*. Small cell lung carcinoma tumours are initially responsive to chemotherapy but multidrug resistance tends to develop on relapse, however, these tumours express relatively low levels of P-glycoprotein. The expression of Bcl-2 in small cell lung carcinoma cell lines was studied by Ikegaki *et al.* (1994) and found to be high in five out of six cell lines and the authors suggest that the high level of expression of Bcl-2 in these cells may account, in part, for the development of multidrug resistance in these tumours. A significant number of ovarian tumours (tumours which also frequently develop resistance) have been shown to express Bcl-2 and the Bcl-2 protein was found to be over-expressed in an ovarian cell line, A2780, developed by continuous exposure to cisplatin (Eliopoulos *et al.*, 1995). The relevance of Bcl-2 in ovarian carcinoma drug resistance *in vivo* is still unknown, however. Human malignant gliomas are largely resistant to current strategies of surgery, chemotherapy, radiotherapy and immunotherapy, Weller *et al.* (1995) described stable *bcl-2* transfected human glioma cell lines with increased resistance to chemotherapeutic drugs such as cisplatin.

High levels of Bcl-2 protein in pre-B human leukaemia 697 cells delayed taxol-induced DNA fragmentation and apoptosis (Tang *et al.*, 1994). Taxol has been shown to induce Bcl-2 phosphorylation (via Raf-1 activation (Blagosklonny *et al.*, 1996) or independently of Raf-1 activation (Ibrado *et al.*, 1997)) in prostate cancer cell lines (Haldar *et al.*, 1996), which results in the inhibition of the hypo-phosphorylated protein binding to Bax. The authors conclude that taxol may be the treatment of choice for Bcl-2 positive tumours. Tu *et al.* (1996) demonstrated that treatment of myeloma cell lines with doxorubicin, etoposide and hydrogen peroxide consistently induced a concentration and time-dependent

up-regulation of Bcl-2 in all cell lines tested. Levels of Bcl-x were not affected. Results found by Lasorella *et al.* (1995) indicate that the over-expression of Bcl-2 detected after retinoic acid-induced differentiation of neuroblastoma cells represents a likely mechanism that contributes to deregulation of the apoptotic response to anticancer agents and drug resistance. (It is possible that tumour cells expressing a more differentiated phenotype resist apoptosis or that therapy itself induces differentiation.) The results found indicate a strong association between differentiation induced by retinoic acid, a diminished apoptotic response to cytotoxic agents, and increased *bcl-2* expression in neuroblastoma cells. Walton *et al.* (1993) showed that a murine IL-3 dependent cell line, FL5.12, transfected with *bcl-2* was two-fold more resistant to both nitrogen mustard and camptothecin, two mechanistically different DNA-damaging drugs. This indicates a *bcl-2*-dependent apoptotic pathway involved in determining the extent of drug-induced death and a novel mechanism of drug-resistance.

Dole *et al.* (1994) transfected *bcl-2* plasmids into a Bcl-2 negative human neuroblastoma cell line (Shep-1) and found that clones expressing high levels of Bcl-2 were resistant to cisplatin and etoposide induced cytotoxicity in a dose dependent manner, supporting the hypothesis that Bcl-2 enhances the malignant phenotype of neuroblastoma by promoting tumour resistance to chemotherapeutic agents. The effects of Bcl-2 expression appeared to be especially important when the cells are exposed to chemotherapeutic agents like cisplatin for short periods of time (as would be the case during therapy). After pulsed treatment with cisplatin (10μg/ml for 4 hours), Bcl-2 expressing cell lines show an enhanced survival and a sustained proliferative advantage as compared to cells which do not express the protein. These findings support the notion that Bcl-2 expression may provide a mechanism for drug resistance *in vivo*.

To examine the relevance of Bcl-2 to drug resistance, Kitada *et al.* (1994) used anti-sense techniques to reduce the levels of steady state Bcl-2 protein levels in t(14;18) containing human lymphoma cell lines. Treatment of the t(14;18)-containing lymphoma line, SU-DHL-4, with 18-mer synthetic *bcl-2*-antisense oligonucleotides produced sequence-specific reductions in the relative levels of *bcl-2* mRNA within one day; a corresponding reduction in protein levels took longer (~ 3 days) probably due to the long half-life of the Bcl-2 protein (~ 10-12 hours). This antisense-mediated reduction in Bcl-2 protein levels in these cells did not accelerate the rate of cell death but rather it rendered cells more prone to apoptosis caused by an additional stress i.e. growth factor loss or treatment with chemotherapeutic drugs. In conclusion, these investigators found that antisense approaches

that resulted in average reductions of 30-40% in the relative levels of Bcl-2 protein enhanced the sensitivity of t(14;18)-expressing lymphoma cell lines to conventional chemotherapeutic drugs such as methotrexate and arabinoside C. Thus, a putative new chemotherapeutic strategy could include the induction of DNA damage with conventional chemotherapeutic drugs, while simultaneously modulating regulators of the apoptotic pathway acting downstream of the conventional cytotoxic drugs, thereby enhancing the relative sensitivity of cancer cells to drug-induced damage.

Previous reports indicate that *bcl-2* is regulated by oestrogen. To study this regulation *in vitro*, MCF-7 (oestrogen receptor positive cell line) cells were used (Teixeira *et al.*, 1995). MCF-7 cells express the *bcl-2* mRNA when grown in media containing oestrogen and mRNA levels are down-regulated when oestrogen is removed from the media, coincidental with this finding was that oestrogen depletion doubled the sensitivity of the MCF-7 cells to the cytotoxic effects of adriamycin. No change in Bax levels were seen. MCF-7 cells transfected with *bcl-2* expression plasmid displayed a marked increase in resistance to adriamycin, in the absence of oestrogen. In the presence of oestrogen, MCF-7 cells expressing *bcl-2* antisense transcripts were partially sensitised to the effects of adriamycin (Teixeira *et al.*, 1995). This result supports the theory that some anti-oestrogens increase the sensitivity of breast cancer cells to adriamycin by decreasing cellular levels of Bcl-2.

Further evidence of the role *bcl-2* plays in drug resistance was shown by Miyashita and Reed (1992). Miyashita and Reed (1992) published evidence to support the theory that Bcl-2 interferes with a (but not the only) pathway for cell death that is activated by multiple drugs used for the treatment of cancer. When cultured for one day in the continuous presence of 10μM methotrexate, 1.5μM 1-β-D-arabinoside or 1μM vincristine, S49.1 and WEHI cells tranfected with the *bcl-2* cDNA underwent much less DNA fragmentation and cell death than the untransfected control cells. Treatment of the same cells with 35μM of VP-16 produced comparable levels of DNA fragmentation and percentage cell death in control and transfected cells, indicating that Bcl-2 did not confer resistance to this drug (at least at the concentration used in this study). The drugs to which Bcl-2 producing S49.1 and WEH17.2 (T-lymphoid clones) cells displayed increased resistance have diverse mechanisms of action thereby showing that bcl-2 functions in a final common pathway of Programmed Cell Death that can be activated by multiple mechanisms. However, treatment of the bcl-2 expressing cells with H_2O_2 resulted in cell death and DNA fragmentation, revealing that bcl-2 does not protect cells from all types of

drugs that induce cell death and that bcl-2 dependent and independent pathways exist for the regulation of cell survival. A study on the effect of etoposide-induced (exposed to 17.3μM for 1 hour) apoptosis revealed that *bcl-2* had little effect on the formation /disappearance of DNA strand breaks (initial DNA damage by the drug) although it did inhibit apoptosis (secondary DNA fragmentation) induced by the cytotoxic agent (Kamesaki *et al.*,1993), supporting the theory that *bcl-2* must work downstream of drug-mediated damage.

Fisher *et al.* (1993) surmised that the survival of DNA damaged cells, and the delay in the onset of apoptosis, promoted by Bcl-2 expression may lead to increased mutation of the genome and the later emergence of cells with greater oncogenic potential and/or drug resistance i.e. may allow for the alteration seen in "typical" drug resistant markers. This may be the reason for the significant correlation of P-glycoprotein levels with Bcl-2 expression in studies by Volm and Mattern (1995).

The data collected on the role of the Bcl-2 family and chemoresistance has been used to a favourable advantage by enforcing the expression of Bcl-2 in bone marrow cells, thereby circumventing myelosuppression (bone marrow cell death) by increasing the resistance of bone marrow cells to apoptosis caused by Topoisomerase inhibitors (Kondo *et al.*, 1994).

4.1.5.1 Bax and role in MDR

Studies have been described to suggest that the ratio of Bcl-2 to Bax (Bax acts as a promoter of cell death (Section 1.7)) is important in chemoresistance (Chresta, 1996; Stoetzer,1996). Those tumours that express high levels of Bcl-2:Bax are most likely to fail in response to chemotherapy (Stoetzer, 1996).

It has been shown that low Bax expression might contribute to the pathogenesis of breast cancer (Bargou *et al.*, 1995) and results by Krajewski and co-workers (1995) indicated that marked reductions in Bax expression occurred in a third of advanced breast cancers which were expected to respond poorly to chemotherapy (Krajewski *et al.*, 1995). Krajewsji *et al.* (1995) hypothesised that Bax may play an important role in the apoptotic elimination of tumour cells following exposure to DNA damaging drugs and radiation. To test this hypothesis, a group of 121 women with metastatic adenocarcinoma of the breast were studied for the expression of the Bax protein, by immunohistochemical methods. The material studied was from archival primary tumours of patients who had enrolled for a

two-arm study designed to compare efficacy and toxicity of combination chemotherapy delivered in either four weekly divided doses or once a month (total drug dose and duration being equal) - the response rate was higher in those who received therapy once a month. Bax status was found to correlate with the response rate in the weekly dose group but not the monthly dose group (also found for Bcl-2). Of the 119 primary breast tumour samples immunostained, 34% had reduced Bax expression in greater than 90% of the cells comprising the invasive component. Bax negative tumours tended to achieve less frequently a partial response or complete response than those with Bax positive tumours (i.e. 21% vs. 43%). Bcl-2 status was not found to be of prognostic significance for response rate or overall survival. Loss of Bax staining within tumour cells infiltrating through the stroma may be of significance to metastatic disease - decreased Bax could represent a mechanism that contributes to acquisition of a metastatic phenotype (decreased tendency of these cells to undergo apoptosis when they lose attachment with extracellular matrix proteins). Elevations in the Bcl-2:Bax ratio did not prevent drug-induced apoptosis but rather shifted the dose response curve so that an increased concentration of drug was required to achieve an equivalent tumour cell kill. Women with Bax negative tumours might benefit, therefore, from more aggressive chemotherapy. Unlike Bcl-2, no correlation was found between Bax expression and oestrogen or progesterone receptor status in this study.

In vitro studies have demonstrated a definite role for Bax and sensitisation to chemotherapeutic drugs. Wagener *et al.* (1996) transfected the *bax*-α gene into the breast cancer cell lines R30C and MCF-7; the transfection did not affect viability by itself but strongly increased chemosensitivity to epirubicin. Reduced expression of *bax* mRNA was seen in cisplatin resistant variants of the IGROV-1 ovarian carcinoma cell line (Perego *et al.*, 1996). In the resistant variants of DLKP analysed in this thesis, elevated levels of *bax* mRNA was seen, however. The carboplatin-resistant variant, DLKP-C14, which showed minimum resistance to adriamycin, vincristine and VP-16 did express the highest level of *bax* mRNA. A high Bax to Bcl-2 ratio was found to be associated with hypersensitivity to etoposide-induced apoptosis in human testicular tumour cell lines (Chresta *et al.*, 1996).

4.1.5.2 Bcl-x and its role in MDR

Studies on Bcl-x levels and response to chemotherapeutic drugs in cancer have been

addressed. The short from of the gene, *bcl-x$_S$*, acts as an inducer of apoptosis whereas the long form, *bcl-x$_L$*, inhibits the apoptotic response of cells. Over-expression of *bcl-x$_S$* should, therefore, result in sensitisation of cells to chemotherapy which was shown by Sumantran *et al.* (1995). Over-expression of *bcl-x$_S$* in MCF-7 cells sensitised the cells to apoptosis induced by low concentrations of VP-16 and taxol (9- and 4-fold respectively) (Sumantran *et al.*, 1995). This result suggests that *bcl-x$_L$* or *bcl-2* may play a role in modulating apoptosis and chemoresistance in these cells.

Increased expression of Bcl-x_L would be expected to confer a survival advantage on cells and a resulting chemoresistant phenotype. Minn *et al.* (1995) showed that the expression of Bcl-x_L could confer a multidrug resistant phenotype on FL5.12 murine cells. Bcl-x_L expression was found to dramatically reduce the cytotoxicty of bleomycin, cisplatin, etoposide and vincristine; its expression did not prevent cells from undergoing cell cycle arrest in response to these drugs rather it prevented the treated cells from undergoing apoptosis. *bcl-x$_L$* can protect cells in G_1, S and G_2/M phase and although it is generally accepted that S-phase cells are more susceptible to apoptosis, this study showed that Bcl-x_L effectively protects cells arrested in S-phase by etoposide and cisplatin. Vincristine-treated cells expressing Bcl-x_L became polyploid after drug removal and the ability of Bcl-x_L to prevent apoptotic death in response to chemotherapeutic induced DNA damage and cell-cycle arrest may allow the accumulation of chromosomal aberrations frequently seen in human tumours. The findings in this report that show Bcl-x_L conferring a multidrug resistant phenotype and allowing cells to remain arrested until they reacquire the ability to proliferate, make Bcl-x_L expression a potential tumour promoter. A spontaneously resistant variant of the murine P388 leukaemia cell line was found to over-express Bcl-x_L (Kühl *et al.*, 1997). This cell line was found to have an unusual phenotype with cross-resistance to MDR and non-MDR related cytotoxins such as camptothecin, alkylators and UV light. Transfection with the *bcl-x$_L$* cDNA also resulted in a similarly broad cross-resistance profile. P-glycoprotein was found to play a moderate role in the resistance of this spontaneously resistant cell line. Over-expression of *bcl-x$_L$* mRNA was seen in all resistant variants of the DLKP cell line which exhibited cross-resistant profile only to the MDR cytotoxins. Dole *et al.* (1995) found that over-expression of *bcl-x$_L$* in a neuroblastoma line, Shep-1, also conferred a survival advantage on cells by inhibiting both cisplatin and 4-hydroxycyclophosphamide-induced cell death; however, after treating these cells with VP-16 only a short-term delay in the onset of apoptosis was seen. In the analysis of the DLKP

variants, the VP-16 selected lines (DLKP-VP3 and DLKP-VP8) expressed the highest levels of *bcl-x_L* mRNA. The finding of Dole and co-workers (1995) may be of therapeutic importance as it suggests that VP-16 may be able to bypass the protective effects of *bcl-x_L* in neuroblastoma tumours. It is possible that an extended exposure to drugs that directly target Topoisomerase II could make transit through mitosis impossible and trigger apoptosis irrespective of the presence of *bcl-x_L*.

4.1.6 Expression of Bcl-2 Family Members in MDR variants of DLKP

Resistant variants of the human squamous lung cell line, DLKP, developed by continuous exposure to MDR (adriamycin, VP-16) and non-MDR (carboplatin) drugs were analysed for the expression of the *bcl-2* family members, namely *bcl-2*, *bcl-x* and *bax*.

Elevated levels of expression of the apoptosis suppressing gene, *bcl-x_L*, mRNA in the multidrug resistant lines analysed in Section 3.3. are expected, as these cell lines are resistant to the apoptosis-inducing chemotherapeutic drugs adriamycin, etoposide and carboplatin. *bcl-2* did not appear to play a part in the resistance mechanism of these cell lines as expression at the mRNA level was undetected.

Increased expression of the apoptosis suppressing gene, *bcl-x_L*, was seen in multidrug resistant variants of the human lung cell line, DLKP, as detected after 23 cycles of PCR. A slight increase was seen in expression of this gene, by RT-PCR analysis, in a carboplatin-selected line, DLKP-C14. Levels of *bcl-x_L* mRNA were increased to 134% of that of DLKP in an adriamycin-selected variant, DLKP-A and to over 200% in VP-16 selected variants (Figure 3.3.1a). No expression of *bcl-2α*, another apoptosis suppressing gene, was detected by RT-PCR after 30 cycles of PCR (Figure 3.3.1b).

Elevated levels of the apoptosis-promoting gene, *bax*, is also seen in the resistant lines, an unexpected result as it has been shown previously that decreased Bax is seen in resistant lines (Chresta, 1996; Perego, 1996; Wagener, 1996). However, it is interesting to note, as the inherent ratio of *bcl-2/bcl-x_L* to *bax/bcl-x_S* determines the cell's susceptibility to death following an apoptotic stimulus (Korsmeyer, 1995) that the least resistant line DLKP-C14 (see Appendix D) expresses the highest *bax* levels and lowest *bcl-x_L* levels. The fact that these cell lines have been selected with different drugs and express different drug resistant markers (e.g. DLKP-A, -VP3 and -VP8 over-express *mdr1* (M.Heenan, 1994) and DLKP-C14 over-expresses metallothioneins (I.Cleary, 1995)) may suggest that these findings are co-incidental and do not affect resistance mechanisms in these cells.

When analysing clonal populations of the heterogenous DLKP-A cell line an increase in mRNA expression of *bcl-x_L* in the DLKP-A5F clone relative to DLKP-A2B (Figure 3.3.1.2a) and a relative increase in *bax* expression in DLKP-A2B relative to DLKP-A5F (Figure 3.3.1.2b) was observed. The inherent ratio of *bcl-2/bcl-x_L* to *bax/bcl-x_S* determines the cell's susceptibility to death following an apoptotic stimulus (Korsmeyer, 1995) and results found in this study indicate that this may be of relevance in the MDR profile of the DLKP-A variants : the increase of *bcl-x_L* and relatively lower *bax* levels may give the DLKP-A5F cells a survival advantage whereas the increase in *bax* levels and relatively lower *bcl-x_L* mRNA levels may make the DLKP-A2B clone more susceptible to apoptosis. Comparable results were obtained at the protein level by Western blotting (L.Connolly, unpublished results); this work is on-going.

Bcl-2 expression was detected in these cell lines (Figure 3.3.1.1(a)), although previous analysis of DLKP-A revealed no expression of the gene in DLKP-A cells (Figure 3.3.1(b)). The trend seen at the cDNA level was increased *bcl-2* expression in DLKP-A2B cells relative to DLKP-A5F cells. Bcl-2 protein was not detected in these cells by Western analysis (L.Connolly, unpublished observations).

4.2 Ribozymes as a Method for Circumventing MDR

The use of chemotherapy in the treatment of some cancers is limited due either to the tumour's inherent resistance to therapy or development of acquired resistance during the course of treatment. Alternative treatments, involving gene therapy, are being actively sought in the treatment of cancer. One method in use is that of "ribozyme cleavage" of key genes identified as playing a role in MDR.

Cech, in 1982, was the first to discover that RNA splicing (removal of introns and ligation of exons to produce processed poly-A tail mRNA) (Freifelder, 1983) could be catalysed by RNA molecules now known as ribozymes (molecules that undergo intramolecular catalysis (e.g. self-splicing) or that act as enzymes (Cech, 1988)). Certain ribozymes can cleave substrate RNAs in a sequence-specific manner (Cech, 1988).

A number of catalytic RNA molecules have now been identified and are characterised by containing stretches of nucleotides that base-pair with a complementary RNA region. Once the ribozyme has bonded to the substrate the catalytic region is in a position to cleave the bound RNA. Ribozymes differ in their three-dimensional shape and have been termed hammer-head, hair-pin or axe-head (Barinaga, 1993).

Ribozyme-mediated reduction of the c-Ha-*ras* oncogene expression in a cultured human bladder carcinoma cell line (EJ) resulted in a reduction in tumour formation and invasion when these cells were implanted in nude mice (Kashani-Sabet *et al.*, 1992), thus supporting the role of c-Ha-*ras* in tumourigenesis. Clinical trials with ribozyme technology in HIV-1 infected patients is underway (Barinaga, 1993). Reversal of multidrug resistance by introducing a hammerhead ribozyme against *mdr1* into a P-glycoprotein positive cell line was shown to be successful previously (Holm *et al.*, 1994a; Daly *et al.*, 1996).

Ribozymes directed against c-*fos* were targetted to drug resistant cell lines in which the gene was over-expressed. The resulting reduction in *fos* gene expression was shown to enhance senitivity to a number of chemotherapeutic drugs (Scanlon *et al.*, 1991). Interestingly, the expression of the c-*fos* ribozme in the human ovarian carcinoma cell line, A2780S, not only decreased the expression of c-*fos* mRNA but also decreased the expression of the *mdr1*, c-*jun* and p53 genes. Reversal of the MDR phenotype by an anti-*mdr* ribozyme occured one-fourth as rapidly as that induced by the c-*fos* ribozyme (Scanlon *et al.*, 1994).

c-*fos* levels were found elevated in DLKP-A cells in this study by RT-PCR analysis. To determine if increased levels of this gene affect drug resistance in the DLKP-A cell line, a ribozyme was transfected into DLKP-A cells. Six clones containing the ribozyme were maintained and assayed for effect on resistance in these cell lines. Adriamycin toxicity assays revealed minimal effect of the ribozyme on toxicity. This ribozyme is in an inducible plasmid and its expression is turned on in the presence of dexamethasone. However, naturally occurring steroids are present in serum which may induce ribozyme expression also. As these assays were set up in medium containing serum, the true effect of the ribozyme may be masked. Studies including analysis in serum-free medium failed as the media used could not support the growth of the cell lines analysed. The DLKP-A cell line is a highly resistant heterogenous population and analysis of c-*fos* expression in clonal populations revealed that the gene is over-expressed only in the more resistant clones (Figure 3.3.1.1(b)). A more accurate representation of the effect of c-*fos* ribozyme-mediated reduction in the expression of this gene may have been realised if, when the ribozyme had been transfected into the DLKP-A5F clonal population, individual DLKP-A5F*fos* ribozyme clones had been isolated. As DLKP-A is highly resistant to many chemotherapeutic drugs (see Appendix D) and over-expresses the *mdr1* gene and protein product (see Appendix E) it may be that c-*fos* has little effect on the MDR phenotype observed in these cells. It is also unknown if c-*fos* mediates its effect on MDR in DLKP-A cells by activating the *mdr1* gene alone or as part of the AP-1 complex. If the latter is the case, a reduction in c-*fos* levels does not necessitate a reduction in AP-1 activity as the gene can be replaced by other family members in the complex; therefore a reduction in c-*fos* levels in DLKP-A cells may have little effect on drug resistance profiles. Kim and Beck (1994) found elevated activity of the AP-1 complex in the human leukaemic cell line, CCRF-CEM, which was due to c-*jun* mRNA induction and not increased c-*fos* levels; in these cell lines it was found that AP-1 activity was preferentially mediated by c-*jun*/Fra-1.

4.3 Development of a Clinically Relevant *in vitro* Model of MDR

To more accurately assess *in vivo* levels of resistance, low-level resistant cell lines act as a more legitimate *in vitro* model. Clinically relevant resistance levels are in the order of 2-8 fold (Antman *et al.*, 1987). Although most resistant lines are developed by continuous exposure of cells to drug over long periods of time (Clynes *et al.*, 1993) in the clinic, patients may develop drug resistance after only several courses of chemotherapy.

To mimic clinical drug resistance, methods of *in vitro* development have altered in the recent past to favour the development of drug resistant cell lines by pulse exposure of cells to clinically attainable levels of drug, over short periods of time. Resistant variant of the human colon tumour line, LoVo, has been developed by pulse-selection with adriamycin (Yang and Trujillo, 1990) and was shown to possess a more stable and different toxicity profile to a variant developed by continuous exposure to the same drug. Lu *et al.* (1988) described the establishment of a cisplatin-resistant cell line following weekly 1 hour exposures to cisplatin for 6 months. Intermittent exposure of the human myeloleukaemic K562 cell line to either epirubicin or vinblastine over a 3-month period resulted in the establishment of resistant variants which were maintained with exposure to the selecting drug for a period of 4 days every 4-5 weeks (Marks *et al.*, 1993); anthracycline resistant variants of this cell line have also been established (Hargrave *et al.*, 1995). Resistant variants of the HL60 promyelocytic cell line were also developed by treatment with low, clinically relevant levels of epirubicin and vinblastine, for 18 hours over a 3-month period (Su *et al.*, 1994). Cisplatin-resistant sub-lines of the human melanoma cell line RPMI-8322 were obtained by repetitive 30min exposures to increasing concentrations of cisplatin during a period of 3 years (Zhao *et al.*, 1995)

4.3.1 Selection of Low-level Resistant Variants from a Clonal Population

To date, most multidrug resistant cell lines have been selected from non-clonal parental populations. Establishing drug resistant cell lines in this manner gives rise to the possibility that inherently resistant cells, or cells with a survival advantage, are being selected from the mixed population. It is not known, therefore, if the resistance seen in the selected variant is due to selection of an inherently resistant cell in the parental population only or if it is due to the effect that the selecting drug had on the genetic make up of the cell. To demonstrate that an initially sensitive clonal cell population exposed to drug could

develop resistance to adriamycin the sensitive DLKP cell line, from which DLKP-A, DLKP-VP3, DLKP-VP8 and DLKP-C14 cell lines were established, was used.
A resistant variant was selected from DLKP-SQ, a clonal population isolated previously in this laboratory from the parental DLKP cell line (S.McBride, 1995), by continuous exposure to adriamycin to a final concentration of 250ng/ml. The selection process was carried out over two to three months. This selection resulted in the development of a resistant cell line, DLKP-SQ/A250c, that was 20 fold resistant to adriamycin and also had increased resisitance to vincristine. Further characterisation of this cell line was not carried out.

Continuous exposure of cells in culture to chemotherapeutic drugs does not accurately reflect drug concentrations to which patients are exposed *in vivo*. Pharmacokinetic studies of adriamycin (a commonly used MDR-drug in the treatment of primary breast and lung cancer) in lung cancer patients (Piscitelli *et al.*, 1993) revealed that drug concentrations of 100 - 500ng/ml are attainable in serum up to 4 hours after drug administration.
It was therefore attempted to use a selecting drug concentration of 250ng/ml adriamycin to develop a resistant variant of DLKP-SQ, by weekly pulse exposure to the drug. A weekly pulse was chosen to allow any genetic mutation establish itself in the genome. Successful selection of a two-fold resistant line (to adriamycin, VP-16, vincristine but not 5-fluorouracil or carboplatin) was achieved after pulse exposure to the selecting drug concentration for 4 hours once every 4 weeks (DLKP-SQ/4x4hADR). Selection with the same drug concentration for 1 hour pulses did not result in resistance; this exposure to drug did not achieve the same cell kill as exposure for 4 hours, as this exposure time may not have allowed sufficient uptake of drug. Exposing cells to drug for 8 hours killed all the cells initially, except for one colony which was not found to have significantly altered resistance.

This pulse exposure method was repeated on DLKP-SQ cells and after four pulses a four fold resistant variant was isolated (DLKP-SQ/A250p). Continuing exposure (to ten pulses) of this cell line to adriamycin resulted in the isolation of the DLKP-SQ/A25010p variant which grew readily following exposure to 250ng/ml adriamycin for four hours. This resistant variant was found to be ten fold resistant to adriamycin, greater than forty fold resistant to vincristine but only three fold resistant to VP-16. No alteration in resistance to 5-fluorouracil was seen for this cell line. Similar trends in toxicity profile was seen for the

highly resistant MDR cell line DLKP-A, which was developed by continuous selection to adriamycin.

Table 4.3.1.1 Fold resistance of DLKP-A

	Adriamycin	Vincristine	VP-16	5-Fluorouracil
DLKP-A	254 ± 119	1504 ± 707	61 ± 8	1.75 ± 0.74

Fold resistance of DLKP-A relative to its sensitive parental line DLKP (analysis M.Heenan, 1994)

To determine if pulse exposure of cells to drug is a stable and reproducible model to induce the development of multidrug resistance, replica selections were carried out with DLKP-SQ and another clone isolated from the heterogenous DLKP population, DLKP-I. Analysis was confirmed by the development of a resistant DLKP-SQ/A25010p[2] and DLKP-I/A25010p cell line with similar trends in toxicity profiles. Clonal populations of DLKP-SQ/A25010p maintain stable resistance to adriamycin (C.O'Loughlin, pers.comm.).

Other laboratories have also reported the selection of resistant lines by pulse-exposure to clinically relevant concentrations of drug.

Hargrave *et al.* (1995) treated the myeloleukemia cell line, K562, with 20ng/ml (34nM) doxorubicin for 3 days over a period of 2 months. The doxorubicin resistant variant (K/DOX) showed only low levels of cross-resistance to epirubicin (1.7 fold), daunoribicin (1.5 fold) and doxorubicin (1.5 fold). No cross-resistance to etoposide was seen and less than 5 fold resistance was seen for vinblastine, taxol and actinomycin D. Intermittent exposure of K562 cells to epirubicin or vinblastine resulted in the establishment of resistant variants with cross-resistance profiles; as the selecting drug concentration increased, so too did the level of cross resistance (Marks *et al.*, 1993). All cell lines were slightly sensitised to VP-16. Highest levels of resistance were seen for vincristine which is in agreement with results found in this and previous studies.

Four treatments of HL60 cells with 8ng/ml epirubicin for 18-20 hours resulted in a 2.8 fold resistant cell line which increased to 30 fold following a fifth treatment (H/E8 subline); this cell line was maintained without loss of resistance for over 12 months without further drug treatment (Su *et al.*, 1994). Increased resistance to idarubicin, vinblastine, taxol, etoposide and actinomycin D was also seen for this cell line. HL60 cells were slower to adapt to growth in vinblastine, however 9 treatments induced a 4 fold

resistant variant (H/V8) (Su *et al.*, 1994).

Lu *et al.* (1988) established a cisplatin resistant variant of the ovarian cell line, A2780. This A2780CDDP (cisplatin-resistant) variant was established by treatment with 50μM cisplatin for 1 hour weekly for 6 months.

A pulse selected LoVo subline (SRA1.2) (Yang and Trujillo, 1990) was obtained by pulsing cells nine times with 1.2μg/ml adriamycin for one hour. The resulting cell line, SRA1.2 LoVo was found to be 2.5 fold resistant to adriamycin and demonstrated a cross-resistant profile to the other MDR drugs analysed. The resistance of the cell line was stably maintained.

The establishment of the DLKP-SQ/A25010p cell line in this thesis demonstrates a low, more clinically relevant, level resistant variant which may act as an *in vitro* model of drug resistance. The cell line was established after ten weekly pulses to adriamycin to mimic clinical scheduling. DLKP-SQ/A25010p demonstrates a cross-resistant profile to adriamycin, vincristine and VP-16 but not 5-fluorouracil. A stable adriamycin resistant phenotype was maintained over a six-month period without further exposure to drug, and is therefore preferential to many of the other pulse-selected variants established in other laboratories (Table 4.3.1.2) which were maintained by intermittent exposure to drug.

Figure 4.3.1.1 Selection of Adriamycin Resistant Variants from DLKP-SQ

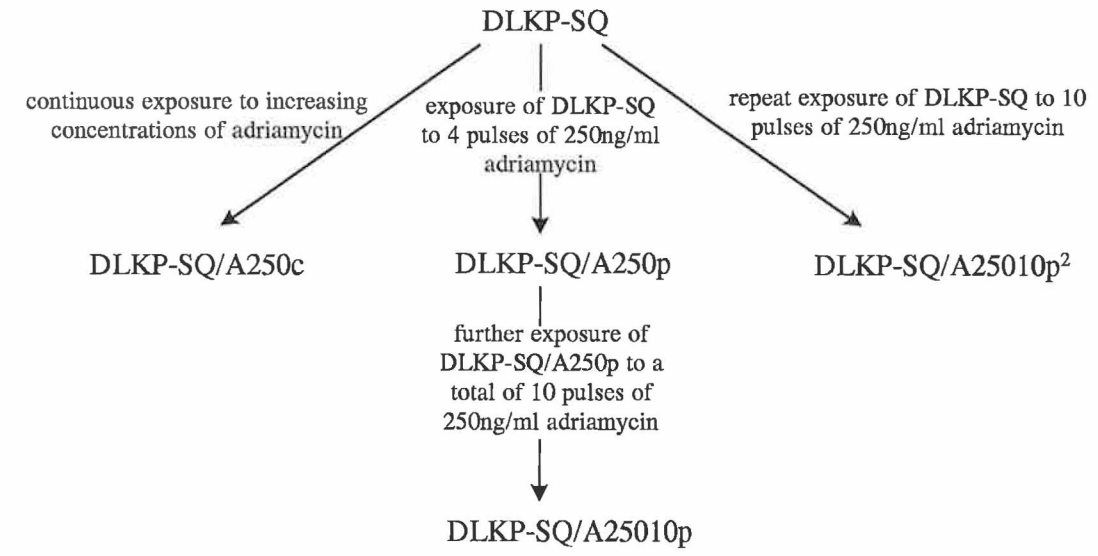
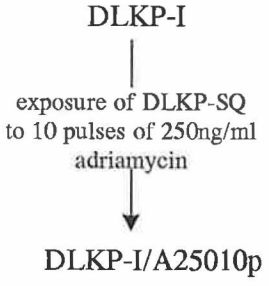


Figure 4.3.1.2 Selection of Adriamycin Resistant Variant from DLKP-I



Cell line	Cell type	Selecting Drug	Drug concentration Time-scale	Fold-resistance (selecting drug)	Stability	Reference
A549	lung	adriamycin	μM, 24 hour	.....	(3 months) stable without drug	Chevillard *et al.*, 1992
K/DNR	myeloleukemic	daunorubicin	35ηM, 3 days 2 months	2.5	intermittent drug exposure	Hargrave *et al.*, 1995
K/DOX	myelolekemic	doxorubicin	34ηM, 3 days 2 months	1.5	intermittent drug exposure	Hargrave *et al.*, 1995
K/EPR	myeloleukemic	epirubicin	34ηM, 3 days 2 months	6.8	intermittent drug exposure	Hargrave *et al.*, 1995
K/IDA	myeloleukemic	idarubicin	36ηM, 3 days 2 months	1.0	intermittent drug exposure	Hargrave *et al.*, 1995
A549(VP)28	lung adenocarcinoma	etoposide	1 hour weekly 28 weeks	8.1	.....	Long *et al.*, 1991
A549(VM)28	lung adenocarcinoma	teniposide	1 hour weekly 28 weeks	6.8	.....	Long *et al.*, 1991
HCT116(VP)35	colon carcinoma	etoposide	1 hour weekly 35 weeks	8.6	.....	Long *et al.*, 1991
HCT116(VM)34	colon carcinoma	teniposide	1 hour weekly 34 weeks	6.6	.....	Long *et al.*, 1991
A2780DDP	ovarian cancer	cisplatin	50μM, 1 hour weekly 6 months	17.2	stable	Lu *et al.*, 1988
K562/E40	myeloleukemic	epirubicin	40ηg/ml, 3-14 days 3 months	3.3	intermittent drug exposure 18 months	Marks *et al.*, 1993
K562/V8	myeloleukemic	vinblastine	8ηg/ml, 3-14 days 3 months	8.0	intermittent drug exposure 18 months	Marks *et al.*, 1993
CCRF-CEM/P	TLL	methotrexate	3μM, 24 hour	.....	.....	Pizzorno *et al.*, 1988
H/E8	promyelocytic	epirubicin	8ηg/ml, 18-22 hours 3 months	2.8 (4 pulse) 30 (5 pulse)	stable without drug 12 months	Su *et al.*, 1994
H/V8	promyelocytic	vinblastine	8ηg/ml, 18-22 hours 3 months	4.3	stable without drug	Su *et al.*, 1994
SRA1.2 LoVo	colon tumour	adriamycin	1.2μg/ml, 9 x 1 hour	2.5	stable without drug 10 months	Yang and Trujillo, 1990

Drug-resistant human cell lines developed by pulse exposure to chemotherapeutic drugs. CCRF/CEM is the human T lympoblast leukemic (TLL) cell line. K = K562 myeloleukemic cell line; H = HL60 promyelocytic cell line. Stability of the drug-resistant phenotype is stated when reported in the literature.

Table 4.3.1.2 Human Drug-resistant Cell Lines selected by Pulse-exposure to Chemotherapeutic Drugs.

4.4 Drug Accumulation Studies in Pulse Variants

Decreased drug accumulation has been implicated as a mechanism of resistance in many animal (Inaba and Johnson, 1978; Skovsgaard, 1978; Breier *et al.*, 1994) and human (Fojo *et al.*, 1985; Wiebe *et al.*, 1992) MDR cell lines and usually occurs as a result of over-expression of the *mdr1* gene, which encodes the P-glycoprotein trans-membrane pump. In addition to a reduction in accumulation, MDR cell lines may also exhibit altered distribution and compartmentalisation of drug in vesicles, away from the nucleus (target site) and into the cytoplasm (Chauffert *et al.*, 1984). Adriamycin is commonly used in drug accumulation studies as it fluoresces under ultraviolet illumination and is readily visualised by fluorescent microscopy. Sensitive cells have been shown to accumulate adriamycin in the nucleus whereas resistant variants contain mainly cytoplasmic accumulation (Willingham *et al.*, 1986; Hindenburg *et al.*, 1987; Versantvoort *et al.*, 1993; Barrand *et al.*, 1993).

Adriamycin accumulation was analysed in the resistant variants of DLKP-SQ and DLKP-I to determine if drug accumulation is involved in the resistance mechanism of these cell lines. In the sensitive parental lines, intense nuclear accumulation was observed, in agreement with previous findings, after 4.5 hours of exposure to 10μM adriamycin. The nucleoli of these cells were free of adriamycin as was the cell cytoplasm. The resistant variants developed by pulse exposure to adriamycin, DLKP-SQ/A25010p and DLKP-I/A25010p, accumulate adriamycin in the cytoplasm, in agreement with other studies on multidrug resistant cell lines. DLKP-SQ/A250c, developed by continuous selection with adriamycin, did not accumulate adriamycin in its nucleus and less drug was seen in these cells' cytoplasm compared to that seen in the pulse-selected variants. Adriamycin accumulation in these cell lines was therefore found to be inversely proportional to the degree of resistance.

Analysis of *mdr1* mRNA levels was carried out in these cell lines and it was found that DLKP-SQ/A25010p, DLKP-I/A25010p and DLKP-SQ/A250c had elevated expression of the *mdr1* gene with highest levels seen in DLKP-SQ/A250c. This is supportive of results of P-glycoprotein mediated drug exclusion. The total exclusion of adriamycin from the nucleus of these resistant variants is interesting as the pulse-selected cell lines are only 10 and 16 fold resistant to adriamycin, respectively. The level of *mdr1* gene expression in

DLKP-SQ and DLKP-I resistant variants was much less than that detected in highly resistant lines such as DLKP-A (see Appendix E) but these DLKP-SQ and DLKP-I resistant variants were as effective in preventing drug accumulation in the cell. Previous adriamycin accumulation studies on highly resistant variants of the DLKP cell line demonstrated reduced drug accumulation in the resistant variants following 2 hour incubations in 10μM adriamycin. In the most sensitive DLKP-A clone, DLKP-A2B, intense nuclear fluorescence was observed in 65% of the cells (I. Cleary, 1995). Level of adriamycin resistance in DLKP-A2B is somewhat similar to that seen in DLKP-SQ/A250c cells although drug exclusion from the nucleus of the DLKP-SQ/A250c cell line was absolute. The most resistant clone, DLKP-A5F, had very faint nuclear fluorescence but intense staining in distinct areas in the cytoplasm (I. Cleary, 1995). As the pulse-selected variants were exposed to similar concentrations of drug for a longer length of time (4.5 hours vs. 2 hours) it was assumed that the lower resistant cell lines were as effective in preventing drug accumulation as the highly resistant DLKP-A variants and that the result was not an artifact of drug exposure time. This implies that much of the P-glycoprotein detected in highly resistant lines may be redundant or that other mechanisms may be involved in drug exclusion in these low-level resistant variants. However, studies on the DLKP-A variants were not carried out at the same time as the DLKP-SQ / DLKP-I variants and, therefore, cannot be used as an absolute standard. Further analysis, which should include a complete study where the DLKP-A variants could be compared to the DLKP-SQ variants.

The sensitive DLKP-I clone was transfected with the *mdr1* ribozyme (S.McBride, 1995) and adriamycin accumulation studies analysed. Greater nuclear accumulation of adriamycin was seen in the *mdr1* ribozyme expressing clone than the parental DLKP-I clone, a novel observation which shows a role for P-glycoprotein in adriamycin uptake studies in sensitive cells. Selection of the *mdr1* ribozyme expressing clone, DLKP-I*mdr1*Rz2D4, by pulse exposure to adriamycin produced the resistant variant DLKP-I*mdr1*Rz2D4/A25010p with a similar cross-resistant profile to that of DLKP-I/A25010p. This variant was shown to express *mdr1* but to a lesser extent than that seen in its sister cell line DLKP-I/A25010p. Adriamycin accumulation studies revealed greater localisation of drug in the cytoplasm than that seen in either the DLKP-SQ/A25010p or DLKP-I/A25010p variants which suggests a role for P-glycoprotein drug exclusion in this system. As no significant difference in toxicity levels were seen between DLKP-

I/A25010p and DLKP-I*mdr1*Rz2D4/A25010p but less *mdr1* mRNA was detected in the ribozyme expressing variant, results may imply that mechanisms other than P-glycoprotein is involved in adriamycin accumulation, such as *bcl-x*$_L$, or else only a small threshold of *mdr1* mRNA is necessary and has been attained by the ribozyme-expressing resistant variant. Some cells in the resistant population shows strong nuclear accumulation of adriamycin suggesting a mechanism other than P-glycoprotein mediated drug accumulation in these cells or that these cells have lost their resistant phenotype. To investigate this, clonal sub-populations should be isolated and analysed for resistance profile and adriamycin accumulation.

As no over-expression of the *mdr1* gene was seen in the 4 fold resistant variant DLKP-SQ/A250p after 25 cycles of PCR and only a slight increase in mRNA levels (relative to DLKP-SQ) were seen when amplification was increased to 30 cycles, future drug accumulation studies should include this cell line as a possible model for non- or low-level P-glycoprotein mediated drug accumulation. Analysis of adriamycin accumulation in the DLKP-SQ/A250p cell line could determine if a lower threshold of P-glycoprotein expression is sufficient to reduce drug accumulation to the same extent as that seen in the DLKP-SQ(I)/A25010p variant. Also to determine if mechanisms other than P-glycoprotein mediated drug accumulation are involved in resistance mechanisms in the DLKP-SQ pulse-selected model, the *bcl-x*$_S$ transfected variant with reduced resistance, DLKP-SQ/A25010pbclx$_S$.3, should be included in such studies as this cell line has no apparent altered *mdr1* levels (results not shown).

4.5 Analysis of Oncogene Expression in Resistant Variants of DLKP-SQ

By time-lapse video-microscopy it was shown that exposure of DLKP-SQ cells to 250ng/ml adriamycin for 4 hours induced an apoptotic pathway in these cells. A cell line that grows readily in this concentration of drug would therefore be expected to have increased resistance to apoptosis and perhaps altered expression of apoptosis-related genes or other genes known to affect cell proliferation.

Initial studies, by RT-PCR analysis, on RNA isolated from DLKP-SQ, DLKP-SQ/A250p DLKP-SQ/A25010p and DLKP-SQ/A250c revealed and increase in *bcl-x*$_L$ (the apoptosis repressing gene) expression in DLKP-SQ/A250p, DLKP-SQ/A250c and to a greater extent in DLKP-SQ/A25010p. Levels of the antagonistic *bax* gene was also elevated in the more highly resistant cell lines but not in the DLKP-SQ/A250p variant. This trend was also seen in the highly resistant DLKP variants studied in Section 3.3 where up-regulation of apoptosis supporting and repressing genes was detected in all resistant variants. Expression of *bcl-2* was not detected in these cell lines by RT-PCR.

The balance of members of the *bcl-2* family (including *bcl-2*, *bcl-x*, *bax*, and others decribed in Section 1.7.1.2) of proteins in the cell determines the cell's inherent ability to undergo apoptosis. Cell death will occur in either of two ways, the first being an increase in apoptosis-supporting genes such as *bcl-x*$_S$ or *bax* or a reduction in apoptosis-repressing genes such as *bcl-x*$_L$ or *bcl-2*.
Previous studies have shown that introduction of *bcl-x*$_L$ or *bcl-2* into cells increases resistance to apoptosis-promoting agents such as chemotherapeutic drugs cDNA (Miyashita and Reed, 1992; Dole *et* al., 1995; Minn *et al.*, 1995). Equally introduction of *bcl-x*$_S$ or *bax* sensitises cells to apoptotic stimulus (Sumantran *et al.*, 1995; Wagener *et al.*, 1996).

As *bcl-x*$_L$ mRNA was found to be over-expressed in the DLKP-SQ/A25010p resistant cell line it was decided to introduce the antagonistic *bcl-x*$_S$ cDNA into this cell population to determine if expression of the gene (which was not detected in the parental cell) could sensitise the multidrug resistant variant to chemotherapeutic drugs.
Five stably transfected clones were isolated and grown as individual cell lines. Toxicity levels of four of these cell lines were analysed as the fifth clone was lost to fungal

contamination. It was found that two of these *bcl-x$_S$* expressing cell lines (DLKP-SQ/A25010p-*bclx$_S$*.1 and .4) were slightly more sensitive to adriamycin, vincristine and VP-16 but not to 5-fluorouracil (all of which are known to induce apoptosis). The DLKP-SQ/A25010p-*bclx$_S$*.2 cell line showed increased resistance to the MDR drugs but not 5-fluorouracil. Therefore *bcl-x$_S$* expression sensitised cells only to drugs that the resistant line was more resistant to. Analysis of DLKP-SQ/A25010p-*bclx$_S$*.3 (shown, by RT-PCR analysis, to express the highest levels of the *bcl-x$_S$* gene) revealed a 66% decrease in adriamycin toxicity levels, a 55% decrease in VP-16 toxicity levels to almost DLKP-SQ sensitive levels but only a 38% decrease in vincristine toxicity levels which in the resistant line were increased 43 fold. As adriamycin and vincristine mediate their toxicity via the P-glycoprotein pump the difference in resistance levels in the *bcl-x$_S$* transfected variant is interesting. No statisitical alteration to 5-fluorouracil toxicity was observed. The lack of effect on 5-fluorouracil toxicity may imply that the apoptotic pathway induced by this pathway is not affected by the *bcl-2* family in this system.

Table 4.5.1 Fold Resistance of DLKP-SQ/A25010p*bcl-xs*.3

	Adriamycin	Vincristine	VP-16	5-Fluorouracil
DLKP-SQ	1	1	1	1
DLKP-SQ/A25010p*bcl-xs*.3	3.4	26.3	1.3	1.1
DLKP-SQ/A25010p	10.0	42.9	2.9	2

Fold resistance of *bcl-xs* expressing cell line relative to the parental resistant DLKP-SQ/A25010p line and sensitive DLKP-SQ cell line.

bcl-x$_S$ cDNA was transfected into the DLKP-SQ cell line and toxicity profile analysed. No change in sensitivity to adriamycin or vincristine, VP-16, 5-fluorouracil (analysis by S.Touhey, pers.comm.) was seen for the DLKP-SQ/*bclx$_S$*.1 clone compared to the parental DLKP-SQ cell line. This observation agrees with the result found for the resistant variant whereby *bcl-x$_S$* only appears to act against the MDR phenotype and not intrinsic/sensitivity levels of the cell lines. As *bcl-x$_S$* only affects drugs to which DLKP-SQ/A25010p has increased resistance it appears that the over-expression of *bcl-x$_L$* in the resistant variant has a functional effect on resistance levels in this cell line.

Analysis of other oncogene products by RT-PCR on resistant variants of DLKP-SQ revealed over-expression of c-*fos* gene in the three resistant variants with greatest

expression seen in DLKP-SQ/A25010p. The *mdr1* gene has previously been shown to have an AP-1 promoter site and to be induced in the presence of AP-1 (c-Fos/c-Jun). In clinical studies on lung cancer, levels of P-glycoprotein were shown to correlate with the expression of c-*fos* and c-*jun* (Volm *et al.*, 1993). Previous analysis on expression of c-*fos* levels in clonal populations of DLKP-A revealed over-expression of *mdr1* in DLKP-A and DLKP-A5F relative to DLKP-A2B; a similar trend was seen for the expression of c-*fos*. Analysis of DLKP-SQ variants demonstrated an over-expression of c-*fos* in DLKP-SQ/A250p and DLKP-SQ/A250c and to a greater extent in DLKP-SQ/A25010p. Analysis of *mdr1* levels was shown to be highest in the DLKP-SQ/A250c variant and therefore expression of elevated expression of c-*fos* did not agree with elevated expression of *mdr1*.

c-*erb*B-2 mRNA levels were over-expressed in the DLKP resistant variants. Analysis of DLKP-SQ variants revealed no over-expression of the gene product in these low-level resistant lines. Further analysis, however, of DLKP-SQ and DLKP-SQ/A25010p revealed up-regulation of c-*erb*B-2 gene expression in the DLKP-SQ/A25010p resistant variant.

4.6 Induction vs. Selection in the DLKP-SQ Model of Resistance

The general opinion in the field is that drug resistance is fundamentally a result of genetic mutations establishing themselves in the genome of inherently sensitive cells. Most resistant cell lines established to date have been selected from heterogenous populations. In this scenario it is unknown if the resulting resistant cell line is due to the selection of pre-existing, intrinsically resistant cells, or cells with a survival advantage, from the mixed parental cell line. To overcome this problem in part, sensitive clonal sub-populations of the DLKP cell line were used to act as a model to develop resistance. Any genetic change which occurs in the resulting resistant population is, therefore, directly caused by exposure to the chemotherapeutic drug. To determine if resistance development in the DLKP-SQ model is a result of drug-induced mutation or induction of a set of resistance-related genes, further studies must be undertaken. Such studies could include the exposure of DLKP-SQ to one pulse of adriamycin and subsequent isolation and propagation of a number of clonal sub-populations. Should all these sub-populations respond to a second pulse with adriamycin in a similar manner, the initial event in the development of resistance is most likely as a result of gene induction, rather than spontaneous mutation.

Gene expression following exposure of DLKP-SQ cells to adriamycin was monitored in these studies to determine if adriamycin could induce the expression of MDR-related genes or oncogenes believed to play a role in drug resistance.

4.7 Gene Induction following Adriamycin administration

To successfully design improved or alternative therapies to overcome the development of drug resistance *in vivo,* it would be advantageous to study the effect chemotherapeutic drugs have on transcription of genes associated with the development of resistance. Sensitive cell lines vary in their ability to develop resistance (Section 3.5.4) and comparison of the ability of these cell lines to respond to chemotherapeutic drugs, at the transcriptional level, may yield valuable information on initial events in the development of drug resistance - prevention being better than cure! Adriamycin was chosen in these preliminary studies to determine what genes, if any, are induced by drug exposure in the DLKP-SQ cell line that readily develops resistance to adriamycin. RT-PCR analysis was used to monitor gene expression.

4.7.1 Induction of *mdr1* Expression Following Adriamycin Administration

Kohno and co-workers (1989) were the first to report the direct activation of the *mdr1* gene promoter by anticancer agents such as adriamycin, daunomycin, vincristine and colchicine in transient expression systems. Later work by Chaudhary and Roninson (1993) analysed the effect of short-term chemotherapeutic drug administration on levels of the *mdr1* gene in a number of sensitive cell lines derived from leukaemias or solid tumours. Most cell lines displayed transient induction of the *mdr1* gene following short-term exposure to adriamycin, daunorubicin, vinblastine, etoposide, cisplatin and other drugs. Induction of *mdr1* in the K562 leukaemia cell line was stable for a number of weeks. Induction in all cell lines analysed was only evident in the presence of morphologically detectable cell damage.

Gekeler and co-workers (1994) also found increased *mdr1* mRNA levels in the T-lymphoblastoid human cell line, CCRE-CEM, following administration of sub-lethal concentrations of actinomycin D or adriamycin. Increased *mdr1* mRNA levels were also seen in the human sarcoma cell line, MES-SA, following treatment with 40nM adriamycin for 14 days (Chen *et al.*, 1994). Hu and co-workers (1995) also found up-regulation of *mdr1* mRNA in a MDR variant of the CCRF-CEM T-cell lymphoblastic leukaemia cell line following 4 hour exposure to 1.5μg/ml daunorubicin and epirubicin and 6 hour exposure to a similar concentration of adriamycin. Previous studies in this laboratory revealed up-regulation of *mdr1* gene expression in OAW42SR cells, but not DLKP cells,

after exposure to 0.1 or 1.0μg/ml adriamycin for 72 hours (O'Driscoll, 1994).

In this thesis, DLKP-SQ cells were exposed to concentrations of drugs used previously in these studies to select resistant variants (Section 3.5.2). Cells were exposed to adriamycin at 25ng/ml continuously and at 250ng/ml for 4hours. RNA was extracted after certain time-spans had elapsed and analysed by RT-PCR for the expression of *mdr1* mRNA. This study did not show induction of the *mdr1* gene under these conditions, suggesting either a failure in detection by RT-PCR or that up-regulation of this gene is not an initialising event in development of resistance in the DLKP-SQ model.

To determine if *mdr1* is important in initialisng resistance in the DLKP model, a previously transfected DLKP-I expressing the *mdr1* ribozyme (S.McBride, 1995) was used as a model for resistance development in the absence of *mdr1* gene up-regulation. Pulse selection of DLKP-I*mdr1*Rz2D4 and its sister cell line, DLKP-I, with 250ng/ml adriamycin for 4hours resulted in the development of resistance variants. The DLKP-I/A25010p was found to be 16 fold resistant to adriamycin whereas the ribozyme variant was only 10 fold resistant (although difference was not statistically relevant). Up-regulation of the *mdr1* gene was seen in both variants but expression in the ribozyme-expressing cell line was reduced relative to DLKP-I/A25010p. These results may indicate the presence of a partially effective ribozyme or that some cells have lost ribozyme expression. Some cells, when analysed for adriamycin accumulation, showed strong nuclear staining whereas others did not. This is the first study in the literature to describe selection from an *mdr1* ribozyme expressing cell line. It is inconclusive from this result whether *mdr1* gene induction is an initialising event in the development of the multidrug resistant phenotype.

4.7.2 Induction of c-*fos* Expression Following Adriamycin Administration

Levels of early transcription factors such as c-*fos*, c-*jun* or c-*myc* have previously been shown to be induced following short-term exposure to chemotherapeutic drugs. Kim and Beck (1994) reported induction of c-*jun* mRNA in the human leukemic lymphoblast cell line, CCRF-CEM, following 6 hour treatment with 10μM VM-26. Administration of cisplatin for 1 hour induced expression of c-*fos* mRNA (Kashani-Sabet *et al.*, 1990a). Treatment of the human prostate PC3 cell line with 100μM VP-16 caused significant increases in c-*myc* expression in a time-dependent manner but had little effect on

expression of c-*jun*, however (Sinha *et al.*, 1995). In contradiction to the up-regulation of c-*myc* by VP-16, down-regulation of c-*myc* levels were seen when MCF-7 cells were exposed to 10μM VM-26 (Orr *et al.*, 1995) or 50nM adriamycin (Fornari *et al.*, 1994) for up to 72 hours and lead to growth arrest (but not apoptotic death) in those cells. Fornari and co-workers (1996) demonstrated that acute exposure of MCF-7 cells to 1μM adriamycin also resulted in a decrease in c-*myc* expression.

c-*fos* is an "immediate early gene" whose expression is up-regulated in response to cellular stress and has also been shown to be important in the development of drug resistance. c-*fos* levels were increased in DLKP-SQ on exposure to adriamycin. Addition of fresh media only, however, induced c-*fos* levels to some extent as well (Figure 3.6.3.1) so the extent of the adriamycin effect would need further interpretation.

Up-regulation of c-*fos* has been shown as an early response to many cellular stress signals including chemotherapeutic drugs (Janknecht, 1995). The response is immediate and expression peaks within 15min; levels of expression are normally returned to basal level within 30min.

To determine the effect of c-*fos* on initialising events in the development of drug resistance, DLKP-SQ cells were transfected with the c-*fos* ribozyme. All clones isolated grew well in serum-supplemented media. Unsuccessful attempts were made to grow the clones in serum-free media. Three clones were used as models in the pulse-selection process. These clones were set up in normal ATCC media (control) and in dexamethasone-supplemented media (which induced the expression of the c-*fos* ribozyme). On pulse exposure to adriamycin none of the clones assayed, either in the presence or absence of dexamethasone survived. Death of the control cells may indicate the induction of the c-*fos* ribozyme by basal levels of dexamethasone in serum (as was shown by RT-PCR analysis, Figure 3.9.2.1); therefore, it appears that c-*fos* expression is an important mechanism of stress response to adriamycin in the DLKP-SQ model.

P-glycoprotein is known to contain an AP-1 site in its promoter region. Recently another promoter site in the *mdr1* promoter region for a newly discovered transcription factor, YB-1, was found (Rubin *et al.*, 1992). Expression of this transcription factor was localised in the cytoplasm in sensitive cells but was detected in the nucleus of drug-resistant cells and was associated with *mdr1* expression (Bargou *et al.*, 1997).

4.7.3 Induction of *bcl-x$_L$* Expression Following Adriamycin Administration

bcl-x$_L$ was chosen to determine if an apoptosis-suppressing gene expressed in resistant variants of DLKP is induced as an early response to chemotherapeutic drug treatment.

Initial studies on the inductive effect of continuous exposure to 25ng/ml adriamycin on *bcl-x$_L$* expression revealed a steady increase of gene levels with time, as studied by RT-PCR. Induction was also seen with pulse exposure to adriamycin (Figure 3.11.1.1). Repeat analysis did not, however, reveal a similar trend, so unfortunately no conclusion can be drawn. Time constraints prevented further repeats.

Bcl-x$_L$ is an apoptosis repressing gene and levels of expression are up-regulated in all resistant variants analysed. Any gene that gives a cell a survival advantage would be favoured in the selection process and may allow for the later development or over-expression of common MDR markers such as P-glycoprotein. Preliminary gene induction studies in this thesis may indicate a role for *bcl-x$_L$* in initial events in the development of the multidrug resistant phenotype. A previous report (Tu *et al.*, 1996) demonstrated the induction of *bcl-2* mRNA in response to doxorubicin (0.2 or 1.0μM) in the 8226 myeloma cell line as early as 4 hours after treatment.

To address the role of *bcl-x$_L$* in initial stages in the development of drug resistance the antagonistic *bcl-x$_S$* gene (which promotes apoptosis) was introduced in the DLKP-SQ cell line. Stable expression of the *bcl-x$_S$* gene was seen in the five isolated clones, without alteration in adriamycin sensitivity. Pulse exposure of these cells to adriamycin resulted in total cell kill of one clone, DLKP-SQ/*bcl-x$_S$*.1; all other cell lines survived and grew readily in repeated pulses with adriamycin. No alteration in IC_{50} values for adriamycin, VP-16, vincristine or 5-fluorouracil was seen between DLKP-SQ and DLKP-SQ/*bclx$_S$*.1 (S.Touhey pers.comm.). As this response was only seen with one out of five clones, further analysis is essential to prove that this effect was indeed due to the forced over-expression of the *bcl-x$_S$* gene. No change in the rate of apoptosis was seen between DLKP-SQ and this *bcl-x$_S$* transfectant. Kitada and co-workers (1994) also found that the similar effect of reducing the *bcl-2/bcl-x$_L$* to bax/bcl-*x$_S$* ratio by *bcl-2* anti-sense technology did not accelerate the basal rate of cell death but rather it rendered the cells more prone to apoptosis caused by an additional stress such as chemotherapeutic drugs.

As *bcl-x$_S$* sensitises cells to apoptotic-inducing agents it would be expected that *bcl-x$_S$* over-expression would sensitise DLKP-SQ to adriamycin treatment. Expression of *bcl-x$_S$*

in the DLKP-SQ cell line prevented the development of resistance in one of the clones and may support the idea that *bcl-*x_L is an initialising event in the development of the multidrug resistant phenotype. The basis of resistance development is in the stable establishment of mutations in the cell's genome. The DLKP-SQ cell line readily adopts mutations caused by adriamycin and develops into the resistant population, DLKP-SQ/A25010p. Resistance was not established in the DLKP-SQ/*bcl*x_S.1 cell line following pulse treatment with adriamycin. The over-expression of *bcl-*x_L in the DLKP-SQ pulse-method may allow mutations to establish in the genome by giving the cells a survival advantage, as proposed for *bcl-2* by Fisher (1993). The fact that the DLKP-SQ transfected with *bcl-*x_S does not develop resistance suggests that this gene is neutralising the effect seen by *bcl-*x_L.

4.7.4 Induction of c-*erb*B-2 Expression Following Adriamycin Administration

c-*erb*B-2 was studied as it is a well accepted marker of poor prognosis in lung and breast cancer. Recent evidence has shown that the protein plays a role in the development of drug resistance and it was also shown in previous work to be over-expressed in all resistant variants of the DLKP line studied (Figure 3.3.2b). c-*erb*B-2 levels were also increased on exposure to adriamycin but repeat analysis did not concur (Figure 3.6.2.1).

4.8 *In vitro* Model used in Search of Chemical Inhibitors of Resistance Development

The oncogenes studied in this thesis are part of signalling cascades whereby a message is received at the plasma membrane and transduced through the cytoplasm to the nucleus. Intracellular signalling pathways mediating the effects of oncogenes on cell growth or transformation offer novel targets for the development of anticancer drugs (Powis, 1994). Most anti-cancer agents developed to date target the cell's DNA and as only 12% of human cancers are cured by chemotherapy treatment a rationale for new approaches to drug design is essential. In the circumvention of the multidrug resistance phenotype minimal success has been realised with agents such as verapamil and cyclosporin A as these agents have significantly toxic side effects (Ford, 1995). To achieve reversal of MDR *in vivo* with most of the common circumventing drugs require doses of modulators near to or even exceeding the maximum tolerated doses of those drugs leading to unacceptable toxicity (Philip *et al.*, 1992). This area of research actively needs new agents to either prevent the development of the resistance phenotype or circumvent the established phenotype thus allowing the common chemotherapeutic drugs to exert their effect.

Protein phosphorylation and de-phosphorylation is fundamental to cellular signalling. The two major signalling pathways in the eukaryotic cell consist of phosphorylation on serine/threonine or tyrosine residues in proteins and are controlled by serine/threonine or tyrosine kinases and phosphatases, respectively. Common mediators of serine/threonine phosphorylation signals are protein kinase C, c-AMP dependent protein kinase A, mitogen-activated protein kinases and cdc2 kinase. Tyrosine kinases include receptor kinases such as *ras* family and non-receptor protein tyrosine kinases such as c-src. Evidence has indicated a role for the calcium- and phospholipid-dependent protein kinase C in the regulation of P-glycoprotein activity (Chaudhary and Roninson, 1992) and some reports indicate that inhibitors of protein kinase C (e.g. staurosporine, calphostin C) increase drug accumulation in MDR cells (reviewed by Skovsgaard, 1994). Over-expression of protein kinase C alone has been shown to produce an MDR phenotype without changes in P-glycoprotein expression (Fan *et al.*, 1992).

4.8.1 Inhibition of the MDR Phenotype by P-glycoprotein Inhibitors

Cyclosporin A, a hydrophobic cyclic peptide of 11 amino acids that is widely used as an

immunosuppressive agent in human organ transplantation, has been found to reverse resistance to anthracyclines, vincristine and VP-16 (but not cisplatin and radiation) in many MDR cell lines (reviewed by Stewart and Evans, 1989). Cyclosporin A has been found to act as a P-glycoprotein substrate and antagonises P-glycoprotein, at least in part, through competitive inhibition of cytotoxic drug efflux (Ford, 1995).

In this study, standard toxicity assays were carried out on DLKP-SQ and DLKP-SQ/A25010p cells to analyse the effect cyclosporin A had on adriamycin toxicity in these cell lines. Concentrations of adriamycin were chosen which were close to the average IC_{50} value for adriamycin i.e. 15ng/ml for DLKP-SQ and 150ng/ml for DLKP-SQ/A25010p. In this assay however, this drug concentration only achieved 10 - 30% cell kill. Three concentrations of each of the co-selecting drugs was used. Increased cell kill was seen in DLKP-SQ and DLKP-SQ/A25010p cells when co-treated with cyclosporin A (results are summarised in Table 4.8.2). This effect was dose-dependent and higher concentrations of cyclosporin A achieved greater cell kill with similar concentrations of adriamycin. The highest concentration of cyclosporin A used achieved only 10-20% kill in control (not treated with adriamycin) cells.

This assay was carried out on triplicate plates on the same day, time constraints prevented further analysis.

4.8.2 Effect of Tyrosine Kinase Inhibitors on the MDR Phenotype

To determine if signalling events are involved in the establishment of the clinically relevant *in vitro* model of drug resistance in the DLKP-SQ cell line, a number of putative inhibitors of the phosphorylation pathways were used. These inhibitors have also been shown to affect common markers of MDR such as MRP (genistein; Versantvoort *et al.*, 1993, 1995, 1995a and 1996) and Topoisomerase II (emodin; Müller *et al.*, 1996).

Genistein is a naturally occurring dietary protein tyrosine kinase inhibitor that is hypothesized to be reponsible for the lower rate of breast, colon and prostate cancer in asian populations (Steele *et al.*, 1995). The chemical has exhibited various biological properties relevant to chemoprevention, including antioxidant and anti-inflammatory activity, anti-angiogenic properties and anti-metastatic activity. Akiyama and co-workers (1987) and Sibley *et al.* (1987) described a role for this chemical in inhibiting tyrosine kinase activity, an activity inherent to approximately one-half of the known oncogenes

(Hunter, 1984). Since then, genistein is frequently employed as a chemical probe to explore signal transduction pathways. Koroma and de Juan (1997) suggest, however, that the efficacy of genistein as an inhibitor of protein tyrosine kinase is very low in endothelial cells. In agreement with this result is the finding by Peterson and Barnes (1996) that genistein inhibited the growth of breast cancer cells in culture without gross inhibition of protein tyrosine kinase activity. Okura and co-workers (1988) reported the inhibition of H-*ras* morphological transformation in NIH-3T3 cells by genistein. Clark *et al.* (1996) demonstrated the inhibition of breast cancer cell proliferation by genistein mediated through the *ras* signalling pathway. Induction of c-*fos* expression by platelet activating factor in human epidermoid A-431 cells could be inhibited by genistein (Tripathi *et al.*, 1992). Inhibition of protein tyrosine kinase activity by genistein caused a decrease in c-*myc* expression in two colon cancer cell lines, HCT8 and SW837 (Heruth *et al.*, 1995). Liu and co-workers (1994) postulated a role for tyrosine kinase activity in induction of apoptosis (and reduction in *bcl-2*α mRNA levels) by the chemotherapeutic drug taxol which could be blocked by genistein in the human OV2008 ovarian tumour cell line.

Genistein, a non-DNA intercalator, also inhibits the activity of topoisomerase II *in vitro* with IC_{50} values around 111μM (Markovits *et al.*, 1989). Versantvoort *et al.* (1993, 1995, 1996) have reported that genistein is a potent inhibitor of MRP- but not P-gp-mediated daunorubicin transport.

Emodin, an anthraquinone with clinical cathartic properties, exhibits a variety of biological effects including immunosuppressive and vasorelaxant activities and is frequently used as a laxative and anticancer agent in Chinese medicine (Su *et al.*, 1995). Su and co-workers (1995) demonstrated an antimutagenic effect of emodin against the carcinogenic nitro-polycyclic aromatic hyrocarbon, 1-NP. Emodin was found to be a potent inhibitor of topisomerase II activity (Müller *et al.*, 1996), by inhibiting the interaction between topisomerase II and DNA, and reduced DNA damage effects caused by etoposide. Jayasuriya and co-workers (1992) found that emodin inhibits the protein tyrosine kinase $p56^{lck}$ isolated from *Polygonum cuspidatum*. Chan *et al.* (1993) reported a growth-inhibiting effect of emodin on *ras*-transformed cells. Zhang *et al.* (1995) demonstrated the preferential inhibition of c-*erb*B-2 tyrosine kinase activity, without changing c-*erb*B-2 protein levels, and thus blocked growth of the c-*erb*B-2 over-expressing human breast cancer cell lines and induced differentiation of these cells. Genistein, at a

concentration known to inhibit EGFR, showed no obvious effect on tyrosine phosphorylation of c-*erb*B-2 protein in this study. Zhang and Hung (1996) also found that emodin decreased tyrosine phosphorylation of c-*erb*B-2 and preferentially suppressed the proliferation of *erb*-B-2 over-expressing non-small cell lung cancer cells. The authors also found that 30μM emodin sensitised NSCLC cells to cisplatin, adriamycin or etoposide. A study by Liu *et al.* (1996) demonstrated the down-regulation of c-*myc* expression by emodin.

Toxicity assays were carried out on DLKP-SQ and DLKP-SQ/A25010p cells to analyse the effect genistein and emodin had on adriamycin toxicity in these cell lines.

The concentrations of adriamycin chosen were 15ng/ml for DLKP-SQ and 150ng/ml for DLKP-SQ/A25010p. These drug concentrations achieved 10-30% cell kill. Three concentrations of each of the co-selecting drugs was used; 3.75, 7.5 and 15μM genistein and 6.25, 12.5 and 25μM emodin. Increased cell kill was seen in DLKP-SQ/A25010p cells, but not DLKP-SQ, when co-treated with genistein (Table 4.8.1). The higher concentrations of emodin and genistein were quite toxic to the cells. Co-treatment of cells with emodin did not increase the toxicity (but rather induced cell growth) of adriamycin in these cell lines (Table 4.8.1). No reduction in adriamycin toxicity profile was seen with emodin in DLKP-SQ/A25010p, perhaps suggesting that c-*erb*B-2 is not over-expressed in this cell line. As it has been shown that the resistant variants of DLKP over-express this protein it may be interesting to test this chemical on cell lines such as DLKP-A.

The expected non-synergistic effect of combination treatment of adriamycin with cyclosporin A, emodin and genistein is shown in Table 4.8.1 for DLKP-SQ and DLKP-SQ/A25010p. This assay was carried out on triplicate plates on the same day, time constraints prevented further analysis.

Table 4.8.1 Effect of Co-treatment on Survival in DLKP-SQ and DLKP-SQ/A25010p cells

% growth	DLKP-SQ* (expected value)	DLKP-SQ* (actual value)	DLKP-SQ/A25010p' (expected value)	DLKP-SQ/A25010p' (actual value)
0.5μg/ml cycA	70.3	63.1 ± 5.0	85.6	60.0 ± 1.2
1.0μg/ml cycA	72.3	58.1 ± 1.2	84.2	20.5 ± 3.0
2.0μg/ml cycA	66.0	46.7 ± 4.7	79.4	4.9 ± 1.4
3.75μM gen.	58.4	41.8 ± 8.3	79.5	57.1 ± 3.8
7.5μM gen.	29.3	24.1 ± 2.5	57.6	38.5 ± 5.4
15μM gen.	0.8	1.4 ± 1.0	20.8	14.4 ± 0.7
6.25μM emodin	62.7	60.2 ± 7.4	83.8	89.9 ± 7.6
12.5μM emodin	39.9	41.6 ± 8.1	66.9	68.7 ± 6.3
25μM emodin	2.1	3.0 ± 2.6	3.2	5.4 ± 0.9

Values are presented as percentage growth relative to 100% control for untreated DLKP-SQ* or DLKP-SQ/A25010p'. The expected percentage growth is given as the sum of the effect of co-treatment of cells with adriamycin and cyclosporin A (cycA), genistein (gen.) and emodin (emodin) without synergistic effect of co-treatment. Actual growth found is the average of three toxicity assays set up on the same day under the same conditions.

Table 4.8.2 Synergistic/Antagonistic Effect of Co-treatment on DLKP-SQ Variants.

% growth	DLKP-SQ*	DLKP-SQ/A25010p**
non-synergistic response	100.0	100.0
0.5μg/ml cycA	89.8	70.1
1.0μg/ml cycA	80.4	24.4
2.0μg/ml cycA	70.8	6.2
3.75μM gen.	71.6	71.8
7.5μM gen.	82.3	66.8
15μM gen.	175.0	69.2
6.25μM emodin	96.0	107.3
12.5μM emodin	104.0	102.7
25μM emodin	143.0	169.0

Values presented in Table 4.8.2 are given as a percentage of the expected (non-synergistic) growth values described in Table 4.8.1. Values less than 100% describe a synergistic effect of co-treatment with adriamycin; values greater than 100% indicate antagonism between the two drugs. Cyclosporin A (cycA) was found to enhance cell kill by adriamycin in DLKP-SQ and DLKP-SQ/A25010p. Genistein (gen.) produced a synergistic effect in DLKP-SQ/A25010p and at lower concentrations only in DLKP-SQ cells. Emodin (emodin) was found to stimulate cell growth in the presence of adriamycin in this analysis.

4.8.3 Effect of Inhibitors on Development of Drug Resistance

Cyclosporin A, a known inhibitor of P-glycoprotein (Ford, 1995) was used at a non-toxic level as a co-treatment with adriamycin. Following two weekly pulses of adria*mycin* and cyclosporin A, cell survival of DLKP-SQ was reduced to 4% compared to 100% for adriamycin only treated cells. Genistein, a tyrosine kinase inhibitor and also shown to inhibit the MRP pump (Versantvoort *et al.*, 1993, 1995, 1996), displayed a similar response, reducing cell survival to 7% of control. Co-selection with emodin (previously shown to inhibit the activity of c-*erb*B-2 (Zhang *et al.*, 1995; Zhang and Hung, 1996)) was not found to increase cell death and seemed to increase cell survival. A non-toxic level of emodin was used during the pulse selection process of DLKP-SQ with adriamycin.

To establish if the decrease in cell survival caused by co-treatment with genistein and cyclosporin A was due to increased toxicity of adriamycin or an actual effect on signalling pathways repeat analysis was carried out where cell growth was analysed after the first and second pulses.

Co-treatment with cyclosporinA after the first pulse reduced survival to 50% and after the second week survival was expected to be 25%. This was not the case, however, as survival was reduced to only 12%. As cyclosporin A increased toxicity in DLKP-SQ cells in 96 well toxicity assays, the combination treatment may be increasing toxicity of adriamycin by increasing the intracellular level of adriamycin; without affecting a biochemical pathway in the development of resistance. Treatment of DLKP-SQ with 250ng/ml adriamycin for eight hours killed all cells except for one colony, therefore if cyclosporinA is merely increasing toxicity of adriamycin by prolonging drug accumulation it is not as effective as an eight hour treatment with the drug.

Co-treatment with genistein after one pulse had no synergistic effect on cell death, however, after a second pulse, in the presence of genistein, cell survival was reduced to 40%. As genistein had no effect on toxicity of adriamycin in 96 well toxicity assays on DLKP-SQ it may imply that genistein is actually sensitising cells to adriamycin by affecting some biochemical pathway involved in the development of resistance. Further work needs to be done to confirm/deny this hypothesis and should include alternative scheduling of drugs etc. To determine if, after the first pulse, genistein has somehow sensitised cells to adriamycin treatment by affecting a signalling pathway, cells could be

grown for one pulse in adriamycin and genistein and in the second pulse in adriamycin alone. If survival remained at 100% (relative to adriamycin-treatment alone) following the first and second pulses then it is unlikely that genistein had sensitised the cells; however, if a similar trend was seen to the results presented in this thesis it would appear that genistein has acted by affecting some signalling pathway.

The use of DLKP-SQ pulse-selection as a model for the development of MDR and assessment of co-selecting agents is confirmed by these results.

4.9 Ability of Cell Lines to Develop Resistance

It has been found in this laboratory that many cell lines readily develop resistance whereas others do not. DLKP is a cell line that readily adapts to growth in chemotherapeutic drugs and variants stably resistant to adriamycin, etoposide and carboplatin have been established. The A549 cell line, in our hands, has failed attempts to develop resistant variants although other laboratories have published variants of this cell line (Long, 1991; Chevillard *et al.*, 1992).

Throughout the course of this work attempts were made to develop resistant variants of a breast cell line, BT-20, by continuous and pulse exposure to adriamycin. Clonal populations of BT-20 and A549 were used in pulse selections but resistant variants could not be selected. A clonal population of MCF-7 (F.O'Sullivan, pers.comm.) was used as well but the concentrations of adriamycin used were not suitable to establish a resistant variant.

In an attempt to determine why DLKP readily develops resistance a number of genetically manipulated cell variants were established and used in the pulse-selection method developed. DLKP-SQ transfected with *bcl-x$_S$* prevented the establishment of a resistant variant from one clone. DLKP-SQ transfected with c-*fos* ribozyme (thus reducing c-*fos* levels) prevented the establishment of a resistant variant from the three clones tested. Selection from an *mdr1* ribozyme transfected clone, DLKP-I, was successful, however, the degree of efficiency of the ribozyme is unknown as the resistant variant expressed *mdr1* mRNA.

RT-PCR analysis was carried out on a group of six cell lines, three of which have been shown to develop resistance readily (e.g. DLKP (Clynes *et al.*, 1992), MCF-7 (Scaddan and Dufresne, 1995) and OAW42SR (Redmond *et al.*, 1993; Moran *et al.*, 1997)) and the other cell lines have not been successfully selected with common chemotherapeutic drugs in this laboratory (see Section 3.5.3). No genes were identified which could be used to predict sensitivity to chemotherapeutic drugs, by RT-PCR.

4.10 Breast Cancer - A Model for Acquired Drug Resistance

35% of women diagnosed with breast cancer die as a consequence of the disease which is now the leading cause of death among women in the Western world (Harris *et al.*, 1992). Tumours are initially sensitive to chemotherapy but frequently relapse giving rise to untreatable disease.

A number of studies have suggested a role for P-glycoprotein in drug resistant breast tumours (for review see Giaccone *et al.*, 1995) and as a marker for a more malignant phenotype. Other common MDR markers such as MRP, LRP, Topoisomerases have also been implicated. Schneider *et al.* (1994) found a significant association between *mdr1* and c-*erb*B-2 expression in operable mammary carcinomas indicating that *mdr1* may be a marker of tumour aggressiveness.

4.10.1 Treatment of Breast Cancer

Anthracyclines are the most effective single agents in the treatment of breast cancer (Bonadonna *et al.*, 1993). These commonly used agents have the unfortunate side effect of resulting in the development of the multidrug resistant phenotype commonly seen in relapsed breast cancer patients. These relapsed patients previously treated with anthracyclines are known to have a very poor prognosis and second line chemotherapy with unrelated drugs e.g. cyclophosphamide : methotrexate : 5-fluorouracil is ineffective. For this reason it is very important that predictive markers be found for patients presenting with primary tumour; this may be done by studying pre- and post-chemotherapy patient tumours and identifying common genetic alterations which could then be used as predictive markers of treatment outcome.

4.10.2 A Cellular Model for Drug Resistance in Breast Cancer

It was attempted in this study to develop a resistant variant of BT-20 (derived from a tumour which had not been treated by chemotherapy) to adriamycin (used to treat primary breast cancer) and to the combination cyclophosphamide, methotrexate and 5-fluorouracil (secondary chemotherapy regimen).

Initial observations when assessing the toxicity profile of this cell line revealed an extraordinarily high resistance to methotrexate but relative sensitivity to other drugs

assayed (Table 3.12.1). This was quite surprising as the patient from whom BT-20 cell line had been established had not been treated with chemotherapy. Combination selection was not attempted on finding this result. Methotrexate toxicity levels in MCF-7 were relatively quite low. Methotrexate is an analogue of folic acid and exerts its cytotoxic effect by competitive inhibition of the cytosolic enzyme dihydrofolate reductase (Waltham *et al.*, 1988). Mechanisms of resistance to methotrexate include increased dihydrofolate reductase activity, altered binding to the enzyme, decreased drug uptake and decreased polyglutamination (for review see Skovsgaard *et al.*, 1994). It will be interesting to study these aspects in the BT-20 cell line.
Selection with adriamycin was also undertaken but proved unsuccessful for both continuous selection process and selection by pulse exposure (Section 3.12).

A previous report states that this cell line is oestrogen and progesterone receptor negative (Koechli *et al.*, 1994), which are predictive of good prognosis (Sirvent *et al.*, 1995). It also expresses the p53 gene but not the *bcl-2* gene. MCF-7 cells, which do develop resistance readily, are known to have the opposite expression of the above markers of prognosis. Comparing the effect of adriamycin on these cell lines, as described in Section 3.11, should be undertaken as a model of gene induction by adriamycin in breast cancer.

4.10.3 Analysis of mRNA levels in archival breast tissue

Tumorigenesis is a multi-factorial problem with alterations to many genes observed both at the transcriptional and translational stages. A similar problem is seen in the phenomenon of multidrug resistance whereby a wide range of gene expression alterations have been identified. To determine clinically relevant markers to predict the development and identification of drug resistant tumours a panel of genes needs to be studied. Clinical specimens are often limiting in size, however, and may prevent the analysis of a broad range of putative MDR markers by traditional approaches such as immunohistochemistry, thereby jeopardising a correct diagnosis.

Analysis of many oncogenes has shown alterations in expression either by amplification or an increase in transcription levels; the same is true for many of the common hallmarks of multidrug resistance. Changes in both gene amplification and transcription are reflected in the resulting mRNA levels and it is for this reason (and the fact that very sensitive techniques such as RT-PCR require small amounts of tissue) that mRNA levels can be used to study gene expression and alterations in drug resistant cell lines and human tumours.

The mRNA levels of specific genes are generally studied using such techniques as 'Northern Blot' (Alwine *et al.*, 1977), 'RNA slot/ dot blot' (Kafatos *et al.*, 1979.), 'RNase protection assay' (Reyes and Wallace, 1987), '*in situ* hybridisation' (review of method see Leitch, 1994) and RT-PCR (for review see Wright and Wynford-Thomas, 1990).

Northern blotting is semi-quantitative but is insensitive and 5-10μg of purified poly (A)+ RNA is required to produce a signal. It is also time consuming as it frequently necessitates the use of ^{32}P-labelled probes. The amount of RNA and the length of time required to carry out analysis by Northern blot is, therefore, unfavourable in a clinical setting where specimen size is limiting and a diagnosis required in as little time as possible.

RNA slot/blot technique also faces criticism for routine use in a clinical situation. The technique is also semi-quantitative but also insensitive, requiring 1-10μg of poly (A)+ RNA to produce a signal. In addition, the method is not dependable for detecting extremely rare sequences because of background problems.

Analysis by RNase protection assays are more sensitive than the Northern or slot/dot

blotting (100ng to 1μg of poly (A)+ mRNA is required for each assay). The method is, however, quite time consuming due to the use of radioactive detection methods and a specific probe for each mRNA.

The most sensitive techniques used for analysis of mRNA levels, especially in a clinical setting, are hybridisation and RT-PCR analysis.

In situ hybridisation can detect 10 to 100 molecules of mRNA in a given cell and in a clinical specimen can be used to detect mRNA in a 3-D environment in the way that immunohistochemistry can be used to detect the 3-D localisation of specific antigens. The main disadvantages of *in situ* hybridisation are that it is technically difficult and, similarly to immunohistochemistry, is time consuming and requires similar amounts of tissue to process a panel of genes. This method does not allow for quantification of transcripts.

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis is 1,000 to 10,000 times more sensitive than traditional blot techniques and can be used to detect multiple mRNA signals in as little as 1 to 1,000 cells. The procedure has the added advantage of speed which would be benificial in a clinical situation and can be used to analyse RNA of extremely rare abundance or in small amounts of total RNA - an obvious advantage if dealing with minimal biopsy material. A major disadvantage of RT-PCR analysis is intrinsic to the procedure itself and that is the exponential nature of the reaction which could lead to false positives. From a diagnostic view-point the major disadvantage of analysis by RT-PCR is the fact that no information on 3-D distribution is seen - this would be important if a specific gene was only expressed in a certain number of cells, it is also very important if this procedure is being used for tumour analysis to insure that the tissue being analysed does not contain normal tissue as well.

An *In situ* - RT-PCR procedure is now being developed to combine the advantages of both techniques, the procedure is as yet in its infancy and great care is needed to minimise on background contamination leading to false positives.

4.10.3.1 Analysis of mRNA by RT-PCR

RT-PCR analysis was first published by Veres *et al.* (1987) as an adaptation of the basic PCR method for the study of mRNA in a manner similar to that of DNA.

mRNA is produced by transcription from DNA in all cells; an enzyme was discovered, however, which allows the reverse of this fundamental molecular control to take place and

produce DNA from an RNA template. This reverse transcriptase enzyme was first discovered by Temin (1972) and was shown to have three enzymatic activities:

(i) copy an RNA molecule to yield double-stranded DNA-RNA, using a primer and joining deoxynucleotide triphosphates in a 3'-5' linkage;

(ii) degrade RNA in a DNA-RNA hybrid;

(iii) copy a primed single strand of DNA to form double-stranded DNA:

on which the RT-PCR method is based.

RT-PCR is a disruptive method (unlike *in situ* hybridisation) whereby RNA is extracted from cells or tissue. Total RNA is sufficient for most RT-PCR analysis, however, some situations demand the use of poly (A)+ mRNA. The extraction of undegraded RNA from cells and tissues can often prove difficult (due to the labile nature of RNA and the presence of active ribonuclease enzymes in the specimen). To prevent this problem cells or tissues are usually lysed in a solution containing a strong protein denaturant e.g. guanidium thiocyanate.

Extracted mRNA is selectively primed to act as a template on which double-stranded DNA (complementary DNA) can be formed by the reverse transcriptase enzyme. The cDNA formed can then be amplified and studied as in a typical PCR reaction for the presence of specific genes. A choice of primers can be used to selectively prime the mRNA and these include:

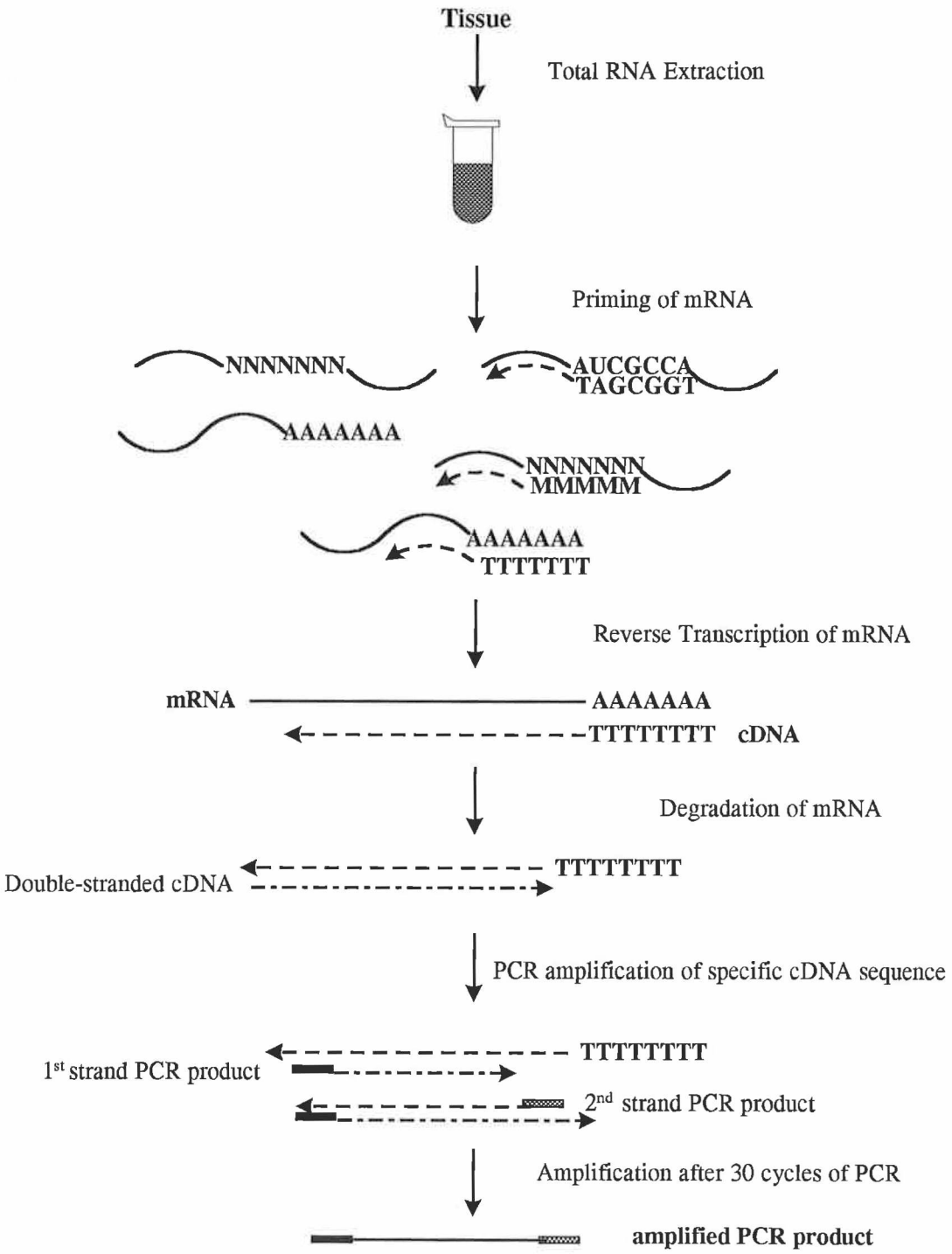
(i) oligo (dT) primers (for eukaryotic RNA);

(ii) random hexanucleotide primers;

(iii) 3' antisense gene-specific primer used in the PCR reaction.

It should be possible to prime undegraded mRNA with oligo (dT) primers only. However, degraded mRNA (from clinical specimens) may need a combination of all of the above primers to produce amplified cDNA by RT-PCR.

Figure 4.10.3.1.1 Analysis of mRNA by RT-PCR



TGGCGAT - hypothetical 3'antisense gene-specific primer used in the PCR reaction
MMMMM - random hexanucleotide primers
TTTTTTT - oligo (dT) primer
- 5'sense gene-specific primer
- 3'antisense gene-specific primer

4.10.3.1.1 RT-PCR Analysis in Clinical Studies

Analysis by RT-PCR of clinical samples suffers from one major flaw and that is its failure to correlate with histological findings. Tumours comprise of heterogenous cell populations some of which express the gene of interest, others may not. An added problem when one analyses a piece of tissue by RT-PCR is that the specimen under analysis may not be pure tumour.

Studies have been published on RT-PCR analysis of fresh tissue and to a limited extent on formalin fixed paraffin embedded archival tissue. It is relatively simple to extract good quality RNA, suitable for RT-PCR, from fresh tissue and a number of commercially avaliable kits are now available for quick extraction of total RNA or poly (A) mRNA e.g. Ultraspec (Biotecx) or Mini-Message Maker (R&D Systems). Extraction of RNA from fixed tissue is much more difficult, however, as the RNA is often degraded by such fixation methods. A number of studies have reported successful amplification of mRNA by PCR from paraffin embedded tissue; these reports have only been successful for the amplification of one or two genes that are expressed at high levels in all cells e.g. β-actin (Ben-Ezra *et al.*, 1991), Aldolase A (Finke *et al.*, 1993), GAPDH and HPRT (Foss *et al.*, 1994). A recent report published by this laboratory (O'Driscoll *et al.*, 1996) demonstrated successful amplification of mRNAs for a variety of MDR-related genes in 10 breast tumours. A study including a larger group of specimens has not yet been published.

4.10.3.1.2 Analysis of mRNA levels in breast tissue by RT-PCR

A method has previously been reported in this laboratory which allowed for the extraction of mRNA from 6 archival paraffin-embedded breast tissue (O'Driscoll *et al.*, 1996) and successful amplification of MDR-related genes. A study was undertaken in this thesis to extract RNA from a broader range of breast tumours, including 15 paired pre- and post-chemotherapy patient blocks. Success was limited in this study as the previously described protocol (O'Driscoll *et al.*, 1996) did not sufficiently digest tissue and therefore a possible source of RNA was lost.

Improvements to the digestion protocol were undertaken and this included increasing digestive enzyme concentrations and frequency of addition (up to every 2 hours). This improved digestion of some tissues, but not all.

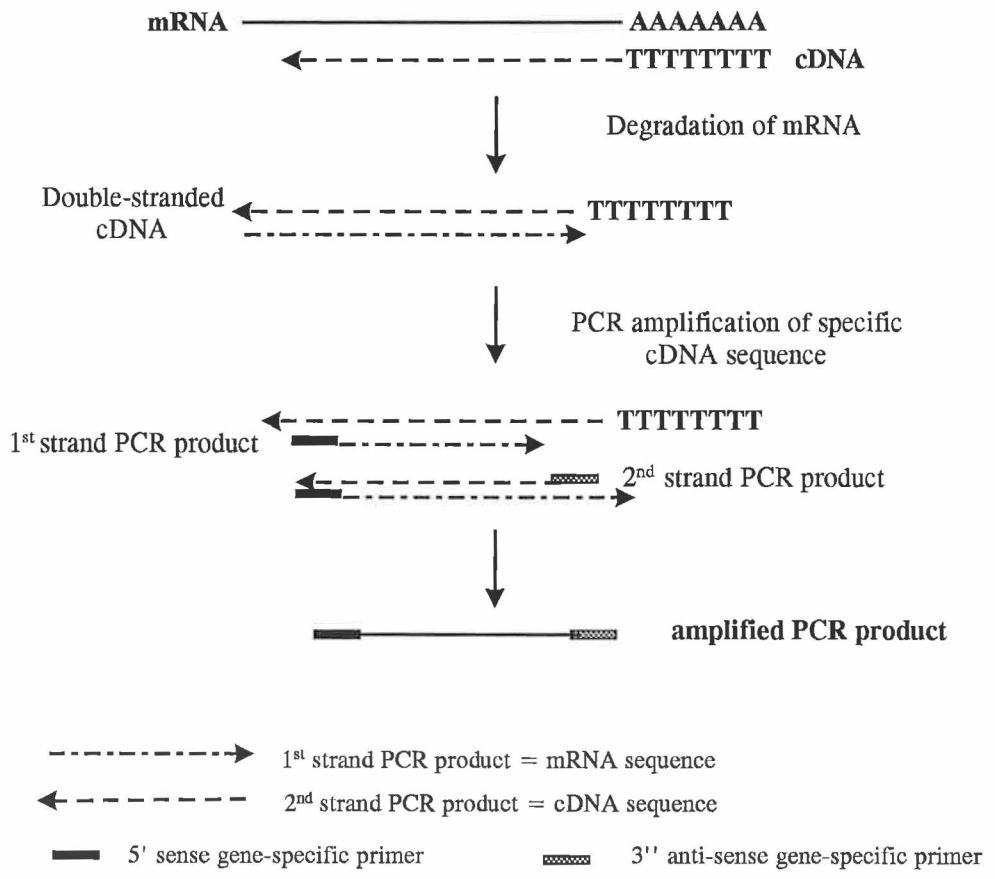
To allow for easier and more efficient tissue digestion, a number of commercially

available digestion or lysate buffers were used. Lysis of a section from tonsil was carried out in a solution containing guanidine and acid phenol (from Ultraspec II kit, Biotecx). RNA was previously isolated from this tissue using standard methods (O'Driscoll *et al.*, 1996) and successfully amplified by RT-PCR for the analysis of Topoisomerase IIα and β-actin. RNA extracted from the tissue by lysis in the guanidine/phenol solution was not amplified successfully by RT-PCR. A milder buffer was then used to digest archival tissue, this buffer (from R&D Systems mini-message maker kit) does not contain guanidine or phenol and digestion of all tissue to date using this buffer has been successful. The buffer was further supplemented with Proteinase K.

Isolation of RNA from tissues using this protocol was not of sufficiently high quality to be reverse transcribed by the method described previously (O'Driscoll *et al.*, 1996) which used random and oligo (dT) primers to reverse transcribe mRNA. These steps take into account the degraded nature of mRNA as random primers bind to sequences throughout the length of the mRNA molecule and not just at the 3' end of a full mRNA, which may be degraded in archival tissue (see Figure 4.10.3.1.1).

It was found, however, that the above rationale was not sufficient to reverse transcribe mRNA isolated in this study. Reverse transcription is used to transcribe, where possible, total mRNA populations. When studying degraded mRNA of particular genes it is possible to home-in on the actual mRNA sequence of the gene to be analysed by PCR and specifically reverse transcribe that mRNA fragment. This can be done using the 3'antisense primer used in subsequent PCR reactions, the justification for this is described in Figure 4.10.3.1.2.1. Using this method of reverse transcription, amplification of a band for *β-actin* (Figure 3.13.1.1) was observed for RNA extracted from a tumour block which did not amplify previously when reverse transcription was carried out using random and oligo(dT) primers only.

Fig. 4.10.3.1.2.1 Reverse Transcription of mRNA - Rationale for Using 3'Specific anti-sense primers



Full length undegraded mRNA is transcribed above using poly(dT) primers. cDNA is formed which is complementary to this mRNA strand. The mRNA strand is then degraded and a second cDNA strand is formed, complimentary to the cDNA to produce double stranded cDNA. This second cDNA strand has the identical sequence to mRNA.
In the PCR reaction a specific 5'sense primer binds to the first cDNA strand and produces a 1st strand PCR product. A specific 3'anti-sense primer binds to the second cDNA strand (identical to the mRNA) and produces a 2nd strand PCR product.
As the 3'anti-sense primer binds to an identical site on the second strand cDNA (identical to mRNA), it will also bind to mRNA and can be used to prime mRNA for reverse transcription.

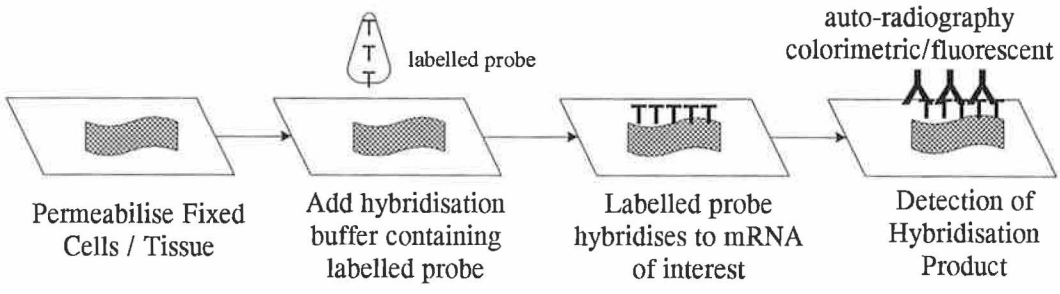
More recently a study was begun to analyse mRNA levels of specific genes isolated from fresh breast tissue. To date success has been achieved on 14 fresh breast tissues for the expression of *β-actin* although analysis of a range of specific genes was only successful on four of these tumours. Similar samples from the same tumours have been processed in St.Vincent's Hospital (Dublin) for paraffin-embedding and studies will be carried out on the recovery of RNA extracted from archival tissue compared to corresponding fresh tissue. Further optimisation of RT-PCR of mRNA extracted from archival tissue is required, however.

4.10.3.2 Analysis of mRNA by *In situ* Hybridisation

Analysis of mRNA by *In situ* hybridisation involves the direct hybridisation (i.e. RNA is not extracted from the specimen) of a specific probe to the material in question e.g. paraffin-embedded archival tissue, frozen sections or cell preparations (cytospins or cell sections cut from embedded agar plugs). The probe can either be single-stranded DNA or anti-sense RNA and is either isotopically (with e.g. ^{35}S) or non-isotopically (Digoxigenin or Biotin) labelled. RNA : RNA duplexes are most stable and therefore a labelled RNA probe is preferred for hybridisation with mRNA. The hybridised probe is then detected *in situ* using isotopic development or fluorescent or colorimetric non-isotopic protocols.

For accurate analysis of mRNA expression by *in situ* hybridisation a number of controls must be set up with the positive slide. These controls should include hybridisation with (i) sense RNA probe, (ii) control probe (gene not present in specimen under investigation), (iii) oligo-dT or *β-actin* probe (to assess mRNA quality throughout the specimen), (iv) pre-treatment with RNase (to ascertain if probe is actually binding mRNA) and (v) hybridisation without labelled probe.

Figure 4.10.3.2.1 *In situ* Hybridisation of Labelled Probe to mRNA



4.10.3.2.1 *In situ* Hybridisation Analysis in Clinical Studies

In situ Hybridisation is a relatively new histological technique to be used as a diagnostic tool and its current uses are mainly for the detection of viral infections within cells and analysis of gene expression in cancer (Lloyd *et al.*, 1994). The technique can be used on frozen or paraffin-embedded sections therefore has the added advantage of being used in retrospective studies.

A number of reports have been published on *mdr1* expression by *in situ* hybridisation on

paraffin-embedded sections (Marie *et al.*, 1992; Vergier *et al.*, 1993; Ramani and Dewchand, 1995).

4.10.3.2.2 Optimisation of *In situ* Hybridisation Protocol for the detection of *mdr1*

Analysis of tumour tissue by RT-PCR has a major disadvantage in that it is a disruptive technique. analysis of mRNA levels by *in situ* hybridisation is used to detect expression of particular mRNAs in intact tissue sections. The technique is, however, time-consuming and technically difficult and thus limits its use as a common diagnostic tool to probe tissues for expression of mRNAs. As RT-PCR analysis from paraffin sections is limited, *in situ* hybridisation may be the method of choice for analysing mRNA levels in archival sections.

It was intended in this study to develop a protocol which would allow specific detection of *mdr1* expression in archival paraffin-embedded breast tissue, pre- and post-chemotherapy, and if possible compare results to that found by RT-PCR.

Optimisation of this protocol (described in Section 2.11.2) on foetal liver sections (known to express *mdr1*) was carried out. Specific staining in the erythroid pre-cursor cells was detected and staining pattern agreed with that seen by immunohistochemistry for P-glycoprotein (Figure 3.13.3.1) expression. *mdr1* expression was also detected by RT-PCR on mRNA extracted from the same foetal liver block (Figure 3.13.1.1(b)). Previous reports have also described detection of *mdr1* expression by *in situ* hybridisation (Marie *et al.*, 1992; Vergier *et al.*, 1993; Ramani and Dewchand, 1995). A study, for the detection of *mdr1* by *in situ* hybridisation, on pre- and post-chemotherapy paired tumours has not been published to date. In this study successful *in situ* on eight tumour blocks from breast cancer patients. These tissue blocks consisted of four paired pre- and post-chemotherapy blocks from patients who had been treated with a CMF regimen. Successful staining with poly(dT) to assess mRNA levels throughout the sections was achieved, although it was observed that mRNA preservation throughout the section was not uniform. *mdr1* expression was detected in all sections analysed. No conclusion can be made as to the significance of this expression as comparison with immunohistological detection of P-glycoprotein levels is awaited.

The surprising conclusion drawn from this study is that mRNA extraction from archival sections is not dependable as the mRNA extracted is highly degraded. Ironically,

therefore, *in situ* hybridisation could be used to detect the presence of the poly-A tail, the "hallmark" of mRNA preservation, in the same tissue sections. *In situ* hybridisation was also successful in detecting a specific gene product, using a probe of approximately 1kb. These results would imply that mRNA analysis in archival sections is best achieved by *in situ* hybridisation, although analysis by this method is time-consuming.

4.11 Summary and Conclusions

1. This thesis investigated the role of oncogene expression, at mRNA level, in multidrug resistance of human cancer. Analysis of oncogene expression in drug resistant variants of the human squamous lung cell line, DLKP, and the human ovarian cell line, OAW42, was carried. Expression of c-*erb*B-2, c-Ha-*ras1*, c-*fos*, c-*myc* and the *bcl-2* family members, *bcl-x* and *bax*, was investigated on adriamycin- (DLKP-A, OAW42A, OAW42A1), VP-16- (DLKP-VP3, DLKP-VP8) and carboplatin- (DLKP-C14) selected variants of the DLKP and OAW42 cell lines. Increased expression of the *bcl-*x_L, *bax* and c-*erb*B-2 genes was seen in all the resistant variants of DLKP analysed. c-*fos* levels were elevated only in the DLKP-A variant. c-*myc* was up-regulated in the carboplatin-selected cell line but was down-regulated, when compared to DLKP, in the other DLKP variants analysed. c-Ha-*ras1* levels were elevated in the DLKP variants, with the exception of the DLKP-VP8 cell line. Elevations of these genes in the OAW42 resistant cell lines was not observed and a decrease in c-*fos* levels was observed in the adriamycin-selected resistant variants, OAW42A and OAW42A1.
2. As the resistant variants of the DLKP and OAW42 cell lines analysed were a heterogenous population, it was decided to analyse the expression of the same oncogenes in clonal sub-populations of the DLKP-A cell line. Analysis was carried out on the most sensitive clone, DLKP-A2B, and the most resistant clone, DLKP-A5F. Analysis revealed a greater *bax*:*bcl-*x_L ratio in the least resistant clone, DLKP-A2B, when compared to DLKP-A5F, suggesting that the sensitivity of the former may, in part, be due to the balance of apoptosis-regulating genes. c-*erb*B-2, c-Ha-*ras1* and c-*fos* expression was also up-regulated in the more resistant DLKP-A5F sub-population. These results suggest that analysis of gene expression in clonal populations of cells may provide greater insight into the mechanisms of drug resistance.
3. Elevated levels of the c-*fos* gene were found in the DLKP-A cell line. c-*fos* had previously been reported in the literature to affect drug resistance with partial sensitivity restored by down-regulation of the c-*fos* gene. In this study, a c-*fos* ribozyme was used to down-regulate the expression of the c-*fos* gene in the DLKP-A cell line. Six clones expressing the c-*fos* ribozyme were isolated from the heterogenous population. The ribozyme is in an inducible plasmid and expression is induced by addition of dexamethasone. The clones analysed had a lower resistance to adriamycin than the parental DLKP-A cell line. Minimal effect of dexamethasone induction was

seen on toxicity levels. Unfortunately, steroids, which can substitute for dexamethasone, are also found at basal levels in serum. In the ribozyme-expressing sub-populations of DLKP-A, ribozyme expression was seen without the addition of supplemented dexamethasone. It is unknown, therefore, if the lowered resistance levels are due to a decrease in c-*fos* levels or whether more sensitive sub-populations had been cloned from the parental DLKP-A cell line. Lower expression of *mdr1* in the ribozyme-expressing clones was also seen. Unsuccessful attempts were made to grow one of the ribozyme-expressing clones in serum-free medium which would remove basal levels of dexamethasone. Transfection of the ribozyme into the clonal sub-population, DLKP-A5F (shown to over-express c-*fos* mRNA), did not affect overall toxicity levels of adriamycin. However, individual colonies were not isolated following transfection.

4. The cell lines used in the initial part of this study were developed by continuous exposure to drug for prolonged periods and are resistant to drug concentrations above those achieved in the clinic. As patients can develop drug resistance after only a few courses of chemotherapy, a more clinically relevant *in vitro* model of drug resistance was developed by pulse exposure of clonal populations of DLKP, DLKP-SQ and DLKP-I, to clinically attainable levels of drug for a short period of time. The resulting resistant variants, DLKP-SQ/A25010p and DLKP-I/A25010p, were developed following ten weekly exposures of the sensitive parental lines to 250ng/ml adriamycin. The resistant variants were found to be ten and sixteen fold resistant to adriamycin, respectively. These cells exhibited a classical MDR phenotype in that increased resistance to vincristine and VP-16 was also demonstrated. No alteration in 5-fluorouracil toxicity level was seen. Decreased drug accumulation was observed in the resistant variants. The resistant variants displayed up-regulated *mdr1* expression.
5. Characterisation of the pulse-selected resistant variant of DLKP-SQ revealed increased expression (by RT-PCR) of the *bcl-x*$_L$, *bax*, c-*erb*B-2 and c-*fos* genes relative to the sensitive parental line. To determine the role expression of the *bcl-2* family of genes plays in this resistant model, *bcl-x*$_S$, the apoptosis-supporting gene, was transfected into the resistant DLKP-SQ/A25010p cell line. Clonal populations stably expressing the *bcl-x*$_S$ gene were analysed and it was found that the clone expressing the most *bcl-x*$_S$ showed the greatest increase in sensitivity to adriamycin, VP-16 and vincristine; no alteration in 5-fluorouracil sensitivity was observed. This result suggests that over-expression of the *bcl-x*$_L$ gene in the DLKP-SQ/A25010p cell line plays a role in the

resistance of that cell line to chemotherapeutic drugs. Initial toxicity work was undertaken to determine if co-treatment of DLKP-SQ/A25010p with agents known to affect cellular signalling cascades and common MDR markers could enhance toxicity of adriamycin in this cell line. Co-treatment with cyclosporin A (a P-glycoprotein inhibitor) enhanced the toxicity of adriamycin in this cell line suggesting that *mdr1* over-expression in the DLKP-SQ/A25010p has a significant role in the resistance seen in this cell line. Co-treatment with genistein (a tyrosine kinase inhibitor) also partially enhanced the toxicity of adriamycin, indicating that a kinase pathway may be involved in the resistance of the DLKP-SQ/A25010p cell line. Co-treatment with another kinase inhibitor, emodin, previously shown to target c-*erb*B-2, did not enhance the toxicity of adriamycin in the DLKP-SQ/A25010p cell line.

6. To determine the initialising events in the establishment of the MDR phenotype in the pulse-selected model, gene induction studies following treatment with adriamycin were carried out on the DLKP-SQ cell line. Initial results indicated an up-regulation of *bcl-*x_L and c-*erb*B-2 mRNA. This result could not be repeated, however. In an attempt to modulate resistance development by molecular methods, the DLKP-SQ cell line was transfected with the *bcl-*x_S cDNA and the c-*fos* ribozyme. Pulse exposure of five *bcl-*x_S expressing clones to adriamycin resulted in growth of four of the clones but one clone, *bcl-*x_S.1, was not susceptible to resistance development. This result may suggest that the up-regulation of *bcl-*x_L in DLKP-SQ is an initialising event in the development of drug resistance in the DLKP-SQ model. DLKP-SQ cells transfected with the c-*fos* ribozyme did not develop resistance when exposed to adriamycin suggesting a role for c-*fos* in the response of DLKP-SQ to cellular stress caused by chemotherapeutic drugs.
7. As *mdr1* levels were also increased in the pulse-selected resistant variants of DLKP, attempts were made to develop a resistant variant of a clonal population of DLKP-I previously transfected with the *mdr1* ribozyme. A resistant line was established which expressed *mdr1* mRNA but to a lesser extent than that expressed in DLKP-I/A25010p. Resistance levels were similar in the resistant variants developed from DLKP-I and the *mdr1* ribozyme expressing clone. This finding, and subsequent adriamycin accumulation studies, suggest that factors in addition to P-glycoprotein over-expression may be involved in the resistant variants of these pulse-selected variants.
8. Molecular studies of gene expression/alteration in the pulse-selected variant of DLKP-SQ suggested that this may be a novel system amenable to the search for inhibitors of resistance development. Initial studies have been carried out in this thesis to determine

if known inhibitors/circumventors of the MDR phenotype or oncogene signalling cascades affected the development of resistance using the DLKP-SQ pulse method. Results indicate that a P-glycoprotein inhibitor (cyclosporin A) and a tyrosine kinase inhibitor (genistein) affect the development of resistance. It remains to be seen if this is due merely to increased toxicity effects or due to inhibition of a signalling pathway. The potential usefulness of DLKP-SQ pulse-selection as a model for the development of MDR and the assessment of co-selecting agents is confirmed by these results.

9. Attempts were made throughout the course of this thesis to develop resistant variants of a number of cell lines, including the lung cell line, A549, and the human breast cell line, BT-20. Failure to establish resistant variants of these two cell lines prompted analysis of gene expression in a group of six cell lines, three of which were known to develop resistance while the other three did not. No consistent alteration in gene expression was observed for the group of cell lines that did vs. did not develop resistance. One interesting finding in analysis of the BT-20 cell line was its extraordinarily high resistance levels to methotrexate, a surprising result as the patient from whom the BT-20 cell line was derived had not been treated with chemotherapy. The fact that a given tumour could be inherently resistant to one drug may account, in some instances, for the lack of response to chemotherapy.
10. As this thesis and other reports have shown that expression of certain oncogenes can affect the development of resistance in human cancer cell lines. It is essential in the clinic that a broad range of prognostic factors such as *mdr1*, *bcl-2* family, c-*fos*, c-*erb*B-2 and other genes are analysed in a given tumour to determine the patient's response to a given chemotherapeutic regimen. As clinical material is often limiting a technique is required which will allow the analysis of many parameters with minimal tissue. RT-PCR would be an acceptable technique; however, extraction of mRNA from routinely-processed archival tissue is quite difficult. A previous report in this laboratory described the extraction and analysis by PCR of mRNA from archival breast tissue. In this thesis attempts were made to expand that study. Analysis of RNA extraction from thirty samples was unsuccessful using this protocol, although further optimisation of the reverse-transcriptase step improved RT-PCR analysis. This finding implies that analysis of mRNA from archival sections could not be relied on as a routine technique. Analysis of mRNA in eight randomly selected tumours out of the thirty by *in situ* hybridisation for *mdr1* did, however, prove successful. Analysis of fresh tumour samples was also carried out by RT-PCR.

4.12 Future Work

1. Further work arising from this thesis must include analysis of oncogene expression in the DLKP and OAW42 variants at the mRNA level, by other techniques such as Northern blotting, and at the protein level using Western blotting techniques. Further characterisation of the c-*fos* ribozyme transfected DLKP-A cell lines could include optimisation of a serum-free media to support the growth of the clones. A comprehensive toxicity profile of the transfected cells should also be compiled.
2. Characterisation of the pulse-selected variants of DLKP-SQ and DLKP-I should be completed. This would include analysis at the mRNA and protein levels for the expression of MDR-related genes and oncogenes discussed in this thesis. A complete toxicity profile including analysis of cisplatin, taxol and other drugs should be analysed and may indicate which mechanism of drug resistance is involved in the resistant cell line. Pulse-selected variants of these cell lines could also be established using other chemotherapeutic drugs to obtain a panel of cell lines displaying resistance to clinically attainable levels of drug.
3. Drug accumulation studies in the DLKP-SQ and DLKP-I resistant variants should be repeated and compared to the highly resistant cell lines such as DLKP-A. These studies could also include co-treatments with agents such as cyclosporin A or verapamil to inhibit P-glycoprotein.
4. Further characterisation of the DLKP-SQ/A25010p-*bclx*$_S$.3 and DLKP-SQ/*bcl-x*$_S$.1 cell line should include complete toxicity profile including drugs such as the heavy metals, taxol and others. Analysis of oncogene and MDR-related gene expression should be carried out at the mRNA and protein levels. Drug accumulation studies could also be analysed. The reduction in resistance levels seen in this variant suggests the DLKP-SQ/A25010p model may be a good system to target by *bcl-x*$_L$ anti-sense technology.
5. As the DLKP-SQ/*bcl-x*$_S$.1 cell line was not susceptible to resistance development by adriamycin may indicate that DLKP-SQ cells targetted by *bcl-x*$_L$ anti-sense technology and exposed to adriamycin could also be prevented from developing resistance. Selection of the DLKP-SQ/*bcl-x*$_S$.1 cell line with clinically relevant levels of other chemotherapeutic drugs should also be undertaken to determine if *bcl-x*$_S$.1 effect is drug-specific, a fact that is not well documented in the literature. DLKP-SQ cells could also be transfected with the *bcl-x*$_L$ cDNA to determine if this results in increased resistance to a similar profile of drugs.

6. Characterisation of the c-*fos* ribozyme expressing DLKP cells should be completed. Exposure of these cells to different drugs may also be interesting. Optimisation of a seum-free medium for these cells would also be useful to ascertain the true effect of ribozyme induction on chemosensitivity levels. As c-*fos* and c-*erb*B-2 levels were over-expressed in drug resistant variants of the DLKP cell line, transfection of DLKP-SQ with these cDNAs may determine if over-expression of these genes increases chemoresistance in the transfected variants.
7. Further characterisation of the *mdr1* ribozyme transfected resistant variant, DLKP-I*mdr1*Rz2D4/A25010p, is required. Further drug accumulation studies should be undertaken, including co-incubation with P-glycoprotein inhibitors. Selection from the sensitive clone with other chemotherapeutic drugs could be analysed.
8. The effect of tyrosine kinase inhibitors on the establishment of the MDR phenotype in the DLKP-SQ model should be analysed. This model could also be used to search for inhibitors of drug resistance development.
9. Gene induction studies following exposure to different chemotherapeutic drugs could be established and oncogene and MDR-related gene expression analysed. Further experiments could be undertaken to determine if resistance development is a result of gene induction or selection.
10. Characterisation of the BT-20 cell line and its inherent resistance to methotrexate should be addressed.
11. Clinical studies in this thesis suggest that further optimisation of mRNA extraction from archival sections is required before it can be used for RT-PCR analysis. Analysis of gene expression in paraffin sections by *in situ* hybridisation may be a more routine method of analysis.

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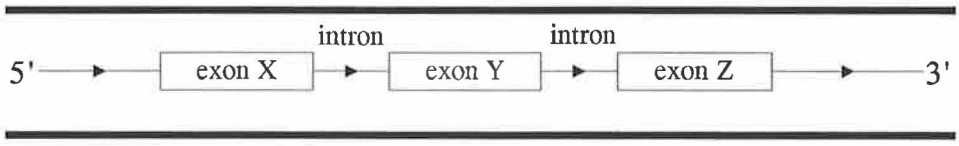
6. Appendices

Appendix A Guidelines used in Choosing Primers for PCR Analysis

To successfully amplify cDNA by PCR, specific primers to the gene of interest must be chosen to prime the cDNA for PCR amplification. a number of guidelines should be followed, when feasible, to design the best primer pair to give successful amplification of a PCR product of suitable size.

Figure A.1 Hypothetical Gene Structure



(i) Complementarity to Template = Specificity

This is the fundamental requirement when chosing a primer. The primer must be chosen to hybridise specifically to the gene of interest and not to any other gene which may be present in the mRNA population. If one primer binds to an extra template the primer is wasted through competition with this sequence; however, if both primers bind to this extra template another region will be amplified thereby making analysis more difficult.

To find the most homologous sequences to be used as primers, DNA data-bases (such as the one held by EMBL) must be searched. When the sequence of interest is sent to the data-base it is compared to all known sequences and the first fifty sequences to which it has greatest homology will be aligned to the sequence of interest, their percentage homology will be calculated and the position on the template shown and returned to the sender.

Care must be taken, however, to avoid areas of secondary structure formation or complimentarity between primers. Long stretches of purines or pyrimidines must also be avoided.

(ii) Primer Length and Composition and Target Length = Efficiency

Primers are normally chosen to be 18-30 base pairs in length, shorter or longer primers could be used if absolutely necessary. A balance must be met when deciding on primer length, however; obviously by increasing the number of base pairs included greater specificity could be achieved but the disadvantage of long primers include increasing the

possibility of introducing secondary strucure into your primer and increasing the annealing temperature (see below) of the primer. The length of a primer pair should be equal if possible.

If possible primers should have a balanced G/C and A/T concentration. The distribution of bases should be random and long stetches of polypurines and polypyrimidines should be avoided - this is to prevent non-specific binding and avoid the possibility of secondary structure formation. Secondary structure could also occur if a primer possesses palindromic sequences and if such sequences are present then this area of the gene should be avoided.A primer pair should not possess complimentary sequences to each other, especially at thier 3' end as this could result in the formation of "primer-dimers" i.e. both primers annealing at thier 3' ends producing a template that will compete with the target of interest.

The base pair composition of any given primer determines its specificity and annealing temperature to the cDNA during amplification. Increasing this temperature reduces the annealing of incorrect primers. Primers should be chosen whose Tm (i.e. the temperature at which half of the duplex is dissociated) is between 55°C and 75°C. The appropriate PCR annealing temperature may be equal to the melting temperature or 5°C below this temperature. The T_m for a given primer can be estimated from the following equation:-

$$T_m = 2°C \text{ (no. of A+T residues)} + 4°C \text{ (no. of G+C residues)}$$

Optimum amplification will be achieved if the difference between the primer pairs is between 180 and 500 base pairs.

(iii) Location on Template = Analysis

The complimentary primers chosen should be designed so that any contaminating DNA could be differentiated from the amplified cDNA. This is most effectively done by picking primers which (i) flank introns i.e. primer (a) from exon X and primer (b) from exon Z or (ii) span introns i.e. primer (a) 5'region in exon X and 3'region in exon Y and primer (b) in exon Z. Either of these methods will show if there is contaminating DNA present as this will present itself as a longer PCR product length than the expected cDNA band, the latter method has the added advantage of primer (a) not hybridising to the DNA.

The primers should frame a sequence as far 3' as possible on the gene - this ensures that cDNA produced by the oligo (dT) priming need not be full length, this is especially important for large genes. Areas in very close proximity to the 3' tail should be avoided as it has been shown that it is from the poly (A)+ tail that degradation of mRNA initially occurs - this is important to note when analysing mRNA from biological specimens.
For further analysis of the PCR product, the primers chosen should frame a sequence with a diagnostic restriction site for validation purposes.

Primer design and nomenclature for *bcl-2* gene family members :

The primers designed in this project to analyse *bcl-2*, *bcl-x* and *bax* were chosen to detect:

(a) *bcl-2α* and *bcl-2β*

Only *bcl-2α* was detected in this work and throughout the course of this thesis the mRNA referred to as *bcl-2* refers to the *bcl-2α* transcript which is translated to the protein product.

(b) *bcl-x_L* and *bcl-x_S*

Where the text refers to *bcl-x* detection, analysis of both forms of the mRNA was sought. Upon analysis it was found that only the long form of the mRNA was expressed in most cell lines and the mRNA detected was termed *bcl-x_L*. Where *bcl-x_S* specific detection was required the gene is referred to as such.

(c) *bax-α* and *bax-β*

Again two forms of *bax* mRNA are transcribed, but only the α-form is translated to protein. Primers were designed to co-amplify *bax-α* and *bax-β* and then *bax-β* only as a means of distinguishing between both transcripts. As no band was detected in initial amplification steps for the *bax-β* transcript it was assumed that the signal detected for the co-amplified *bax-α* and *bax-β* was as a result of *bax-α* expression only. Therefore in further experiments *bax* mRNA expression refers to *bax-α* only.

All primers chosen were 100% homologous to the specific gene and maintained minimal or no homology to other family members.

Appendix B Use of DNA Data Bases

The most important criteria in the choice of primers for PCR is uniqueness to the template to be analysed. It is essential that the primers do not cross-react with sequences likely to be present in the mRNA of cells/tissues being studied. DNA data bases (e.g. EMBL, Genbank) are used to check the homology of a sequence to all those sequences deposited by researchers in the data-base.

EMBL DNA data bases were accessed easily via E-mail by linking to large mainframe computers (e.g. VAX) and connecting to a UNIX system run through the Bioinformatics Centre, Trinity College, Dublin (co-ordinator A.T.Lloyd). Having accessed this system a wealth of excellent research facilities was available allowing the extraction of a deposited gene sequence, homology searching, and restriction mapping to name but a few, through a package called GCG.

The following is an example of how to access this system via e-mail:
(Note Acer is case sensitive)

$ **telnet acer.gen.tcd.ie**
connected to acer
Username:**rnamhl**
Password :**XXXXXX**
rnamhl@acer : **gcg**

To extract a sequence from the data-base the ***Query*** *programme is used. If the accession number, sequence deposited name or other identification is known the procedure is simple and fast.*
rnamhl@acer :**query**
find
L220121
<Return>
<Return>
extract

list1

.....list1 contains the extracted sequence which is extracted as a "simple extraction"; to make the result accessible to analysis on the GCG package the sequence is saved in GCG format.

.....the extracted sequence is saved as hsbclxl.seq

.....to read the extracted sequence type the following at the prompt:

rnamhl@acer : **cat hsbclxl.seq**

*The **FastA** programme was used for homology searching of sequences.*

rnamhl@acer : **fasta**

FastA of what sequence ? : **hsbclxl.seq**

Beginning at ? : **345** *(the first base of the sequence to be searched)*

Ending at ? : **364** *(the last base of the sequence to be searched)*

Search what data-base ? : EMBL (a choice of many is given)

.....the sequence is then searched against all deposited sequences and the results of the 50 most homologous sequences are returned aligned to the search query. The result is called hsbclxl.fasta. The "cat" command is used again to view the result.

*The **map** programme is used to create a restriction digest map of the required sequence. The programme will search the sequence for all known restriction sites or it can be prompted to search for particular sites. The programme will list at the bottom those enzymes that do and don't cut the particular sequence.*

rnamhl@acer : **map**

map of what sequence? **hsbclxl.seq**

beginning at ? **345**

ending at ? **740**

.....the user is then prompted to select the enzymes with which the search is to be carried out and also to specify if protein translation is required in the result. The result is saved as hsbclxl.map and again is viewed from the user's directory as cat hsbclxl.map.

Appendix C PCR Primers Used for the Detection of MDR Genes

Gene	Primer length	A+T:G+C	T_m	Amplified cDNA length (bases)	Amplified DNA sequence length (bases)	Location on template
mdr1	20	10:10	60	157	1,257	2596-2615
	20	12:18	56			2733-2752
mrp	21	13:8	58	203	N.K.	1317-1337
	21	11:10	62			1499-1519
Topo.IIα	21	11:10	62	139	N.K.	4052-4072
	26	12:14	80			4165-4190
GST π	18	8:10	56	270	749	57-81
	24	14:10	68			304-327
β-actin	29	16:13	84	383	590	660-688
	22	12:10	64			1021-1042
β_2-microglobulin	20	11:9	58	114	1,263	222-241
	20	12:8	56			316-335

.....for more information see O'Driscoll *et al.*, 1993

Appendix D Fold Resistance of DLKP and OAW42 Variants

Table D.1 Fold Resistance of DLKP Resistant Variants

	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKP-A	254 ± 119	1.46 ± 0.42	1504 ± 707	61 ± 8	1.75 ± 0.74
DLKP-A2B	37 ± 16	0.57 ± 0.07	228 ± 71	18 ± 8	1.28 ± 0.77
DLKP-A5F	331 ± 69	1.39 ± 0.03	1275 ± 625	51 ± 8	0.98 ± 0.47
DLKP-VP3	90 ± 13	0.32 ± 0.17	1089 ± 161	58 ± 2	0.47 ± 0.38
DLKP-VP8	272 ± 38	0.22 ± 0.06	1737 ± 348	101 ± 7	0.90 ± 0.43
DLKP-C14	1.67	12.2	1.12	1.9	0.77

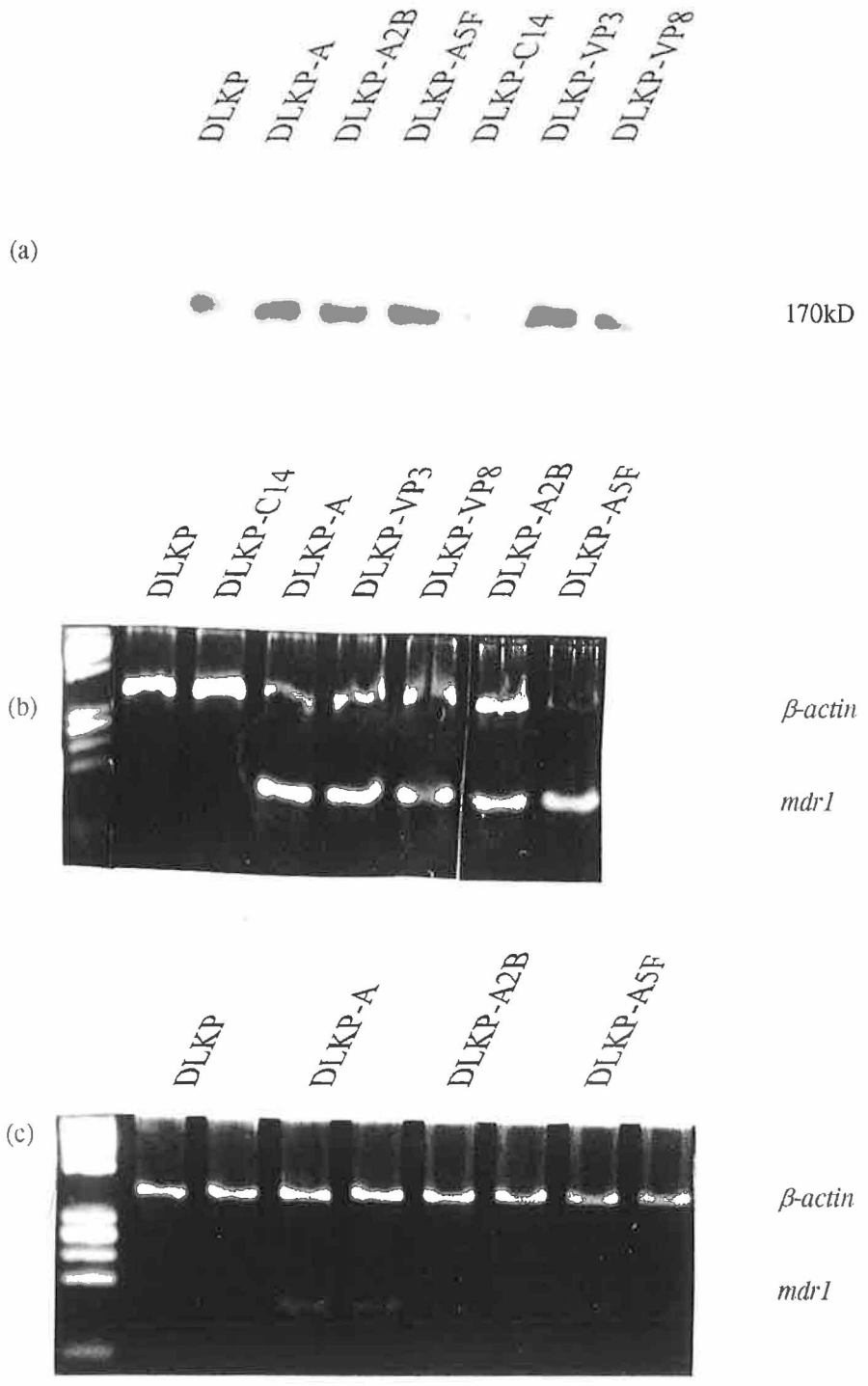
This analysis was carried out by Dr.M.Heenan and Dr.I.Cleary

Table D.2 Fold resistance of OAW42 Variants

	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
OAW42SR	8.3	0.66	20	2.1	0.52
OAW42A1	14	0.92	14	2	0.75
OAW42A	25.8	1.55	40	7.5	0.99

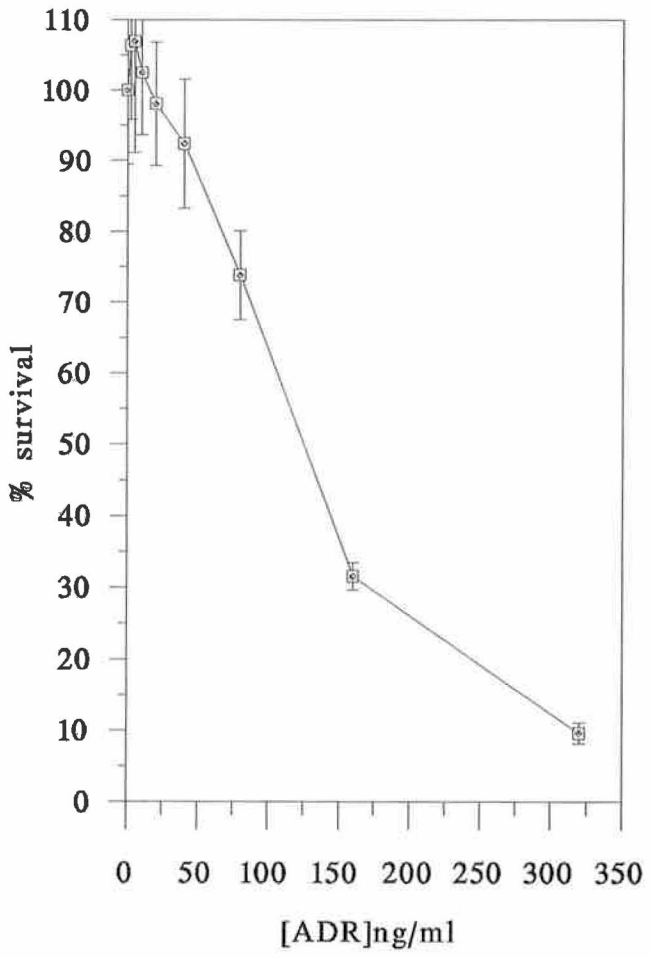
This analysis was carried out by Dr.I.Cleary

Appendix E P-glycoprotein/*mdr1* Levels in Resistant Variants of DLKP



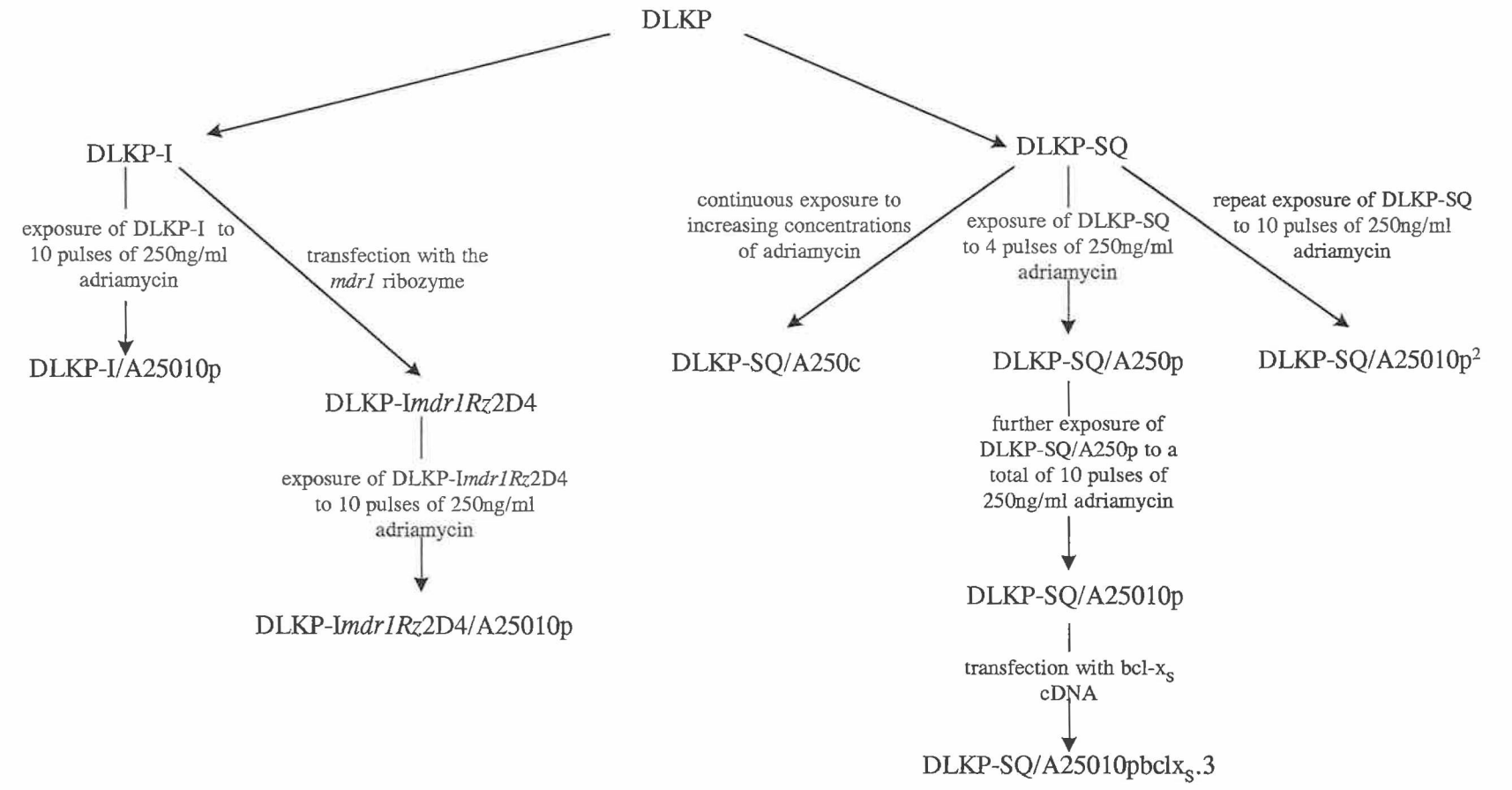
(a) Western blot analysis of P-glycoprotein levels in DLKP variants and *mdr1*mRNA expression in DLKP variants following (b) 25 cycles and (c) 20 cycles of PCR.

Appendix F Calculation of IC_{50} Values



IC_{50} of adriamycin from above graph	=	125ng/ml
	=	125×10^{-6}g/l
Molecular Weight of adriamycin	=	580
Calculation	=	$\frac{125 \times 10^{-6}}{580}$
IC_{50} value	=	215nM

Appendix G Resistant Variants of DLKP-SQ and DLKP-I



Selection of drug resistant cell lines from clonal populations of the parental DLKP cell line.

Appendix H Table of Figures

Appendix I Abbreviations

1-β-D-ara	1-β-D-arabinoside
ADR	Adriamycin
AIF	Apoptosis Inducing Factor
AMP	Ampicillin
ATCC	American Tissue Culture Collection
ATF	Activating Transcription Factor
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BHFR1	Epstein-Barr Virus Protein
BSA	Bovine Serum Albumin
CAF	Cyclophosphamide: Adriamycin: 5-Fluorouracil
CBP	Carboplatin
cdc	Cell Division Cycle
CDK	Cyclin Dependent Kinase
cDNA	complementary DNA
CMF	Cyclophosphamide:Methotrexate: 5-Fluorouracil
CMP	Cyclophosphamide (mafosphomide)
CPT	Cisplatin
CsCl	Caesium Chloride
Cyc A	Cyclosporin A
DEPC	Diethyl Pyrocarbonate
DHFR	Dihydrofolate Reductase
DIG	Digoxigenin
DMEM	Dulbeccos Minimum Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNDP	Deoxynucleotide Diphosphate (where N= A,C,G,T,U)
dNMP	Deoxynucleotide Monophosphate (where N= A,C,G,T,U)
dNTP	Deoxynucleotide Triphosphate (where N= A,C,G,T,U
DSP	Dual Specific Phosphatases
DTT	DiThiothretol
ECACC	European Collection of Animal Cell Culture
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EM/Emod	Emodin
EMBL	European Molecular Biology Laboratory
ER	Oestrogen Receptor
ERK	Extracellular Signal Regulated Protein Kinase
FCS	Fetal Calf Serum
FRK	Fos Regulated Kinase
5-FU	5-fluorouracil
GAP	GTPase Activating Protein
GAPDH	Glyceraldehyde-6-phosphate Dehydrogenase
GDI	GDP Inhibition Protein

Gen	Genistein
GIP	GTPase Inhibiting Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor.
GnSCN	Guanidine Thiocyanate
Grb-2	Growth Factor Receptor Binding Protein -2
GST	Glutathione-S-Transferase
HBS	Hepes Buffer Saline
HIV	Human Immunodeficiency Virus
HPRT	Hypoxanthine-guanine phosphopribosyltransferase
IC_{50}	Inhibitory Concentration 50 Percent
ICE	Interleukin-β-1-Converting Enzyme
IGF	Insulin-like growth factor
IL-3	Interleukin -3
JNK	c-Jun N-terminal Kinase
LB broth	Luria-Bertani broth
LRP	Lung-resistance Related Protein
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MEM	Minimum Essential Medium
MKP	MAKP Phosphatase 1
MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase
mRNA	Messenger RNA
MRP	Multidrug Resistance-associated Protein
MTX	Methotrexate
NBT	4-Nitro blue tetrazolium chloride
NCTCC	National Cell and Tissue Culture Centre
NDP	Nucleotide Diphosphate (where N= A,C,G,T,U)
NF-1	Neurofibromatosis tumour suppressor gene
NISH	Non-isotopic *in-situ* hybridisation
NMR	Neuclear Magnetic Resonance
NSCLC	Non-Small Cell Lung Cancer
NTP	Nucleotide Triphosphate (where N= A,C,G,T,U)
O.D.	Optical Density
ORF	Open Reading Frame
P-gp	P-Glycoprotein
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PKC	Phosphokinase C
PMSF	Phenylmethyl sulfonyl fluoride
r.p.m.	Revolutions per minute
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase

SDS	Sodium Dodecyl Sulphate
SOS	Son of Sevenless Protein
SRE	Serum Response Element
SSC	Sodium Saline Citrate
TBE	Tris Boric Acid, EDTA Buffer
TBS	Tris Buffere Saline
TGFβ 1	Transforming Growth Factor β 1
TLL	T- lymphocyte Leukaemia
T_m	Melting Temperature
TNF	Tumour Necrosis Factor
TS	Thymidylate Synthase
UV	Ultra Violet
VEGF	Vascular Endothelial Growth Factor
VM-26	Teniposide
VNC	Vincristine
VP-16	Etoposide

Appendix J Molecular Weights of Compounds Used in Thesis

5-Fluorouracil	130.1
Adriamycin	580.0
Carboplatin	371.26
Cyclosporin A	1202.6
Emodin	270.24
Genistein	270.2
Mafosfamide	500.5
Methotrexate	508.5
Vincristine	923.0
VP-16	588.6

Appendix K Buffers

4M Guanidium Thiocyanate:

Guanidium thiocyanate (Sigma G-6639)	50g
N-lauroyl sarcosine (Sima L-5125)	0.5g
1M Na-citrate, pH 7	5ml

Bring to 100ml with H_2O and check that pH is approximately 7.
Filter through 0.45μm filter and store at room temperature in the dark.
Before use add:

β-mercaptoethanol (Sigma M-6250)	700μl/100ml
Antifoam A (30%) (Sigma A-5758)	330μl/100ml

5.7M Cesium chloride:

CsCl (Sigma C-3032)	95.8g
1M Na-citrate, pH 7	2.5ml

Bring to 100ml with H_2O.
Filter sterilise, DEPC-treat and autoclave.
Store at room temperature.

1M Na-citrate:

Na-citrate (RDH 32320)	29.4g

Bring to 80ml with H_2O and pH to 7 with HCl.
Bring to 100ml and filter sterilise.
Store at room temperature.

10XPBS

NaCl	40g/500ml
KCl	1g/500ml
$Na_2HPO_4.7H_2O$	5.75g/500ml
KH_2PO_4	1g/500ml

20XSSC

3M NaCl	87.66g/500ml
0.3M Na-citrate	44.12g/500ml

20XTBS/BSA

1M Tris, pH 7.6	60.5g/500ml
3M NaCl	87.66g/500ml
40mM $MgCl_2$	1.91g/ml
2%(w/v) BSA	10g/500ml

100XTE

1M Tris/HCl, pH 8.0	12.11g/100ml
0.1M EDTA, pH 8.0	3.722g/ml

10XTBE
54g Tris/HCl
27.5g Boric Acid
20ml 0.5M EDTA pH 8.0

10XMOPS
0.25M MOPS
0.05M Na-acetate

0.01M EDTA, pH 7.0

LB Broth
5g Yeast
10g Bactotryptone
10g NaCl
in 1L H_2O

Terrific Broth (TB)
12 g Bactotryptone
24g Bactoyeast extract
4ml Glycerol
100ml 0.17M H_2PO_4
0.72 M K_2HPO_4

1XHBS
50mM Hepes, pH 7.1
280mM NaCl
1.5mM Na_2HPO_4