Investigation of apoptosis-related gene expression in multi-drug resistant cells.

A thesis submitted for the degree of Ph.D.

by

Deirdre Cronin B.Sc. Hons

The experimental work described in this thesis was carried out under the supervision of Professor Martin Clynes Ph.D. at the

National Cell & Tissue Culture Centre,

School of Biological Sciences,

Dublin City University,

Glasnevin,

Dublin 9,

Republic of Ireland.

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.

Signed: Deirdre Cronin **ID No.:** 97970433

Date: 6th November 2002.

Abstract.

The RNase protection assay (RPA) was used to identify changes in gene expression accompanying alterations in drug resistance levels in lung carcinoma cells. Based on this analysis, mcl-1 and bax were selected for transfection into drug sensitive cells to establish whether or not their expression influences drug resistance levels. The RPA was also used to investigate alterations in expression of bcl family genes as a consequence of transfection of a bcl-xL ribozyme.

Previous research in this laboratory, as well as results of the RPA analysis, indicated that caspase-3 levels were reduced in more resistant cell lines. To investigate the role of caspase-3 in drug resistant cells, the first reported ribozyme to human caspase-3 was designed and transfected into a drug-resistant variant (DLKP-A5F) of a human lung carcinoma cell line (DLKP). By both *in vitro* cleavage and stable and transient transfection in drug resistant DLKP-A5F cells, this ribozyme was shown to be effective at down-regulating human caspase-3 mRNA and protein levels. Initial results in stable transfectants indicated an increase in drug resistance, but on repeated subculture, these resistance levels reverted back to those of parent cells. Analysis of the multi-drug resistance protein, P-gp, revealed that its level had also decreased in some of the clones. These results indicate the importance of analysing P-gp levels in transfection studies where drug resistance is being monitored and suggest the possibility that caspase-3 expression may somehow regulate P-gp protein level.

DNA microarray technology was used to investigate differences in gene expression between two resistant variants (one high-level, one low-level) of doxorubicin-treated DLKP cells. Results indicated changes in the gene expression of some ABC transporter proteins, multi-drug resistance gene (mdr-1), apoptosis-related genes (galectin-1, apoptosis-associated protein kinase), calpain-1, retinoic acid receptor-α as well as metastases-related genes.

Finally, the expression of apoptosis-related genes was examined in a panel of archival breast tumour biopsies. The expression of these genes (bcl-1, bax, mcl-1 and bag-1) was correlated with clinicopathological parameters with the aim of identifying their

prognostic significance. Bcl-2 correlated with ER status, bag-1 expression and five-year relapse-free and overall survival. Bax expression did not associate with any other parameter. Mcl-1 correlated with tumour grade and had borderline significance with lymph node status and survivin δ3 expression.

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On reading this thesis, I hope people will think of those who suffer from cancer, especially those individuals whose tumour tissue was used in this work. I hope that in some small way, this study will contribute to more successful treatments for this devastating disease.

This thesis is dedicated with love to Mum, Dad, Máire and Don.

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Abbreviations

Ab	-	Antibody
ABC	-	ATP Binding Cassette
AMP	-	Adenosine Monophosphate
ATP	-	Adenosine-triphosphate
ATCC	-	American Tissue Culture Collection
BSA	-	Bovine Serum Albumin
cDNA	-	Complementary DNA
CPP-32	-	Caspase-3
Da	-	Daltons
DEPC	-	Diethyl Pyrocarbonate
DMEM	-	Dulbecco's Minimum Essential Medium
DMSO	-	Dimethyl sulfoxide
DNase	-	Deoxyribonuclease
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxynucleotide triphosphate (N= A, C, T, G or U)
DTT	-	Dithiothreitol
ECM	-	Extracellular matrix
EDTA	-	Ethylene diamine tetracetic acid
ER	-	Estrogen receptor.
FCS	-	Fetal Calf Serum
GAPDH	-	Glyceraldehyde-6-phosphate dehydrogenase
GSH	-	Glutathione
GST	-	Glutathione-S-Transferase
GS-X Pump	-	GSH-conjugate export carrier
HEPES	-	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
H-SFM	-	Ham's F12 Serum Free Medium
IC_{50}	-	Inhibitory Concentration 50%
Ig	-	Immunoglobulin
IMS	-	Industrial Methylated Spirits
IVC	-	In vitro cleavage
kDa	-	Kilo Daltons
LRP	-	Lung Resistance-related Protein
MAb	-	Monoclonal Antibody
MDR	-	Multiple Drug Resistance
MRP	-	Multidrug Resistance-associated Protein
MEM	-	Minimum Essential Medium
min	-	Minute(s)
MMLV-RT	-	Moloney Murine Leukemia Virus-Reverse Transcriptase
mRNA	-	Messenger RNA

Mr marker	-	Molecular Weight Marker
NCTCC	-	National Cell & Tissue Culture Centre
NRK	-	Normal Rat Kidney
NSCLC	-	Non-Small Cell Lung Carcinoma
OD	-	Optical Density
Oligos	-	Oligonucleotides
P	-	Passage
PBS A	-	Phosphate Buffered Saline A
PEG	-	Polyethylene Glycol
PCR	-	Polymerase Chain Reaction
P-gp	-	P-glycoprotein
PKA	-	cAMP-dependant protein kinase
PO	-	Phosphodiester
PS	-	Phosphorothioate
RA	-	Retinoic Acid
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
RPA	-	RNase protection assay
RNasin	-	Ribonuclease Inhibitor
rpm	-	Revolution(s) Per Minute
RT-PCR	-	Reverse Transcriptase-PCR
Rz	-	Ribozyme
Rz1R	-	Reverse ribozyme
SCLC	-	Small Cell Lung Carcinoma
SDS	-	Sodium Doedecyl Sulphate
sec(s)	-	Second(s)
SF	-	Serum-Free
SFM	-	Serum-Free Medium
TBE	-	Tris-boric acid-EDTA buffer
TBS	-	Tris Buffered Saline
TE	-	Tris-EDTA
Topo II	-	Topoisomerase II
Td	-	Thymidine
TEMED	-	N, N, N', N'-Tetramethyl-Ethylenediamine
Tris	-	Tris(hydroxymethyl)aminomethane
TUNEL	-	Terminal deoxynucleotidyl transferase-mediated X-linked nick-end labelling.
UHP	-	Ultra high pure water
v/v	-	volume/volume
w/v	-	weight per volume

Section 1.0: Introduction.

1.1 Chemotherapy in the treatment of cancer.

Acquired multiple drug resistance is the biggest obstacle in the treatment of human malignancies. This multiple drug resistance phenotype enables tumours to evade death induced by a variety of anticancer drugs. As well as the development of multi-drug resistance, standard chemotherapy for neoplastic disease is accompanied by systemic toxicity. Cancer chemotherapeutic drugs have a relatively low toxic to therapeutic dose with the drugs failing to discriminate between normal and malignant cells.

The main aim of cancer chemotherapy is to cause a maximal level of destruction in tumour cells. A number of cytotoxic drugs with antitumour efficacy have been discovered, many of which are used routinely to treat patients. These drugs include the anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, pyrimidine analogues and platinum compounds. Ongoing research continues to recruit new therapies and improve existing ones.

1.1.1 The Anthracyclines.

The antimitotic antibiotics, anthracyclines, were isolated from different species of Streptomyces. Included in this family of compounds are actinomycin, daunorubicin and doxorubicin (adriamycin). They consist of an anthracycline four-ring structure linked by a glycosidic bond to daunosamine, an amino sugar. Daunorubicin and doxorubicin are most widely used in cancer chemotherapy and differ in their structure by a hydroxyl or hydrogen at position 14 on the anthracycline ring.

The antineoplastic activity of these drugs has been mainly attributed to their strong interactions with DNA in the target cells. They are thought to function by intercalating with the DNA that distorts the DNA template and therefore prevents nucleic acid synthesis by interfering with the template DNA strand-breakage-reunion reaction of topoisomerase 2. The anthracyclines can also precipitate the formation of reactive oxygen species leading to free radical damage of the ribose moiety of RNA and DNA. The anthracycline chromophore contains an iron-chelating site (hydroxyquinone) which combines with the DNA and catalyses the transfer of electrons from glutathione to oxygen resulting in formation of reactive oxygen species. Radicals such as the hydroxyl

radical (OH*) can be generated which can attack cell components and stop cell growth (Priestman, 1979, Pratt et al., 1994).

The drugs are administered parenterally as they become inactive in the gastrointestinal tract. They can be reduced to their semiquinone form by biological reducing agents, such as NADH and NADPH. Active forms are daunorubicinol and doxorubicinol. Daunorubicin has a half-life of 40 minutes, followed by a slow decline over 50 hours. Doxorubicin has a biphasic half-life of 10 minutes and 30 hours, as the principal circulating form is the unmetabolised drug.

Daunorubicin has been found to be effective against acute nonlymphocytic leukaemia, while doxorubicin has been widely used in the treatment of carcinomas and sarcomas. Both drugs, however, induce severe toxicity to malignant as well as normal cells. These side effects include bone marrow and gastrointestinal tract toxicity and alopecia. Myelosuppression, leukopoenia, thrombocytopoenia & anemia also occur, and cardiotoxicity due to damage to the cardiac muscle cells has been reported years after treatment is completed (Pratt et al., 1994). Total dose for daunorubicin is 20mg/ kg body weight and for doxorubicin is 550mg/m^2 (Priestman, 1979).

1.1.2 The Vinca Alkaloids.

The alkaloid drug family includes cocaine, morphine and atropine, however, it's anti-cancer members are the vinca alkaloids, vincristine and vinblastine (which were derived from the *Caranthus rosea* plant) and sythetic derivatives, vindesine & vinorelbine. These compounds bind specifically to free tubulin dimers. Upon binding, they cause formation of paracrystalline aggregates containing equimolar amounts of drug and tubulin dimers. They therefore disrupt the balance between the microtubule polymerisation and depolymerisation which results in the net dissolution of microtubules, destruction of mitotic spindle and the arrest of the cells in metaphase. They also interfere with the cells' ability to synthesize DNA and RNA (Cyberbotanica, 2002). Resistance to these drugs usually occurs due to overexpression of P-glycoprotein (Pratt et al., 1994).

These drugs are used in the treatment of leukaemias and lymphomas (vincristine) and also for Hodgkin's disease, other lymphomas and in combination with other drugs for

the treatment of solid tumours, such as ovarian, testicular, breast cancers (Priestman, 1979).

Drugs are administered by intravenous bolus injections. All have similar α and β half-lives (2-5 minutes and 50-150 minutes). Vincristine has a terminal half-life of 85 hours and vindesine and vinblastine have one of 24 hours. Side effects include peripheral neuropathy as well as bone marrow damage, etc. These drugs do not usually display cross-resistance and so can be used to replace each other where resistance to one has occurred (Pratt et al., 1994; Priestman, 1979; Cyberbotanica, 2002).

1.1.3 Taxanes.

The taxanes are a group of drugs including paclitaxel (Taxol) and docetaxol (Taxotere). Taxol was originally isolated from the *Taxus brecifolia* yew tree, but was in short supply, taxotere, however, was found to have similar properties and was in much greater supply.

Their mechanism of action involves causing a stabilisation of ordinary cytoplasmic microtubules and formation of abnormal bundles of microtubules. The cancer cells therefore become so clogged with microtubules that they cannot proliferate (NCI, 2001).

Both drugs are administered in short (1-6 hours) and long infusions. Taxol has a half-life of 5 hours. The drugs are used to treat ovarian, breast and lung cancers, as well as Kaposi's sarcoma seen in AIDS patients. Side effects of these drugs include neutropenia, hypersensitivity, reversible neurotoxicities, gastrointestinal toxicity and alopecia (Pratt et al., 1994; NCI, 2001).

1.1.4 Epipodophyllotoxins.

Etoposide (VP-16) and teniposide (VM-26) are cytostatic glucosides. They are derived from podophyllotoxin, and extract of the May apple plant (Priestman, 1979). They block the cell cycle at two stages: G1 phase and S phase (Cyberbotanica; 2002). VP-16

targets topoisomerase 2 by stabilising the complex between topoisomerase 2 and DNA, thereby interfering with the enzyme's activity and resulting in DNA double strand breaks and cytotoxicity. It also prevents the activation of protein kinase p34 (cdc2) which ordinarily becomes activated at the end of the G2 phase of the cell cycle (Pratt et al., 1994).

VP-16 is used to treat testicular cancers and small-cell lung cancers among others. It has a half-life of 4- 8 hours. VM-26 is taken up faster than VP-16 and is retained for longer due perhaps to it being a lipophyllic compound. VM-26 is used to treat lymphomas. Both are administered orally or intravenously, and approximately 30-50% is recovered in urine of treated patients. Side effects associated with both include alopecia, nausea, anorexia, leucopenia and thrombocytopenia (Cyberbotanica, 2002; Pratt et al., 1994; Priestman, 1979).

1.1.5 Pyrimidine Analogues.

The pyrimidine analogues are compounds which are structurally similar to cellular compounds. Pyrimidine analogues such as 5-Fluorouracil (5-FU), inhibit the methylation reaction of deoxyuridylic acid to thymidylic acid, thus preventing the synthesis of nucleic acids.

5-FU has a biphasic half-life of 5-20 minutes and 20 hours. It is commonly used to treat cancers of the rectum, stomach, colon, pancreas and breast. It is used in combination with other antineoplastic drugs for the treatment of cancers such as Duke's stage C colon cancer where it is combined with levamisole. It is administered intravenously and side effects include bone marrow depression and gastrointestinal toxicity (Pratt et al., 1994; Delmar website, 2001).

1.1.6 Platinum Compounds.

The platinum compounds were discovered by Rosenberg and coworkers (1969) and were found to be effective agents at preventing cell division in bacteria. The compounds were also effective at eliminating tumours, although their use was somewhat limited due

to toxic side effects. The platinum compounds bind to DNA base pairs, leading to the formation of intrastrand cross-links. The resulting distortion of the DNA prevents effective repair.

Cisplatin is used for a wide variety of cancers including breast, lung, head and neck, germ-cell, etc. It is administered intravenously and has a half-life of 58 to 72 hours. Side effects include renal toxicity, emesis, neurotoxicity, bone marrow suppression and hearing loss (Pratt et al., 1994, Cisplatin factsheet, 2002, Priestman, 1979).

To minimise the serious side effects, carboplatin is sometimes administered to patients. It promotes less nephrotoxicity and is sometimes used in treating ovarian and lung carcinomas (Chemotherapy Protocols- Lung cancer factsheet).

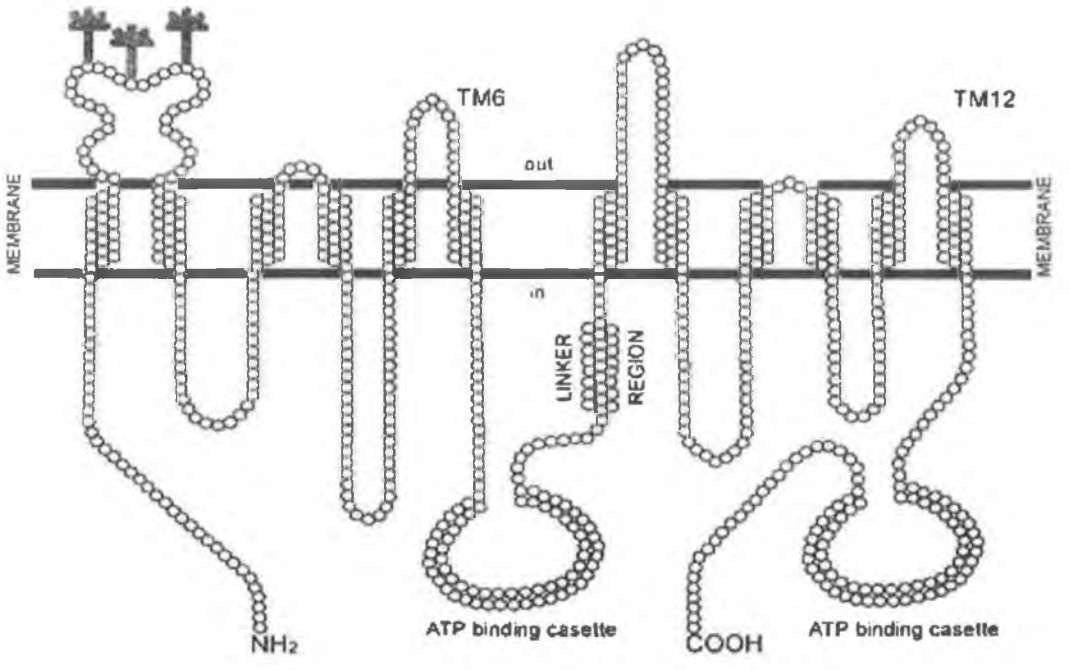
1.2 Multiple Drug Resistance in Cancer.

The ability of tumours to evade cytotoxic therapy is the cause of death in the majority of cancer patients. Failure of chemotherapy is frequently due to tumours overexpressing proteins such as MDR-1, MRP, LRP & Gluthathione-S-transferase. These proteins are overexpressed in a wide variety of drug resistant cancers and cultured cell lines.

1.2.1 P-Glycoprotein.

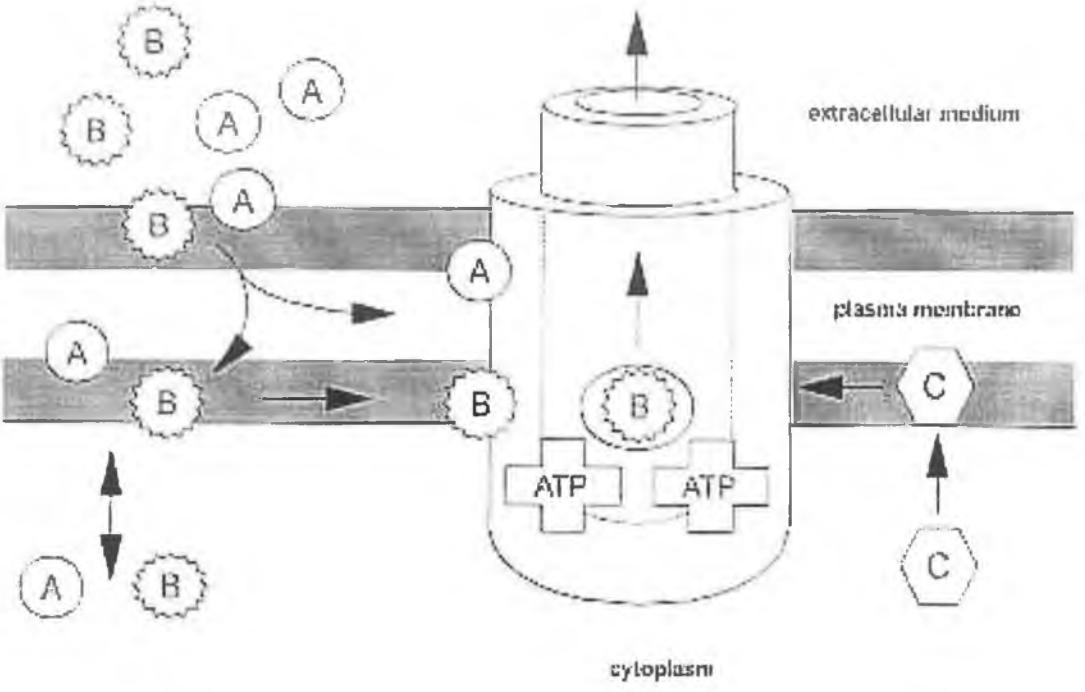
P-glycoprotein is the most frequently expressed drug resistance-associated protein in drug-resistant cultured cancer cells. This is a member of the ATP-binding cassette transporter protein family that is responsible for transporting drug out of cells (Schurr et al., 1989). The P-glycoprotein is a 170kDa protein which consists of 12 transmembrane domains, six domains linked to a similar six domains via a nucleoside binding domain (Juliano & Ling, 1976; Croop, 1993). There are two classes of MDR proteins, the first which includes proteins thought to be responsible for multiple drug resistance, while class two proteins have no effect on drug resistance but are thought to play a role in lipid transport (Germann, 1993).

Figure 1.1 Schematic diagram of P-glycoprotein showing its 12 transmembrane domains (Lehne, www.bentham.org).



In non-neoplastic cells, mdr-1 is thought to play a role in xenobiotic efflux and it is expressed normally in liver, kidney, pancreas, adrenal gland, etc. It is also thought to act as a chloride channel, an ATP channel and has a role in cholesterol biosynthesis (Germann, 1993). Pgp is frequently overexpressed in post-chemotherapy tumours where it hinders the cytotoxic response to drugs, thus somewhat desensitizing the cells. The protein has been found in membrane enriched fractions of cells and is phosphorylated on serine residues (Schurr et al., 1989). An increase in this phosphorylation is thought to cause an increase in Pgp-mediated drug efflux activity whilst inhibition of phosphorylation decreases this activity. Drug enters the cells via a non-energy dependent mechanism and leaves via this active ATP pump (Germann, 1993).

Figure 1.2 Model of the transport of P-glycoprotein illustrating removal of drugs directly from the plasma membrane either as uncharged drug (A) diffusing through the membrane or as positively charged drug (B) embedded in the inner leaflet of the plasma membrane. Pgp probably effluxes endogenous substrates (C) in a similar manner (Lehne, www.bentham.org).



The overexpression of MDR-1 confers increased resistance to drugs such as the anthracyclines, the epipdophyllotoxins, and the vinca alkaloids but cells remain sensitive to the platinum compounds and pyrimidine analogues, etc. (Schurr et al., 1989). Thus its expression confers cross-resistance to a variety of structurally and functionally unrelated drugs. It has been demonstrated that P-glycoprotein can bind to drugs such as vinblastine (Pratt et al., 1994). Pgp can be blocked by calcium channel blockers, such as verapamil. However, due to the pharmacological properties of such compounds, they are unsuitable for combination therapy.

1.2.2 MRP.

The multi-drug resistance-associated protein is a 190kDa phosphoglycoprotein located in the plasma membrane and endoplasmic reticulum of drug-resistant cells. It is a highly phosphorylated protein and some protein kinase inhibitors such as chelerythrine can prevent phosphorylation of MRP-1 and reverse this resistance.

MRP-1 is distributed in the cytoplasm of normal tissues (Flens et al., 1996). These tissues include kidney, brain, lung, bladder, liver, ovary etc. (Cole et al., 1992, Kool et al., 1997, Loe et al., 1996). MRP-1 overexpression has been detected in solid tumours including breast, ovarian and prostate cancers, small cell and non-small cell lung carcinomas as well as in hematological malignancies (Loe et al., 1996, Hipfner et al., 1999). It is thought to have prognostic significance in primary breast cancer where its expression is associated with poor prognosis (Huang et al., 1998). In small cell lung cancer, it was also found to be a marker of poor response to therapy (Campling et al., 1997).

Both Pgp and MRP are members of the ABC-protein family (ABC-binding cassette protein family). These proteins function as transport ATPases hydrolysing ATP in conjunction with transporting their substrate molecules through cellular or intracellular membranes. Although Pgp and MRP-1 have only 15% sequence homology, both function in an energy requiring drug efflux system which reduces intracellular accumulation of drug. While Pgp is capable of binding and transporting free drug, it is unclear as to whether MRP can bind free drug or not. It is capable of binding and transporting glutathione-S-conjugate LTC4 in isolated membrane vesicles, and of transporting in vitro oxidated glutathione, cholecstatic glucuronidated steroids and chemotherapeutic drugs, such as vincristine and daunorubicin (Center, 1998). Due to its ability to transport glutathione conjugates of drugs out of cells, it is often identified in non-Pgp multi-drug resistance. It is speculated that MRP-1 may confer initial resistance to drugs, while Pgp overexpression develops at higher concentrations (Cathal Elliott, PhD 1997).

Since its discovery in 1990 (Marquandt et al., 1990), a number of MRP-related proteins have been identified. These include hMRP-2 (c-MOAT a liver canalicular multispecific organic anion transporter), and genes which were identified based on human expressed

sequence tags (ESTs) which include hMRP-3, hMRP-4, hMRP-5 & hMRP-6 (Varadi et al., 1998). They are each implicated in a number of signalling processes. MRP-1 is pump involved in effluxing xenobiotics out of the cell. MRP-2 extrudes organic anions into bile. MRP-3 transports a bile constituent (glycocholate). MRP-4 and MRP-5 confer resistance to purine and pyrimidine analogs. The function of MRP-6 has yet to be confirmed (Kruh et al., 2001).

1.2.3 Glutathione & Glutathione-S-transferases.

Glutathione (GSH) is a sulphydryl-containing tripeptide compound present at high concentrations in mammalian cells which has cellular reducing capacity. These cells include those of the kidneys, brain, lung and intestine (Tsuchida and Sata, 1992). The –SH group, of GSH, is strongly nucleophilic and confers to the molecule a unique ability to react with cytotoxic electrophile drug derivatives and to react with drug-generated reactive oxygen compounds (Ishikawa et al., 1998; Pratt et al., 1994). It therefore plays a critical role in the cells detoxification response to a wide variety of cytotoxic agents.

The glutathione-S-transferases (GSTs) are a family of enzymes which catalyse the conjugation of electrophilic compounds to F+GSH. These enzymes are divided into three classes (α, μ & π) according to the enzyme's amino-terminal amino acid sequence, substrate specificity, sensitivity to inhibitors and immunological techniques. The 24-29kDa subunits can form homodimers or heterodimers with members of the same class and each monomer has a kinetically independent active site (Tahir and Mannervik, 1986). Each active site is composed of a G-site for glutathione binding and a H-site for binding the hydrophobic electrophile.

While other enzymes have high level specificity for their substrates, enzymes of detoxification, such as the GSTs, are usually expressed at high levels but without high specificity so they react with a wide variety of agents (Gulick & Fahl, 1995). GSH and GSTs have been found to be overexpressed in a wide variety of cancers (colon, bladder, oesophagus, stomach, pancreas & ovary) and also in a number of drug-resistant cell lines. It is the GST-π enzyme that appears to be increased in most cases of drug resistance. An MCF-7 breast cancer cell line which was treated with adriamycin was 200-fold resistant to adriamycin and had a 45-fold increase in glutathione (Batist et al., 1986). An ovarian adenocarcinoma cell line with sensitive and resistant variants had

increased levels of GST in the resistant line. (Lewis et al., 1988). GST has also been reportedly increased in an adriamycin-resistant colon cell line (SW620) (Chao et al., 1992). Resistance to cisplatin in cells has also been associated with increased levels of glutathione in a small-cell lung cancer cell line (Fram et al., 1990). Interestingly, transfection of GST into MCF-7 cells showed no change in resistance and a cotransfection experiment involving GST and MDR-1 resulted in no additive effects in resistance (Moscow & Dixon, 1989 & 1993, Fairchild et al., 1990).

The studies suggest that a critical role for these enzymes but the exact mechanism of how they contribute to resistance in these neoplastic tissues is unclear. There are a number of compounds which are natural inhibitors of glutathiones; i.e. BSO (butathionine sulfoximine), ethacrynic acid, etc. (Gulick & Fahl, 1995). BSO has been found to restore sensitivity to melphalan in a melphalan-resistant leukemia cell line (Pratt et al., 1994), however as for Pgp and MRP, the challenge remains to selectively inhibit glutathione and its associated enzymes in tumour cells exclusively, without threatening the constitution of normal cells.

1.3 Introduction to Apoptosis.

Apoptosis is a normal, continuous process of selecting cells to die. It is a highly regulated physiological mechanism where cell death occurs at a specific time during the development of an organism. Cells die due to exposure to external or internal stimuli, including DNA damage (i.e. exposure to ionising radiation, etc.), growth factor deprivation (serum withdrawal of cells in culture), heat, developmental programmes, and extremes of temperature. The intensity of the stress, its concentration and duration, determines which form of cell death occurs, i.e. by necrosis or apoptosis. Necrosis involves cellular response to injury whereas during apoptosis, cells are induced to die by suicide (Kanduc et al., 2002).

Injurious stimuli, such as mechanical insult or exposure to toxic chemicals, induce cells to undergo a series of changes characteristic of necrosis. These include the disruption of ATP generation affecting the cellular ion pumps. This has downstream effects leading to increases in Ca2+ levels within cells, and disruption of the Na+/K-ATPase pump. The plasma membrane is unable to control the passage of ions and water across it and the cells and their organelles swell and lyse. Nuclear changes are late in the process due to activation of hydrolytic enzymes. Cell lysis leads to release of cell contents resulting in damage to neighbouring cells and inducing an inflammatory response. Therefore, during necrosis a number of cells are affected (Kanduc et al., 2002).

In contrast, apoptosis occurs when the cell stress is less severe. Apoptosis is tightly regulated and genetically controlled by distinct morphological and biochemical changes. It is a continuous process and occurs more commonly than necrosis (Kiechle & Zhang, 2002, Kinloch et al., 1999).

During apoptosis, a suicidal cell detaches from its neighbouring cells and its cell volume decreases with preservation of organelles (mitochondria stay intact), followed by cell membrane blebbing. Small membrane-wrapped cell fragments break off from the "blebbed" cell called apoptotic bodies. Surrounding scavenger cells and macrophages recognise these cells due to cell surface changes (lectin receptors, vitronectin receptors, flipping of the phosphatidyl serine receptor and appearance of cell surface antigens such as the 61D3 antigen) (Kanduc et al., 2002, Desagher & Martinou, 2000). Phagocytes engulf the apoptotic bodies and inflammation is not induced.

Underlying all the morphological changes, are intricate biochemical changes. As mentioned earlier, DNA degradation is a hallmark of apoptosis. Ca2+ and Mg2+ dependent endonucleases cleave DNA resulting in chromatin condensation. Nuclease enzymes Dnase I and Dnase II and CAD (caspase-activated Dnase) are thought to be involved (Kiechle & Zhang, 2002, Cohen, 1997). The DNA degradation is an irreversible step and commits the cell to die. DNA is nicked and then cleaved into high molecular weight fragments (~50kbp), via cleavage at looped domains, and then into low molecular weight fragments (~200bp), via cleavage at internucleosomal regions. This is of practical importance in DNA laddering experiments (Kiechle & Zhang, 2002).

Other biochemical changes include cleavage of 28s rRNA and protein degradation. Among the proteins cleaved are, PARP, lamin, topoisomerase, DNA-dependent protein kinase, all of which are nuclear proteins, and also structural proteins, including β-actin and α-fodrin (Martin et al., 1995). Transglutaminase enzymes are activated and these catalyse formation of ε (γ-glutamyl) lysine crosslinks between proteins (Aeschlimann & Thomazy, 2000). This process is calcium dependent and makes apoptotic bodies resistant to mechanical disruption and chemical attacks through a protein net which may play a role in limiting leakage of cellular contents. Inhibiting the transglutaminase enzymes leads to death by necrosis rather than by apoptosis (Schulze_osthoff & Stroh, 1998, Kanduc et al., 2002). Activation of the proteolytic caspase cascade and the electron transport chain means that the process is ATP dependent (Section 1.4).

1.3.1 The role of apoptosis.

Just as mitosis is needed for continued cellular regeneration, apoptosis is needed to balance cell number and prevent uncontrolled cell generation, leading to diseases such as cancer.

Apoptosis is needed for proper development. During immune cell development, the positive and negative selection of B and T lymphocytes ensure that self-reactive clones are removed from the immune system (Grodzicky & Elkon, 2002). During fetal development, the formation of digits and synapses between neurons requires that surplus cells be eliminated by apoptosis.

Apoptosis is needed to eliminate cells that pose a threat to the integrity of the organism. Cells with damaged DNA have increased production of p53, a potent inducer of apoptosis. Many cancer cells have mutated p53 that has lost its ability to induce apoptosis (Oren et al., 2002, Sharpless & DePinho, 2002).

The importance of apoptosis is especially seen in diseased states. In cancer cells, too little apoptosis enables the cell to survive and accumulate a number of mutations leading to uncontrolled cell growth (Hickman, 2002, Mullauer et al., 2001). In neurodegenerative diseases, such as Alzheimer's, too much apoptosis occurs leading to loss of essential neurological functions. In virally infected cells, such as in HIV infected CD4+ T-cells, cells are induced to die by apoptosis, leading to depletion of CD4+ immune cells (Tatton & Olanow, 1999).

Tissue homeostasis is a balance between apoptosis (or cell loss) and mitosis (or cell gain). A cell's decision on whether or not to commit suicide depends on the withdrawal of positive signals and the receipt of negative signals. Positive signals are essential for the survival of cells, whereby cells receive and donate continuous stimulation from other cells; e.g., growth factors for neurons, and IL-2 an essential factor for the mitosis of lymphocytes. Negative signals include increased levels of oxidants within the cell, damage to DNA by these oxidants or other agents like UV light, X-rays, chemotherapeutic drugs, and binding of death activators to specific receptors on the cell surface, signalling to the cell to begin the apoptosis program, (TNF binds to TNF-R;

lymphotoxin binds to the TNF-R; and Fas ligand binds to Fas or CD-95) (Guo & Hay, 1999).

1.3.2 The Identification of the Apoptotic Pathway.

Initial insight into the apoptotic process came from studies on the nematode worm, *C. elegans* (Liu & Hengartner, 1999, Joza et al., 2002, & references therein). These studies gathered evidence that apoptosis in animals is caused by the activation of a suicide programme. During development of the worm, 1090 somatic cells are formed, and 131 of these die by apoptosis at specific times. These cells are predetermined to die as the same 131 cells die in every worm. Through loss of function and gain of function experiments, it was discovered that three ced (**ce**ll **d**eath) genes were of utmost importance in the normal development of the worm. These genes are:

- *Ced-3*: a pro-apoptotic protein with homology to ICE in mammalian cells.
- *Ced-4*: a pro-apoptotic protein thought to be homologous to apaf-1 (apoptosis activating factor-1) in mammalian cells.
- *Ced-9*: An anti-apoptotic protein homologous to the bcl-2 family.

Ced-9 acts upstream of ced-3 and ced-4 (del Peso et al., 2000). Ced-4 binds ced-3 and via a cleavage reaction, promotes its activation. Ced-9 can counteract this interaction by binding ced-3. In doing so, it inhibits both the long (ced-4L) and short (ced-4S) forms of ced-4 from binding (which inhibit and promote apoptosis, respectively). Ced-4 regulates the interaction between ced-3 and ced-9. In non-apoptotic cells, ced-9 is complexed with ced-3 and ced-4. This prevents apoptosis as ced-4 is unable to activate ced-3. On receipt of an apoptotic stimulus, ced-9 dissociates, ced-3 is activated and apoptosis follows.

Mutations in the ced genes, lead to irregularities in apoptosis (Reddien et al., 2001, del Peso et al., 2000). Loss of ced-3 or ced-4 leads to decreased apoptosis and increased cell survival. Overexpression of ced-9 leads to decreased apoptosis while loss of ced-9 function leads to increased cell death and greater cell loss. The mammalian homologue

of ced-9, bcl-2, can be substituted for ced-9 loss of function and wild type levels of programmed cell death resume.

Ced-4 has been demonstrated as a link between bcl-2 and caspases when transfected into mammalian cells as it has a death effector domain resembling that seen in caspase-8 (FLICE/MACH). The mammalian homologue of ced-4, Apaf-1, appears to act in a similar manner; however, Apaf-1 activation of caspases requires the presence of Apaf-3 and cytochrome c (Lauber et al., 2001).

1.3.3 The Death Receptors.

Death activators binding to receptors at the cell surface can also initiate apoptosis. These receptors include the Fas receptor and the TNF (tumour necrosis factor) receptor (Sartorius et al., 2001). Both are integral membrane proteins with their receptor domains exposed at the surface of the cell. The binding of the complementary death activator (FasL and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase-8, which in turn initiates a cascade of caspase activation leading to the death of the cell (Sartorius et al., 2001, Kondo et al., 1997).

The Fas receptor is also known as CD-95 (Scaffidi et al., 1998). Fas is a 45 kDa glycosylated transmembrane receptor. It is a member of the TNFR family. It is found on both lymphoid and non-lymphoid cells. Its corresponding ligand, fas-L, is a 40kDa glycosylated transmembrane protein and is also a member of the TNF family. Its distribution is more limited, found on cells involved in killing (T-cells, NK-cells and macrophages) (Sartorius et al., 2001). The main role of the fas pathway is in T cell development and in the killing of virus infected cells (Sharma et al., 2000).

Fas ligand is a homotrimeric molecule (Huang et al., 1999). It exists complexed with two other fas ligands. The three death domains cluster together and bind to the fas receptor. An adaptor molecule, FADD (fas-associated death domain) then binds via its death domain to the clustered receptor death domains. FADD's death effector domain binds to a repeated DED on caspase-8 (aka FLICE/ MACH). Caspase-8's DED is called a CARD (caspase recruitment domain). When FADD binds caspase-8, caspase-8 oligomerises and drives its own activation through self-cleavage. Caspase-8 then

activates caspase-9 and commits the cells to apoptosis (Scaffidi et al., 1998, Sartorius et al., 2001).

Another signalling system inducing apoptosis involves the TNF-R1. It acts in a similar way to fas. TNF trimerises TNFR-1 upon binding, again leading to the clustering of the receptor's death domains. An adapter protein called TRADD (TNFR-associated death domain) binds through its death domain to the clustered receptor death domains. TRADD recruits several signalling molecules to the activated receptor; namely, TRAF-1 and RIP. (These can in turn stimulate pathways leading to the activation of NF-κB and JNK). FADD can also bind TRADD and apoptosis ensues (French & Tschopp, 2002, Velthuis et al., 2002).

1.4 The Bcl-2 Family.

Bcl-2 was the first mammalian homologue of the ced genes to be found. It was originally identified in patients with Burkitt's **B c**ell **l**ymphoma where the gene is the product of a translocation between chromosome 14 and 18 (Tsujimoto & Croce, 1986). In cancerous B cells, the portion of chromosome 18 containing the bcl-2 gene has undergone a reciprocal translocation with the portion of chromosome 14 containing the antibody heavy chain locus. The t(14;18) translocation places the bcl-2 gene close to the heavy chain gene enhancer. Because B cells are involved in making large quantities of antibodies, this enhancer is very active and overexpression of bcl-2 is seen in these t(14;18) cells.

By preventing cell death, bcl-2 acts as a proto-oncogene. The overexpression of bcl-2 creates a backdrop of extended cell survival thus allowing the accumulation of additional genetic mutations and increasing the probability of tumorigenic conversion. In this way, it leads to prolonged cell survival is not achieved by increasing the rate of cell proliferation but by reducing the rate of cell death. The bcl-2 protein is produced at high levels in many types of cancers; i.e. 90% of colorectal cancers, 30-60% of prostate cancers, 70% of breast cancers, 20% of non-small cell lung carcinomas as well as 65% of lymphomas (Rutledge et al., 2002). *In vivo*, the expression of bcl-2 represents a poor response to therapy.

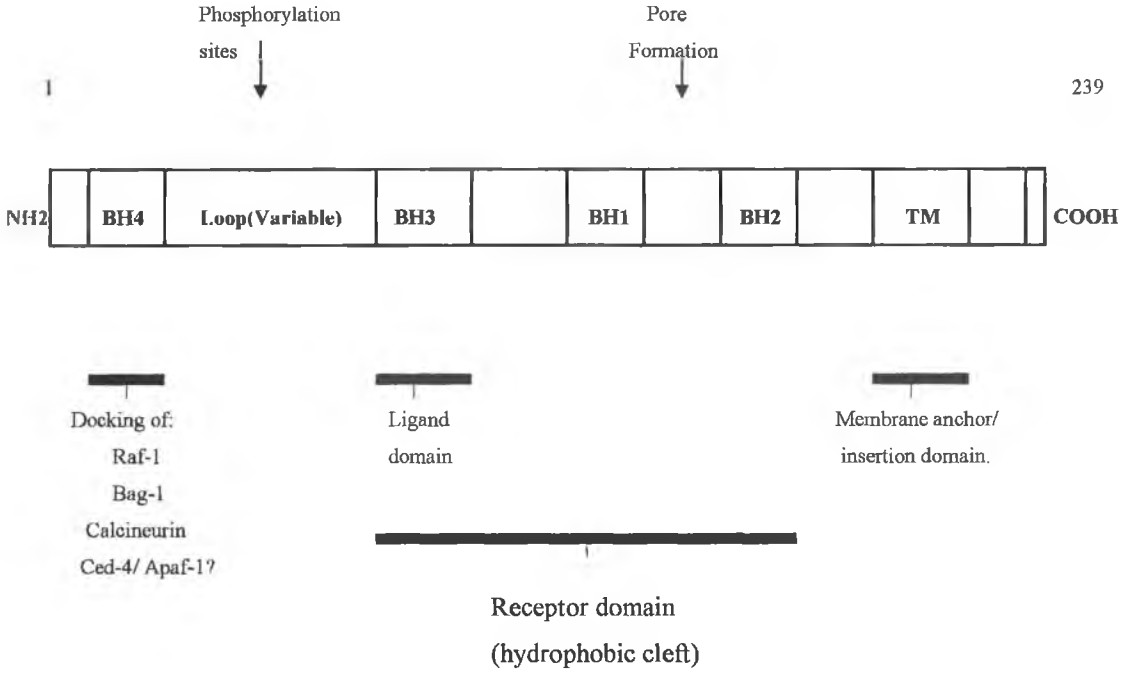
Bcl-2 is a 25kDa transmembrane protein with a C-terminal hydrophobic domain. This domain allows it to insert itself into membranes, namely, the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear membrane.

Structurally, bcl-2 has a unique sequence which provides few clues as to the exact biochemical process by which it blocks apoptosis. The protein is organised into a number of conserved domains. These are highly conserved right across the spectrum of bcl-2 family proteins and each domain appears to have a specific function. (See figure 1.3 for organisation of bcl-2 protein).

The BH1 and BH2 domains are involved in pore formation (Reed, 1998). In cell death antagonists, they allow heterodimerisation of bcl-2 with bax to repress apoptosis (Kelekar & Thompson, 1998, Lutz, 2000). The BH3 domain is involved in binding of

pro-apoptotic proteins to death antagonists. Some pro-apoptotic proteins are homologous only to other bcl-2 family proteins by stretches of amino acids within their BH3 domains, and yet they still retain the ability to interact with death suppressors and induce cell death, e.g. bak and bad (Kelekar & Thompson, 1998, Lutz, 2000, Griffiths, 1999). The BH4 domain allows proteins to interact with death regulatory proteins such as raf-1, bad, calcineurin and apaf-1. This domain is found in all anti-apoptotic proteins (Huang et al., 1998, Reed, 1998).

Figure 1.3: ***Organisation of Bcl-2 protein.***



There are multiple bcl-2 homologues discovered based on structure and their effects on apoptosis. They can be divided into two groups based on their ability to induce or suppress cell death. Whether or not apoptosis proceeds in a cell depends on the ratio of pro-apoptotic versus anti-apoptotic protein levels. The bcl-2 family encompasses a wide range of genes which when translated into proteins interact with each other forming homo- and hetero-dimers. It appears that the "decision" to activate this intrinsic suicide

mechanism depends on those interactions. Excess death antagonists promote survival whereas excess death agonists promote apoptosis (Reed, 1998, Rutledge et al., 2002).

Table 1.1 Pro- and Anti-apoptotic genes.

Suppress apoptosis	**Induce apoptosis**
Bcl-2	Bcl-xS
Bcl-xL	Bak
Mcl-1	Bax
Bfl-1/A-1	Bad
Bcl-w	Bik (NBK)
Bag-1	Bcl-xβ
E1B-19K	Hrk
BHRF-1	Mtd/Bok
Ced-9	DIVA
Bcr-abl	Bim
Mutant p53	

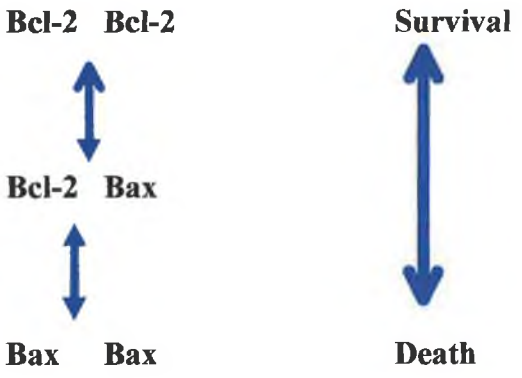
Differential splicing of individual genes can be an important mechanism for generating lineage-specific proteins that carry out distinct, but related biological functions. Bcl-XL and Bcl-XS are alternative splice forms with opposing functions (Boise et al., 1993, Grillot et al., 1997). Bcl-XL, like bcl-2, can induce significant resistance to apoptotic cell death. Bcl-XS inhibits the anti-death effect of bcl-2 and bcl-xL. As well as bcl-xL and bcl-xS, bcl-x is alternatively spliced into bcl-xβ, bcl-$x_{\delta TM}$ and bcl-xγ (Yang et al., 1997, Ban et al., 1998, Shiraiwa et al., 1996).

Many of the bcl-2 family genes have alternative splice sites generating products with opposing functions. These include bcl-xL and mcl-1 (Grillot et al., 1997, Bingle et al., 2000). Alternative splicing has also been reported in other bcl-2 family members. These

include bcl-2, where bcl-2β is a 22kDa protein that diverges from the 26kDa protein after the BH2 domain due to alternative splicing, (Hanada et al., 1995, Shiraiwa et al., 1996). There are also spliced variants of bax at the mRNA level, and variants of bag-1 at the protein level (Bingle et al., 2000, Yang et al., 1999, Cutress et al., 2002). Ced-4, a protein in C- elegans, which corresponds to mammalian APAF-1, also undergoes alternative splicing producing two distinct proteins with opposing physiological effects. The shorter ced-4 RNA promotes apoptosis, while the longer ced-4 promotes cell survival, (Shaham & Horvitz, 1996).

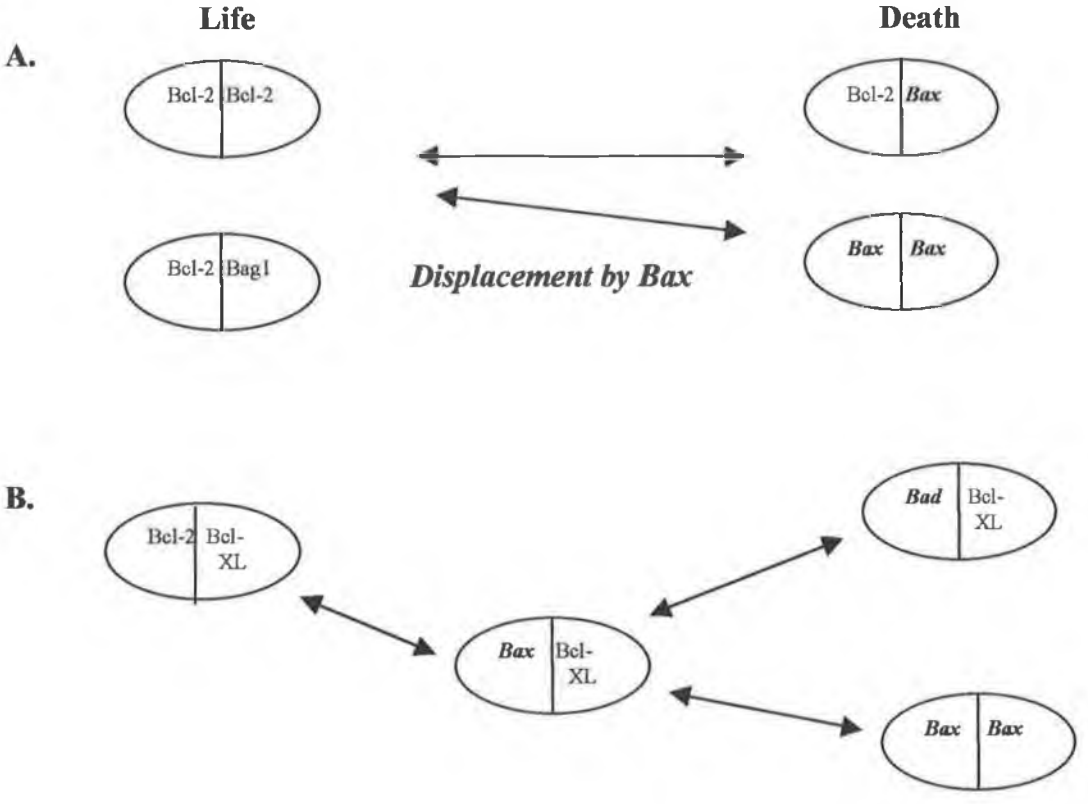
1.4.1 Dimerisation of Bcl-2 Family Proteins.

The bcl-2 family exists as a multi-component protein complex and the relative concentration of pro- and anti-apoptotic members will determine whether the cell will live or die. (See example figure 1.4). Bcl-2 family proteins have the ability to dimerise with eachother (Ottilie et al., 1997). Bax is a central protein in these reactions and it has been found that the tendency of a cell towards apoptosis depends to a large extent on the ratio of bcl-2 versus bax expression. Bax appears to counter the death repressor activity of bcl-2 (Boise et al., 1993, Schmitt et al., 1997, O'Gorman & Cotter, 2001). Due to the transmembrane domains of both proteins, their sequence homology and secondary structures, they are likely to be inserted in the membrane in the same orientation. However, both membrane and cytsolic forms of bcl-2 can associate with bax. In its interactions with bcl-2, if bax is in excess, bax homodimers dominate and apoptosis occurs. However, when bcl-2 is in excess, cells are protected (Ottilie et al., 1997, Reed, 1998).



When bax or another pro-apoptotic protein is overexpressed in some cell lines it alone does not cause programmed cell death. The tendency of a cell to die depends on another death signal, such as cytokine deprivation, to initiate the death pathway. Thus the bcl-2/ bax association represents an endogenous regulator of cell death, against a backdrop of environmental signals.

Figure 1.4 Dimerisation of bcl-2 family members determines whether a cell will live or die. Bad can displace Bax, allowing Bax:Bax homodimers to form, tipping the balance towards apoptosis. Pro-apoptotic proteins are indicated in bold italic font.



1.4.2 Mechanism of action of Bcl-2 Family Proteins:

To understand and investigate the action of any protein, its location within the cell must be known. Through a series of experiments, bax, bcl-2 and bcl-xL have been localised to three specific organelles within the cell. Bcl-2 is a transmembrane protein that inserts itself into the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes (Mandal et al., 1996, Sartorius et al., 2001, Desagher & Martinou, 2000). This insertion is facilitated by a single predicted transmembrane segment located at the COOH-terminus of the protein and is flanked on either side by positively charged residues (Nguyen et al., 1994). The COOH 22-amino acid domain functions as a signal anchor sequence that targets and inserts the protein into the mitochondrial outer membrane in an N_{cyto}.- C_{in} orientation where the bulk of the protein faces the cytosol.

The ability of bcl-2 to heterodimerise with bax resides within the cytosolic domain of bcl-2 and strongly correlates with bcl-2 function. As bax is a soluble protein, it can interact with the cytosolic portion of bcl-2 (Reed, 1998, Sartorius et al., 2001, Desagher & Martinou, 2000).

The association of bcl-2 and bax is mediated by interactions between their BH domains. A study by Sedlak and coworkers in 1995, found that intact BH1 (residues 138- 154 of bcl-2) and BH2 (residues 188- 196) domains appeared to be essential for dimerisation. Mutations in these residues restricted the potential partners of a particular protein. In BH1, glycine, and in BH2, tryptophan 188, if mutated, prevented bcl-2 from dimerising with bax (Sedlak et al., 1995).

The BH3 domain, however, appears to be the only essential component for protein-protein interaction (Lutz, 2000). It was reported essential for bax to function as a death protein (Simonen, Keller and Heim, 1997). BH3 alone, or in combination with the N terminus was sufficient for strong interaction with bcl-2, bcl-xL and bax itself. The BH3 only proteins are thought to be critical at regulating protein interactions. A study by Virdee (2000) found that phosphorylation of the bad protein within its BH3 domain resulted in loss of its pro-apoptotic activity. The transmembrane region appeared to be necessary for activity perhaps because bax interacts with membrane-bound proteins. However even without this domain, bax could associated with mitochondria in mammalian cells (Hsu et al., 1997).

The proteins appear to relocate themselves when apoptosis is induced. Hsu et al (1997) observed a shift in subcellular location of bax from the cytosol to either crude membrane or nuclear compartments after treatment with dexamethosone. Bcl-xL also relocated itself and the protein was embedded in the membranes. However, no change was observed in bcl-2 location. Mandal et al. (1998) showed that inactive bax was mainly in the soluble form in both the cytosolic and nuclear compartments. Again, during apoptosis bax redistributed itself to its membrane bound form.

Bax, bcl-xL and bcl-2 have the ability to form pores in cell membranes, which is pH dependent (Antonsson et al., 1997). They are voltage-gated channels and the F_0/F_1 ATPase proton pump is required for bax-induced death. Bax has been found to co-purify with the mitochondrial megapore/ permeability transition pore complex (PTPC). This is a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability (Marzo et al., 1998). Overexpression of bax in Jurkat cells induces the regulated opening of this large non-specific pore in the inner mitochondrial membrane (Pastorino et al., 1998).

As well as affecting the mitochondrial membrane permeability, the pro-apoptotic proteins function by generating reactive oxygen species. In *E. coli* cells, 0.01% of bax protein was sufficient to cause cell death. Co-expression of bcl-xL, caused a modest inhibitory effect on cell death caused by bax. Bax increased oxygen consumption, triggered the generation of superoxide radicals which led to nicked DNA and bacterial cell death. Just as bax promotes cell death via generation of oxygen radicals, bcl-2 may counteract this by inhibiting the anti-oxidant pathway (Asoh et al., 1997).

1.4.3 Bax.

Bax is probably the most potent of the apoptosis-inducing proteins. Its interactions with other family members and other proteins determine to a large extent the fate of the cell.

Bax is a 21kD protein that shows extensive amino acid homology with bcl-2. Oltvai and coworkers analysed the bax gene in murine cells and found it to have extensive

homology with bcl-2. Both have ORFs of 576bp and are 89.4% identical to one another. Both ORFs encode a 192 amino acid protein with a molecular weight of 21 kD. Murine and human bax proteins are 96% homologous and have seven serine/ threonine residues which may represent sites of phosphorylation. The C-terminal transmembrane domain indicates that bax exists as an integral membrane protein (Oltvai et al, 1993).

Structurally, the bax gene consists of six exons within a 4.5kb region. Alternative splicing of the gene produces three distinct transcripts, ie;

~ a 1.0 kb α RNA encodes the 192 amino acid 21kd protein with the transmembrane segment.

~ a 1.5kb β RNA possesses an unspliced intron. It encodes a 218 amino acid 24kd protein that lacks a hydrophobic terminus (ie a transmembrane domain) and may be a cytosolic form.

~ a γ RNA which exists in 1.0 and 1.5kb forms due to alternative splicing in intron 5. These lack a small 53bp exon 2 which shifts the reading frame to exon 3. The γRNAs, if translated, would predict a protein of 41 amino acids with a molecular weight of 4.5 kd.

The splicing of the bax gene may prove to be significant in its activity as well as its tissue localization. The expression of the various RNA species differs from tissue to tissue. Bax is not lymphoid restricted but is in fact expressed in a variety of tissues. Both 1.0 kb and 1.5 kb forms are found in the lungs, stomach, kidneys, thymus, lymph nodes, bone marrow, and spleen. The 1.0kb RNA was preferred in the heart and smooth muscle and was most abundant in the pancreas. The 1.5kb RNA expression was recorded in the duodenum and the brain, along with a higher molecular weight species in the latter (Oltvai et al, 1993).

Homology exists between bax and bcl-2, especially in bax at the juncture of exons 4 and 5, and in bcl-2 at the juncture of exons 2 and 3. Moreover, the bax α (membrane form) and the bax β (cytosolic form) parallel to the bcl-2α (integral membrane form) and the bcl-2β (cytosolic form) (Tsujimoto and Croce, 1986).

The interactions of bax with other family members and other proteins determine to a large extent the fate of the cell. Bax antagonises bcl-2 function. The relative amounts of bcl-2 and bax determine cell viability during growth factor deprivation. Bax also has the ability to dimerise with bcl-Xl, Mcl-1 and bfl/A1 genes.

Bax protein levels are altered in several clinically relevant settings where cell death occurs. Elevated levels of bax are seen in neurons following cerebral ischaemia, myocardiocytes following acute myocardial infarction, and in tumours during responses to chemotherapy and radiation. Many tumours have mutations in the bax gene giving them survival advantages. A study by Gil and coworkers (1999) found missense mutations in gastrointestinal cancers that prevented the proapoptotic activity of bax. These mutations are commonly found in colon cancers also. It is thought that these mutations aid tumour progression (Ionov et al., 2000).

Bax deficiency or mutation promotes oncogenic transformation. Its expression is therefore a determinant of chemosensitivity. Mutations that decrease bax in tumours contribute to drug resistance enabling tumour-promoting mutations to occur in cells with survival advantages afforded by the expression of bcl-2/ bcl-xL (McCurrach et al., 1997). P53 is a regulator of bax expression and is expressed in mutated form by many cancer cells. A potential anticancer therapy, curcumin is thought to induce apoptosis in human breast cancer cells by inducing bax expression via a p53 regulatory pathway (Choudhuri et al., 2002).

Thus bax is a key regulator protein, interacting with other cellular proteins, to maintain cellular homeostasis.

1.4.4 Bcl-x: Bcl-2 associated X Protein.

The bcl-x gene is a bcl-2-related gene that can function as a bcl-2 independent regulator of programmed cell death. In itself, it is a blueprint for the production of up to five different proteins. As mentioned, a number of isoforms of bcl-x have been isolated, namely bcl-xL, bcl-xS, human bcl-xβ, bcl-xδTM and bcl-xγ (Boise et al., 1993, Grillot et al., 1997, Yang et al., 1997, Shiraiwa et al., 1996, Ban et al., 1998). Indeed the bcl-x gene alone may represent a subfamily of genes involved in apoptosis regulation within

the larger confines of the bcl-2 family. Each spliced form has distinct domains and functions justifying its position as a unique member of an ever-increasing family.

Bcl-xL, bcl-xβ and bcl-xδTM are found in tissues that are "long-lived", including the brain, eyes, heart, intestine, kidney, liver and thymus (Yang et al, 1997; Grilllot et al., 1997; Boise et al., 1993). In contrast to this, bcl-xS expression is confined to tissues with a high rate of turnover, e.g. thymic T-cells (Boise et al., 1993). The most recently discovered member of the bcl-x group, bcl-xγ, is found in thymus, lymph node and eye, but not liver, brain, kidney, intestine or heart (Yang et al., 1997).

Bcl-x and bcl-2 exhibit sequence homology. The genomic organisation of bcl-x details a three exon structure (Grillot et al., 1997). Exon-1 is non-coding, exon-2 contains the BH1, BH2, BH3 & BH4 domains, and exon-3 encodes the carboxyl terminus for both bcl-xS and bcl-xL. Individual bcl-x genes differ in their homologous regions and each should be considered separately.

The molecular basis detailing the exact formation of the bcl-xL and bcl-xS mRNAs has yet to be elucidated. Reports have indicated up to five promoter sites in the bcl-x gene, that potentially five rise to at least five mRNAs (Pecci et al., 2001, Grillot et al., 1997). A study by Pecci (2001) indicated that these promoters had tissue-specific activity and that these promoters determine the balance of bcl-x isoforms (Pecci et al., 2001).

Bcl-xL, the long isoform, and bcl-xS, the short form, contain distinct open reading frames flanked by identical 5' and 3' UTR sequences. Bcl-xL is generated by the juncture of exons 2 and 3 through a splicing reaction (Grillot et al., 1997). It is a 241 amino acid protein, which has 43% sequences homology with bcl-2 (Sato et al., 1994). Bcl-xL contains BH1, BH2, BH3 and BH4 domains, similar to those of bcl-2. Like bcl-2, the bcl-xL protein can induce significant resistance to apoptotic cell death and furthermore, in some studies, it has been found to be more efficient than bcl-2 in preventing cell death (Shimizu et al., 1995).

Bcl-xS, due to alternative splicing has a deletion of 63 amino acids in the region of greatest homology to bcl-2 (Nunez et al., 1994, Chalfont et al., 2002). It antagonises the anti-death effect of bcl-2 and bcl-xL (Zhang et al., 1995). Bcl-xS mRNA is generated by a donor splice site located within exon 2 at position 375. This alternative GT donor site

is spliced to an AG acceptor site in exon 3, forming mature bcl-xS mRNA (Grillot et al., 1997). Bcl-xS, therefore, lacks the BH1 and BH2 domains of bcl-2. It encodes a 170 amino acid protein. It acts as a dominant regulator of cell death over bcl-2 and bcl-xL (Boise et al., 1993). This may be due to the formation of an inactive heteromeric bcl-2/ bcl-xS complex. The BH1 domain and membrane-anchoring C-terminus of the bcl-2 protein are essential for its interaction with bcl-xS (Zhang et al., 1995). It has been found that certain compounds e.g. ceramide can induce apoptosis by increasing bcl-xS and decreasing bcl-xL (Chalfant et al., 2002).

A novel form of the bcl-x gene was identified in T-cells, namely, bcl-xγ (Yang et al., 1997). This unique gene is expressed primarily in T cells. It is specifically connected to TCR ligation and is essential for resistance to TCR-dependent apoptosis. It is up-regulated at the RNA levels after T cell activation and was accompanied by enhanced expression at the protein level. Failure of bcl-xγ expression after CD3 ligation represents a genetic marker for programmed cell death, while activated T cells that express bcl-xγ are spared. This may represent the genetic mechanism by which the interaction between peptide ligand and the TCR is converted into a signal leading to successful activation and positive selection in the immune system's process of thymic selection (Yang et al., 1997). Bcl-xγ encodes a 235 amino acid isoform characterised by a unique C-terminus of 47 amino acids. In contrast to bcl-xL and bcl-xS, bcl-xγ lacks an obvious hydrophobic domain flanked by charged residues. Therefore, bcl-xγ is present mainly in the cytosol rather than embedded in the membrane.

Another product of splicing of bcl-x is bcl-xδTM. It was isolated from a mouse pre-B-cell library. It has a 72 base pair deletion at the carboxy-end region DNA of bcl-xL, ie, a deletion of the transmembrane domain. It may be a product of splicing within exon 3 sequences (Grillot et al., 1997). Bcl-xβmRNA is generated by an unspliced mRNA sequence. It was identified in rats and promotes apoptosis in promyeloid cells, (Shiraiwa et al., 1996). It encodes a protein of 209 amino acids, (Yang et al., 1997). Both bcl-xβ and bcl-xδTM have been described in rat and/ or mouse but their physiological functions remain unclear, (Grillot et al., 1997).

Manipulation of the alternative splicing of bcl-x has been carried out in a number of studies. Mercatante and coworkers (2001) used oligonucleotide antisense sequences to

target the 5' splice site of bcl-xL. This resulted in shifting the balance towards increased expression of bcl-xS. In prostate cancer cells, this induced cell death, however in breast cancer cells (MCF-7), apoptosis was induced to a lesser extent (Mercatante et al., 2001). This approach was also used by Taylor (1999) and resulted in increased apoptosis in response to UV irradiation and chemotherapeutic drugs.

1.4.5 Mcl-1

Mcl-1 is an anti-apoptotic protein. It is normally expressed in hematopoietic and lymphoid tissues. The mcl-1 gene was originally identified in ML-1 leukaemia cells. It is induced at the early stages of differentiation in normal hematopoietic cells. When ML-1 cells were induced to differentiate by TPA, mcl-1 expression increased from one to three hours after exposure to the differentiating agent (Reynolds et al., 1994). Mcl-1 expression has also been induced through anti-apoptotic cytokine-mediated pathways in ML-1 cells through interleukin and CSF pathways (Epling-Burnette et al., 2001).

Like bcl-2 the full-length mcl-1 protein has BH1, 2 & 3 domains and transmembrane domain, but lacks a BH4 domain, essential for the anti-apoptotic functions of bcl-2 (Moulding et al., 2000). Full-length mcl-1 has three coding exons. The first is a G/C rich region encoding the first 229 amino acids. This exon encodes the PEST and BH3 domains. Exon two encodes BH1 and a portion of BH2 domains. Exon three encodes the rest of BH2 and the C-terminal transmembrane domain. Mcl-1 has an intron just downstream of BH3 in addition to the conserved intron further downstream in BH2. Other bcl-2 family members do not contain an intron comparable to mcl-1 intron except for the pro-apoptotic member, bax. The carboxyl terminus of mcl-1 has highest sequence similarity to bcl-2 and to the viral protein BHRF-1. This is the membrane-spanning domain of the proteins (Kozopas et al., 1993, Reed, 1998).

The protein also contains two PEST sequences, which are proline, glutamate, serine and threonine motifs. These sequences are typical of proteins that are rapidly turned over. The half-life of mcl-1 is therefore very short. Reports estimate between 30 minutes, to one hour to three hours. This contrasts to that of bcl-2, whose half-life is 10 hours in B-cells (Schubert et al., 2001, Kuo et al., 2001, Moulding et al., 2000, Katoh et al., 1998, Leuenroth et al., 2000, Kozopas et al., 1993, Chao et al., 1998, Fukuchi et al., 2001). As

well as the PEST sequences, four pairs of arginines are found in the mcl-1 protein, which are present in a variety of oncoproteins and other proteins that are transiently expressed and undergo rapid turnover (Kozopas et al., 1993).

Bcl-2 protein lacks PEST sequences, and so has greater stability than mcl-1. Mcl-1 is rapidly induced following an apoptotic stimulus and rapidly turned over. It is thought that this protein with its short half-life provides initial protection against cell death stimuli by giving cells time to marshal a further response. This was seen where epithelial cells were induced to die by IL-6 treatment. Mcl-1 levels had increased by three hours, but other anti-apoptotic proteins had increased at eight hours (Kuo et al., 2001). It is also speculated that the mcl-1 protein, which is reportedly induced during differentiation, serves to protect cells from apoptosis when they are particularly susceptible to apoptosis. DNA damaging agents such as UV irradiation or cytotoxic drugs induce bcl-2 to decrease in the first few hours following exposure, whereas mcl-1 mRNA levels are rapidly but transiently increased (Johnson, 1999).

Recently, alternatively spliced mcl-1 transcripts have been identified. A short form of mcl-1 containing the BH3 and transmembrane regions has been found to be a pro-apoptotic protein, mirroring findings in the bcl-xL/ bcl-xS genes (Bingle et al., 2000). This shorter form of mcl-1 is a product of splicing exon one to exon three, thus excluding exon two. Upstream of the deleted region, the two transcripts were identical in sequence. Therefore the first 229 amino acid residues are in frame and encode the PEST and BH3 domains. However, the remaining 42 amino acid residues are out of frame leading to the loss of BH1, BH2 and TM domains (Bingle et al., 2000). Despite the functional studies on effects of transfection with mcl-1, both long and short variants, it remains to be elucidated how these isoforms function and are co-ordinated in vivo (Bae et al., 2000).

Mcl-1 has a more widespread distribution than bcl-2. It is found in mitochondrial and non-mitochondrial compartments and localises to the nucleus and cytoplasm and to membrane-bound organelles (Leuenroth et al., 2000). It is also found on light intracellular membranes where bcl-2 is not (Moulding et al., 2000).

Yang and coworkers (1996) studied the intracellular distribution of mcl-1 and bcl-2. They found that mcl-1 expression overlapped with but was not identical with that of

bcl-2. Mcl-1 is similar to bcl-2 in that the mcl-1 protein has prominent mitochondrial localisation, and it associates with membranes through its carboxyl hydrophobic tail. It differs from bcl-2, however, in its relative distribution among other nonmitochondrial compartments. Mcl-1 is abundant in the light membrane fraction of immature ML-1 cells while bcl-2 is abundant in the nuclear fraction (Yang et al., 1996).

In mcl-1 transfected cells, suppressing the potassium channels in membranes lead to decreased viability after VP-16 treatment. Therefore the integrity of the potassium channels appears to be essential to the ability of mcl-1 to prevent apoptosis (Wang et al., 1999a).

It associates with pro-apoptotic but not anti-apoptotic bcl-2 family proteins (Johnson, 1999). In a yeast cell system, mcl-1 was found to inhibit bax-induced cell death. In a study using murine FDC-P1 cells, mcl-1 co-immunoprecipated with bax, but to a lesser extent than bcl-2. Mcl-1 heterodimerises with bax and neutralises cytotoxicity induced by bax (Sato et al., 1994, Zhou et al., 1997). It forms dimers with other pro-apoptotic proteins, namely, bad, bak, bok, bik and bod (Johnson, 1999). It appears to participate in the regulation of caspase-3, whose activation is an early event in apoptosis. Mcl-1 expression induced by treating leukaemia cells with VP-16 resulted in decreased caspase-3 activity (Katoh et al., 1998).

1.4.6 Bag-1

Bag-1 is an anti-apoptotic gene that localises to chromosome 9 (Takayama et al., 1995, Yang et al., 1999). It has no significant homology to bcl-2 or any other bcl-2 protein. However when bag-1 and bcl-2 are co-expressed, they provide markedly increased protection from cell death induced by several stimuli (Brimmell et al., 1999, Cutress et al., 2002). Bag-1 represents a novel type of anti-death gene and routes of apoptosis induction previously attributed to bcl-2 independent pathways may instead reflect a need for combination of bcl-2 and bag-1 (Takayama et al., 1995, 1996, 1997).

There are at least six isoforms of the bag-1 protein generated by alternative translation initiation (Yang et al., 1999, Takayama & Reed, 2001). They differ in their subcellular location. The largest protein, bag-1L localises to the nucleus via its unique N-terminal

domain while smaller proteins localise to the cytoplasm (bag-1S) and between cytoplasm and nucleus (bag-1M) (Yang et al, 1999). It appears that the subcellular location of the isoforms and which isoform is expressed has proved important in clinical studies. Reports have estimated that up to two thirds of breast cancers or non-small cell lung cancers had cytoplasmic bag-1 expression (Tang et al., 1999, Turner et al., 2001, Rorke et al., 2001).

The protein interacts with several steroid hormone receptors such as the androgen, oestrogen and glucocorticoid receptors. It also interacts with retinoid receptors and influences the transcriptional activation and apoptosis induced by steroid hormones and retinoids (Brimmell et al., 1999, Yang et al., 1999, Turner et al., 2001). Bag-1 has been found to bind the tyrosine kinase growth factor receptors (platelet derived growth factor receptor and hepatocyte growth factor receptor) and thereby enhances their ability to suppress apoptosis. It may functionally act as an adaptor between tyrosine kinase receptors and anti-apoptotic machinery of the cell. Wang et al have reported that bag-1 interacts with raf-1's catalytic domain. They also speculate that raf-1 is found in a tri-molecular complex with bag-1 and bcl-2. Bag-1 may also be a novel raf-1 interacting protein where it may recruit other proteins to activate the kinase or protect it from inactivation by phosphatases. Bag –1 also binds heat shock proteins such as hsp/ hsc 70 (Cutress et al., 2002).

The exact role that bag-1 plays in tumour progression has yet to be elucidated. Its overexpression suppresses caspase activation and apoptosis in response to a variety of stimuli including chemotherapeutic drugs, which may contribute to its ability to promote tumour progression (Takayama & Reed, 2001, Cutress et al., 2002). Some studies in breast cancer patients report that its expression is increased in tumorigenic tissue, but its association with other prognostic factors such as tumour size & grade, receptor status, disease-free and overall survival are under investigation (Tang et al., 1999).

1.4.7 Other bcl-2 family members.

1.4.7.1 Bad: Bcl-2 associated death protein.

Bad is a death agonist. It restores apoptosis when bound by pro-apoptotic proteins such as bcl-2 and bcl-xL. This protein can be post-translationally modified through the presence of an extracellular influence, such as a cytokine. In the presence of IL-3, bad is phosphorylated on two serine residues (Virdee et al., 2000). Phosphorylated bad is found bound to a signal transduction protein 14-3-3. It is thought that 14-3-3 is a chaperone protein that binds bad and keeps it sequestered in the cytosol and may prevent it being targeted by phosphatases (Zha et al., 1996). Such post-translational modification of a bcl-2 protein illustrates how its death-agonist activity is regulated. Phosphorylated bad does not bind bcl-xL leaving bcl-xL free to promote cell survival (Kelekar et al., 1997, Downward et al., 1999). Unphosphorylated bad counteracts the apoptosis inhibition induced by bcl-xL expression but not that by bcl-2. Unphosphorylated bad can bind bcl-xL at the membrane displacing bax. This would enable bax to form homodimers, resulting in cell death (Kelekar et al., 1997).

1.4.7.2 Bak: Bcl-2 homologous antagonist/ killer.

Bak promotes apoptotic cell death by counteracting the protective effects of bcl-2 expression (Griffiths et al., 1999). Through studies in bax and bak knock-out mice, bax and bak have been found to be essential for TNF-α-mediated apoptosis, but act independently of each other (Degenhardt et al., 2002). Bak is expressed in a wide variety of tissues including skeletal muscle, heart, liver, pancreas, kidney and brain. It shows extensive homology to human bcl-2 and bcl-xL proteins, particularly at BH1 and BH2 domains. It shares 53% amino acid sequence identity with bcl-2 and overall is 25, 22 and 19% identical to bcl-2, bcl-xL and bax respectively. The bak protein contains a C-terminal hydrophobic transmembrane domain indicating that it exists as an integral membrane protein, (Kiefer et al., 1995). The BH1 and BH2 domains have proved to be indispensible to bak function and are thought perhaps to contribute to the regulation of bak or facilitate interactions with bcl-2 homologues or other proteins (Finnegan et al., 2001, Chittenden et al., 1995, Elangovan et al., 1997).

1.4.7.3 Bik: Bcl-2 interacting killer.

Bik functions to promote cell death in a mechanism similar to that of other death-inducing proteins, bax and bak. However despite this, it has very little homology with these proteins. It lacks the trademark BH1 and BH2 domains common to the other members of the family (Boyd et al., 1995). It has the BH3 domain in common with these proteins and it was through the identification of bik that the importance of the BH3 domain came to be realised (Kelekar & Thompson, 1998). While BH3 is found in all bcl-2 family members, it has been found that BH3 is essential to the death-promoting activity of bik, bax and bak (Germain et al., 2002, Puthalakath & Strasser, 2002). Through deletion mutation analysis of this domain in these proteins, the ability of these proteins to interact with bcl-xL was lost. The death-promoting activity of bik can be inhibited by coexpression of bcl-2, bcl-xL, BHRF-1 or E1B-19K proteins. It is likely that these proteins antagonise the activity of bik. Thus bik may be a common target for the cellular and viral apoptosis inhibiting proteins of the bcl-2 family (Boyd et al., 1995). Bik has been shown to be a potent tumour suppressor gene (Zou et al., 2002) and its expression has antagonized drug resistance due to bcl-2 overexpression in breast cancer cell lines (Radetzki et al., 2002).

1.4.7.4 HRK: Harakiri/ DP5.

HRK is a pro-apoptotic gene that does not interact with any other pro-apoptotic proteins such as bax, bcl-xS, bak, etc. HRK, like bik, lacks bcl-2 homology domains BH-1 and BH2. It is classified within the family based on 8 amino acids in the BH3 domain, which have homology to other proteins (Kelekar & Thompson, 1998). It needs a stretch of 16 amino acids in BH3 domain to interact with anti-apoptotic proteins. Structurally, it resembles the pro-apoptotic protein bik/NBK. Its expression pre-dominates in bone marrow, spleen, and also pancreas, liver, kidney, lung and brain (Inohara et al., 1997, Imaizumi et al., 1999, Imaizumi et al., 1997).

1.4.7.5 MRIT-9: MACH-related inducer of toxicity.

This is pro-apoptotic gene that interacts with caspases and the bcl-2 family. It has sequence homology with FLICE links death domain receptors of TNF to downstream caspases. It activates caspase dependent death. It associates with caspases FLICE, CPP-

32/YAMA and FADD. It simultaneously and independently interacts with bcl-xL and FLICE. Bcl-2 and bcl-xL can block MRIT-induced apoptosis in MCF-7 cell line. The exact in vivo function of MRIT is still unknown (Han et al., 1997).

1.4.7.6 p28 BAP 31.

This is a 28kDa pro-apoptotic protein. Similar to MRIT, it bridges the apoptosis-initiating caspase with anti-apoptotic regulators bcl-2 or bcl-xL. Bax prevents bcl-2 from associating with p28 (Ng et al., 1998). It is a component of a putative apoptotic signalling complex in the ER that also includes bcl-2/bcl-xL and procaspase-8 (pro-FLICE). It may be responsible for activating receptor-associated procaspase-8 and initiating the caspase cascade. It is possible that p28 is a bcl-2-regulated component of an apoptotic signalling pathway, possibly involving coordinated ER-mitochondrial events. In the absence of bcl-2, p28 becomes the target of the FLICE/ICE-related caspase upon induction of apoptosis. A cytosolic element of p28 is removed and the resulting product, p20, is capable of inducing apoptosis. Caspase-1 and caspase-8, but not caspase-3 catalyse this cleavage reaction in vitro (Annaert et al., 1997).

1.4.7.7 MTD.

Mtd is a pro-apoptotic gene. It does not homodimerise and lacks the ability to bind to the death antagonists. It is a naturally occuring bcl-2 relative that promotes apoptosis in the absence of direct interactions with survival-promoting bcl-2 and bcl-xL. It has the BH1, 2, 3 and 4 domains (Inohara et al., 1998).

1.4.8 Bcl-2 Genes and Chemotherapy.

Chemotherapeutic drugs mediate cytotoxicity by initiating apoptosis. They induce alterations in apoptosis-related gene expression (Backus et al., 2001, Fujito et al., 1998b, Fang et al., 1998). Bcl-2 has long been associated with drug resistance as it provides a backdrop of prolonged cell survival enabling mutations to form in cancer cells. Indeed it has emerged that bcl-2 itself is a direct target of some anticancer agents, but only in certain types of cancer cells (Johnstone et al., 2002).

Firstly, VP-16 or etoposide has been reported by Fujita et al. (1998a), to cause bcl-2 cleavage resulting in the production of a pro-apoptotic fragment of the protein. VP-16 is thought to activate caspase-3 (CPP-32), which cleaves bcl-2 from the intact p26 protein to a p23 cleavage product, in U937 leukaemic cells. This p23 cleavage product was expressed in the cytosolic fraction of the cells undergoing apoptosis but not in the non-apoptotic cells. VP-16-activated caspase-3 cleaves bcl-2 between Asp^{34} and Val^{35}. The resulting NH-2-terminal truncated protein is similar to bax as it lacks a BH4 domain, necessary for the interaction of the protein with bad, Raf-1, ced-4 and calcineurin.

Taxol, paclitaxel and docetaxel are used to treat prostate cancer. Such cancers often display bcl-2 and bcl-xL overexpression conferring resistance to chemotherapy. The effectiveness of the taxane drugs in treating prostate cancers is thought to lie in their ability to induce mitotic arrest and to inactivate bcl-2 and bcl-xL by phosphorylation (Pienta, 2001, Inoue et al., 2001, Huang et al., 1999). A study by Fang et al. (1998), reports that taxol induced a serine phosphorylation of bcl-2 and bcl-xL, which was associated with its anti-apoptotic effects. The phosphorylation is thought to either regulate the pre-apoptotic mitochondrial release of cytochrome c into the cytosol or alter the mitochondrial membrane permeability.

Bcl-2 and bcl-xL contain loop domains of approximately 60 amino acids. The loop domains may act as negative regulatory regions that can be targets for post-translational modification. A number of researchers have found that bcl-2 is phosphorylated on serine 70 when exposed to taxol. Loop-deletional mutants of bcl-2 and xL are not phosphorylated by taxol. Taxol has been shown to induce an increase in free: bound bax rendering cells sensitive to apoptosis (Fang et al., 1998, Ito et al., 1997, Inoue et al., 2001, Haldar et al., 1996).

A study by Haldar (1996) investigated the effectiveness of etoposide or taxol on inducing apoptosis in prostate cancer cells. Etoposide treatment did not induce any phosphorylation of bcl-2. The response of prostate cancer cells to taxol appeared to depend on their bcl-2 expression levels. Their results showed that LNCaP prostate cancer cells which express bcl-2, die on treatment with taxol, but in DU145 cells that do not express bcl-2, taxol does not induce cell death. They also found that phosphorylated bcl-2 was prevented from dimerising with bax but there were no changes in levels of bax (Haldar et al., 1996).

Paclitaxel induces microtubule bundling associated with a marked increase in the percentage of cells in the G2-M phase of the cell cycle, followed by fragmentation and morphological features of apoptosis. Paclitaxel also induces phosphorylation of bcl-2. Ibrado and coworkers (1996) looked at the effects of paclitaxel on HL-60 cells. One cell line was transfected with bcl-xL, which therefore over-expressed xL and the other translated with neomycin. They found that phosphorylation of bcl-xL caused activation of CPP32β by cleaving it into p20 and p12 subunits.

Porchynsky et al., (1998) reported that the post-translational modification of bcl-xL by tubulin-targeting agents is a general response in many tumours of diverse origin, including breast, colon, non-small cell lung carcinoma and ovarian. Bcl-xL was phosphorylated in malignant cells after treatment with paclitaxel, vincristine, vinblastine, colchicine and nocodazole. Paclitaxel resistant ovarian carcinoma cells with mutations in tubulin failed to exhibit phosphorylation of xL after paclitaxel exposure. This phosphorylation was mediated by raf-1. The raf-1 kinase can be recruited to a mitochondrial location via an association with bcl-2's BH4 domain. A decrease in raf-1 by treatment of cells with geldanamycin, correlated with a decrease in bcl-xL phosphorylation.

This study also reports that the phosphorylation of bcl-2 when cells were treated with tubulin-targeting drugs was cell density dependent. At high cell density, less phosphorylation was seen than at low cell density. This is probably because microtubule active drugs are schedule dependent and are more toxic to actively dividing cells. Therefore, at low cell density, drugs were more effective (Porchynsky et al., 1998).

The anti-apoptotic proteins have been reported to be increased in cells resistant to other chemotherapeutic drugs. Human small cell lung cancer cells cultured in subtherapeutic concentrations of etoposide illustrated marked increases in bcl-2 expression. In addition, these resistant cells were cross-resistant to cisplatin and doxorubicin (Sartorius & Krammer, 2002). Noutomi and coworkers (2002) reported increased bcl-xL expression in cells resistant to carboplatin.

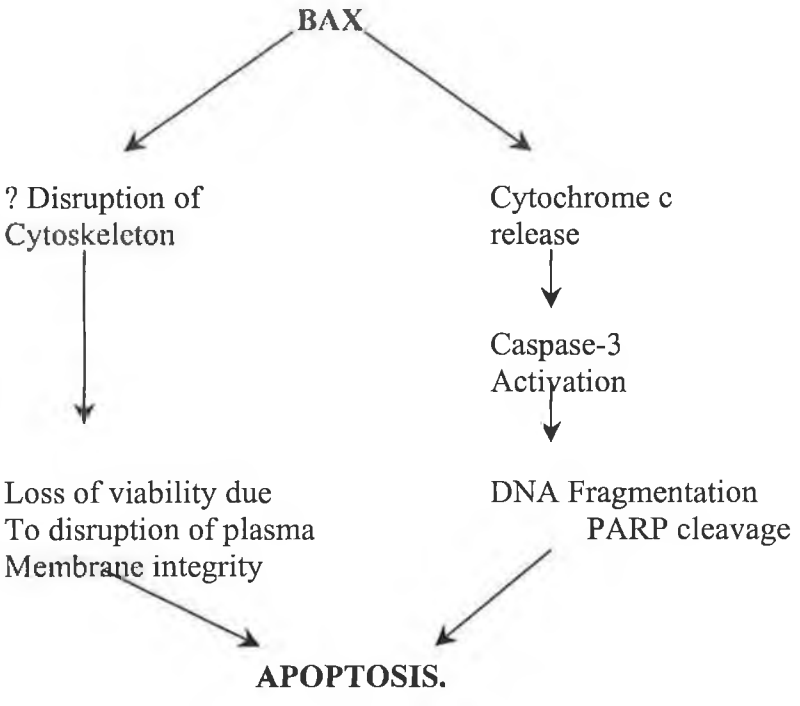
The introduction of pro-apoptotic genes into drug resistant cells can often render the cells sensitive to drugs. There are numerous reports whereby transfection of a pro-apoptotic gene into drug resistant cells induces apoptosis. Ohi and coworkers (2000) observed a decrease in bcl-xS expression in MRP-overexpressing nasopharyngeal cancer cells. However, reintroduction of the gene via transfection, increased the sensitivity of the cells to adriamycin, vincristine and vindesine. Xiang and coworkers (2000) observed a significant increase in chemotherapy-induced cytotoxicity in ovarian cancer cells transfected with the bax gene.

The observation that BH3 domains are essential for the pro-apoptotic activity of bcl-2 family proteins is now being exploited for design of new cancer drugs (Baell & Huang, 2002). As only a short sequence in the BH3 domain is necessary for binding to anti-apoptotic proteins rendering them inactive, it may be possible to develop peptides mimicking these pro-apoptotic proteins. Such small molecules aim to block the anti-apoptotic function of bcl-2 and bcl-xL (Huang, 2000). Recent reports have indicated that such BH3 peptides can access the BH3 binding pocket of bcl-xL (Degterev et al., 2001, Wang et al., 2000).

Thus the chemotherapeutic drugs and small molecule peptides display a certain amount of success at inducing apoptosis in cancer cells and the effectiveness of the drugs largely depends on the bcl-2 family protein ratio becoming imbalanced. However, it is the caspase family proteins that execute cell death.

1.4.9 The Bcl-2 Family and the Caspase- Family.

Figure 1.4.9.1 gives an overview of the mechanism of how pro-apoptotic proteins, such as bax, induce apoptosis (Pastorino et al., 1998, Mullauer et al., 2001, Reed, 1998, Parone et al., 2002).

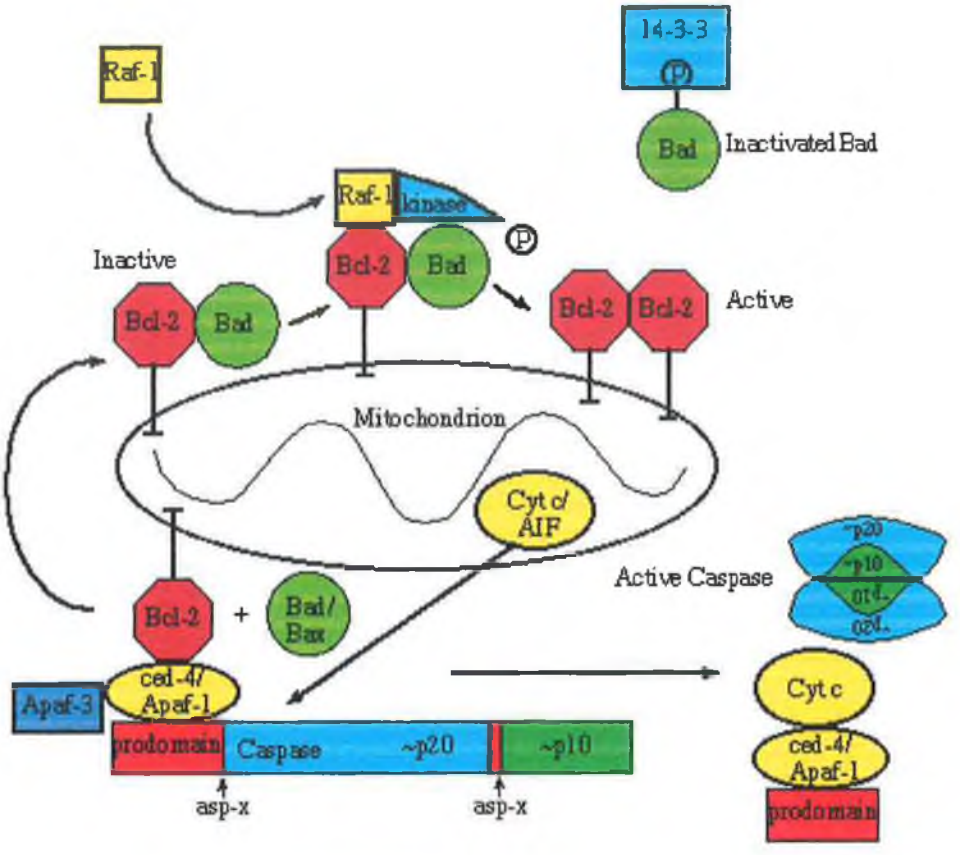


As can be seen from the diagram, bax induces the accumulation of cytochrome c in the cytosol. Although only approximately 20% of total cytochrome c is released from the mitochondria, it appears to be sufficient to activate caspase 3 and initiate cell death. The rest of the cytochrome c remains associated with the inner mitochondrial electron transport chain (Desagher & Martinou, 2000, Sartorius et al., 2001).

Caspase inhibitors and bcl-2 do not affect bax-induced cytochrome c release, indicating that bax indirectly activates caspases and that bcl-2 interferes with bax-induced killing downstream of cytochrome c release. In addition, caspase inhibitors delay but do not prevent bax-induced cytotoxicity, indicating that there is a pathway to cell death that

cannot be blocked by caspase inhibitors. It is thought that bax may play a key role in this route to cell death (Rosse et al., 1998).

Figure 1.5.5: Overview of Apoptosis and Mitochondrial involvement. (www.cord.ubc.ca/%7Esteeves/%7Echris/mitoch.htm).



Bax heterodimerises with bcl-xL which prevents bcl-xL from binding ced-4/ APAF-1, leaving ced-4 free to activate caspases. Bax, therefore, may induce release of cytochrome c and loss of the membrane permeability transition by interfering with bcl-xL and allowing caspase activation to occur (Jurgensmeier et al., 1998, Reed, 1998).

Kitanaka et al., (1997) overexpressed bax in COS-7 cells. This overexpression induced apoptosis without any additional stimulus. In this study it was found that bcl-xL

prevented apoptosis induced by overexpressed bax but that bcl-2 did not. Bax and other pro-apoptotic proteins, may act in two ways to induce cell death. Firstly, mediating death effector activity via the activation of z-VAD-fmk-inhibitable caspases. The other mechanism leading to membrane-permeability cell death which is insensitive to zVAD-fmk. These results mean that bax can activate cell death via activation of caspase-3 (Kitanaka et al., 1997).

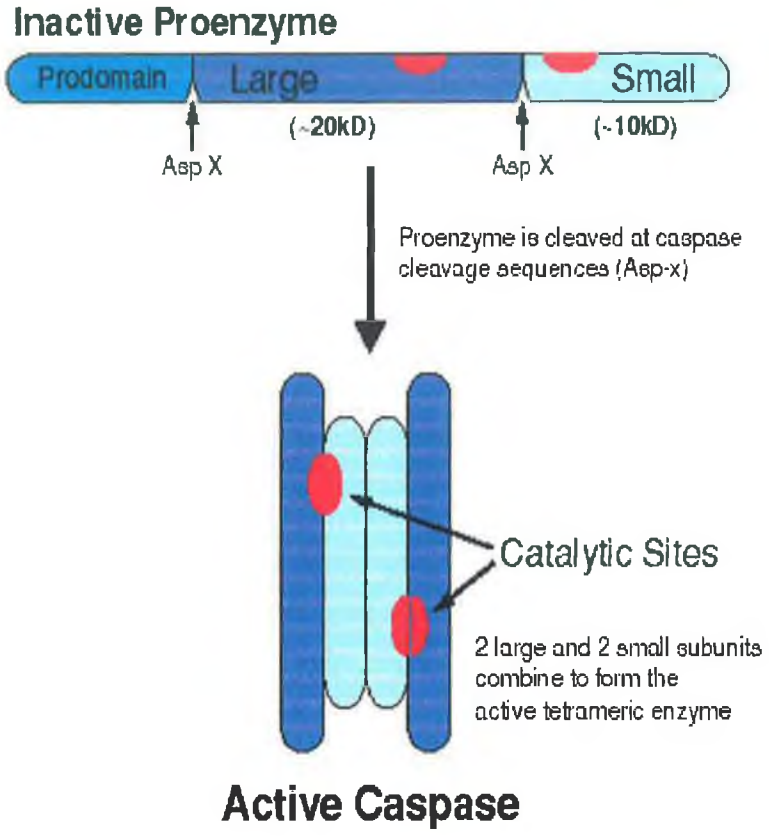
As already mentioned, bax, bcl-2, -xL, etc, act upstream of the caspase cascade. Once caspase-3 is activated, having been proteolytically cleaved from procaspase-3, it then cleaves the chaperone inhibitor of caspase activated deoxyribonuclease, releasing the DNAase activity. This accounts for the DNA fragmentation seen in the nuclei of apoptotic cells (Sethi et al., 1999).

1.4 The Caspase Family.

Many cell death stimuli, including growth factor withdrawal, receptor ligation, drug treatment, and virus infection, have been shown to activate the cellular family of cysteine proteases known as caspases (Slee & Martin, 1998, Zheng et al., 2000, Cohen, 1997, Weber et al., 2001). Once activated, these proteins initiate and execute a number of apoptotic pathways in cells. Like the bcl-2 family, this protease family was originally discovered because of its homology to genes from the nematode worm, C. elegans. The ced-3 gene, essential for apoptosis induction in the worm, was found to be homologous to ICE (interleukin-β-converting enzyme) or caspase-1 (Fernandes-Alnemri et al., 1994, Zhivotovsky et al., 1996, Shaham & Horvitz, 1996). To date, fourteen caspase proteins have been identified and named according to the order in which they were isolated (Slee & Martin, 1998).

This tightly regulated protein family exists in cells as inactive precursor enzymes, also known as zymogens. However, activation by a death stimulus causes processing of each enzyme into a cleaved or active form (Cohen, 1997, Fadeel et al., 2000, Song & Steller, 1999, Kumar, 1999). The zymogen contains an N-terminal pro-domain which is separated by an aspartate cleavage site from an interdomain linker sequence containing one or two aspartate cleavage sites which separates the larger prodomain from the smaller subunit of the enzyme. Caspases are cleaved into two subunits, usually 20kDa and 10kDa, at these aspartate residues. These domains then form a heterotetramer; the active enzyme. These activating events can be carried out by other caspases and are thought to represent a major regulatory step in the apoptosis pathway (Cohen, 1997). Procaspases reside in the cytoplasm as inactive precursor molecules that are activated by proteolytic cleavage (Bowen et al., 1999).

Figure 1.6.1: Caspase cleavage and activation.
(www.cord.ubc.ca/%7Esteeves/%7Echris/cspstrfn.htm)



All caspases contain the amino acid sequence QACxG (tryptophan-aspartate-cysteine-x-glutamate) which contains the active cysteine. The protease activity of the caspase family is unique in that they cleave following aspartate residues (asp x). This means that active caspases can autoactivate other caspases following an initial activating stimulus as well as cleaving themselves. In this way, caspases are thought to participate in a cascade in which a caspases at the apex of the cascade activates the next caspase and this in turn can activate the next caspases and so on. Therefore the proteases can regulate their own feedback through positive and negative feedback (Cohen, 1997, Fadeel et al., 2000, Song & Steller, 1999, Kumar, 1999).

Each active site contains a positively charged S1, which binds substrate's negatively charged P1 aspartate. Substrate-specificity varies as the S2-S4 substrate binding sites vary resulting in varied substrate specificity in P2-P4 position but there's an absolute requirement for aspartate at the P1 residue. Substrate specificity determines which caspase subfamily an individual protein can be classified into. Caspase-1, -4, & -5 are ICE-like proteases prefering YxxD substrates, whereas caspases-2, -3, -6, -7, & -8 prefer DxxD substrates (Stennicke et al., 1997, Nicholson, 1999).

Long prodomain caspases (i.e. caspases-2, -8, -9 & -10) act upstream of small prodomain apoptotic executioners (caspases-3, -6, & -7). Caspases act at two levels, initiator caspases are activated in response to signals and in turn activate effector caspases to execute the death sentence (Barinaga, 1996, Slee & Martin, 1998).

Caspase-1 was the first member of the caspase family to be identified. It was discovered based on the sequence similarity to the *C. elegans* death gene, ced-3 (Fernandes-Alnemri et al., 1994, Shaham & Horvitz, 1996). Caspase-1 was originally labelled ICE (for interleukin-1-beta-converting enzyme). Mice deficient in caspase-1 seem to develop normally and their cells are capable of undergoing apoptosis. Subsequently an entire gene family was unveiled. Of this family, caspase-3 was shown to have the highest similarity to ced-3. Caspase-3 is the key executioner. It is a 32kDA protein widely distributed in tissues. Mice deficient in caspase-3 die at 1-3 weeks with defective brain development but some of their cells are still capable of undergoing apoptosis (Zheng et al., 1999). Recently, the nomenclature of this family has been changed to simplify things. The term caspase was created to denote the **C**ysteine requiring **ASP**artate prote**ASE** activity of these enzymes.

The human homologue for the **ced-4** protein was then discovered (Zou et al., 1997). In C. elegans, ced-4 is complexed with ced-3 and can also bind to ced-9. Therefore the search for a human protein that can bind to bcl-2 and also to caspases revealed the **a**poptosis **p**rotease **a**ctivating **f**actors (**apaf**). Three apaf proteins have been identified to date. These are apaf-1 (a 130kDa protein with high similarity to ced-4); apaf-2 (human cytochrome c, which is released from mitochondria into the cytosol during apoptosis), and apaf-3 (caspase-9) (Ruiz-Vela et al., 2002, Cohen, 1997).

Apaf-1 exists complexed with proforms of caspase-9 (Weber & Vincenz, 2001, Ruiz-Vela et al., 2002, Zheng, 2000). This binding occurs at a homologous region called the CARD (**ca**spase **r**ecruitment **d**omain). During apoptosis, cytochrome c is released from the mitochondria. Bcl-2 can inhibit the release of cytochrome c and hence block apoptosis. (Bax can activate it). Bcl-2 is thought to act as a pore-forming protein in the mitochondria where its hydrophobic domains facilitate the formation of a pore. (The same can be said for the bcl-XL protein, which has α-helical regions spanning its transmembrane domain, similar to those seen in the pore-forming domain of the diphtheria toxin). Bcl-2 also binds to the pro-caspase-apaf-1 complex. There appears to be a dual role for bcl-2 in apoptosis, firstly, it forms pores to block the release of cytochrome c and secondly, it sequesters pro-caspase-apaf-1 complexes in the cytosol. In the absence of bcl-2, cytochrome c can enter the cytosol. This released cytochrome c is essential to form the apoptosome which consists of cytochrome c, apaf-1 and pro-caspase-9. The formation of the apoptosome results in the activation of caspase-9 through a cleavage reaction. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation) which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and the death of the cell (Cain et al., 2002, Almond & Cohen, 2002, Cecconi, 1999, Sartorius et al., 2001, Joza et al., 2002).

Caspases can be activated by autoactivation (Renatus et al., 2001). Studies in bacteria have shown that the overexpression of proteins can lead to self-cleavage and autoactivation (Polverino & Patterson, 1997). Caspase-8 possesses enough activation in its zymogen to achieve autolytic proteolytic maturation. Following a signal for activation, clustering of pro-caspase-8 occurs (Stennicke et al., 1998).

Initiation of the death machinery can also be facilitated by trans-activation or proteolysis by other proteinases (Cohen, 1997, Li et al., 1997). Transactivation is via adapter molecules which have death domains (DD), death effector domains (DED) or caspase recruitment domains (CARD) (Sartorius et al., 2001, Huang et al., 1999). Examples of adapter molecules include FADD (fas associated death domain). This protein can couple fas receptor to caspase-8 and -10 via its death domain and its death effector domains. When FADD binds caspase-8, caspase-8 oligomerises and drives its

own activations through self-cleavage. Caspase-8 then activates caspase-9 and commits the cell to apoptosis. TNF-R1 acts in a similar way to fas.

Apaf-1 (apoptosis protease activating factor) also acts as an adapter molecule (Lauber et al., 2001). Cytochrome c, released from the mitochondria, can bind apaf-1. This causes a conformational change in apaf-1, exposing its CARD, which enables it to recruit pro-caspase-9. Pro-caspase-9 oligomerisation facilitates caspase auto-activation (Wolf, 1999). Other adapter molecules, such as RAIDD (a CARD-containing adapter) can couple pro-caspase-2 to death receptors via CARD-CARD interactions.

Active caspases can autoactivate other caspases following an initial activating stimulus as well as cleaving themselves. Thus they can regulate their own activity through positive and negative feedback (Cohen, 1997, Li et al., 1997, Nicholson, 1999, Slee & Martin, 1998). Through transactivation, caspases activate each other. Caspase-9, (an initiator caspase) activates caspase -3 and -7 (effector caspases), leading to a cascade of reactions culminating in cell death. Other proteases can activate caspases, i.e. granzyme B (a cytotoxic T-cell proteinase) is an aspartate-specific serine protease which can activate pro-caspase-3 and -7 leading to the apoptotic cascade of reactions.

Active caspases are responsible for the cleavage of many cellular proteins (Salvesen & Dixit, 1997, Cohen, 1997). Caspase-3 cleaves PARP (an enzyme responsible for inactivating endonucleases by ADP-ribosylation) and ICAD releasing CAD, leading to DNA fragmentation and chromatin condensation (Wolf & Greene, 1999). Caspase-3 also cuts gelsolin (an actin-binding protein) and cleaved gelsolin fragments sever actin filaments, hence the cell blebbing and formation of apoptotic bodies during apoptosis (Kothakota, 1997). Fodrin which associates with phosphatidyl serine, is an actin-binding protein and can be cleaved by caspases. This leads to the exposure of phosphatidyl serine on the outer plasma membrane during apoptosis (Depraetere & Golstein, 1998).

As previously mentioned, the bcl-family proteins can also be cleaved by activated caspases. Caspase-3 cleaves bcl-2 and bcl-xL releasing their pro-apoptotic C-terminus. Caspase-8 cleaves bid, inducing cytochrome c release from the mitochondria. Caspase-8 has also been shown to cleave p28Bap31 (a protein which normally complexes with bcl-2 family members and procaspase-8 at the surface of the endoplasmic reticulum). In the

absence of anti-apoptotic or the presence of pro-apoptotic members, p28Bap31 is cleaved to a p20 subunit that induces cell death. In disease situations, caspases can cleave neurological proteins at low levels which uncontrolled, can lead to neurodegenerative diseases.

In some tissues patterns of bcl-2 and caspase-3 expression were inversely correlated. In immature hematopoietic cells, high bcl-2 and low CPP-32 was detected, whereas in granulocytes, the reverse was true, although this pattern was not seen generally (Krajewski et al., 1997). Differences in levels of CPP-32 in cells may influence the relative sensitivity or resistance to apoptosis. It has also been reported that survival factors negatively regulate the expression of caspase-3 (Miho et al., 1999). Treatment of embryonal carcinoma cells with basic fibroblast growth factor causes phosphorylation of bad. This cannot bind bcl-xL, therefore bcl-xL homodimers form, preventing the apoptosome from forming. Hence the cleavage and activation of caspase-3 is prevented.

1.6.1 Caspase-3.

Caspase-3 is the key executioner enzyme in apoptosis. It has high similarity to ced-3. Its expression has been detected in a wide variety of tissues (whereas caspase-1 has only been found in monocytes and macrophages). Purified caspase-3 can cleave PARP, DFF (DNA fragmentation factor), fodrin, sterol-regulatory element-binding proteins (SREBPs), U1-associated 70kDa protein, huntingtin, DNA-dependent protein kinase, etc (Liu et al., 1997 & references therein). Inhibiting caspase-3 can block apoptosis in a variety of systems (Polverino & Patterson, 1997).

Caspase-3 or CPP-32 was originally identified based on its sequence homology to ced-3 and ICE active sites. CPP-32α is a 831bp cDNA which translates to a 277amino acid protein. Its molecular weight is 32 kDa. Evidence for alternative splicing in CPP32 exists due to the identification of two other isoforms. CPP-32β has a deletion of nucleotides 43-209 and contains two nucleotide substitutions at positions 395 and 794. The third isoform of CPP-32 has similar length to CPP-32α but contains substitutions at bases 395 and 794 (Fernandes-Alnemri et al., 1994). Multiple variants of caspase-3 appeared by 2D-electrophoresis when HL-60 cells were induced to die by etoposide. As

well as alternative splicing, these enzymes could arise from alternative processing of proenzymes and/ or post-translational modification of processed subunits. Whether or not these caspase-3 species are functionally active requires further study (Martins et al., 1997).

Caspase-3 contains the highly conserved pentapeptide sequence, QACRG. Other conserved residues include Cys 285 of the active site, along with amino acid residues which are involved in substrate binding and catalysis, namely, arg-179, his-237 and arg-341. The side chains of the former two are involved in recognising aspartates at the P1 position in substrates (Fernandes-Alnemri et al., 1994).

1.6.2 Activation of Caspase-3.

The addition of active caspase-3 to normal cytosol activates the apoptotic program (Enari et al., 1996). Caspase-3 is therefore necessary and sufficient to trigger apoptosis. Caspase-3 activation can occur by a variety of complex interactions. Death receptors, once bound by ligand, can recruit and activate caspase-6, which in turn cleaves caspase-3. Alternatively, cytochrome c release can cause caspase-9 cleavage and this then activates caspase-3 (Bowen et al., 1999). Despite this, caspase-3 activation is said to be an early event in apoptosis. For this reason, CPP-32 probably acts in a positive feedback loop, recruiting other caspases to enhance apoptosis. Keane et al. (1997) report the cleavage of CPP-32 to precede PARP cleavage and DNA laddering in neurons induced to die by staurosporine.

Like all caspase enzymes, caspase-3 is post-translationally regulated (Stennicke et al., 1998). The zymogen is proteolytically cleaved by enzymes, such as caspase-8, to produce two active subunits. During processing of the pro-enzyme, the initial cleavage reaction occurs at an aspartate residue within an IETDS sequence. This sequence is located around bases 515. This cleavage reaction produces two fragments, p20 and p12. The p20 subunit then undergoes another cleavage, removing its prodomain. This second cut occurs at the ESMDS sequence around bases 75. At this stage the two active subunits of caspase-3 have been produced, p20 has been cleaved into p17. Two p17 subunits then heterodimerise with two p12 subunits. Together they form a structural conformation which contains the active site (Meergans et al., 2000).

The removal of the short prodomain of caspase-3 is essential to the activation of the enzyme. Meergans and coworkers (2000), used constructs of the caspase-3 protein and transfected them into HeLa Tet-off cells. They examined the effects of different expression levels of the prodomain on the cells by varying the tetracycline-concentration in the media. At all expression levels, constructs lacking the prodomain displayed the highest levels of caspase-3 activity. Results were mirrored using synthetic fluorescent substrates (DEVD-afc) where cleavage of the substrate was lower in cells containing wild-type pro-caspase-3 than in those lacking the prodomain of caspase-3 (hence constitutively active caspase-3). These and other data underline the critical role of the prodomain in preventing CPP-32 activation in unstimulated cells.

A study by Stennicke et al. (1998), used recombinant zymogens and active proteases in a defined system compared to zymogens in cytosolic extracts to study activation of pro-CPP-32 by caspases-8 and -10. They showed that caspase-8 and -10 rapidly induced processing of CPP-32, with caspase-8 being the faster of the two. However, granzyme B, from cytotoxic T-lymphocytes, was found to be the most efficient activator of CPP-32.

The amino terminal peptide sequence does not appear to influence the protein's catalytic activity. In one study, a number of mutant CPP-32 proteins were constructed. All had variations of the N-terminus. The effects of these mutants on rates of hydrolysis of Ac-IETD-AFC and Ac-DEVD-AFC were analysed. Results obtained showed that the catalytic apparatus didn't appear sensitive to the presence or absence of the N-peptide. All mutants cleaved PARP without out significant variation in kinetics. Thus in this system, the N-peptide appears only to minimally influence activity of the enzyme or its activation (Stennicke et al., 1998).

1.6.3 Caspase-3 Expression.

Northern blot analysis has shown that caspase-3 levels are highest in cell lines of hematopoeitic origin, especially lymphocytic and promyelocytic cells, as well as in those of brain and embryonic origins (Fernandes-Alnemri et al., 1994). Immunohistochemical analysis has shown that the CPP32 protein is highly expressed in peripheral blood mononuclear cells, and tissue from tonsil, colon, prostate and testes.

Moderate expression levels were found in pancreas, adrenal gland, kidney, liver, temporal and frontal cortex, uterine myometrium, and skeletal muscle, while low levels were detected in ovary and breast tissues (Krajewski et al., 1997). A wide variety of CPP32 expression was found in cells of the pulmonary system. For example, pseudostratified columnar epithelial cells lining the trachea and bronchi of the lungs displayed moderate intensity cytosolic CPP32 immunostaining. The stratified epithelium of the larynx was found to be very positive for CPP32 but more apical cells near the surface of the epithelium was CPP-32 negative. It is thought that CPP-32 levels may vary in these cells due to the state of activation or differentiation of the cells.

One would expect that down-regulating CPP-32 expression, a key executioner, would increase the resistance of cells to apoptosis. Kuida et al. (1996) found that CPP-32-knockout mice were smaller than their littermates, had neurological abnormalities and visible masses on their heads. Mice had excessive accumulation of neuronal cells owing to a lack of apoptosis in the brain. These mice only survived 4-5 weeks (Kuida et al., 1996). In CPP-32 neg. cells, cells had increased resistance to CD-95 and CD-3 induced cell death. In addition, neutrophils, negative for CPP-32, were resistant to apoptosis stimulated by treatment with cyclohexamide (Woo et al., 1998). Hepatocytes negative for CPP-32 had altered morphological changes (Woo et al., 1999). During fas-mediated apoptosis, there was no cytoplasmic blebbing, no detectable nuclear fragmentation, irregular chromatin clumping and slower breakdown of the DNA. Similar findings were seen in CPP-32 negative thymocytes (Zheng et al., 1998).

In all these studies, despite the absence of CPP-32, apoptosis could still proceed, due possibly to other caspases compensating for the lack of CPP-32 or perhaps via a mechanism where the caspases are redundant in function. Exactly what proteins are recruited and how apoptosis can precede in the absence of caspase involvement,is currently under review.

1.6.4 Caspase-3 Substrates.

Stennicke et al. (1997 & 1998), found that caspase-9 processing occurred after the addition of caspase-8 to a 293 T-cell extract. Therefore, they speculated that caspase-8 activates CPP-32 via a mitochondrial route where caspase-9 activates CPP-32. They discovered that a 48kDa caspase-9 zymogen was cleaved into a 36kDa protein due to

processing at aspartate 330 and then into a 34 kDa protein. In MCF-7 cells transfected with CPP-32, caspase-9 processing occurred but no processing occurred in parent cells. For this reason, caspase-9 is thought to be a downstream effector protein, activated after CPP-32.

There are a number of caspase activity assays available. Based on substrate specificities, the caspases can be subdivided into three classes; 1) CED-3-like proteins, including CPP-32, Mch-2, -3 & -4; 2) ICE family members; and 3) NEDD-2 family members. Distinguishing between activated caspases in cell extracts exploits these specificities. YVAD-AMC is a synthetic substrate preferred by ICE, while DEVD-AMC is a synthetic substrate preferred by CPP-32 because of the presence of tyrosyl and aspartyl residues at the N-terminal (P4 position) of each tetrapeptide respectively. (Keane et al., 1997). In extracts from neuronal cells induced to apoptose by a range of diverse stimuli, including staurosporine, serum withdrawal or ceramide, there was increased hydrolysis of DEVD-AMC when compared with untreated cell extracts. Incubation of extracts with YVAD-AMC did not result in cleavage of the substrate. This work suggested that apoptosis was due to the activation of a CPP-32-like protein. Suspicions were confirmed by the appearance of a p17 peptide on Western Blots. This p17 peptide represented a subunit of activated CPP-32 (Keane et al., 1997).

1.6.5 Drug resistance & Caspase-3.

Anti-cancer drugs work by inducing apoptosis in cells. This is achieved through activating cascades of reactions that culminate in the activation of caspases. Caspase-3 appears to be a downstream target of these cytotoxic drugs. Taxol induces apoptosis by increasing caspase-3 activity (Weigel et al., 2000). Similar to bax, the caspase-3 protein is not essential to apoptosis, as cells negative for this protein, can still undergo apoptosis; ie., neurons, endothelial cells, pneumocytes and breast cancer cell line, MCF-7 (Krajewski et al., 1997). Treating bladder cancer cells causes activation of caspases-3, -8 & -9 (Ono et al., 2001). It has also been observed that the down-regulation of caspase-3, through treatment with caspase-3 inhibitor, in adriamycin-resistant pancreatic carcinoma cells (PANC-1) prevented adriamycin-induced cytotoxicity (Sasaki et al., 2001).

There are many studies that document a down-regulation of caspase-3 in Pgp overexpressing cells (O'Loughlin et al., PhD 1999; Johnstone et al., 1999; Ruefli et al. 2002). Studies in DLKP cell lines have revealed a number of interesting findings. Doxorubicin induced apoptosis in DLKP-SQ, A250-10p#7, A2B and A5F cell lines which are sensitive, 10, 30 and 300-fold resistant to doxorubicin respectively. These cells have varying levels of caspase-3. The sensitive line having the highest CPP-32 levels and the most resistant having lowest levels. Apoptosis in response to doxorubicin proceeded with no PARP cleavage, but fodrin was cleaved in all cell lines. In addition, in vitro cleavage of DEVD-AMC was very low, demonstrating very low CPP-32 activation. No increases in the levels of pro-caspases occurred during apoptosis, and caspase inhibitors did not protect the cells from apoptosis. These findings suggest that doxorubicin treatment of these DLKP variants proceeded via a caspase-independent mechanism (O'Loughlin Ph.D. 1999).

Ruefli et al., found that Pgp+ cells were less sensitive to those nuclear apoptotic events that occur following caspase activation. It was found that this was due to decreased caspase activity in the drug resistant cells. Johnstone and coworkers (1999) have also found that the inhibition of Pgp function restored caspase-3 activation upon crosslinking of cell-surface Fas. They reported that treatment of Pgp+ and Pgp- cells with soluble, recombinant FasL also resulted in caspase-dependent apoptosis of Pgp- cells, whereas Pgp+ cells were relatively resistant (Johnstone et al., 1999). As well as Pgp-overexpressing cells, the overexpression of bcl-xL in cisplatin-resistant cells resulted in failure to activate caspsae-3. In addition, cells resistant to adriamycin or vincristine also exhibited overexpression of bcl-xL and failed to respond to cisplatin and the antimetabolite, arabinofuranosyl-cytosine (ara-C). In these cells, caspase-3 was not activated (Kojima et al., 1998). These results suggest that decreased caspase activity may have a role in certain forms of drug resistance.

1.7 Gene Therapy & Ribozymes.

As well as drug resistance in chemotherapy, the physical make up of the tumour makes it difficult to penetrate with cytotoxins. Tumours have a poor blood supply with variable rates of blood flow and larger intercapillary distance than those found in normal tissues (Tunggal et al., 1999). Therefore for enhanced effectiveness of chemotherapy, better delivery systems are being investigated and more specific, gene-targeting therapies being developed. Hence the emergence of antisense and ribozyme technology.

The perturbing, or even silencing, of disease-related genes is an idea that seems applicable to a vast number of illnesses, perhaps especially to complex ones, including most neoplasms. Many hematologic and other malignancies are currently incurable, and are treated only by highly toxic interventions. With their prospect of exquisite specificity, nucleic-acid therapeutics continues, therefore, to be highly desirable.

Basically, gene expression may be interrupted at either of two stages. The first is transcription, the stage at which a gene's code is transferred to a messenger RNA that leaves the cell nucleus. One idea is to engineer antisense capable of interacting with a specific stretch of the DNA double helix, thereby creating a triplex (a triple-strand helix) within a gene or in one of its control elements. This in turn may prevent the gene's code from being read.

Alternatively, gene therapy disrupts nuclear material at the translational level within cells, where the messenger acts as an instruction tape enabling the cell--in particular, a ribosome--to synthesize protein. The idea is to attack the messenger. If an antisense strand can hybridize with a specific messenger, the resulting duplex may cause a jam in any ribosome reading the message. A related idea is the ribozyme, an RNA that can cleave RNA. The cleaving motif is placed in a strand whose ends are antisense chosen to hybridize with specific messenger code (Gewirtz et al., 1998).

Techniques to selectively inhibit the expression of certain genes have been developed. These target nucleic acids at the RNA level, as in the case of ribozymes, and at the DNA level, using antisense. Antisense sequences are short strands of DNA which are designed to switch off gene expression by interfering specifically with the translation of the encoded protein at the mRNA level. They target and bind to specific sequences in

the gene of interest and prevent translation. In theory, antisense treatment aims to render the cell deficient in only the specific protein, while other proteins are still being actively transcribed. However, antisense can be a transient effect and difficulties arise while attempting to get a stable oligodeoxynucleotide into the cell long enough for it to target the gene of interest. Therefore other techniques such as use of ribozymes are being explored.

Ribozymes are RNA-enzymes that catalyse RNA-cleavage in a sequence-specific way. Cleavage depends on the presence of divalent metal ions at neutral or higher pH and results in the production of two truncated RNA molecules. They can be synthesised as tools to inhibit the expression of specific RNA transcripts. Their structures are based on naturally occurring site-specific, self-cleaving RNA molecules. They have the ability to cleave deleterious RNAs or repair mutant cellular RNAs, and so have potential therapeutic benefits. They were originally discovered by Cech (1981) from his research into intron splicing in *Tetrahymena thermophila*. Cech found that strands of purified RNA had the ability to self-cleave, despite a lack of protein enzymes in the mix. Altman also found only the RNA component of Rnase P to be necessary for the cleavage of RNA. Subsequent to these studies, a number of other RNAs with enzymatic activity were discovered. These were classified into the following groups:

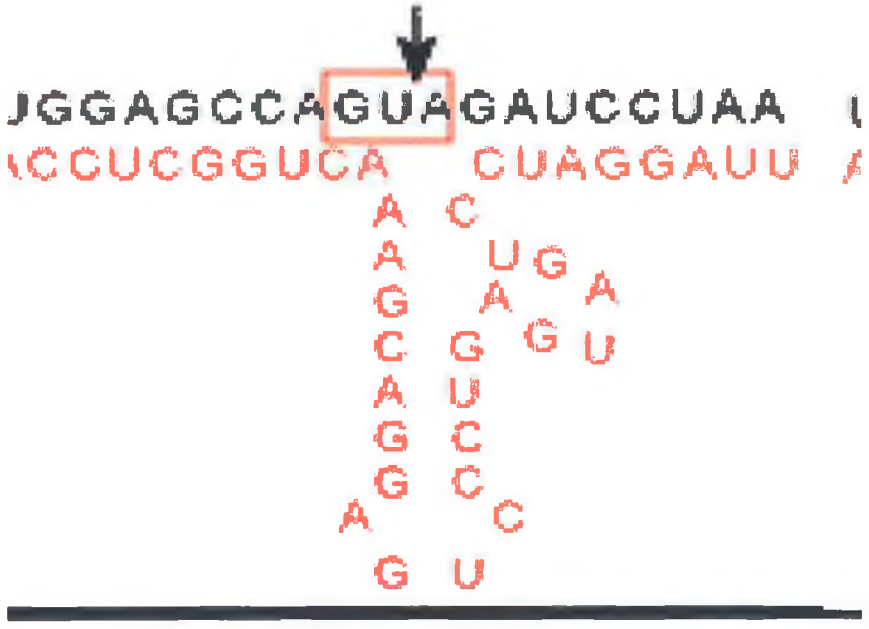
- Self-splicing group 1 and 2 introns (*Tetrahymena thermophila*)
- Rnase P (RNA moiety of the protein-RNA complex),
- Self-cleaving RNAs; found in small pathogenic RNAs. These include hammerhead ribozymes, hairpin ribozymes, trans-active hepatitis δ virus ribozymes & the Neurospora VS ribozyme. (The latter are naturally cis-occurring ribozymes and have been converted to trans-active ribozymes by splitting the catalytic core from the substrate sequence).

Ribozymes cleave their target RNA at NUX sites, where X= C, U or A, and N= any nucleotide. The GUC triplet is the preferred site. Surrounding these NUX sites, are sequences complementary to the target RNA.

1.7.1 Hammerhead Ribozymes.

The hammerhead ribozyme is the smallest of the ribozymes and therefore can be easily synthesised. Hammerhead ribozymes are so-called due to their "hammer-like" appearance. They consist of two stems flanking either side of the NUX site. These RNA molecules possess the activity of restriction enzymes and recognise NUX sequences and cleave them. Most efficient cleavage occurs after a GUC triplet (Palfner et al., 1995, Esteban et al., 1998). The sequences of the adjacent stems determine the specificity of these molecules for their target (James & Gibson, 1998; Gewirtz et al., 1998). The specificity of a ribozyme is essential to its ability to cleave target RNA. A mismatched base will decrease effectiveness.

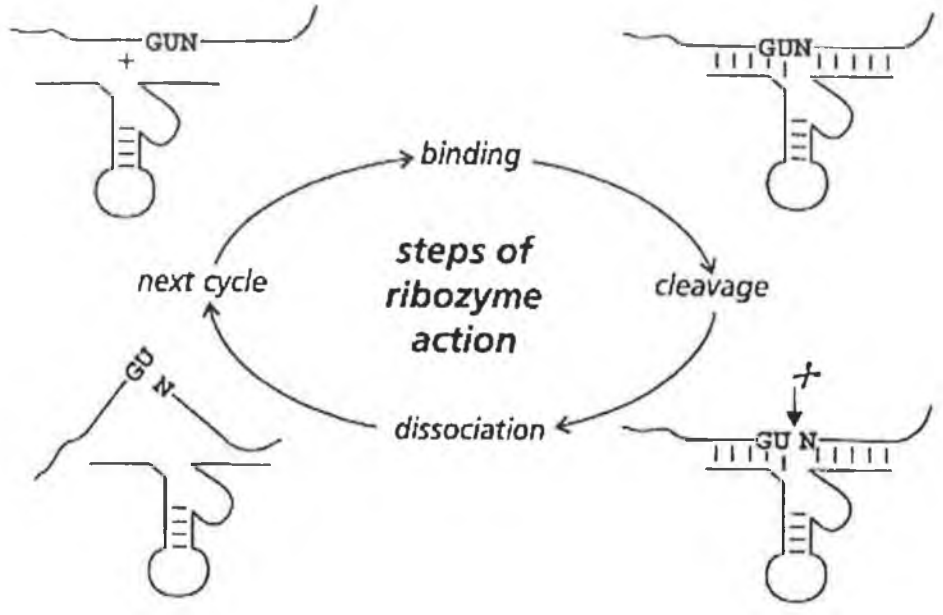
Figure 1.8.1: Structure of Hammerhead Ribozyme.
(http://academic.brooklyn.cuny.edu/chem/zhuang/QD/toppage1.htm)



Ribozyme efficacy is positively correlated with shorter stem regions (Amarzguioui et al., 2000). A balance therefore between the length of the hybridizing arms and their specificity for the target region must be found so as to provide for easy dissociation of

ribozyme from target once the target has been cleaved. This enables the ribozyme to bind to further RNA segments and continue cleaving.

Figure 1.8.2: Mechanism of action of Hammerhead Ribozyme. Turnover cycle of RNA cleavage by a hammerhead ribozyme. Binding of the enzyme and substrate results in the catalytically active structure. After cleavage the two product strands dissociate and ribozyme strand can go into the next cycle.
(http://dragonzoo.utoronto.ca/~jlm2001/J01T0501D/gene_therapy2.htm)



Ideally, cleavage of target RNA should occur in the presence of 500mM magnesium. However, the intracellular environment has much lower concentrations of magnesium, estimated to be at approximately 500uM (Gewirtz et al., 1998). During a cleavage reaction, target RNA forms base pairs with stems 1 and 2. The magnesium ions cleave a phosphodiester bond in the bound substrate and the cleaved fragments dissociate from the ribozyme. Once cleavage has occurred, the detached ribozyme is free to bind more target mRNA.

Aminoglycosides such as neomycin B and the tetracyclines have been shown to inhibit the activity of hammerhead ribozymes (James & Gibson, 1998, Clouet-d'Orval et al., 1995, Stage et al., 1995, Rogers et al., 1996, Olive et al., 1995, Murray & Arnold, 1996, Hermann & Westhof, 1998). It is believed that through displacing the divalent metal

ions essential for catalysis, antibiotics such as neomycin B, peptides and tetracyclines reduce the effectiveness of ribozymes (Rogers et al, 1996). Neomycin stabilises the ground state rather than transition state of the cleavage complex. Ammonium ions are instrumental for antibiotic-hammerhead interaction (Stage et al., 1995). Few antibiotics have illustrated a positive influence on ribozyme activity. However a study by Olive and coworkers (1995), found that the antibiotic viomycin enhances the cleavage reaction of a hammerhead ribozyme against Neurospora VS RNA by decreasing the amount of MgCl2 need for the reaction.

Ribozyme should be selected with a target NUX site in an area of single-stranded RNA. The NUX site is therefore within a loop region of the RNA. RNA structure can be predicted using M-fold outlining potential folding patterns of mRNAs (Zuker et al., 1991 & m-fold program). The single-stranded region allows target to base pair with its complementary sequence on the ribozyme, and also ensures that the catalytic moiety of the ribozyme, (NUX site), can access the target region (Amarzguioui et al., 2000; Zhao & Lemke, 1998).

The effectiveness of the ribozyme also depends on the length of the stem regions since shorter stems improve the ability of the ribozyme to be released and reattach itself to new RNA strands. As well as being more expensive to synthesise, longer flanking sequences have been found to interfere with ribozyme activity and to retard the kinetics of RNA cleavage (Qiu et al., 1998, Gewirtz et al., 1998). This interference is due to an increased chance that short runs of complementary bases may hybridize if larger intervening sequences are looped out. Such duplexes then interrupt ribozyme function. An important consideration in ribozyme selection is that a high number of uridine residues in hybridizing regions flanking the catalytic core. These residues can build U:G base pairs as well as A:U base pairs. They form a number of stable catalytic inactive secondary structures which could decrease the overall catalytic activity of a given ribozyme. This was reported by Palfner (1995) when they compared different mdr-1 ribozymes.

1.7.2 Delivery of Ribozymes.

Despite the simplicity of the ribozyme-target interaction, the therapeutic potential of ribozymes can be hindered by a number of factors. One of the most notable of these is the problem of efficient delivery of ribozyme into target cells. Not only does the ribozyme have to access its target site RNA, it also has to enter the target cell and this can be the biggest challenge in ribozyme gene therapy. In addition, as with many potential cancer therapies, there are potential side-effects due to ribozyme targetting the gene of interest in normal as well as neoplastic cells.

There are two methods of delivery. Ribozymes can be introduced into cells as preformed ribozymes (exogenous delivery) or as a ribozyme gene (endogenous delivery). For the former, the transfection method (i.e. electroporation or lipofection) is important and ribozymes are added to the cell medium complexed to cationic lipids such as lipofectin or DOTAP. Such compounds facilitate transport across lipid-containing cell membranes.

Endogenous delivery involves inserting the ribozymes into untranslated regions of genes which are transcribed by RNA polymerase 2 (James & Gibson, 1998). Cells can be transfected with a coding vector and then selected for ribozyme expression (Heidenreich et al., 1996).

There have been searches for modifications to improve ribozyme stability. This is due to the potential of ribozymes to be cleaved by intracellular nucleases or by nucleases in the serum. It is therefore essential to manufacture ribozymes that have increased resistance against nuclease degradation. Many ribozymes are synthesised with phosphorothioate linkages in combination with 2'-amino- and 2'-fluoro-modified pyrimidine nucleosides (Heidenreich et al., 1996).

1.7.3 The Therapeutic Potential of Ribozymes.

Disrupting gene expression by using short fragments of nucleic acids, both ribozyme and antisense, have important therapeutic benefits. Such techniques are potentially applicable to a number of diseases including neoplasms and viral diseases, and have been heralded as a "magic bullets". As well as the development of multi-drug resistance in cancer cells, standard chemotherapy for neoplastic disease is accompanied by

systemic toxicity. Cancer chemotherapeutic drugs have a relatively low toxic to therapeutic dose with the drugs failing to discriminate between normal and malignant cells. Therefore the genes behind tumour development are now being targeted by such methods as antisense and ribozyme technology. Gene therapy is a logical way to treat genetic diseases. Such approaches in cancer therapy could, perhaps, erradicate some of the unwanted effects of chemotherapy by increasing the specificity of treatment. However, the problem with using gene therapy for cancer treatment is that cancer is rarely due to a monogenetic defect. Instead multiple mutations have been isolated in each cancer type.

In cancer therapy, an obvious target for gene knockout studies is the frequently overexpressed protein encoded by the MDR-1 gene. Drugs such as cyclosporin and verapamil have shown to reverse the MDR phenotype, however, their use is limited due to toxic drug effects and lack of specificity (Thierry et al., 1993). For this reason, MDR-1 is an ideal target of a more specific therapy. There are numerous studies where this gene has been inhibited either by antisense or ribozyme technology. One such study was by Zhenqiang et al (1997). In their work, they identified that CEA is expressed on lung adenocarcinomas. They used a retroviral vector which had a CEA promoter coupled to an MDR-1 ribozyme gene. When this was introduced into drug resistant lung adenocarcinoma cells (GAOK) and in HeLa cells, the ribozyme was exclusively expressed in the GAOK cells. In addition, reduction of MDR-1 lead to a 91.5% reduction in resistance to doxorubicin, vinblastine and colchicine. There were no effects on the growth of the HeLa cells. This study illustrated how ribozyme technology can be manipulated to target cancer cells specifically (Zhenqiang et al., 1997). Workers in this lab also developed a ribozyme to mdr-1 which, when expressed in doxorubicin-resistant lung adenocarcinoma cells, was effective at reducing drug resistance virtually to that of the parent cells (Daly et al., 1996). Kobayashi et al (1994) found ribozyme to MDR-1 reduced resistance levels to vincristine in MOLT-3 cells by 35 fold.

c-Fos is a transcription factor that promotes cell growth, differentiation and transformation. It is a primary modulator of apoptosis. It functions along with other modulators c-myc, E2F-1, cdc-2, cyclin A and p53 to activate apoptosis (Palut et al., 1998). C-Fos has a very short half-life which enables c-fos mediated signals to be switched off very quickly (Wang & Templeton, 1998).

In some studies, the effects of cytotoxic drugs on c-fos expression have been investigated. Ritke et al., report that VP-16 stimulated c-jun and c-fos mRNA expression in human leukaemia cell lines, K562 & HL-60 cells (Ritke et al., 1994) When Scanlon et al., transfected c-fos ribozyme into cells, they found it increased the sensitivity to chemotherapeutic agents, including cisplatin, and significantly reduced dTMP synthase, DNA polymerase-b, topoisomerase 1 and hMT-2-A genes (Scanlon et al., 1991).

The gene has also been targeted by antisense technology. A study by Gillardon et al., used antisense to c-fos to investigate its role in UV induced apoptosis in rat skin. They found that the loss of c-fos did not appear to play a major role in sunburn or speculated that other members of the fos/jun family compensated for its loss (Gillardon et al., 1994).

Ribozymes have been used to target members of the ErbB family, a group of tyrosine kinases that are frequently overexpressed in carcinomas. A study by Tang et al, down-regulated ErbB4 using ribozymes. Elevated levels of this gene have been seen in many breast cell lines. Their hammerhead ribozymes caused a decrease in ErbB4 tyrosine phosphorylation in a 32D model system. In addition, transfection of the ribozyme into T47D human breast carcinoma cells reduced ErbB4 expression and anchorage-dependent colony formation (Tang et al., 1998).

The targets of ribozymes have ranged from genes overexpressed in cancer cells to foreign genes that cause infectious diseases. Viral targets include segments on the HIV genome. Weerasinghe et al (1991) found that MT4 cells transformed with vectors expressing ribozyme to HIV-1 were resistant to varying degrees of infection by the virus. The HIV-1 tat gene was also down-regulated using ribozyme and antisense by Lo et al., however, they found antisense to be more effective at inducing resistance to viral infection (Lo et al., 1992). As well as HIV, ribozymes to other viruses such as hepatitis B, hepatitis C and influenza viruses are being investigated for their effectiveness at decreasing viral load (Beck & Nassal, 1995, Sakamoto et al., 1996, Tang et al., 1994). Clinical trials are ongoing to assess the effectiveness of ribozymes to hepatitis B ("HepBzyme") and hepatitis C ("Heptazyme"). (Ribozyme Pharmaceuticals Inc.). This company has also designed ribozymes to VEGFR ("Angiozyme") and the Her-2

receptor ("Herzyme"), and clinical trials in colorectal cancer and breast cancer patients have begun.

Members of the bcl-2 family of genes are, in theory, good targets of antisense and ribozyme therapy. The anti-apoptotic genes are responsible for prolonging tumour cell survival and creating a backdrop against which many mutations in other genes can accumulate. Indeed, bcl-2 itself has been the target of many antisense *in vitro* trials, and is now being targeted *in vivo* (Ziegler et al., 1997; Jansen et al., 1998; Scheid et al, 1998; White et al., 1997; Piche et al., 2001; Kitada et al., 1994, Bloem & Lockhurst., 1999, Leech et al., 2000).

In neoplastic disease, the concept has reached clinical trials. In hematological disease, patients have been treated with antisense to the bcl-2 gene. Early results proved that the treatment was successful at delaying disease progression and death and one patient showed complete response to therapy while others had reduced tumour size, lower levels of circulating lymphoma cells and improvement in symptoms. Finbarr Cotter did report that following antisense therapy to bcl-2, there was an upregulation of bcl-xL, but also bcl-xS to compensate for this (Cotter, 1997, Webb et al., 1997). Thus, antisense therapy has potential to be used as a single agent in the treatment of minimal residual disease. A more important application may be to overcome chemoresistance or to enhance the effectiveness of chemotherapy. Perhaps after first round chemotherapy, at remission, antisense should be administered followed by high dose chemotherapy, as antisense is thought to have a sensitising effect. This could therefore lead to prolonged remission.

It has been reported that small cell lung carcinomas over-express bcl-2, and the ectopic expression of bcl-2 has been shown to confer resistance to etoposide, cisplatin and doxorubicin. Zangemeister-Wittke et al. (1998 & 2000) and Koty et al. (1999) have carried out bcl-2 antisense treatment in non-small cell lung cancer cell lines. Both groups found that treating NSCLC cells with antisense to bcl-2 markedly increased spontaneous cell death. Upon antisense treatment, the bcl-2 to bax ratio was disrupted, leading to bax homodimerisation and cell death.

Despite the acceleration of bcl-2 antisense therapy into clinical trials, ribozyme technology has not moved as quickly. When a hammerhead ribozyme targeting bcl-2

was introduced into chronic myeloid leukaemia cell lines, there was a slight tendency towards apoptosis. On flow cytometry, a slight decrease in bcl-2 protein content was observed. This study, by Scheid et al, concluded that bcl-2 targeted ribozymes do not induce apoptosis by decreasing bcl-2 protein levels but by a presently unknown mechanism (Scheid et al., 1998).

In contrast to this, a study by Dorai et al. (1997) introduced divalent hammerhead ribozymes into LNCaP cells (prostate cancer cells). Within 18 hours, they saw a reduction in bcl-2 mRNA and protein levels. In low-bcl-2 expressing variants of LNCaP, apoptosis was induced but in high-bcl-2 expressing variants, it was not sufficient to induce apoptosis. The decrease in bcl-2 mRNA was due to its destruction by the ribozyme rather than by a simple antisense effect.

The role of bcl-2 in oral cancer cells has also been investigated by Gibson and coworkers (2000). They inserted an anti-bcl-2 ribozyme into oral cancer cells via an adenoviral vector system and found a reduction in cell growth. Surprisingly Northern blots showed no decrease in mRNA but Westerns displayed downregulated protein levels.

In the cancer setting, there are few papers that use gene therapy to reduce levels of pro-apoptotic genes from the bcl-2 family or from the caspase family. However, in neurodegenerative disease studies, hammerhead ribozymes have been constructed to rat caspase-3. Eldadah et al (2000) transfected such a ribozyme into cerebellar granule cells where it conferred protection against apoptosis at 24hour post-transfection. Another group also constructed a hammerhead ribozyme to rat caspase-3 and found it could protect PC12 cells from apoptosis induced by low levels of 6-hydroxydopamine (Xu et al., 2001). There are no ribozymes to human caspase-3 used to investigate neuordegenerative or neoplastic disease.

Ribozymes and antisense therapy have therefore provided a new approach to disease treatment. The promising results to date seen in clinical trials indicate that perhaps these therapies could be used in combination with traditional cancer & infectious disease therapy to improve prognosis. The challenge remains to exclusively target diseased cells with minimal effects on normal tissue.

1.8 DNA Microarrays.

Biological systems involve an intricate web of simultaneous interactions. Most experimental data has up to recently been obtained on a "one gene one experiment" basis. However the advent of DNA microarray technology, has made it possible to make a "snap-shot" of cellular events that involves up to 25000 genes in one single experiment. Microarray technology makes use of the sequence resources created by the genome projects and other sequencing efforts to answer the question, what genes are expressed in a particular cell type of an organism, at a particular time, under particular conditions.

Microarrays exploit the preferential binding of complementary single-stranded nucleic acid sequences. A microarray is typically a glass, nylon or plastic slide, on to which DNA molecules are attached at fixed locations or spots. Each DNA molecule or oligonucleotide is approximately 80 bases in length allowing high hybridisation efficiency. The arrays are probed with radiolabelled cDNA generated from target RNA samples of interest. After hybridisation, gene expression is identified by correlating "spots" with assigned positions on the array. There may be tens of thousands of spots on an array, each containing a huge number of identical DNA molecules (or fragments of identical molecules).

The microarray technology is still rapidly developing, therefore it is natural that currently there are no established standards for microarray experiments and how the raw data should be processed.

To date, some of the experiments which have used microarray technology include panels of biopsied tumour specimens which were examined to determine the expression of prognostic factors, as well as the identification of genetic changes in panels of drug resistant cell lines (van't Veer et al., 2002; Zembutsu et al., 2002; Ahr et al., 2002; Dong et al., 2002; Heiskanen et al., 2000).

Tools such as DNA microarrays have the potential to eventually lead to "personalised chemotherapy" for individual patients, as well as the identification of novel drugs to overcome tumour resistance.

1.8 Breast Cancer.

Malignant breast carcinoma develops in 10% of women and 50% of these women will die due to the disease. Most breast cancers arise from hyperplasias in ducts and lobules. Breast cancers are classified as non-invasive carcinomas that lack the ability to invade through the ducts, or invasive carcinomas. Traditionally, diagnosis depends on three criteria, tumour size, nodal involvement and evidence of metastases. This is referred to as TNM staging (Breast cancer factsheet).

Breast cancer is highly treatable with surgery, radiation therapy, chemotherapy, hormonal therapy and most recently antibody therapy, and is most often curable when detected in the early stages. Traditional therapy selection criteria include patient age, stage of the disease, pathological characteristics of the primary tumour including the presence of tumour necrosis, oestrogen and progesterone receptor levels in the tumour tissue, and measures of proliferative capacity. Possibly the most important prognostic factor in primary operable disease is lymph node involvement. Approximately 20-30% of node negative patients develop metastatic disease by ten years, compared to 60-70% of those with one to three positive nodes, and 85% of patients with four or more involved nodes.

There is evidence that ER status as well as proliferative capacity have important independent predictive value. Other factors such as tumour microvessel density, c-erbB-2, c-myc, p53 expression and peritumoral lymphatic vessel invasion may also be prognostic indicators in patients with breast cancer (PDQ Treatment Health Professionals). Therapies such as herceptin aim to target tumours with c-erb-B2 expression.

Each patient's tumour mass is assessed and the most common chemotherapy regimens are combination therapies CMF, CAF or CA. These are cyclophosphamide, methotrexate and fluorouracil; or cyclophosphamide, doxorubicin and fluorouracil; or cyclophosphamide and doxorubicin. The taxane group of drugs are also proving to be active agents in early stage as well as in patients with previous exposure to CMF therapy (Chemotherapy protocols). Substantial toxic effects including severe neutropenia, fever, alopecia, stomatitis, myalgia, neuropathy, asthenia, emesis, and sometimes allergic reactions can occur.

Tamoxifen is one of the most effective treatments for breast cancer. Like many cancer therapies, it acts by inducing apoptosis in tumour cells. However, not all patients can be considered for such therapy as it is an anti-oestrogenic compound. It can be used as a chemopreventive agent to reduce the incidence of cancer in high-risk women or as a treatment to improve overall survival and decrease recurrence in women with invasive breast cancer. The ER status can predict response to tamoxifen as generally, ER negative tumours rarely respond to this therapy (Ciocca et al., 2000).

Approximately 5% of all women with breast cancer have a germ-line mutation in the BRCA1 gene, which is localised to chromosome 17q21. Such a mutation increases the life-time risk of development of breast cancer and also ovarian cancer. A mutation in the BRCA2 gene, which localises to chromosome 13q12-13 also confers a high-risk of these two cancers. Screening programmes are now also examining the expression of these two predictive markers so as to prevent disease development.

There is substantial evidence to suggest that tumours acquire anti-apoptotic mutations in addition to inappropriate proliferation to survive and propagate. The identification of important functional markers which play a causative role in the development and progression of human tumours and which are targets of or able to favour the activity of anticancer agents is essential to treating each cancer as an individual entity. The pre-treatment detection of such markers which could influence clinical tumour responsiveness may be used to select chemical and physical agents and to modulate their activity in individual patients. Estimating the prognosis of a patient is extremely important as it strongly influences therapeutic strategies. As mentioned in section 1.4.2, many drugs act on cancer cells by activating apoptosis. However, the significance of apoptotic gene expression in breast cancer cells is unclear. The overexpression of anti-apoptotic members of the bcl-2 family has been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as bax, promote apoptosis and sensitise tumour cells to various anticancer therapies (Krajewski et al., 1999). Bcl-2 expression has been widely studied in human tumours. Bcl-2 has been shown to protect tumour cells from chemotherapy and radiation induced apoptosis. The significance to bcl-2 expression, however, is controversial. In breast and endometrial adenocarcinomas, bcl-2 expression is frequently found. In these oestrogen dependent carcinomas bcl-2 expression is associated with a positive receptor status and a low extent of apoptosis

(Soini et al, 1999). However, it is regarded as a marker of favourable prognosis (Castiglione et al., 1999).

Tumours progress by resisting the tendency to die. This behaviour is prompted by an accumulation of genetic mutations culminating in a "super-cell" phenotype, resistant to even the most toxic chemicals. Applicative research on cancer now no longer focuses solely on the proposition of prognostic factors but also on the identification of predictors of response to specific treatments. Medical oncologists are increasingly interested in identifying reliable prognostic factors for breast cancer in order to distinguish subsets of patients who can benefit from optimised therapeutic approaches. With the advent of technologies such as DNA microarrays, RT-PCR, Rnase protection assay, as well as proteomics technology, such objectives cannot be unobtainable.

1.10 Aims of this Thesis.

Observations in our laboratory and in the literature have suggested that, as well as affecting expression of the multi-drug resistance genes such as mdr-1, mrp and GST-π, drug resistance can be associated with the expression of apoptosis-related genes. This thesis aimed to analyse the expression of apoptosis-related genes in a panel of drug resistant cell lines in comparison to drug-sensitive controls. It also aimed to assess the role of genes that might influence resistance levels by upregulation (bax and mcl-1) or downregulation (caspase-3) of their expression via cDNA and ribozyme transfection, respectively.

Another method of gene expression analysis that has recently become available is DNA microarray. This was used with the objective of comparing expression of a wide range of genes in two drug-resistant variants of DLKP cells, DLKP-A2B (low level resistance) and DLKP-A5F (high level resistance). This aimed to evaluate the reproducibility of the technique, to identify genes for future study and to optimise the technique for routine use in the laboratory.

Finally, this thesis aimed to analyse expression, at mRNA level, of the apoptosis-related genes, bcl-2, bag-1, mcl-1 and bax, in panels of archival breast tumour biopsies and to correlate their expression with clinicopathological parameters.

Section 2.0: Materials and Methods.

2.1 Preparation for cell culture

2.1.1 Water

Ultrapure water was used in the preparation of all media and 1x solutions. Pre-treatment, involving activated carbon, pre-filtration and anti-scaling was first carried out. The water was then purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP). This system is designed to produce purified water from a suitable municipal water supply. The system utilises a semi-permeable reverse osmosis membrane to remove contaminants from the feed water. This results in water which is low in organic salts, organic matter, colloids and bacteria with a standard of 12-18 MΩ/cm resistance.

2.1.2 Glassware

Solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles (and lids) and all other glassware used for any cell-related work were prepared as follows; all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1 hour. This is a deproteinising agent which removes proteinaceous material from the bottles. Following scrubbing and several rinses in tap water, the bottles were washed twice by machine (Miele G7783 washer/disinfecter) using Neodisher GK detergent and sterilised by 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 all thermostable solutions were sterilised by autoclaving at 121°C for 20 min under 15 p.s.i. pressure. Thermolabile solutions were filtered through a 0.22μm sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all protein-containing solutions. Acrodisc (Pall Gelman Laboratory, C4187) 0.8/0.2μm filters were used for non-serum/protein solutions.

2.1.4 Media Preparation

The basal medium used during routine cell culture was prepared according to the formulations shown in Table 2.1.1. 10x media was added to sterile ultrapure water, buffered with HEPES (Sigma, H-9136) and $NaHCO_3$ (BDH, 30151) and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media was filtered through sterile 0.22 μm bell filters (Gelman, 121-58) and stored in 500ml sterile bottles at 4°C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.2.7.

The basal media were stored at 4°C up to their expiry dates as specified on each individual 10x medium container. Prior to use, 100ml aliquots of basal medium was supplemented with 2mM L-glutamine (Gibco, 25030-024) and 5% foetal calf serum (PAA laboratories, A15-042) and this was used as routine culture medium. This was stored for up to 2 weeks at 4°C.

Table 2.1.1 Preparation of basal media

	DMEM (Dulbecco's Modified Eagle Medium) (mls) (Sigma, D-5648)	Hams F12 (mls) (Sigma, N-6760)
10X Medium	500	Powder
Ultrapure H_2O (UHP)	4300	4700
1M HEPES[1]	100	100
7.5% $NaHCO_3$	45	45

[1] 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 adjusted to 7.5 with 5M NaOH.

Ham's F12 medium was supplemented with 5% FCS. For most cell lines, (DLKP, DLKP-A2B, DLKP-A5F, DLKP-SQ) ATCC (Ham's F12/ DMEM (1:1)) supplemented with 5% FCS and 2mM L-glutamine was routinely used.

2.2 Routine management of cell lines

2.2.1 Safety Precautions

All routine cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet). Any work involving toxic compounds was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. Both laminar flow cabinets and cytoguards were swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the experiment. Each cell line was assigned specific media and waste bottles and only one cell line was worked with at a time in the cabinet which was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, T.H.Goldschmidt Ltd.), as were the incubators. A separate Laboratory coat was kept for aseptic work and gloves were worn at all times during cell work.

2.2.2 Cell Lines

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.2.1. Lines were maintained in 25cm^2 flasks (Costar, 3050), 75cm^2 flasks (Costar, 3075) or 175cm^2 flasks (Nulge Nunc, 156502) at 37°C and fed every two to three days.

Table 2.2.1 Cell Lines used in study (See appendix for IC50 values).

Cell Line	Source of Cell Line	Media	Cell Type
DLKP	NCTCC	ATCC*	Poorly differentiated human Lung squamous carcinoma
DLKP-SQ	NCTCC	ATCC*	Squamous clonal sub-population of DLKP
DLKP-A	NCTCC	ATCC*	Adriamycin resistant variant of DLKP.
DLKP-A2B	NCTCC	ATCC*	Clonal sub-population of DLKP-A.
DLKP-A2B-1C7	NCTCC	ATCC^	Clonal sub-population of DLKP-A2B transfected with ribozyme to MDR-1.
DLKP-A5F	NCTCC	ATCC	Clonal sub-population of DLKP-A.
DLKP-SQ-A250-10p	NCTCC	ATCC	Adriamycin resistant, pulse-selected variant of DLKP-SQ.
Bcl-xL ribozyme transfectants of DLKP-SQ and A250-10p.	NCTCC	ATCC supplemented with geneticin 600ug/ml	

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

NCTCC = National Cell and Tissue Culture Centre.

ATCC^ = DLKP-A2B-1C7 cells were supplemented with 500ug/ml geneticin to maintain expression of the plasmid containing mdr-1 ribozyme.

2.2.3 Subculture of Adherent Lines

During routine subculturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment.

Cell culture flasks were emptied of waste medium and rinsed with a pre-warmed (37°C) trypsin/EDTA (Trypsin Versene - TV) solution (0.25% trypsin (Gibco, 25090-028), 0.01% EDTA (Sigma, E-5134) solution in PBS (Oxoid, BR14a)). The purpose of this was to inhibit any naturally occurring trypsin inhibitor that would be present in residual serum. Fresh TV was then placed on the cells (4ml/25cm^2 flask, 7ml/75cm^2 flask or 10ml/175 cm^2 flask) and the flasks incubated at 37°C until the cells were seen to have detached (5-10 min). The flasks were struck once, roughly, to ensure total cell detachment. The trypsin was deactivated by addition of an equal volume of growth medium (*i.e.* containing 5% serum). The entire solution was transferred to a 20ml sterile universal tube (Greiner, 201151) and centrifuged at 1,200 rpm for 3 min. The resulting cell pellet was resuspended in pre-warmed (37°C) fresh growth medium, counted (Section 2.2.5) and used to re-seed a flask at the required cell density or to set up an assay.

2.2.4 Cell Counting

Cell counting and viability determinations were carried out using a trypan blue (Gibco, 15250-012) dye exclusion technique.

1. An aliquot of trypan blue was added to a sample from a single cell suspension in a ratio of 1:5.
2. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed.
3. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically. An average number per corner was calculated with the dilution factor being taken into account and final cell numbers were multiplied by 10^4 to determine the number of cells per ml. The volume occupied by sample in chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* 0.0001cm^3 (therefore cell number x 10^4 is equivalent to cells per ml). Non-viable cells were those that stained blue while viable cells excluded the trypan blue dye and remained unstained.

2.2.5 Cell Freezing

To allow long-term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below –180°C. Once frozen properly, such stocks should last indefinitely.

1. Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 50 - 70% confluent) and counted as described in Sections 2.2.4.
2. Pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1:9, v/v) (Sigma, D-5879). This solution was slowly added dropwise to the cell suspension to give a final concentration of at least $5x10^6$ cells/ml. This step was very important, as DMSO is toxic to cells. When added slowly, the cells had a period of time to adapt to the presence of the DMSO, otherwise cells may have lysed.
3. The suspension was aliquoted into cryovials (Greiner, 122 278) that were quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C). After 2.5 to 3.5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required.

2.2.6 Cell Thawing

1. Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution, which is toxic at room temperature.
2. The cryovial was removed and thawed quickly under hot running water.
3. When almost fully thawed, the DMSO-cell suspension was quickly transferred to the media-containing universal.
4. The suspension was centrifuged at 1,200 rpm. for 3 min, the DMSO-containing supernatant removed, and the pellet re-suspended in fresh growth medium.
5. A viability count was carried out (Section 2.2.4) to determine the efficacy of the freezing/thawing procedures.
6. Thawed cells were then placed into $25cm^2$ tissue culture flasks with 7mls of the appropriate type of medium and allowed to attach overnight.

7. After 24 hours, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

2.2.7 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated either into TSB (Oxoid CM129) (incubated at 20-25°C) or thioglycollate broth (Oxoid, CM173) (and incubated at 30-35°C). Both sets were incubated at their specific temperature for up to 2 weeks checking for turbidity and sedimentation. TSB supports the growth of yeasts, moulds and aerobes, while thioglycollate supports the growth of anaerobes and aerobes. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 2 days prior to use by incubating samples at 37°C and checking as before.

2.2.8 *Mycoplasma* Analysis

Mycoplasma examinations were carried out routinely (at least every 3 months) on all cell lines used in this study.

2.2.8.1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells. These cells were incubated with supernatant from test cell lines and examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

1. NRK cells were seeded onto sterile coverslips in sterile Petri dishes (Greiner, 633185) at a cell density of $2x10^3$ cells per ml and allowed to attach overnight at 37°C in a 5% CO_2 humidified incubator.

2. 1ml of cell-free (cleared by centrifugation at 1,200 rpm for 3 min) supernatant from each test cell line was inoculated onto an NRK Petri dish and incubated as before until the cells reached 20 - 50% confluency (4 - 5 days).
3. After this time, the waste medium was removed from the Petri dishes, the coverslips washed twice with sterile PBS, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3) for 10 mins.
4. The fixative was removed and after air-drying, the coverslips were washed twice in deionised water and stained with 2 mls of Hoechst 33258 stain (BDH) (50ng/ml) for 10 mins.

From this point on, work proceeded in dimmed light to limit quenching of the fluorescent stain.

1. The coverslips were rinsed three times in PBS.
2. They were then mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate.
3. Examination was carried out using a fluorescent microscope with a UV filter.

Prior to removing a sample for mycoplasma analysis, cells should be passaged a minimum of 3 times after thawing to facilitate the detection of low-level infection.

- Cells should be subcultured for 3 passages in antibiotic free medium (as antibiotics may mask the levels of infection).
- Cell lines routinely cultured in the presence of drugs should be sub-cultured at least once in drug free medium before analysis (some drugs such as adriamycin lead to background level of autofluorescence).
- Optimum conditions for harvesting supernatant for analysis occur when the culture is in log-phase near confluency and the medium has not been renewed in 2-3 days.

2.2.8.2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 20% serum, 10% yeast extract (Oxoid L21, 15% w/v) and 10% stock solution (12.5g D-glucose, 2.5g L-arginine and 250mls sterile-filtered UHP). This medium

optimised growth of any contaminants and incubated at 37°C for 48 hours. Samples of this broth were streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401), which had also been supplemented as above and the plates were incubated for 3 weeks at 37°C in a CO_2 environment. The plates were viewed microscopically at least every 7 days and the appearance of small, "fried egg" -shaped colonies would be indicative of a mycoplasma infection.

2.3 Specialised techniques in cell culture

2.3.1 Miniaturised *in vitro* toxicity assays

2.3.1.1 *In vitro* toxicity assay experimental procedure

Due to the nature of the compounds tested in the assays, precautions were taken to limit the risks involved in their handling and disposal. All work involving toxic compounds was carried out in a Gelman "Cytoguard" laminar air-flow cabinet (CG Series). All chemotherapeutic drugs used by this researcher were stored and disposed of as described in Table 2.3.1.

Table 2.3.1 Chemotherapeutic drugs used in study

Cytotoxic drug	Supplier	Inactivation	Storage
Vincristine	David Bull Laboratories Ltd.	Autoclave	Store at 4^0C
Adriamycin	Farmitalia	Hyperchlorite inactivation followed by autoclaving	Store at 4^0C
VP16 (Etoposide)	Bristol-Meyers Squibb, Pharm. Ltd.	Incineration	Store at RT
Cisplatin	David Bull Laboratories Ltd.	Incineration	Store at RT
5-Fluorouracil			Store at RT
Taxol	Bristol-Meyers Squibb, Pharm. Ltd.	Incineration	Store at 4^0C

1. Cells in the exponential phase of growth were harvested by trypsinisation as described in Section 2.2.3.
2. Cell suspensions containing $1x10^4$ cells/ml were prepared in cell culture medium. Volumes of 100 µls of these cell suspensions were added in to 96 well plates (Costar, 3599) using a multichannel pipette. The plates were divided so that each variable was set up with 8 repeats and 12 variables per plate. A control lane, one to which no drug would be added, was included on all plates. Plates were agitated

gently in order to ensure even dispersion of cells over a given well. Cells were incubated overnight at 37°C in an atmosphere containing 5% CO_2.

3. Cytotoxic drug dilutions were prepared at twice their final concentration in cell culture medium. 100 µl volumes of the 2x drug dilutions were added to each well using a multichannel pipette resulting in the final drug concentration being 1x. Plates were mixed gently as above.
4. Cells were incubated for 6 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80% confluency.
5. Assessment of cell survival in the presence of drug was determined by acid phosphatase assay (Section 2.3.1.2). The concentration of drug which caused 50% cell kill (IC_{50} of the drug) was determined from a plot of the % survival (relative to the control cells) versus cytotoxic drug concentration.

2.3.1.2 Assessment of cell number - Acid Phosphatase assay

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

2.3.1.2 Cytotoxicity assays in combination with caffeine.

DLKP-SQ, Bax-120 and Bax-200 cells were set up in 25cm^2 flasks at 2x10^5 cells/ml and were grown in 4mM caffeine for 48hours. They were then set up in standard toxicity assays in 96-well plates as per section 2.3.1.

2.4 Detection of Apoptosis.

2.4.1 Assessment of α-fodrin cleavage products by Western blotting.

Cells were set up in 25cm2 flasks and transfected at 24hours (as per section 2.5.4.2). Cells were treated with drug (adriamycin at concentration of 100μg/ml) for two hours and refed with fresh ATCC. Cells were harvested for protein extraction at 24 and 48 hours and Western blotting was carried out as per method outlined in section 2.5.1.

2.4.2 TUNEL analysis.

Drug treated cells or untreated (control) cells, as described in section 2.4.1, were trypsinised at the desired times into a single cell suspension, and washed twice with sterile PBS. Cytospins were prepared using a cell suspension of $0.5x10^5$ cells/ml. Cytospins were analysed for the presence of apoptotic cells by TUNEL analysis. DNA strand breaks were identified using an *In Situ* Cell Death Detection Kit with a fluorescein tag (Roche, 1 684 817).

2.5 Analytical Techniques

2.5.1 Western Blot analysis

2.5.1.1 Sample preparation

Cells were grown in flasks until they reached 80-90% confluency. They were then trypsinised and centrifuged at 1,200 rpm. for 5 min. The pellet was washed in PBS and re-pelleted twice. The tube was inverted and drained of supernatant.

2.5.1.1.1 Lysis of cell pellet

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.

2.5.1.1.2 Sonication of cell pellet

One protease inhibitor tablet from Complete™ Protease Inhibitor (Boehringer Mannheim, 1 697 498) was added to 2 mls UHP. This was then diluted 1/25 and 200 µls of this diluted solution was added to the pellet. The mix was sonicated in a Labsonic U (Braun) 2-3 times at a repeating duty cycle of 0.5s, while checking under a microscope to make sure all the cells had been lysed. Before loading on to an SDS-PAGE gel, 2 µls of the sonicated sample was removed and diluted to 10 µls with UHP for protein quantification. Sonicated cell extracts were either used immediately in Westerns or were stored at –80°C.

2.5.1.2 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) solutions as standards. A stock solution of 25 mg/ml BSA was used to make a standard curve. 10 µl samples were diluted into eppendorfs in a stepwise fashion from 0 – 2 mg/ml BSA. The Bio-Rad solution was first filtered through 3MM filter paper (Schleicher and Schuell, 311647) and then diluted 1/5 with UHP as it was supplied as a 5-fold concentrate. The diluted dye reagent (490 µls) was added to each standard and sample eppendorf and the mixtures vortexed. The 500 µl samples were diluted out in 100 µl aliquots onto a 96-well plate (Costar, 3599). After a period of 5 min to 1h, the OD_{570} was measured, against a reagent blank. From the plot of the OD_{570} of BSA standards versus their concentrations, the concentration of protein in the test samples was determined. From this, a relative volume for each protein sample was determined for loading onto the gels. Usually 10-20 µg protein per lane was loaded.

2.5.1.3 Gel electrophoresis

Proteins for western blot analysis were separated by SDS-polyacylamide gel electrophoresis (SDS-PAGE). Resolving and stacking gels were prepared as outlined in Table 2.5.1 and poured into clean 10cm x 8cm gel cassettes, which consisted of 1 glass and 1 aluminium plate, separated by 0.75cm plastic spacers. The plates were cleaned by first rinsing in RBS, followed by tap water and finally UHP. After drying, the plates were wiped down in one direction using tissue paper soaked in 70% Industrial Methylated Spirits (IMS). The spacers and comb used were also cleaned in this way. After these had dried, the resolving gel was poured first and allowed to set for 1 hour at room temperature. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in aluminium foil and stored at 4°C for 24 hours.

1X running buffer (14.4g Glycine, 3.03g Tris and 1g SDS in 1L) was added to the running apparatus before samples were loaded. The samples were loaded onto the stacking gels, in equal amounts relative to the protein concentration of the sample. The loading buffer (New England Biolabs, 7709) was prepared by adding 1/10 volume 30X

Reducing agent to 1 volume 3X loading buffer, and this mix was added at ½ volume to each of the test samples. The samples were loaded including 7µl of molecular weight colour protein markers (New England Biolabs, 7708S). The gels were run at 250V, 45mA for approximately 1.5 hours. When the dye front was seen to have reached the end of the gels, electrophoresis was stopped.

Table 2.5.1 Preparation of electrophoresis gels

Components	Resolving gel (7.5%)	Resolving gel (12%)	Stacking gel
Acrylamide stock[*2]	3.8 mls	5.25 mls	0.8 mls
Ultrapure water	8.0 mls	6.45 mls	3.6 mls
1.875M-Tris/HCl, pH 8.8	3.0 mls	3.0 mls	-
1.25M-Tris/HCl, pH 6.8	-	-	0.5 mls
10% SDS (Sigma, L-4509)	150 µls	150 µls	50 µls
10% Ammonium persulphate (Sigma, A-1433)	60 µls	60 µls	17 µls
TEMED (Sigma, T-8133)	10 µls	10 µls	6 µls

2.5.1.4 Western blotting

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine (Sigma, G-7126) pH 8.3-8.5 without adjusting) for 10 min. Protein in gels were transferred onto PVDF membranes (Boehringer Mannheim, 1722026) by semi-dry electroblotting. Eight sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a

[2] 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 (L-4509), NH_4-persulphate (A-1433) and TEMED, N,N,N,N'-tetramethylethylenediamine (T-8133).

semi-dry blotting apparatus (Biorad). Excess air was removed from between the filters by rolling a universal over the filter paper. A piece of PVDF membrane, cut to the same size of the gel, was prepared for transfer (soaked for 30 secs. in methanol, 2 mins. in UHP and finally 5 mins. in transfer buffer) and placed over the filter paper, making sure there were no air bubbles. The acrylamide gel was placed over the PVDF membrane and eight more sheets of presoaked filter paper were placed on top of the gel. Excess air was again removed by rolling the universal over the filter paper. The proteins were transferred from the gel to the PVDF membrane at a current of 34mA at 15V for 24-25 mins.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus (Stovall, Bellydancer) to ensure even exposure of the blot to all reagents. The PVDF membranes were blocked for 2 hours at room temperature with fresh filtered 5% non-fat dried milk (Cadburys, Marvel skimmed milk) in Tris-buffered saline (TBS) or Phosphate-buffered saline (PBS) with 0.5% Tween (Sigma, P-1379) pH 7.5. After blocking, the membranes were rinsed once in 1X TBS (or PBS) and incubated with 10 mls primary antibody. The specific conditions for the antibodies are outlined in Section 2.5.1.4.1, below. Bound antibody was detected using enhanced chemiluminescence (ECL).

2.5.1.4.1 Procaspase-3

Protein samples assayed for the presence of pro-caspase-3 were treated with a 1/1000 dilution of anti-human CPP-32 mAb (Transduction Laboratories; C31720). The CPP-32 antibody was diluted 1:1000 in 1X PBS and the membranes were incubated shaking overnight at 4°C. The primary antibody was removed and the membranes rinsed 3 times with PBS/0.5% Tween. The secondary antibody was a 1/10,000 dilution of rabbit anti-rat-HRP immunoglobulin (Dako, P450). The membranes were washed 3 times in PBS/0.5% Tween and developed as outlined in Section 2.5.1.5.

Table 2.5.2 Antibodies, suppliers and conditions for Western blotting technique.

Antibody	Supplier	Concentration
P-gp C219	Alexis 801-002-C100	1/1000 in PBS
Procaspase-3	Transduction Laboratories; C31720	1/1000 in PBS
Bax	SantaCruz N20 sc493	1/1000 in PBS
Mcl-1	Pharmingen M037032	1/1000 in PBS
α-Fodrin	Chemicon MAB 1622	1/1000 in TBS

2.5.1.5 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions.

The blot was removed to a darkroom for all subsequent manipulations. A sheet of parafilm was flattened over a smooth surface, *e.g.* a glass plate, making sure all air bubbles were removed. The membrane was placed on the parafilm, and excess fluid removed. 1.5mls of ECL detection reagent 1 and 1.5mls of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane wrapped in cling film. The membrane was exposed to autoradiographic film (Boehringer Mannheim, 1666916) in an autoradiographic cassette for various times, depending on the signal (30s – 5 mins.). The autoradiographic film was then developed.

The exposed film was developed for 5min in developer (Kodak, LX24, diluted 1:6.5 in water). The film was briefly immersed in water and fixed (Kodak, FX-40, diluted 1:5 in water), for 5min. The film was transferred to water for 5 min and then air-dried.

2.5.2 RNA Analysis

2.5.2.1 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.

- 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 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.

2.5.2.2 RNA Isolation

Total RNA was extracted from all cultured cell lines. Human tumour and normal specimens throughout the course of these studies were also analysed using the technique outlined below. The size of the flasks varied, but the method remained the same.

A standard method of extracting RNA from cells was as follows: cells were seeded into 175cm^2 flasks (Nulge Nunc, 156502) at a density of approximately 2x10^6 per flask and allowed to attach and form colonies for 48-72 hours at 37^{0}C. The cells were trypsinised and the pellet was washed once with PBS. The cells were pelleted and lysed using 1ml

of TRI REAGENT™ (Sigma, T-9424). The following procedure is that outlined in the protocol for TRI REAGENT™. The samples were allowed to stand for 5 mins. at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2 mls of chloroform was added per ml of TRI REAGENT™ used and the sample was shaken vigorously for 15 sec and allowed to stand for 15 min at room temperature. The sample was centrifuged at 13000rpm for 15 mins. at 4°C in a microfuge. This step separated the mixture into 3 phases with the RNA contained in the colourless upper aqueous layer. The DNA and protein fractions resulting from the total RNA isolation were retained in case they were required at some future date. The aqueous layer was transferred to a new Eppendorf and 0.5 mls of 100% isopropanol was added per ml of TRI REAGENT™ originally used. The sample was mixed and allowed to stand at room temperature for 10-15 mins. before being centrifuged again at 13000rpm for 10 min at 4°C. The RNA formed a precipitate at the bottom of the tube. The supernatant was removed and the pellet was washed with 1ml of 75% ethanol per ml of TRI REAGENT™ used and centrifuged at 4°C for 5 mins. at 13000rpm. The supernatant was removed and the pellet was allowed to air-dry for 10-15 mins. 20-30 µls of DEPC water was added to the RNA to resuspend the pellet.

2.5.2.3 RNA Quantitation

RNA was quantified spectrophotometrically at 260nm using the following formula:

$$OD_{260nm} \times \text{Dilution factor} \times 40 = \mu g/ml \text{ RNA}$$

An A_{260}/A_{280} ratio of 1.8-2 is indicative of pure RNA, although RNA with ratios from 1.7 – 2.1 were routinely observed and used in subsequent experiments. 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,1989). In these studies 200 µg RNA per 175cm^2 flask was retrieved. RNA samples were diluted to 500 ng/µl and stored at –80°C.

2.5.2.4 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.

2.5.2.5 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) analysis of isolated RNA

2.5.2.5.1 Reverse Transcription of isolated RNA

Reverse transcriptase (RT) reactions were set up on benches using micropipettes, which were specifically allocated to this work.

To form the cDNA, the following reagents were mixed in a 0.5ml eppendorf (Eppendorf, 0030 121 023), heated to 70°C for 10 min and then chilled on ice.

1μl oligo (dT) primers (1μg/μl)
2μl depc UHP
2μl RNA (500ng/μl)

After chilling on ice, the following reagents were added:

4μl of a 5x buffer (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100) (Sigma, B-0175)
2μl DTT (SigmaD-6059)
0.5μl RNasin (40U/μl) (Sigma, R-2520)
1μl dNTPs (10mM of each dNTP) (Sigma, DNTP-100)
6.5μl DEPC water

To this, 1μl Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/μl) (Sigma, M-1427) were added. The solutions were mixed and the RT reaction was carried out by incubating the Eppendorfs at 37°C for 1 hour. The MMLV-

RT enzyme was inactivated by heating to 95°C for 3 mins. The cDNA was stored at -20°C until required for use in PCR reactions as outlined in Section 2.5.2.6.2.

2.5.2.5.2 Polymerase Chain Reaction (PCR) amplification of cDNA

The cDNA formed in the above reaction was used for subsequent analysis by PCR
A standardised polymerase chain reaction (PCR) procedure was followed in this study. Standard Eppendorf tubes were used, as for the RT reactions. All reagents had been aliquoted and were stored at -20°C and all reactions were carried out in a laminar flow cabinet. A complete list of all PCR primers and reaction conditions are included in Appendix A.

A typical PCR reaction contained the following:
14µl UHP
5µl 5x buffer (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100) (Sigma, P-2317)
2µl 25mM-$MgCl_2$ (Sigma, M-8787)
1µl each of first and second strand target primers[3] (250ng/ml)
1µl each of first and second strand endogenous control primer (250ng/ml) (β-actin)
5µl cDNA

Taq/dNTP mixture	1µl dNTPs (10mM each of dATP, dCTP, dGTP and dTTP)
	0.5µl of 5U/µl *Taq* DNA polymerase enzyme (Sigma, D-4545)
	18.5µl UHP

The samples were mixed by pipetting two or three times. A typical reaction would be:

	95°C for 3 min	-	denaturation
Taq/dNTP mixture added here			
30 cycles:	95°C for 30 sec.	-	denaturation
	X[4]°C for 30 sec.	-	annealing

[3] All oligonucleotide primers used throughout the course of this thesis were made to order on an "Applied BioSystems 394 DNA/RNA Synthesiser" by Oswel DNA service, Lab 5005, Medical and Biological Services building, University of Southampton, Boldrewood, Bassett Cresent East, Southampton, SO16 7PX.
[4] Temperature dependent on primer type

72°C for 30 sec. - extension

And finally,

72°C for 7 min. extension

Following amplification, the PCR products were stored at 4°C for analysis by gel electrophoresis

2.5.2.6 Electrophoresis of PCR products

A 2% agarose gel (Sigma, A-9539) was prepared in 1X TBE (10.8g Tris base, 5.5g Boric acid, 4 mls 0.5M EDTA, 996mls UHP) and melted in a microwave oven. After allowing to cool, 4 µls of a 10mg/ml ethidium bromide solution was added per 100mls of gel which was then poured into an electrophoresis apparatus (Bio-Rad). Combs were placed in the gel to form wells and the gel was allowed to set.

4µl of 6X loading buffer loading buffer (50% glycerol, 1mg/ml bromophenol blue, 1mM EDTA) was added to 20µl PCR of each sample and this was run on the gel at 80-90mV for approximately 2 hours. When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a transilluminator and photographed.

2.5.2.7 Densitometric analysis

Densitometric analysis was carried out using the MS Windows 3.1 compatible Molecular Analyst software/PC image analysis software available for use on the 670 Imaging Densitometer (Bio-Rad. CA) Version 1.3. Developed negatives of 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 was set and scanned and a value was taken for the Optical Density (O.D.) of each individual pixel on the screen. The average value of this O.D. (within a set area, usually cm^2) is normalised for background of an identical set area. The normalised reading is taken as the densitometric value used in analysis. As a result, these O.D. readings were unitless.

2.5.2.8 RNase Protection Assay.

Probe Synthesis.

1. The following reagents were brought to room temperature and added to a 1.5ml Eppendorf tube:

   1ul Rnasin (40U/ul) (Pharmingen, 45008Z)

   1ul GACU pool (GAC:2.75mM each & U:61uM) (Pharmingen, 45005Z)

   2ul DTT (100mM) (Pharmingen, 45006Z)

   4ul 5X transcription buffer (Pharmingen, 45007A)

   1ul RPA Template Set (Pharmingen, hAPO-2c 45609P or hAPO-1c 45607P)

   10ul [α-^{32}P] UTP (500uCi/mmol) (ISIS-NEN, NEG007H)

   1ul T7 RNA polymerase (20U/ul) (kept at -20°C until use) (Pharmingen 45009Z)

   These were mixed and incubated at 37°C for one hour.

2. The reaction was terminated by adding 2ul of DNase (1U/ul) (Pharmingen, 45010Z). After mixing, this was incubated for 30 minutes at 37°C.

3. The following reagents were added in order to each Eppendorf tube:

   26ul 20mM EDTA (45012Z)

   25ul Tris-saturated phenol (Sigma P-4682)

   25ul chloroform:isoamyl alcohol (50:1)

   2ul (2mg/ml) yeast tRNA (45011Z)

   After mixing, this was centrifuged for 5 minutes at room temperature.

4. The upper aqueous phase was transferred to a new 1.5ml Eppendorf tube and 50ul of chloroform:isoamyl alcohol (50:1) was added. This was mixed by vortexing and spun for 2 minutes at room temperature.

5. The upper aqueous phase was transfered to a new 1.5ml Eppendorf tube to which 50ul 5M ammonium acetate (Pharmingen, 45013Z) and 250ul ice-

cold 100% ethanol was added. This was incubated at -80°C for at least 30 minutes. Samples were then spun for 15 minutes at 4°C.

6. The supernatant was removed and 100ul of ice-cold 90% ethanol was added to the pellet. Tubes were spun for 5 minutes at 4°C.

7. The supernatant was removed and the pellet was air-dried for 5 to 10 minutes. The pellet was solubilised by adding 50ul of 1x hybridisation buffer and vortexing for 20 seconds, followed by a quick spin in the microfuge.

8. Duplicate 1ul samples were quantitated in the scintillation counter. The probe was stored at -20°C until needed.

RNA Preparation and Hybridisation

9. RNA was extracted from cells as described previously in section 2.5.2.2.

10. RNA was freeze-dried in a Consol 4.5 freeze-drier.

11. 8ul of hybridisation buffer was added to each sample and RNA was solubilised by gentle vortexing for 3-4 minutes followed by a quick spin in the centrifuge.

12. The probe was diluted with hybridisation buffer to the appropriate concentration. 2ul of diluted probe to each RNA sample and mixed by pipetting. A drop of mineral oil was added to each tube followed by a quick spin in the microfuge.

13. The samples were placed in heat block pre-warmed to 90°C. The temperature was allowed to ramp-down slowly to 56°C and incubated 12-16 hours. The heat block was turned to 37°C for 15 minutes.

RNase Treatments:

14. RNase cocktail was prepared (per 20 samples):

2.5ml 1x RNase buffer (Pharmingen, 45016A)

6ul RNase A + T1 mix (A:80ng/ul & T1:250U/ul) (Pharmingen, 45017Z)

RNA samples were removed from the heat block and 100ul of RNase cocktail was pipetted under the oil into the aqueous layer. This was spun for 10 second and incubated for 45 minutes at 30°C.

15. Before the RNase digestion was completed, the proteinase K cocktail was prepared (per 20 samples):

390ul 1x Proteinase K buffer (Pharmingen, 45018A)

30ul Proteinase K (10mg/ml) (Pharmingen, 45019Z)

30ul yeast tRNA (2mg/ml) (Pharmingen, 45020Z)

After mixing, 18ul aliquots were added to new Eppendorf tubes.

16. The RNase digest were extracted from underneath the oil and transferred to the tubes containing the Proteinase K solution. After a quick vortex, quick spin, samples were incubated for 15 minutes at 37°C.

17. 65ul of Tris-saturated phenol (Sigma, P-4682) and 65ul chloroform:isoamyl alcohol (50:1). This was vortexed into an emulsion and spun for 5 minutes at room temperature.

18. The upper aqueous phase was transferred to a new tube. 120ul 4M ammonium acetate (Pharmingen, 45021Z) and 650ul of ice-cold 100% ethanol (Merck, K29539083 131) was added. Tubes were mixed by inversion and incubated at -80°C for at least 30 minutes. Tubes were spun for 15 minutes at 4°C.

20. The supernatant was spun and the pellet was air-dried completely. 5ul of 1X loading buffer (Pharmingen, 45022A) was added and vortexed for 2-3 minutes followed by a quick spin in the microfuge. Prior to loading, samples were heated for 3 minutes at -90°C and then placed immediately in an ice bath.

Gel Resolution of Protected Probes

21. A set of gel plates was cleaned thoroughly with water followed by ethanol. The short plate was siliconised and cleaned again. The gel mold was assembled with 0.4mm spacers.

22. The following was combined to give a final concentration of 5% acrylamide:

74.5ml acylamide solution (final 19:1 acrylamide/bis):

8.85 ml 40% acrylamide (Gibco BRL, 35722-024)

9.31ml 2% bis acrylamide

7.45ml of 10X TBE

35.82g Urea (Sigma, U-5378)

QS to 74.5ml with UHP

450ul ammonium persulphate (10%) (Reidel de Haen, 11222)

60ul TEMED

The gel was poured immediately into the gel mold, air bubbles were removed and a 5mm comb was inserted.

23. After polymerisation, the comb was removed and the wells were flushed thoroughly with 0.5X TBE. The gel was placed in a vertical rig and pre-run at 40 watts constant power for 45 minutes, with 0.5X TBE as the running buffer. The gel temperature was 50°C.

24. Prior to loading the wells were flushed again with 0.5X TBE and samples were loaded. A dilution of the probe set was loaded to serve as a size marker. The gel was run at 50 watts until the leading edge of the bromophenol blue dye reached 30cm.

25. The gel mold was disassembled and the short plate removed. The gel was absorbed onto filter paper. The gel was covered with Saran wrap and layered between two additional pieces of filter paper. The gel was placed in the gel dryer under vacuum for one hour at 80°C. The dried gel was then placed on film (Kodak X-AR) in a cassette with an intensifying screen and developed at -70°C.

26. The X-rays were developed as described in section 2.5.6.5.4.

27. Using the undigested probes as markers, a standard curve was plotted on semi-log graph paper, of migration distance versus log of nucleotide length. The identity of the RNase-protected bands was established.

28. Densitometric analysis was carried out as described previously.

2.5.3 Plasmid DNA manipulation

2.5.3.1 Plasmids and oligonucleotides used

The CPP-32 ribozyme was cloned into the pTARGET expression plasmid by Cambridge Biosciences. The Bax-α cDNA was cloned into the pCEP-4 plasmid and was kindly donated by Dr. Richard Bertrand (Schmitt et al., 1997). The Mcl-1 cDNA was cloned into the pCMV-neo plasmid and was kindly donated by Dr. Ruth Craig (Reynolds et al., 1994).

2.5.3.2 Transformation of Bacteria

100µl of competent JM109 bacterial cell suspension (Promega, L2001) 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 ((10g Tryptone (Oxoid, L42), 5g Yeast Extract (Oxoid, L21) 5g NaCl (Merck, K1880814))/litre LB, autoclaved before use) 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 (LB agar containing appropriate antibiotic conc.) and incubated overnight at 37°C. Single colonies, which grew on these selecting plates, were further streaked onto another selecting plate and allowed to grow overnight at 37°C.

2.5.3.3 DNA miniprep of plasmid DNA

To determine and select the colonies containing the inserted DNA sequence, a miniprep of the plasmid DNA was carried out.

1. Single colonies were selected off the plates and incubated in universals containing 5 mls LB/Amp shaking at 180rpm at 37^{0}C overnight. White colonies generally contain inserts, but inserts may also be present in blue colonies. For this reason, a number of white colonies, blue colonies and white-blue colonies (white colonies with a blue centre) were selected for incubation. The positive control reaction yielded all blue colonies, which were too numerous to count.

2. After 16-24 hours incubation, 1.5 mls of culture was removed from each of the incubated samples and spun down at 8500rpm in a microfuge. The supernatant was decanted and another 1.5 mls of culture was added and again spun down.
3. The samples were subjected to a plasmid miniprep, as outlined in the Stratagene Clearcut™ Miniprep kit (Stratagene, 400732). The cell preps were each resuspended in three 105 µls of Solution 1.
4. 125 µls of Solution 2 was added, and the eppendorf was mixed gently by inversion.
5. 125 µls of Solution 3 was added, and the eppendorf was mixed gently by inversion. The eppendorf was then placed on ice for 5 mins.
6. The eppendorf tubes were then spun down in a microfuge at 8500rpm and the supernatants were carefully transferred to fresh tubes, leaving cell debris behind.
7. The kit-supplied DNA binding resin was mixed by vortexing until resuspended. 15 µls of this resin was added to each collected supernatant. The tubes were then mixed by inversion.
8. The samples were loaded into kit-supplied spin cups. The cups were spun down in a microfuge at 13,000rpm for 30 secs., which retained the resin and plasmid DNA.
9. A kit-supplied wash buffer was prepared for each sample by diluting 200 µls of 2X wash buffer with 200 µls of 100% (v/v) ethanol. 400 µls of this wash buffer was added to the spin cup and the cups spun at 13,000rpm again for 30 secs. This step was repeated for all samples.
10. The spin cups were transferred to fresh eppendorfs. 50 µls of UHP was loaded into each spin cup to elute the DNA and the samples spun down at 13,000rpm again for 30 secs. The plasmid DNA was stored at -20^0C in the eppendorfs.

2.5.3.4 Restriction enzyme digestion of plasmid DNA

5 µls of each isolated plasmid sample was run out on a 2% agarose gel to check for degradation. All digestions were carried out using the recipe as outlined in Table 2.5.3.

Table 2.5.3 Standard DNA digestion mix

Component	Volume (µls)
DNA sample	10
undiluted enzyme	1
10X Multi-core reaction buffer (Promega, R9991)	1.5
UHP	2.5

All 15 µls were run out on a 1% agarose gel, together with 3 µls loading dye. From this information, samples were selected for large-scale plasmid preparation.

2.5.3.5 Preparation/Purification of plasmid DNA

1. From the appropriate culture universal from Section 2.5.3.2, 50µls of culture was removed and spread on LB/Ampicillin plates. The plates were incubated at 37^0C overnight.
2. The following day, single colonies were selected and were incubated shaking in universals containing 10 mls LB/Ampicillin media at 37^0C overnight.
3. Plasmid DNA was isolated using the Wizard™ *Plus* Minipreps DNA Purification System (Promega, A7100). 3 mls of the culture was pelleted at 1,400 x g for 10 mins. The pellet was resuspended in 400 µls cell resuspension solution and then in 400 µls Cell Lysis solution in an eppendorf. The tube was mixed and 400 µls Cell Neutralisation solution was added. The lysate was centrifuged at 13,000rpm in a microfuge for 30 mins. The plasmid was then purified without using a vacuum manifold.
4. A Wizard™ minicolumn was set up in the usual way and the cleared lysate from the cleaned plasmid samples was added to the barrel of the minicolumn/syringe assembly. The lysate was pushed into the column using the plunger. The lysate was then washed in 2 mls column wash solution and centrifuged at 13,000rpm in a microfuge for 2 mins. The plasmid DNA was eluted by the addition of 50 µls UHP followed by centrifugation at 13,000rpm in a microfuge for 2 mins.

2.5.3.6 Large scale plasmid preparation.

A single colony (Section 2.4.4.3) was inoculated into 10ml of LB Amp 50μg/ml and grown overnight; 2ml of this suspension (1% inoculum) was added to 200ml of TB (2.4g Tryptone, 4.8g Yeast Extract, 0.8 mls Glycerol, 0.17M KH_2PO_4 and 0.72M K_2HPO_4) Amp 50μg/ml and left to grow overnight at 37°C for large scale isolation of plasmid from JM109 cells.

2.5.3.6.1 For pCEP-4Bax and pCMV-Mcl-1 plasmids:

The following day the cells were pelleted and lysed 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 35,000xg. for 1h at 4°C. The supernatant was then 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 35,000xg. for 30min at 20°C after which the supernatant was discarded. The pellet was 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 (25:24:1). 10M ammonium acetate was added to the aqueous phase to a final concentration of 2.0M. 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 13,000rpm 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 13,000rpm in an epifuge, washed in 70% ethanol and resuspended in TE. The DNA concentration was determined by measuring the OD_{260nm}.

2.5.3.6.2: For pTARGET-CPP-32Rz1 & pcDNA3.1-CPP-32cDNA.

The following day cells were treated as per the procedure outlined in Qiagen EndoFree Plasmid kit – Maxi Protocol (Cat. # 12361).

2.5.4 Transfection of mammalian cells with exogenous DNA

2.5.4.1 Optimisation of plasmid transfection protocol

Before full transfections involving the various DNA fragments into the different cell lines could proceed, transfection protocols were first optimised for each of the parameters involved. The DNA used was the pCH110 plasmid which codes for beta-galactosidase activity.

The target cell line was trypsinised in the usual fashion (Section 2.2.3) and set up in the container of interest (i.e. 24/6-well plate, 25-75 cm^2 flask) at several different cell concentrations, which were arbitrarily chosen. Following incubation overnight at 37°C, the cells were transfected according to the transfection protocol for the transfectant used. Only the volumes of transfectant and conc. of DNA were altered to ascertain the most efficient combination. Cells were transfected either in the presence of serum overnight or for four hours in the absence of serum, both at 37°C. After transfection, the cells were washed 2X with PBS and fixed by the addition of fix solution (0.4mls 25% glutaraldehyde (Sigma, G-7526), 10mls 0.5M Sodium Phosphate buffer (pH 7.3), 2.5mls 0.1 EGTA (pH 8.0)(Sigma, E-0396), 0.1mls 1.0M $MgCl_2$ (Sigma, M-8266), 37mls UHP) for 10 mins. The cells were then washed for 10 mins. in wash solution (40mls 0.5M Phosphate buffer (pH 7.3), 10mls 1.0M $MgCl_2$ (Sigma, M-8266), 20mg Sodium deoxycholate (Sigma, D-4297), 40µls Nonidet P-40 (Sigma I-3021), 160mls UHP). Staining was carried out on the cells using 2.5mls of stain solution (10mls rinse solution, 0.4mls X-gal (Sigma, B-4252) (25mg/ml in dimethylformamide), 16.5mg potassium ferricyanide (Sigma, P-8131), 16.5mg potassium ferrocyanide (Sigma, P-9387)) overnight at 37°C. After staining, the cells were washed with 10mls rinse

solution and examined microscopically. Positive cells were those stained blue - the combination resulting in the most blue colonies was adjudged to be the most efficient association and was thereafter used for that cell line.

2.5.4.2 Transfection of DNA using lipofection reagents

On the day prior to transfections, the cells to be transfected were plated from a single cell suspension (Section 2.2.3) and seeded into 25cm^2 flasks at 3x10^5 cells per flask. On the day of the transfection, the plasmids to be transfected were prepared along with the lipid transfection reagents according to the manufacturers protocols (DOTAP - Boehringer Mannheim, 1 202 375; Lipofectin – GibcoBRL, 18292-011; Lipofectamine, GibcoBRL, 10964-013; Fugene6 - Boehringer Mannheim, 1 814 443). The cells were either transfected for four hours in the absence of serum after which the media was replaced with serum containing media, or for 24h to 48h in the presence of 10% FCS. For all transfections the cells were incubated at 37°C. This procedure is the same for plasmids coding for ribozyme expression as well as normal gene expression.

For transient transfection of cDNA or ribozyme, cells were set up in duplicate 25cm^2 flasks at 1x10^5 cells/ml and treated in exactly the same way as for ribozyme transfections. After 24 and 48 hours, cells were harvested for RNA and protein.

In the case of drug treatments on transient transfections, drug was added 24 hours post-transfection. The cells were then reincubated at 37°C. Cells were harvested for RNA and protein at 24 and 48 hours post-drug treatment.

2.5.4.3 Estimation of transfection effect

Examining the effect of transfections took different forms regarding the type of DNA used. Ribozyme transfections involved the selection and establishment of stably-transfected clonal cell lines, where RNA, protein and drug profiles were only assayed once the new cell line(s) were obtained. Results were obtained from the transfected cells immediately, without the cloning out of transfected cells.

For expression plasmids, (pCH110), as has already been explained, transfection efficiency was achieved by staining for the plasmid, in which transfected colonies stained blue. These could then be counted and the relative efficiency of the transfection calculated from the number of transfected vs. untransfected cells.

In the case of ribozyme transfections, 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 3-4 times the levels of geneticin normally required to kill 50% of the cells transfected (e.g. IC_{50} for DLKP-A5F cells is 65μg/ml; cells were fed with media containing 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 out in 96-well plates (Costar, 3596) at a clonal density of one cell/well. Clonal populations were propagated from these wells, as transfected cells were periodically challenged with geneticin to maintain stability of transfectants and prevent cross-contamination with non-transfected cells.

2.5.5 Isolation of RNA from Tumour/Normal Samples

1. Breast tumour samples (both Tumour (T) and Normal (N)) were obtained from Dr. Susan Kennedy, St. Vincent's Hospital, Mount Merrion.
2. These were archival tumour biopsies and were stored in tinfoil at -80^0C until RNA isolation.
3. All manipulations of the human material were carried out inside a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) to prevent contamination. The floor of the cabinet was lined with two sheets of tin foil and then covered with two large plastic autoclavable sheets.
4. All implements (e.g. scissors, forceps, tin foil, etc.) used in the RNA isolation were baked @ 200^0C overnight prior to use.
5. The Tumour/Normal sample was removed from the tinfoil wrapping.

6. Pieces of tissue were removed with a forceps to a Braun potter S886 homogenisation chamber. 2 mls of Tri Reagent was added, the pestle was inserted and the cells were homogenised on ice for ~5 mins. at medium speed.
7. After homogenisation, the cell homogenate was removed to two eppendorfs. These were spun @ 1300 rpm for 2 mins. in a bench-top microcentrifuge to remove large cell debris.
8. The samples then underwent the TRI REAGENT™ protocol for RNA isolation (Section 2.5.2.2).

2.5.6 *In-vitro* cleavage of Caspase-3 Ribozyme

2.5.6.1 Generation and purification of the Ribozyme and target DNA templates:

1. Two primers were chosen which would anneal to each other and amplify up a DNA copy of the Ribozyme together with a T7 RNA polymerase Promoter and Leader sequence. Another pair of CPP32 primers were selected which amplified up a stretch of the CPP32 cDNA that contained the Rz cleavage site. The sequence of the primers is included in the Appendix.
2. A standard PCR protocol was carried out using both sets of primers separately as outlined in Section 2.5.2.5.2.
3. The PCR samples containing the Ribozyme were freeze-dried for two hours until completely desiccated. The dried samples were resuspended in a smaller volume of UHP and purified using a Qiagen kit (Qiagen, 20021).
4. The PCR samples containing the target DNA for cleavage were purified directly using the Qiagen kit.

2.5.6.2 Ribozyme in-vitro Cleavage assay (IVC):

All radiolabelled RNA sequences were generated using the T7 Riboprobe® system (Promega, P1440).

1. The plasmid DNA was first linearised using a standard Xba-I restriction enzyme which cut only once in the vector and not at all in the insert.
2. The DNA was cleaned up using the Wizard™ *Plus* Minipreps DNA Purification System (Promega, A7100).
3. The mix for the transcription protocol was made up as outlined in Table 2.5.7

Table 2.5.7 Components for transcription of [α-^{32}P]rCTP-labelled ribozyme

Component	Volume (µls)
Transcription optimised 5X Buffer	4
DTT, 100mM	2
Recombinant RNasin® Ribonuclease Inhibitor (40U/µl)	0.5
rATP, rGTP and rUTP[5] (2.5 mM each)	4
100 µM rCTP	2.4
Linearised target plasmid DNA[6]	1
[α-^{32}P] rCTP (50 µCi at 10 µCi/µl)	5
T7 RNA Polymerase	1

4. The eppendorf was incubated for 1 hour at 37^{0}C.
5. 1 µl of RQ1 DNase (Promega, M6101) was added to the tube and incubated for 15 mins. at 37^{0}C.

2.5.6.3 Purification of target DNA for Ribozyme cleavage

1. 25 µls TE-saturated phenol:chloroform was added to the tube. The tube was vortexed and spun down at 13,000rpm in a microfuge.
2. The yellow upper aqueous phase was then transferred to a fresh tube and 25 µls of chloroform:isoamylalcohol (24:1) was added. The tube was vortexed, spun down and the upper phase transferred to a fresh tube as before.

[5] Prepared by adding 1 µl of each of 10mM rATP, rGTP, rUTP and UHP.
[6] Plasmid DNA must have a conc. of at least 0.5 µg/µl.

3. 12.5 µls of 7.5M ammonium acetate and 75 µls of 100% ethanol were added. The tube was mixed and placed at -70^0C for 30 mins.
4. The tube was then centrifuged at 13,000rpm for 20 mins.
5. The supernatant was removed and the pellet was washed with 0.5 mls 70% ethanol. The pellet was dried at room temperature for 10-15 mins. and finally resuspended in 20 µls UHP.
6. A Quick Spin™ column (Boehringer Mannheim, 1273990) was removed from packaging and prepared as instructed. The contents of the tube were added to the column, which was placed in a collection tube.
7. The column was spun down in a Beckman SW28 rotor at 2600rpm for 2 mins.
8. The eluate was stored at -20^0C and the column was discarded.

2.5.6.4 *In-vitro* Cleavage reactions with Ribozyme and Target DNA:

1. Cleavage reactions were set up in four eppendorfs as outlined in Table 2.5.8

Table 2.5.8 List of components for ribozyme *in-vitro* cleavage (IVC) reactions

Component	Volume (µls)
Target RNA	1
50mM Tris pH 8.0	1
Ribozyme	4
$MgCl_2$(25mM)	1
Recombinant RNasin® Ribonuclease Inhibitor (40U/µl)	1
UHP	2

2. The UHP, 50mM Tris, target RNA and Ribozyme were added together first. The tubes were heated to 90^0C for 3 mins. and placed on ice for 1 min.
3. $MgCl_2$ and RNasin® were added to the tubes, and the samples were incubated at 37^0C.
4. The tubes were taken off the heating block at different time points and 10 µls of sample loading dye was added to them to stop the reaction. The time points chosen were (mins.) 0, 30, 60, 180. All samples were stored at -20^0C.

2.5.6.5 Polyacrylamide gel analysis for RPA/ *in vitro* Cleavage reactions:

The products of the Ribozyme *in-vitro* cleavage reactions were separated on a 12% Acrylamide gels as outlined here.

2.5.6.5.1 Preparation of gel apparatus

Before pouring, the plates were washed in RBS to remove all traces of gel, rinsed firstly with tap water and then UHP. These were then dried with tissue, treated with 10% SDS, followed by more UHP and wiped again. The plates were then wiped in one direction using 70% Industrial Methylated Spirits (IMS). The plastic seal (*in vitro* cleavage gels) and comb were also rinsed in tap water, UHP and then 70% IMS.

2.5.6.5.2 Gel composition (for all gels):

63g Urea (Sigma, U-5378) was dissolved in 15 mls 10X TBE in a microwave, together with 10 mls UHP and heated to 60°C. Once in solution, 30 mls 40% Acrylamide (Gibco BRL, 35722-024) was added and this was made up to 150 mls with UHP. Once gel cast had been assembled, to 50 mls gel of this gel mix was added 250 µls of freshly made 10% APS (Reidel de Haen, 11222) and 50 µls TEMED. The gel mix was then ready to pour.

2.5.6.5.3 Assembly, Pouring and running of gels:

For sequencing gels, a plastic sealing strip was lined on both sides of the casting tray. The gel tray was laid flat, and the gel mix was poured onto it, starting from the bottom. The front plate was gently pushed forward from the bottom, while continuously adding the gel mix, until both plates were laid exactly on top of each other. The gel cast was then fully assembled, with the gel sealed in using clips on both sides. The comb was then inserted and the gel was left to set for 30 mins. 2l of 1X TBE was heated to 60^0C

and added to the gel apparatus. The comb was removed after the gel had set and a syringe was used to wash the wells of residual urea. Sample running buffer (1xTBE) was added to a number of the wells and the gel was pre-run at 1700 volts for 1-1.5 hours or until the temperature of the gel reached 40-50^{0}C, after which it was ready for loading of sequencing samples. The samples were heated to 90^{0}C for 3 mins. before being loaded on the gel. The gel was run for 2hrs, or until the loading dye (bromophenol blue/xylene cyanol) had reached the bottom of the gel.

For *in-vitro* cleavage gels, the base plate was laid flat and the outside was lined with the plastic seal. The front plate was placed directly over the base plate. The cast was kept in place with the use of bullclips. The seal was tested by the addition of UHP into the cast to check for leaks. Once no leaks were detected, the gel was poured, the comb was inserted and left to set. Once set, the bullclips were removed and the gel was placed upright in the running apparatus. 1X TBE was used as the gel running buffer, the comb was removed and a syringe was used to wash the wells of residual urea. The sample running buffer was added to a number of the wells and the gel was pre-run at 1700 volts for 20 mins., after which it was ready for loading of cleavage reaction samples. The samples were heated to 90^{0}C for 3 mins. before being transferred to ice for 1 min., after which they were loaded on the gel. The gel was run at 1700 volts for 1 hour, or until the loading dye had approached (within 2 cm of) the bottom of the gel.

2.5.6.5.4 Disassembly and Developing of acrylamide gels:

For sequencing gels, the plates were separated and 3MM filter paper (cut to size) was placed on top of the gel, avoiding bubbles. The paper was lifted gently, taking the gel with the paper. This was then covered with cling film and placed on top of another sheet of 3MM filter paper in the gel drier, with the cling film facing up. The gel was dried at 80^{0}C for 2 hours. When dry, the gel was placed in a cassette and in the darkroom the cling film was removed and a sheet of X-ray film was placed on the gel (Kodak, X-OMAT S, 500 9907). The cassette was sealed shut and the gel was exposed to the film for 48 hours. The film was removed from the cassette in the darkroom, placed (and agitated) in developer (Kodak, 5070933) for 5 mins., UHP (1 min.), fixer (3 mins.) (Kodak, 5211412) and finally UHP again for 1 min. before being left to drip-dry overnight.

For *in-vitro* cleavage gels, the plates were separated and a sheet of cling film was placed over the gel. The gel was placed behind a perspex screen and put inside a cupboard in the darkroom. A sheet of X-ray film (Kodak, 5211412) was placed over the cling film and exposed for 48 hours. After exposure, the film was developed in the same way as for the sequencing gels.

2.5.7 DNA microarray analysis on DLKP-A2B and DLKP-A5F cells

2.5.7.1 RNA Extraction.

The RNeasy RNA extraction is based on guanidine thiocyanate method of extraction. The procedure was carried out according to the RNeasy protocol (Qiagen, Cat.# 74104).

2.5.7.2 DNA microarray.

RNA from both cell lines was analysed by DNA microarray analysis. The procedure followed was as outlined by the manufacturer, "Atlas Human Cancer 8k Array Gene" (Clontech, 7905-1). In brief, the procedure involved the generation of a radioactively-labelled complementary DNA (cDNA) copy of the isolated RNA samples. These labelled cDNA samples were each hybridised to a separate DNA array membrane and the membranes were rinsed of excess label. These hybridised membranes were then read using a Cyclone™ Phosphorimager and analysed with AtlasImage™ 2.01 software. Altered expression of genes observed between the two membranes were identified and quantified by computer analysis.

Section 3.0: Results.

3.1 Analysis of apoptosis-related gene expression in sensitive and resistant variants of DLKP.

An investigation was carried out to establish the expression level of members of the bcl and caspase gene families in a range of cell lines displaying varying resistance to chemotherapeutic drugs. Previous studies have shown that altering the levels of these genes contributes to neoplastic transformation (Section 1.4).

The RNase protection assay (Riboquant, Pharmingen) is a technique that has the capacity to simultaneously quantify several mRNA species in a single sample of total RNA (See materials & methods, section 2.5.2.8). Two multi-probe template sets were selected which target the bcl family and caspase family genes. For each cell line, 10ug of RNA was hybridised to the radioactive probe. The results indicated that two bcl family genes, mcl-1 and bax, had highest expression in the DLKP variants.

These cells were developed by a number of researchers (see below Table 7.2 Appendix section) at NCTCC. Details of the origin of each cell line and how each was developed are displayed in Table 3.1.1. They each displayed varying levels of drug resistance as a result of drug selection and were therefore chosen for analysis by RPA to determine changes in bcl family gene expression. Figure 3.1.1-3.1.4 show RPAs for a number of drug sensitive and resistant variants of DLKP. The graphs in Figure 3.1.2 display densitometric profiles of anti- and pro-apoptotic genes in the cell lines. Tables 3.1.2 and 3.1.3 outline the gene expression profiles in the cell lines. Figure 3.1.5 shows bcl family gene expression DLKP-SQ and A250-10p cells.

The pro-apoptotic gene bax was highest in DLKP, DLKP-I, adriamycin-selected variants - A2B, A2B-1C7, A6B, and DLKP-SQ-10p-fosRz. The anti-apoptotic gene, mcl-1, had highest levels in DLKP, DLKP-A2B, DLKP-Melph-10p, A6B, SQ-10p-fosRz and VP-3. Of the anti-apoptotic genes, there was no change in bcl-2 or bfl-1 levels. Bcl-xL levels had decreased in all cell lines examined except for A250-10p cells. Bcl-w had decreased levels in most cell lines relative to DLKP cells. Examination of the pro-apoptotic genes revealed that most had decreased in expression in the drug resistant cells. Bad, bik and bak were decreased in most cells relative to DLKP. Bax had decreased except for in A250-10p cells where it was increased. Bcl-xS did not display

any particular trend in expression. It did appear to have no change or be increased in adriamycin-resistant cells and have decreased in other cells selected to other drugs.

Bax and mcl-1 were selected for further investigation based on information from the literature as well as previous studies in this lab.

Table 3.1.1 Cell lines used in this thesis.

Cell Line	Clinical/ Cell History.
DLKP	Established from a lymph node biopsy on a 52 yr, old male diagnosed with poorly differentiated squamous cell lung carcinoma.
DLKP-A	DLKP variant selected by exposure to increasing conc. of adriamycin (final con. 2.1μg/ml)
DLKP-A10	DLKP variant selected by exposure to 10μg/ml adriamycin, almost identical to DLKP by fingerprinting.
DLKP-A2B	Clonal sub-population of DLKP-A. (36-fold increased resistance to adriamycin wrt DLKP)
DLKP-A5F	Clonal sub-population of DLKP-A. (330-fold increased resistance to adriamycin wrt DLKP)
DLKP-A6B	Clonal sub-population of DLKP-A. (95-fold increased resistance to adriamycin wrt DLKP)
DLKP-A11B	Clonal sub-population of DLKP-A. (84-fold increased resistance to adriamycin wrt DLKP)
DLKP-A2B-1C7	Clonal sub-population of DLKP-A2B transfected with mdr-1 ribozyme. (Resistance levels similar to those of DLKP)
DLKP-I	Intermediate subpopulation cloned from DLKP-p5, about 25% of DLKP shows DLKP-I morphology.
DLKP-M	Mesenchymal-like sub-population clone from DLKP-p5, about 5% of DLKP shows DLKP-M morphology.
DLKP-SQ	Squamous sub-population cloned from DLKP-p5, about 70% of DLKP shows DLKP-SQ morphology.
DLKP-SQ-A250-10p	DLKP-SQ variant selected by 10 pulses with adriamycin 250ng/ml for 4hours once/week.
SQ-10p-fos-Rz	DLKP-SQ-A250-10p variant transfected with fos ribozyme.
T1-10	DLKP-SQ variant selected by one or two rounds of pulsing with 30ng/ml taxol for 4hours once/wk. for 10 weeks. (Approx. 1.6-fold resistant to taxol).
T2-10	DLKP-SQ variant selected by one or two rounds of pulsing with 30ng/ml taxol for 4hours once/wk. for 10 weeks. (Approx. 50-fold resistant to taxol).

Table 3.1.1 continued.

Cell Line	Clinical/ Cell History.
DLKP-VP-3	DLKP variant selected by exposure to 3μg/ml VP-16.
DLKP-VP-8	DLKP variant selected by exposure to 8μg/ml VP-16.
DLKP-Melph-10p	DLKP variant selected by 10 pulses of 6μg/ml melphalan for 4hours once wk. for 10 weeks.
DLKP-Melph-LT	DLKP variant selected by long-term treatment with melphalan to a final concentration of 6.5μg/ml.
HMEC	Human Mammary Epithelial Cells isolated from 50year old Causcasian female. These cells were bought from BioWhittaker Labs (HMEC 1001-10). They are "normal" cells that grow up to p20.
DLKP-SQ-xL-Rz	A number of clones of DLKP-SQ-bcl-xL-Rz are used in this thesis.
A250-10p-xL-Rz	A number of clones of DLKP-SQ-bcl-xL-Rz are used in this thesis.
CP-50-10p	DLKP-SQ variant pulse selected with 50μg/ml of carboplatin for 4 hours once wk. for 10 weeks.
MCF-7	Breast carcinoma cell line developed from pleural effusion from a 69yr old female diagnosed with breast carcinoma.
SKOV-Tet-off	Ovarian carcinoma cell line transfected with a tet-off reporter vector.

Figure 3.1.1: Expression of bcl family genes in sensitive and resistant variants of DLKP cells. 10ug of total RNA was hybridised to probe for each cell line. Protected fragments were separated on 12% acrylamide gels. Legend shows the position of each protected fragment.

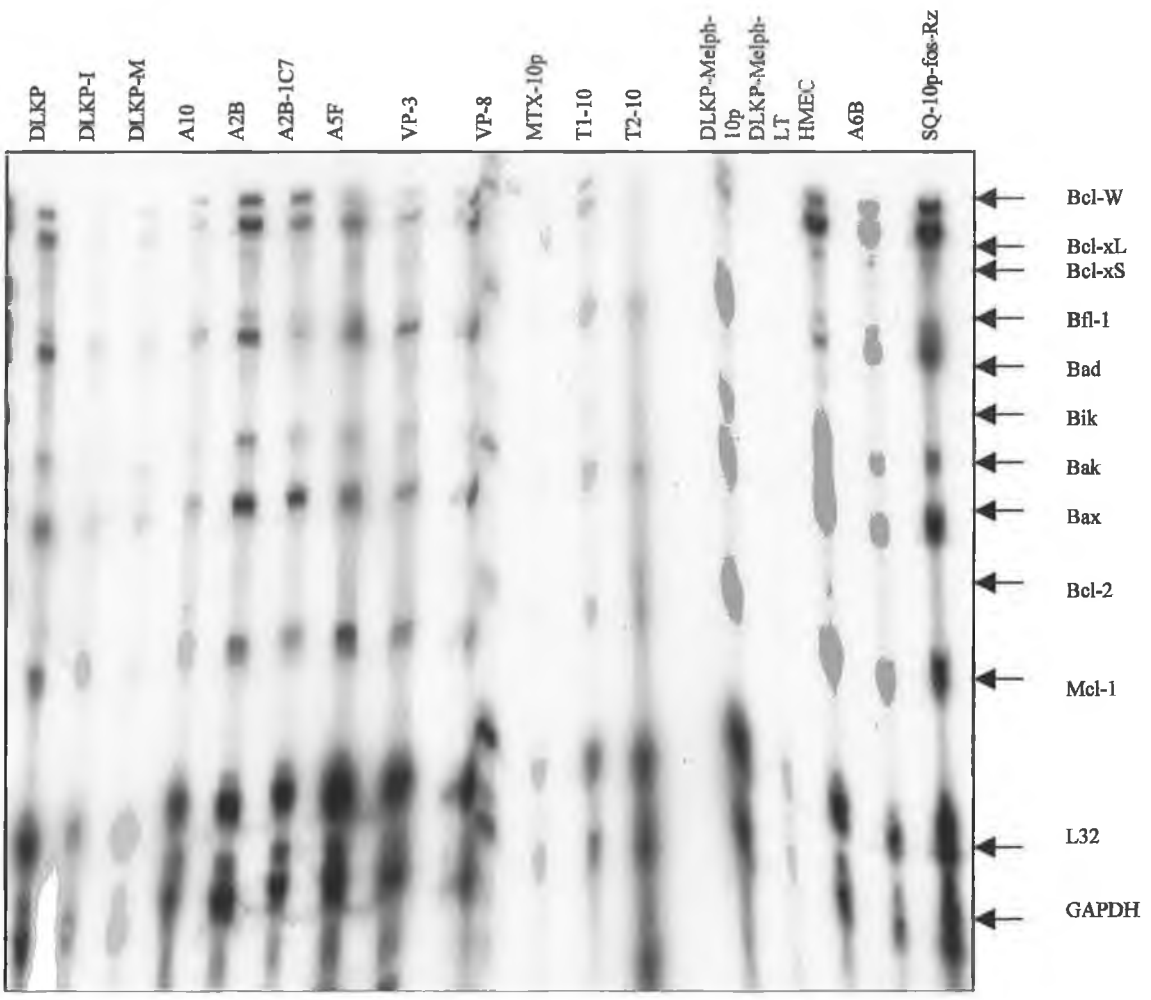


Figure 3.1.2a: Graph showing anti-apoptotic gene expression in sensitive and resistant variants of DLKP.

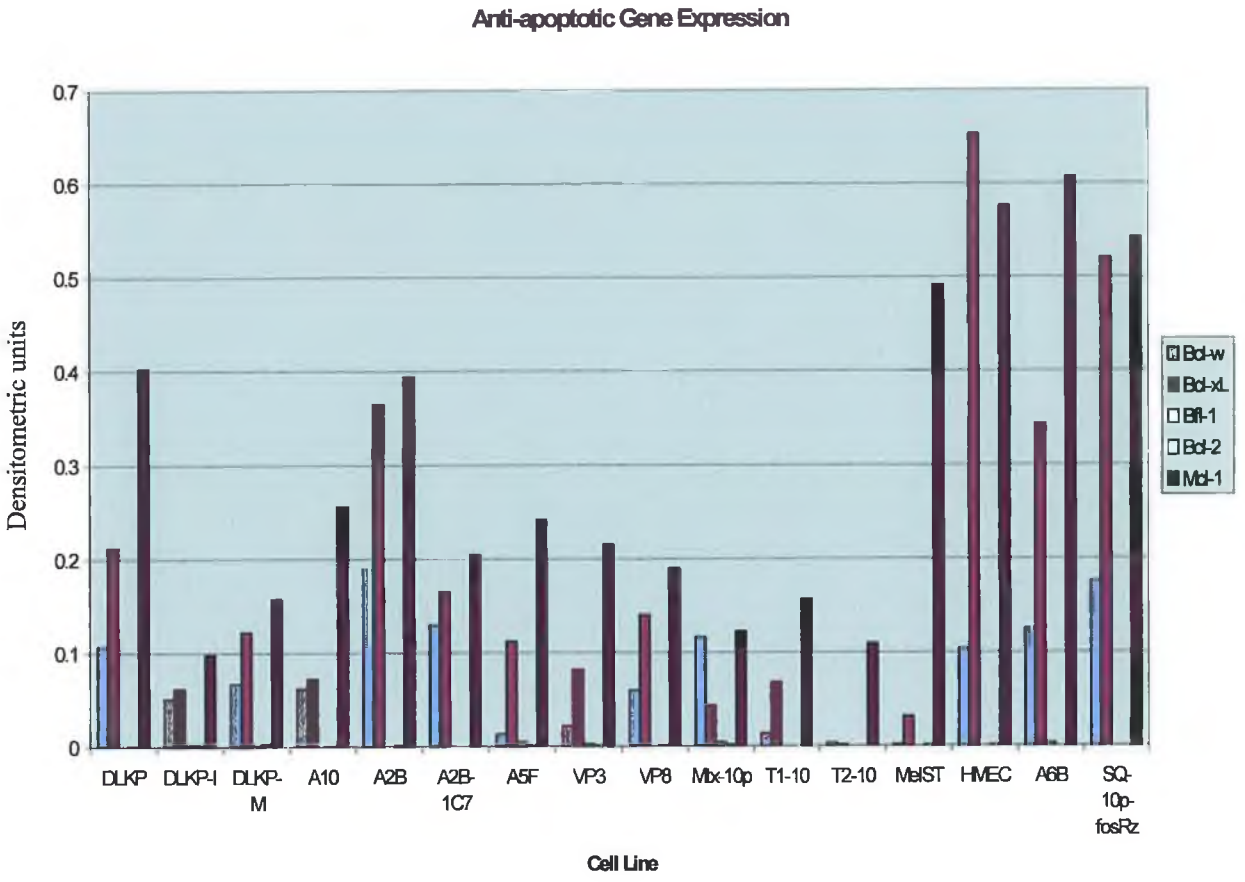


Figure 3.1.2b: Graph showing pro-apoptotic gene expression in sensitive and resistant variants of DLKP.

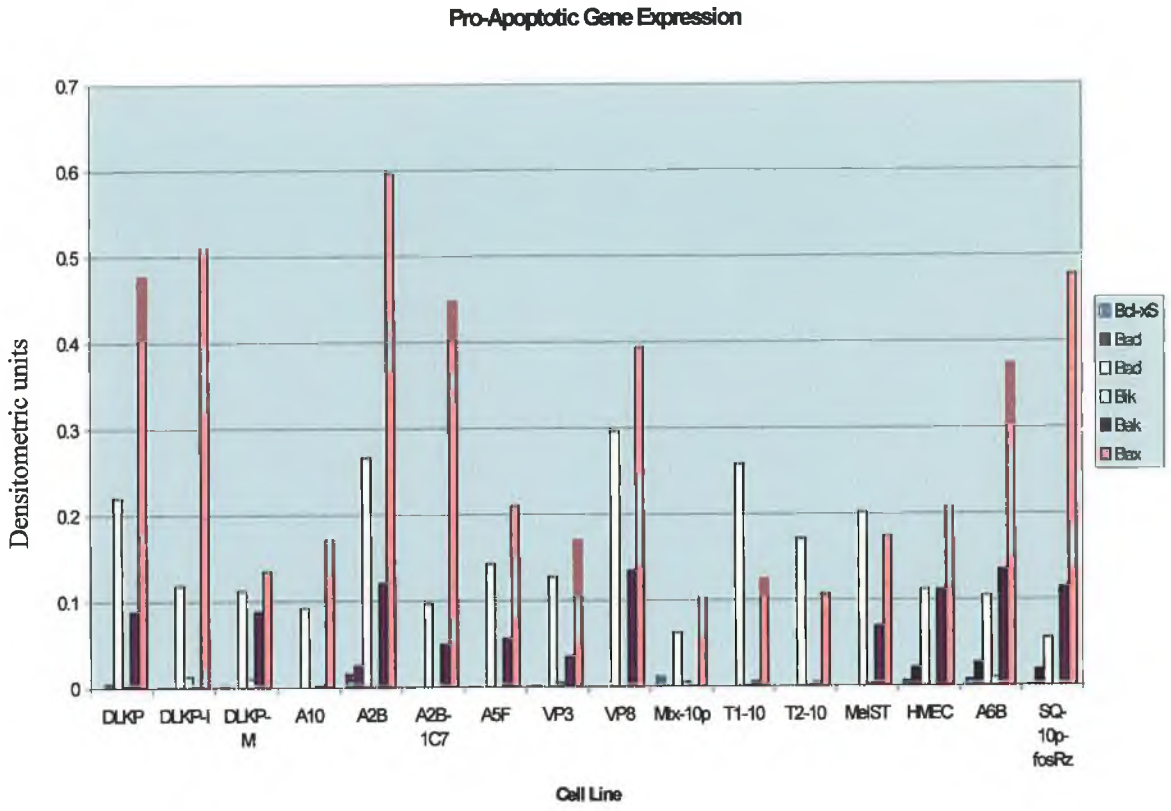


Figure 3.1.3: Repeat Bcl family RPA on DLKP variants. 10ug of RNA from each cell line was hybridised to probe. Samples were separated on 12% acrylamide gel. Legend shows the position of each protected fragment.

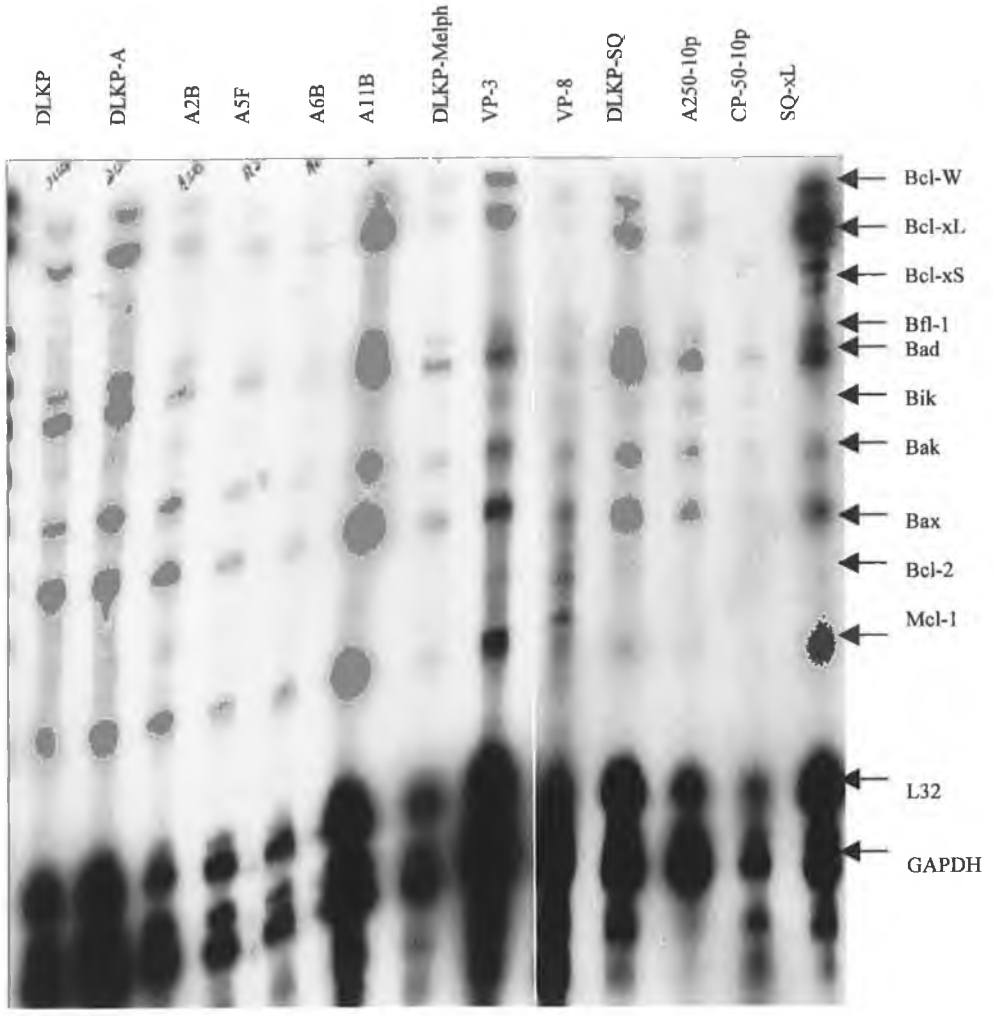


Table 3.1.2: Gene Expression Profile in Experiment 1 (top line) & 2 (bottom line) in Adriamycin treated DLKP variants. All trends in expression are relative to that of parent, DLKP cells.

Cell Line	DLKP-A	DLKP-A10	A2B	A2B-1C7	A5F	A6B	A11B
Gene							
Bcl-w	Down		Down		Down	Down	Down
		Down	Up	Same	Down	Same	
Bcl-xL	Down		Down		Down	Down	Down
		Down	Up	Same	Down	Up	
Bfl-1	Same		Same		Same	Same	Same
		Same	Same	Same	Same	Same	
Bcl-2	Same		Same		Same	Same	Same
		Same	Same	Same	Same	Same	
Mcl-1	Down		Down		Down	Down	Up
		Down	Same	Down	Down	Up	
Bcl-xS	Same		Same		Down	Same	Same
		Same	Up	Same	Up	Up	
Bad	Down		Down		Down	Down	Down
		Down	Up	Down	Down	Up	
Bad	Down		Down		Down	Down	Down
		Down	Up	Down	Down	Down	
Bik	Same		Down		Down	Down	Down
		Same	Same	Same	Same	Up	
Bak	Down		Same		Down	Down	Down
		Down	Same	Down	Down	Same	
Bax	Down		Down		Down	Down	Down
		Down	Up	Same	Down	Down	

Table 3.1.3: Gene Expression Profile in Experiment 1 (top line) & 2 (bottom line) in melphalan (DLKP-Melph), etoposide (VP-3 & VP-8), methotrexate (Mtx-10p) or taxol (T1-10 & T2-10) treated DLKP variants. All trends in expression are relative to that of parent, DLKP cells.

Cell Line	**DLKPMelph**	**VP-3**	**VP-8**	**Mtx-10p**	**T1-10**	**T2-10**	**Mel-ST**
Gene							
Bcl-w		Down	Down	Same	Down	Down	Down
	Down	Same	Down				
Bcl-xL		Down	Down	Down	Down	Down	Down
	Down	Same	Down				
Bfl-1		Same	Same	Same	Same	Same	Same
	Same	Same	Same				
Bcl-2		Same	Same	Same	Same	Same	Same
	Same	Up	Up				
Mcl-1		Down	Down	Down	Down	Down	Up
	Down	Up	Up				
Bcl-xS		Same	Down	Down	Down	Down	Down
	Down	Down	Down				
Bad		Down	Down	Down	Down	Down	Down
	Down	Down	Down				
Bad		Down	Up	Down	Up	Down	Down
	Down	Down	Down				
Bik		Same	Same	Same	Same	Same	Same
	Down	Down	Same				
Bak		Down	Up	Down	Down	Down	Down
	Down	Down	Down				
Bax		Down	Down	Down	Down	Down	Down
	Down	Down	Down				

Figure 3.1.4: Bcl family expression in sensitive DLKP-SQ and resistant DLKP-SQ-A250-10p cells. 10ug of total RNA was loaded for each cell line. Samples were separated on 12% acrylamide gels. Legend shows position of the protected fragments. A slight displacement is observed on this gel due to physical manipulation of the gel when lifting it from the glass plates.

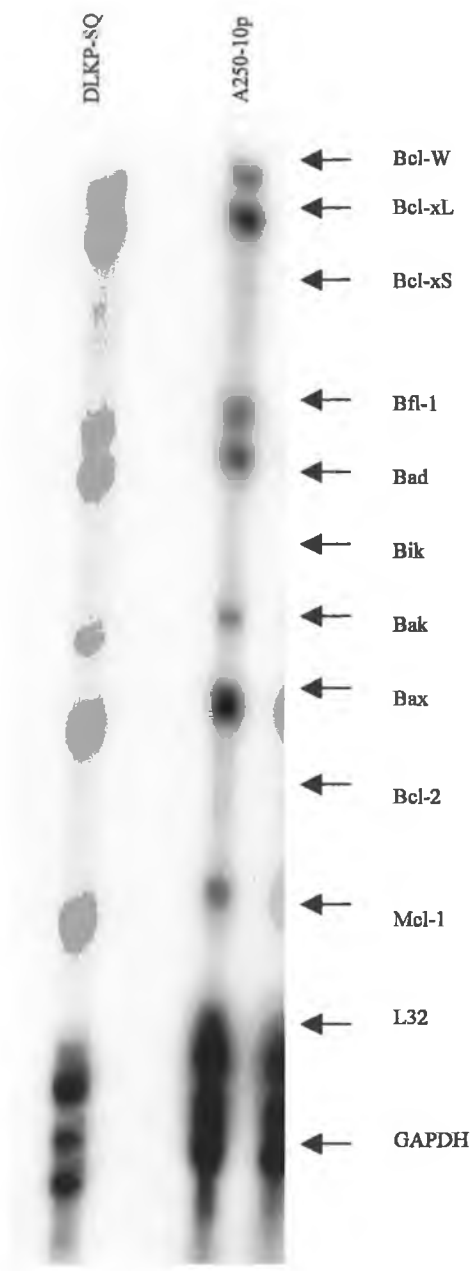
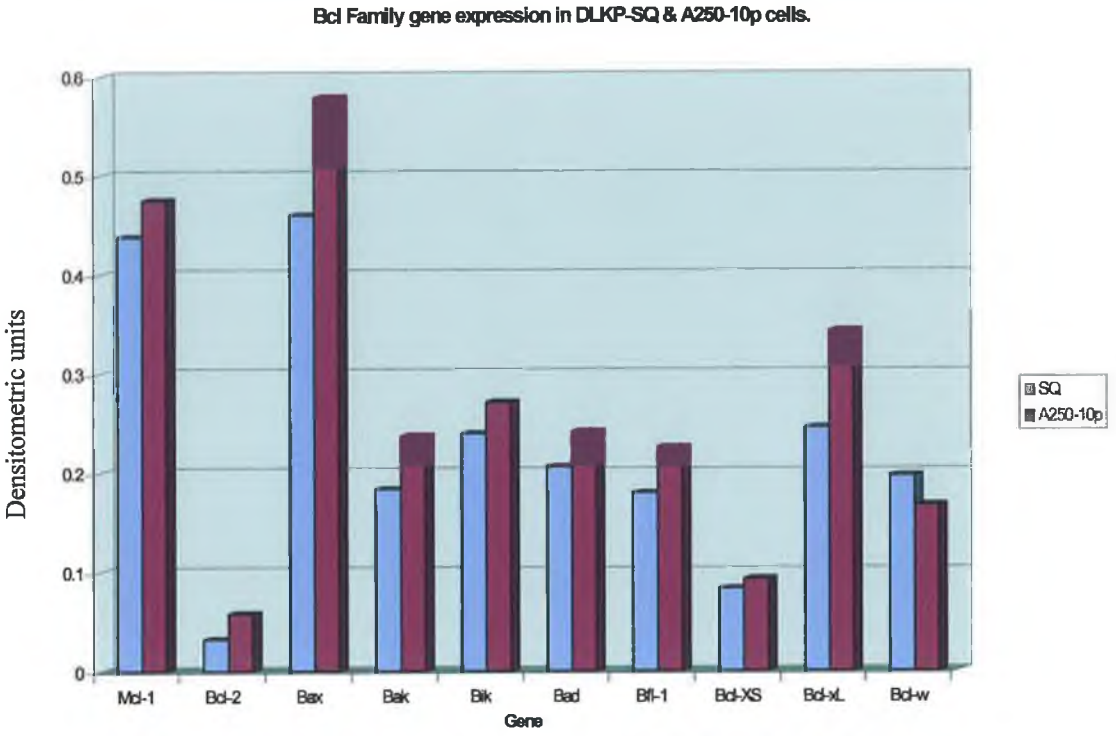


Figure 3.1.5: Bcl family gene expression in DLKP-SQ versus A250-10p cells.



3.1.2 Conclusions on the RPA analysis of DLKP variants.

The RPA was initially used to assess the expression of apoptosis-related genes in the cell lines. It provided a spectrum of gene expression across the cell lines. Mcl-1 had highest expression of the anti-apoptotic genes and bax was highest among the pro-apoptotic genes. As expected, bax was decreased in most of the resistant cell lines relative to parent DLKP cells, however it was increased in adriamycin-pulse selected cells, A250-10p, relative to their corresponding cells, DLKP-SQ. Unexpectedly, mcl-1 levels were mostly down in the resistant cells. This was a surprising result as previous studies have indicated that it is increased after cells have been exposed to cytotoxic drug (Section 1.4.5).

3.1.3 Transfection of bax into DLKP-SQ cells.

As mentioned, RPA analysis showed bax expression was highest in DLKP, DLKP-I, A2B, A2B-1C7, A6B and SQ-10p-fozRz. Expression was also increased in A250-10p relative to DLKP-SQ. Previous studies in this lab found that bax was upregulated in adriamycin-selected DLKP-SQ cells, named A250-10p (Roisin NicAmhlaoibh, PhD 1997). These cells had increased resistance to a number of chemotherapeutic drugs and were approximately 10-fold more resistant to adriamycin. Examination of the bcl family genes revealed that both bcl-xL and bax had increased expression.

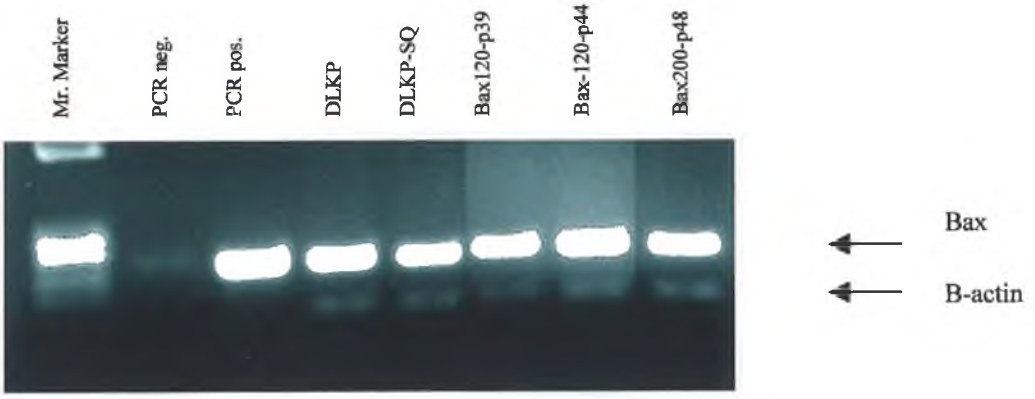
To investigate the contribution of bax to drug resistance in these cells, DLKP-SQ was transfected with bax cDNA, kindly donated by Richard Bertrand (Schmitt et al., 1997). Two mixed populations were isolated as studies by Schmitt et al. found that varying the level of selection agent, hygromycin, resulted in varying levels of the bax protein. Therefore, two mixed populations were isolated at 120ug/ml (Bax-120) and 200ug/ml (Bax-200) hygromycin.

As Figure 3.1.6 shows, there was no consistent increase in bax mRNA expression in either sets of mixed populations. In addition, no consistent change could be detected in bax protein (see Figure 3.1.7).

Toxicity tests revealed that drug resistance levels had not altered in either mixed population. A study by Dubrez et al. (2001) showed that caffeine increased the tendency of bax transfectants to undergo apoptosis. Toxicity assays in the presence and absence of caffeine on Bax120 and Bax200 however did not establish any change in resistance levels. All cell lines, including parent cells, DLKP-SQ, became more sensitive to drug post caffeine treatment, however relative IC50 values did not change.

It is possible that the heterogeneous nature of the mixed population was masking the emergence of interesting clones within both populations. Given that no consistent change in bax levels had occurred in transfected cells, no further analysis was carried out and clones were not isolated.

Figure 3.1.6: RT-PCR for bax expression in parent (DLKP-SQ) and bax transfectants (Bax-120 and Bax-200). Bax amplification yields a 240bp product and β-actin yields a 142pb product. The molecular weight (Mr marker) is "ϕ-X174" HaeIII digest (Promega: G1761). Graph below shows densitometric analysis.



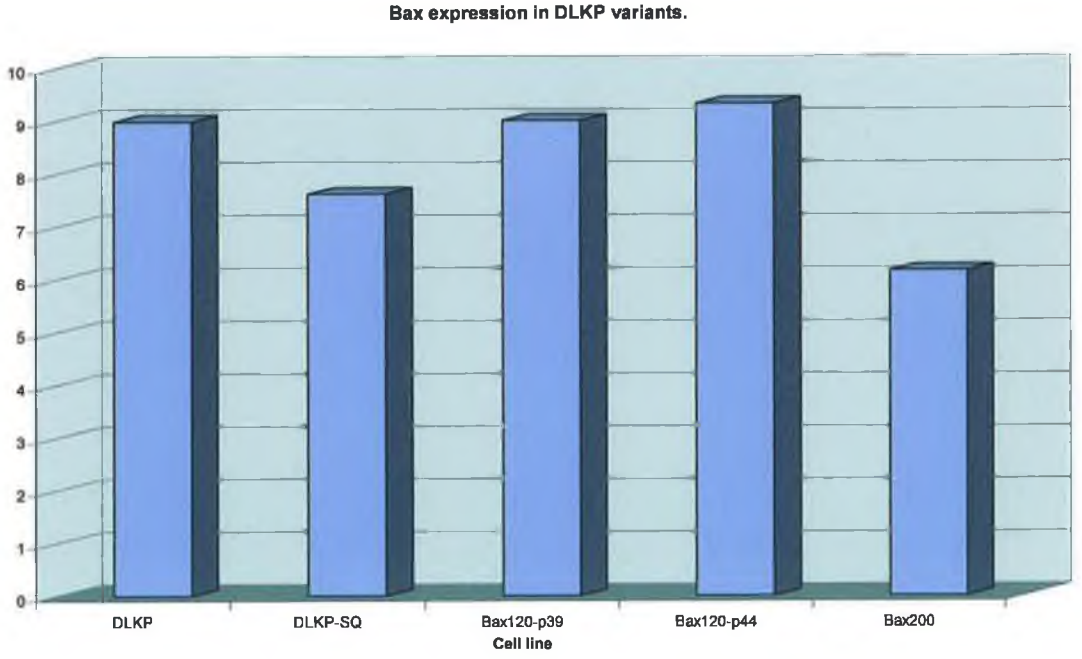
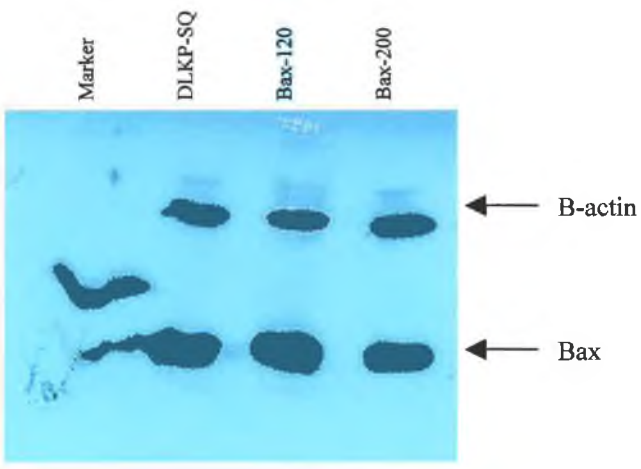
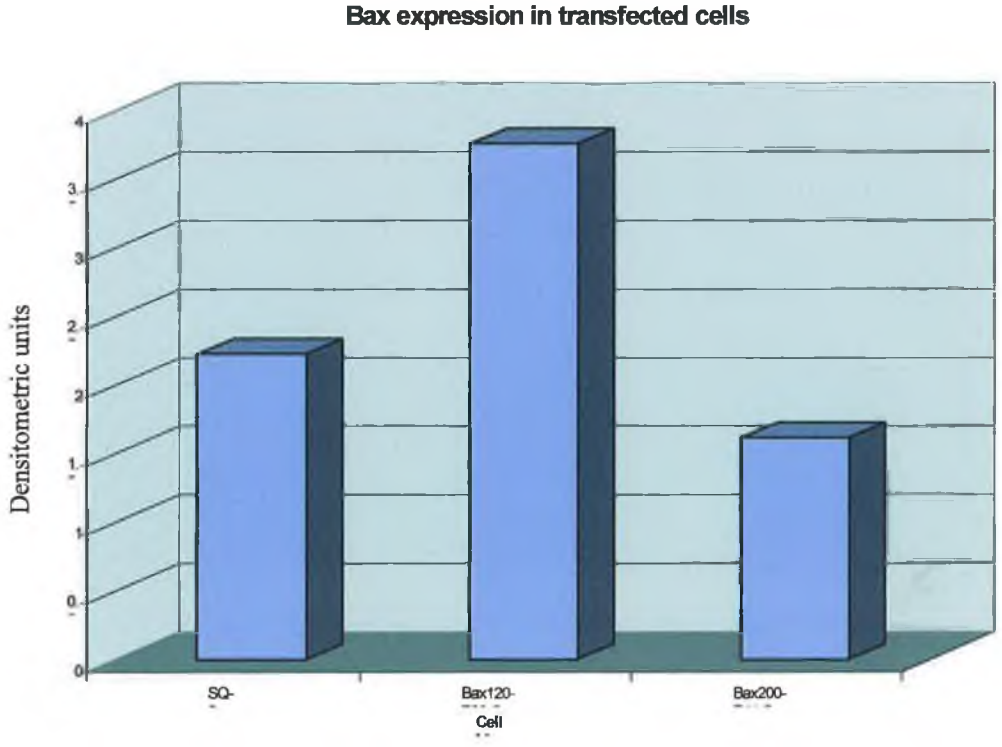


Figure 3.1.7: Western blot showing bax expression levels in parent (DLKP-SQ) and transfected (Bax-120 & Bax-200) cells. Bax is a 21kDa protein and β-actin is a 42kDa protein. The molecular weight marker is colour marker (Sigma C3437). Graph below shows densitometric analysis.



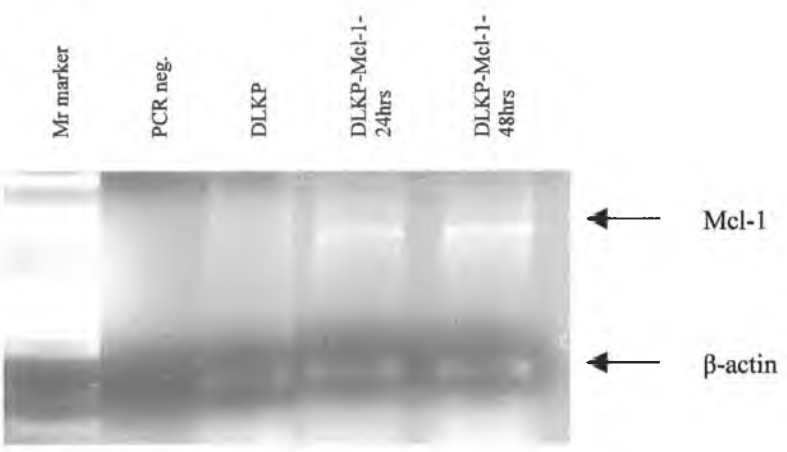


3.1.4 Transfection of mcl-1 into DLKP cells.

Mcl-1 expression was highest of the anti-apoptotic genes when analysed by RPA. Initially, some of the drug-resistant variants appeared to have increases in mcl-1 expression, however this was not found consistently. Highest levels were found in DLKP, A2B, Mel-ST, A6B, SQ-10p-fozRz and VP3. A number of studies have reported that mcl-1 is increased after exposure to drugs such as etoposide where mcl-1 protects against apoptosis, however almost all studies have been carried out on hematopoietic cell lines, from which the gene was originally identified.

To assess its contribution to drug resistance in lung cancer cells, mcl-1 cDNA was transfected into DLKP cells. Mcl-1 has a very short half-life of approximately three hours (see Section 1.4.5) and as such, is thought to act as a rapid response protein, allowing time for bcl-2 and other anti-apoptotic proteins to be recruited and promote cell survival. Mcl-1 expression was upregulated by a transient transfection of cDNA into DLKP cells whereby an increase in mcl-1 expression was observed at 24 and 48 hours (See Figure 3.1.8 showing RT-PCR results). The effects of mcl-1 expression on the phenotype of cells were not estimated as a suitable drug resistance assay could not be optimised and toxicity analysis was not carried out.

Figure 3.1.8: RT-PCR showing mcl-1 expression in transiently transfected DLKP cells. The mcl-1 product is approximately 508bp in this reaction and β-actin is at 142bp in this reaction. The molecular weight (Mr marker) is "ϕ-X174" HaeIII digest (Promega: G1761).

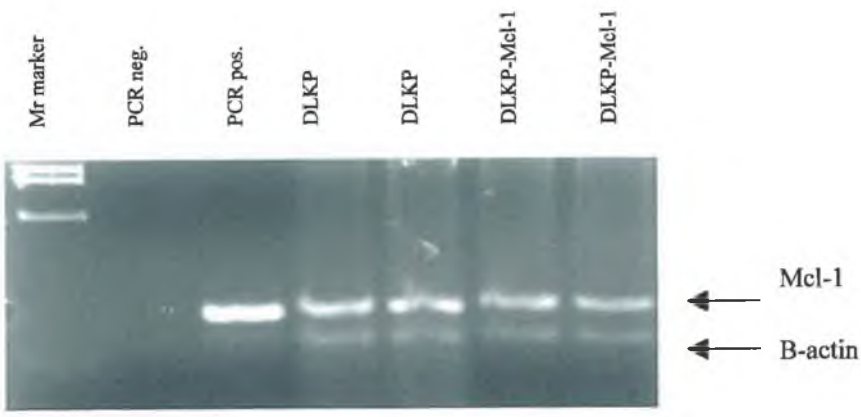


3.1.4.1 Stable transfection of mcl-1 into DLKP cells.

RT-PCR revealed no up-regulation of mcl-1 mRNA in the stable transfectants compared to parent cells (See figure 3.1.9). However, Western blot analysis displayed an increase in mcl-1 protein expression in transfected cells (See figure 3.1.10). The Western blot shows two different concentrations of protein loaded for each cell line. The mcl-1 protein is 39-42kDa in size indicated by the arrow. At each protein concentration, there was an increase in mcl-1 expression in the transfectants when compared with parent cells.

Due to increases at the protein level, toxicity analysis was carried out to assess the contribution of the induced gene expression to drug resistance. Because mcl-1 is an anti-apoptotic protein, it was expected that drug resistance levels would increase in the transfected cells. Transfected cells showed very slight increases in resistance levels. (See table 3.1.4 & 3.1.5). An unexpected increase in P-gp protein levels in the transfected mixed population may explain the increased resistance levels observed (See figure 3.1.11).

Figure 3.1.9: RT-PCR for Mcl-1 expression in parent (DLKP) and DLKP-Mcl-1 cells. Mcl-1 amplifies yielding a 217bp product and β-actin yields a 142bp product. The molecular weight (Mr marker) is "ϕ-X174" HaeIII digest (Promega: G1761).



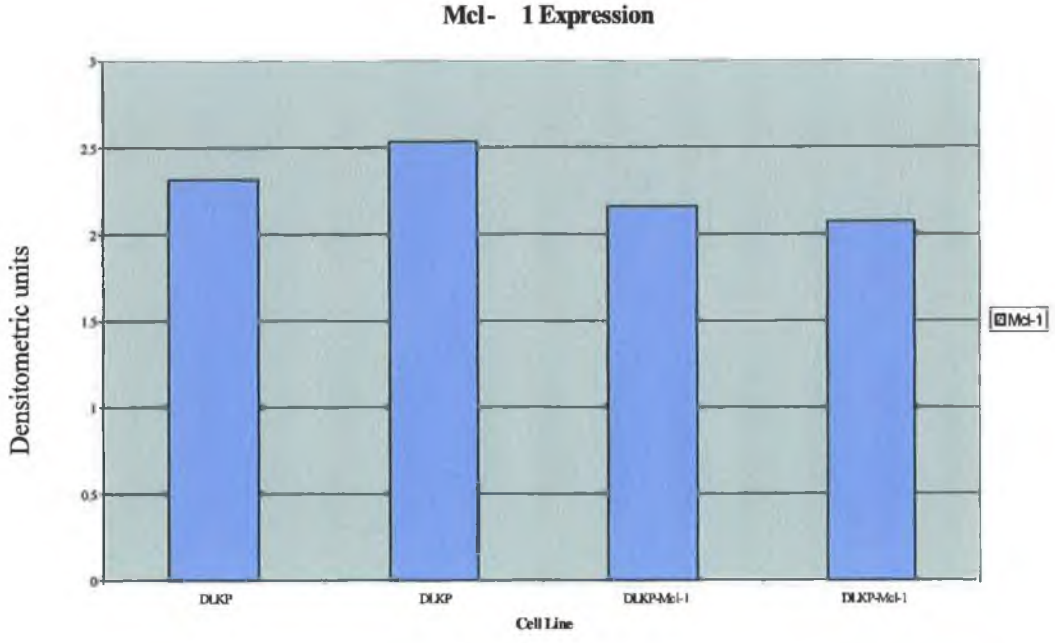
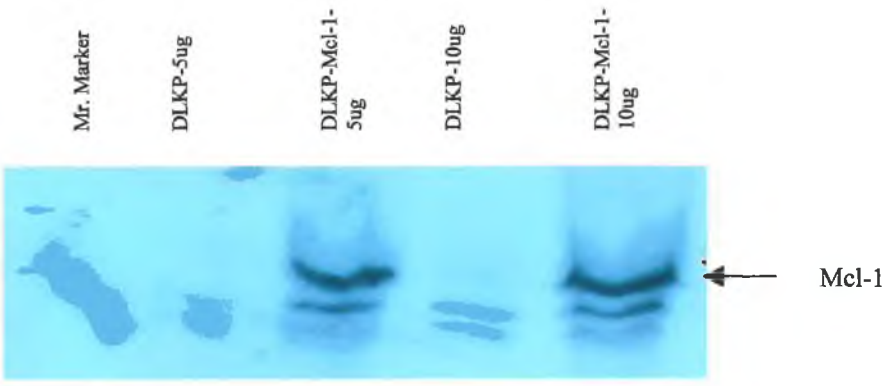


Figure 3.1.10: Western blot showing Mcl-1 expression in parent (DLKP) and transfected (DLKP-Mcl-1) cells. Mcl-1 is a 39-42kDa product. Molecular weight marker is colour marker (Sigma C3437).



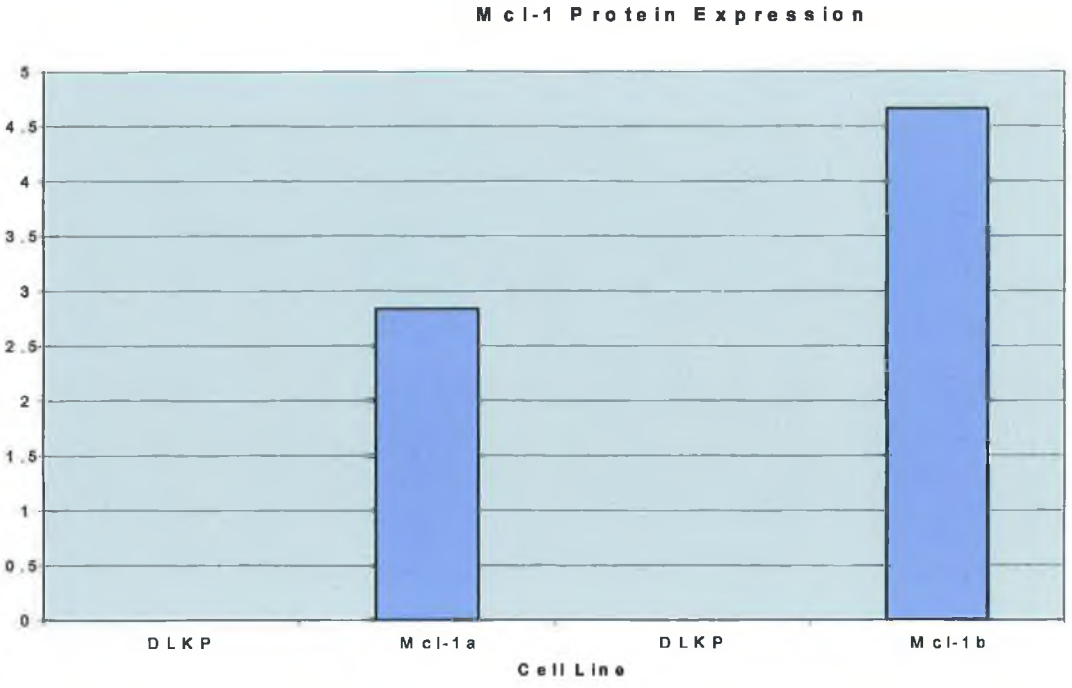


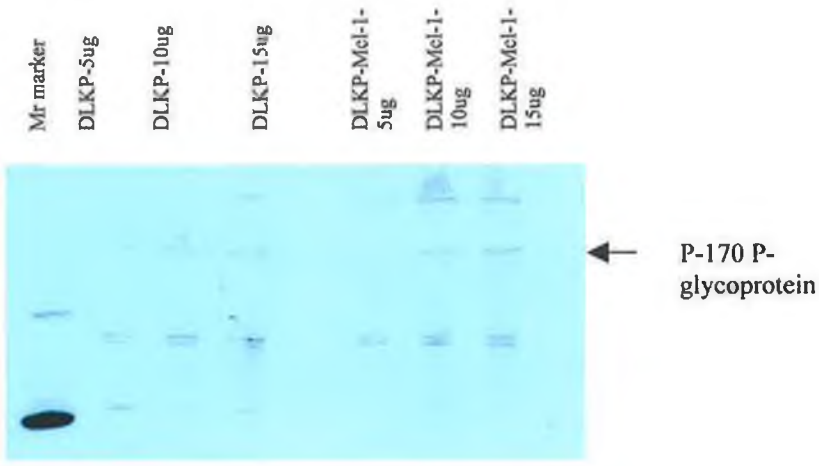
Table 3.1.4: IC50 values in DLKP-Mcl-1 transfectants (Results from two independent sets of toxicity tests). Mean ± standard deviation, n=2.

Drug	DLKP-p16	DLKP-Mcl-1-p24
ADR ng/ml	10.45 ± 0.45	22.95 ± 1.05
VP-16 ng/ml	84.6 ± 11.4	156.1 ± 38.9
5-FU ug/ml	1.552 ± 0.048	1.527 ± 0.023
Cispl. Ug/ml	0.91 ± 0.03	1.01 ± 0.09
VNC ng/ml	1.2 ± 0.1	1.58 ± 0.08

Table 3.1.5: Fold resistance in DLKP & DLKP-Mcl-1 cells.

Drug	DLKP-p16	DLKP-Mcl-1-p24
ADR	1	2.2
VP-16	1	1.85
5-FU	1	1
Cispl.	1	1.1
VNC	1	1.32

Figure 3.1.11: Western blot showing P-glycoprotein expression in DLKP and DLKP-Mcl-1 transfectants. P-gp is a 170kDa protein. Three different concentrations of protein were loaded for each cell line. Molecular weight marker is colour marker (Sigma C3437).



3.1.5 Conclusions on Bcl family RPA work and subsequent transfections.

The RPA did not give fully repeatable results. Initial findings indicated that both bax and mcl-1 had varying expression levels in the DLKP variants. Previous studies in this lab had focused on bcl-2, xL & xS. Bax, along with bcl-xL, had increased in expression in an adriamycin-pulse-selected variant of DLKP-SQ (NicAmhlaoibh et al., 1999). Literature searches suggested that mcl-1 expression increased resistance levels to etoposide (Zhou et al., 1997, Moulding et al., 2000, Wang et al., 1999a). Therefore cDNAs to bax and mcl-1 were obtained and transfected into sensitive variants of DLKP.

No major changes in drug resistance emerged following transfection of bax into DLKP-SQ cells. Despite two separate concentrations (i.e.120ug/ml and 200ug/ml) of selection agent (hygromycin) there did not appear to be a consistent change in bax expression. Bax120 cells did appear to have slight increases in bax mRNA and protein, however Bax200 cells did not. Toxicity analysis in the presence or absence of caffeine revealed no change in resistance levels. It was observed that caffeine treatment did enhance the cytotoxicity of drugs investigated, but this toxicity was also enhanced in parental cells.

In the DLKP-Mcl-1 transfectants, mcl-1 protein levels were increased. However, P-glycoprotein levels were also increased. Presumably this increase in P-gp contributed to the increases in resistance to adriamycin and etoposide. However, P-170 levels have been expected to alter resistance to vincristine also. Future work may serve to investigate this further by using a P-gp inhibitor such as cyclosporin-A to establish if all of the increased resistance can be attributed to overexpression of P-gp. Despite numerous attempts, clones of this transfected cell line could not be isolated. Unfortunately, the DLKP-Mcl-1 cells became contaminated with mycoplasma and as a result no further analysis was done.

It is possible that in both mixed populations their heterogeneous nature resulted in interesting clones were being masked (i.e. those with large changes in gene expression and perhaps resistance levels). As can be seen in section 3.2 this occurred with the caspase-3 ribozyme transfectants where mixed population contained clonal populations with varying levels of the gene and as a result, varying levels of resistance.

The emergence of changes in resistance levels in the mcl-1 transfectants could be attributed to the increase in P-gp expression in the cells. This research highlighted the importance of routine investigation of P-gp in transfected cells where changes in resistance levels occur.

3.1.6 Analysis of cell death-related genes in bcl-xL-ribozyme transfected variants of DLKP.

3.1.6.1 Introduction to the bcl-xL ribozyme project.

Previous studies in this lab had generated a resistant variant of DLKP-SQ cells, A250-10p (NicAmhlaoibh et al., 1999). A250-10p were derived by pulse selecting parent cells (DLKP-SQ) with 250ng/ml adriamycin once weekly for four hours. This resulted in a population which was approximately 10-fold resistant to adriamycin and which overexpressed bcl-xL and, surprisingly, the proapoptotic gene, bax.

In order to investigate the role of bcl-xL in drug-induced apoptosis in these cell lines, bcl-xL mRNA was targeted by a bcl-xL specific hammerhead ribozyme. Two ribozymes were constructed. Ribozyme 1 cleaves bcl-xL at position 548 while ribozyme 2 targets bcl-xL at position 616. Both were transfected into DLKP-SQ and A250-10p cells, and a number of clones were isolated. Most work was carried out on clones transfected with ribozyme 2, however a number of clones containing ribozyme 1 also showed phenotypic changes. In DLKP-SQ transfectants, drug resistance profiles did not change, however growth rate was slowed down considerably (results not shown). A number of clones were isolated from the DLKP-SQ-xLRz population. These include Rz1C2, Rz1C14, Rz2C6, Rz2C8.

A250-10p cells transfected with bcl-xL ribozyme displayed a reduction in drug resistance (results not shown). In some clones resistance levels reverted back to those of DLKP-SQ cells. Isolated clones include Rz2A9, Rz2A10, Rz2A11, Rz2A12, Rz2B4, Rz2D6 & Rz1E4. Growth rate was not affected in these clones.

Despite changes in growth rate and resistance levels, no detectable change in bcl-xL expression at either the mRNA or protein level was evident in ribozyme clones of either cell line. Northern blot analysis revealed an alteration of bcl-xL expression in some clones. The ribozyme-expressing clones displayed an additional band at approximately 1kb. This extra band corresponded to the expected size of one of the cleavage products of the ribozyme. Detection of cleavage products has proved elusive in many studies probably due to the rapid degradation of short RNAs. However, this extra band was sufficiently stable to allow detection by Northern blot analysis. Table 3.1.6 shows the

clones in which the extra cleavage band could be detected by Northern blot analysis. Table 3.1.7 summarises the PCR, Northern blot and drug resistance changes in ribozyme transfectants.

To eliminate the possibility that this band represented another known bcl-2 family member, and to investigate if another bcl-2 family member was being upregulated in the ribozyme transfectants, we used RNase protection assay (Pharmingen). Probes to the bcl and caspase family were hybridised to RNA extracted from bcl-xL ribozyme clones (see section 3.1.6.2).

Table 3.1.6 DLKP-SQ clones. Table shows IC50 value to adriamycin, ribozyme expression by RT-PCR and Northern blot result.

Clone	Adriamycin IC50	Rz Expr (RT-PCR)	Northern blot.
DLKP-SQ	16.3		
Rz1C2	12.3	++	XL expression plus lower band.
Rz1C14	11.3	+	Lot of xL expression, trace of lower band.
Rz2C6	7.11	++	Lots of lower band and some xL.
Rz2C8	11.9	++	Lots of extra band and in some instances no xL, other instances some xL. (7 repeats)
Rz2C23	10.5	++	XL expressed and lower band present.

Table 3.1.7 A250-10p clones. Table shows IC50 value to adriamycin, ribozyme expression by RT-PCR and Northern blot result.

Clone	Adriamycin IC50	Rz Expr. (RT-PCR)	Northern blot
A250-10p	246		
DLKP-SQ	19		
Rz2A9	98.2	++	Trace of xL expression, lots of extra band
Rz2A10	118.8	+	Not done
Rz2A11	106.9	++	Some xL expression, lots of extra band
Rz2A12	22.1	-	Not done
Rz1B4	17	++	Not done
Rz2D6	238.6	-	Not done
Rz1E4	119.8	++	Not done

3.1.6.2 Analysis of bcl-xL family gene expression in bcl-xL ribozyme transfectants.

Ribozyme 1 cuts bcl-xL mRNA at position 548 and ribozyme 2 cleaves bcl-xL at position 617. The Pharmingen bcl-xL probe protects RNA from base 185 to base 548. Therefore it protects the 5' end of the gene.

Table 3.1.8 shows the expression of bcl family genes in the DLKP-SQ ribozyme transfectants. Bcl-xL expression is itself increased in these clones. The reasons for this are not known. Table 3.1.9 shows the expression of bcl family genes in the A250-10p ribozyme transfectant clones. Bcl-xL does appear down-regulated in these clones. As both tables show, there is no one bcl family gene emerging as up or downregulated. If the cells are compensating for ribozyme expression, it is likely that there are multiple bcl family members involved. It is important to note that these are only trends in gene expression. In some cases, they involve four repeats of the experiment.

Figure 3.1.12 Expression of bcl family genes in cells transfected with bcl-xL ribozyme.

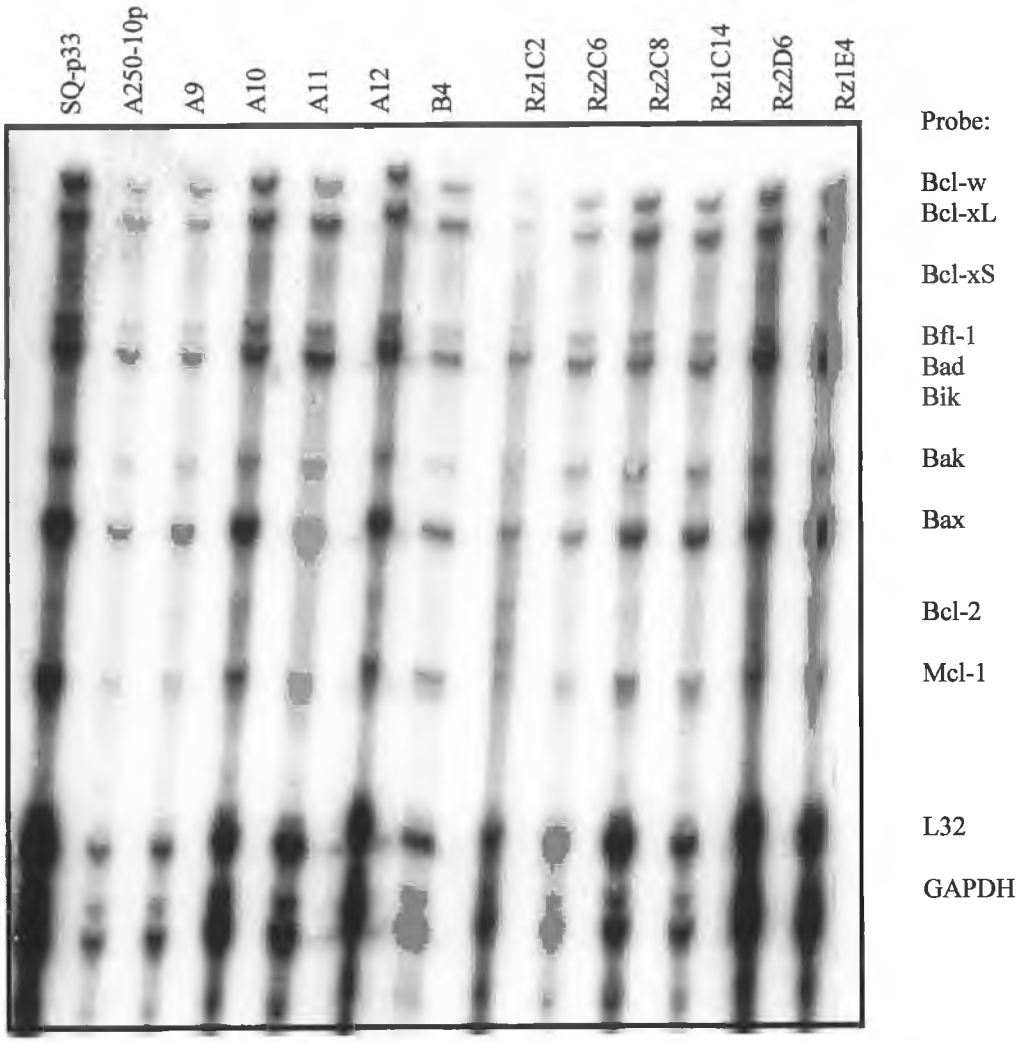


Table 3.1.8 Trends in bcl-2 family expression in DLKP-SQ-bcl-xL Rz clones. (Results are taken from trends seen in four different sets of experiments).

Cell	Rz1C14	Rz2C6	Rz2C8	Rz2C23
Bcl-w	Down	Same	Down	Down
[redacted]	Up	Up	Up	Up
Bfl-1	Same	Same	Same	Same
[redacted]	Slightly up	Slightly up	Up	Up
Mcl-1	Down	Down	Down	Down
[redacted]	Down	Down	No result	No result
Bad	Up	Up	Up	Up
[redacted]	Up	Up	Up	Up
Bik	Same	Same	Same	Same
[redacted]	Down	Up	Up	Up
Bax	Down	Up	Up	Up
[redacted]				

Table 3.1.9 Trends in bcl-2 family gene expression in A250-10p-bcl-xL Rz Clones. (Results are taken from trends seen in four different experiments).

Cell	A9	A10	A11	A12	B4	D6	E4
Bcl-w	Slightly down	Same	Down	Down	Down	No result	Down
Bcl-xL	Down	Down	Down	Down	Down	Down	Down
Bfl-1	Slightly up	Slightly up	Slightly up	Down	Slightly down	Slightly down	No result
Bcl-2	Slightly up	Up	Same	Up	Same	Slightly up	Slightly up
Mcl-1	Slightly down	Slightly up	Up	Up	Up	Slightly up	Up
Bcl-xS	Slightly down	Up	Slightly down	Down	No result	Slightly down	Slightly down
Bad	Down	Up	Down	Same	Slightly down	No result	Down
Bad	Down	No result	Slightly down	Down	Slightly down	Slightly down	Down
Bik							
Bak	Slightly down	Up	Slightly down	Down	Down	Slightly up	Slightly up
Bax	Up	Up	Slightly down	Slightly up	Slightly up	Slightly up	Slightly up

3.1.6.3 Conclusions on bcl-xL ribozyme project.

The results obtained using the RPA did not indicate that any other bcl-2 family member was compensating for loss of bcl-xL expression. The technique did not detect any change in bcl-xL expression in the DLKP-SQ clones, however it did detect down-regulation in the A250-10p clones. There did not appear to be any consistent changes in the expression of any other bcl-2 family members compensating for the loss of bcl-xL in ribozyme transfected cells.

The bcl-xL ribozymes have been subcloned into tetracycline-inducible plasmids and have subsequently been transfected into a number of cell lines. The emergence of the extra band on the Northern blot in these transfectants is currently being analysed.

Interestingly, the RPA analysis showed that levels of bad and mcl-1 were reasonably consistent and opposite in DLKP-SQ and A250-10p clones. In DLKP-SQ, bad levels tended to be up while mcl-1 was decreased. The opposite was true for the A250-10p clones. The significance of these opposite expression levels is not known but may be investigated further in the tetracycline-inducible clones.

3.1.7.1: Caspase family gene expression in DLKP variants.

Sensitive and resistant variants of DLKP cells were analysed by RPA to study expression of caspase family genes. Results displayed changes in caspase-3 expression between some of the adriamycin resistant variants. A gradual decrease in caspase-3 was evident in cells with increasing resistance to the drug, i.e. DLKP-SQ< A250-10p< DLKP-A5F cells. Figure 3.1.13 shows caspase family RPA in DLKP variants. These values are graphically represented in figure 3.1.14. Caspase-3 levels are shown separately in Figure 3.1.15. These results are consistent with previous studies where a decrease in caspase-3 protein levels was seen in cells with increasing resistance (Colette O'Loughlin, PhD 1999).

Included in this analysis were the bax transfectants (Bax120 and Bax200), a melphalan-selected variant of DLKP (DLKP-Melph), and an etoposide-resistant variant of DLKP (VP-3), as well as the adriamycin-resistant variants. The drug resistant variants showed decreased levels of the other caspases investigated relative to the sensitive cells, DLKP-SQ. The bax transfectants also had reduced levels of the caspases when compared to parent, DLKP-SQ cells.

Figure 3.1.13: Caspase family gene expression in DLKP variants. 10ug of total RNA was used from each cell line. Legend shows the position of the protected fragment.

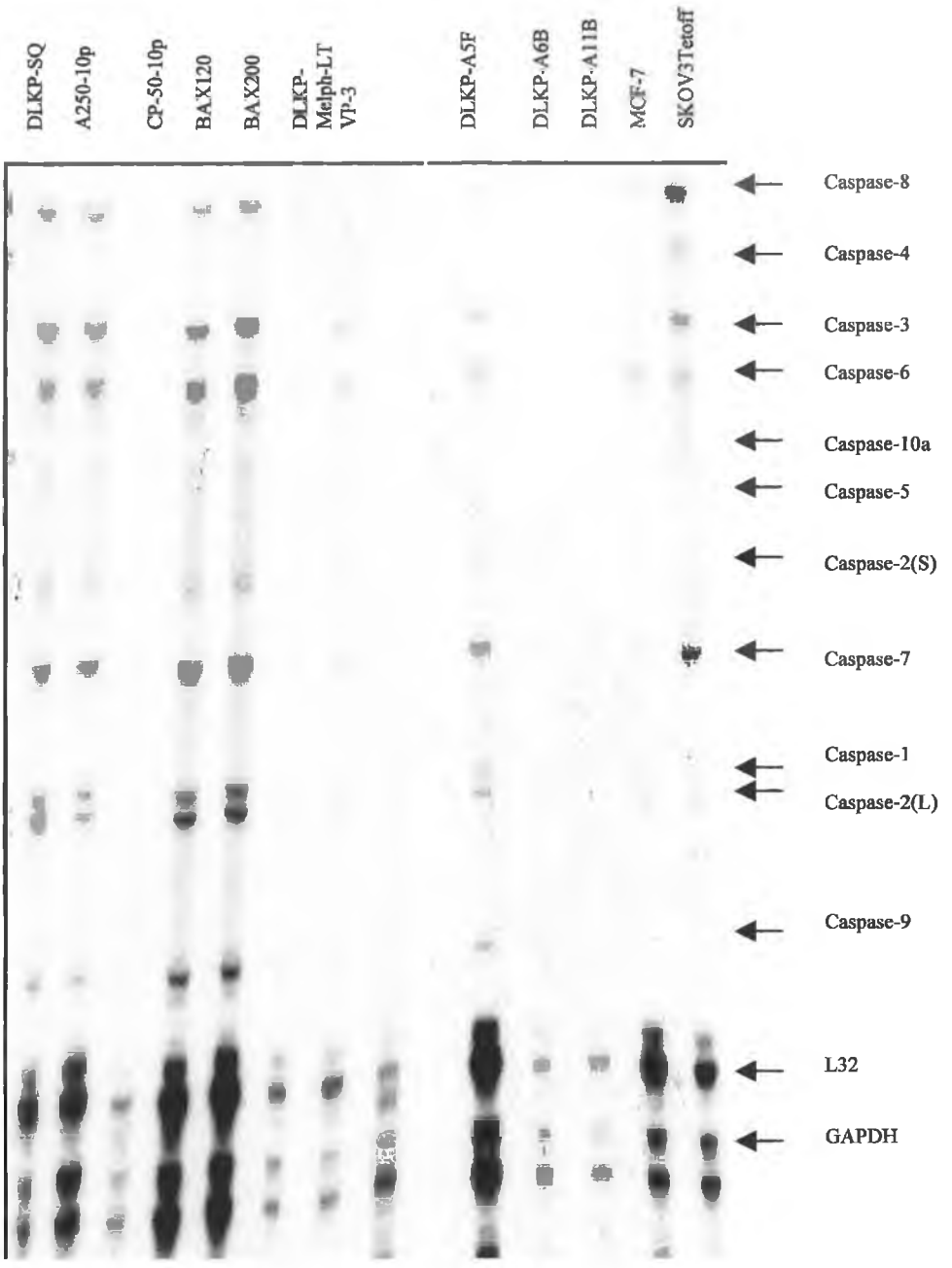


Figure 3.1.14: Graph of Caspase family gene expression in DLKP variants.

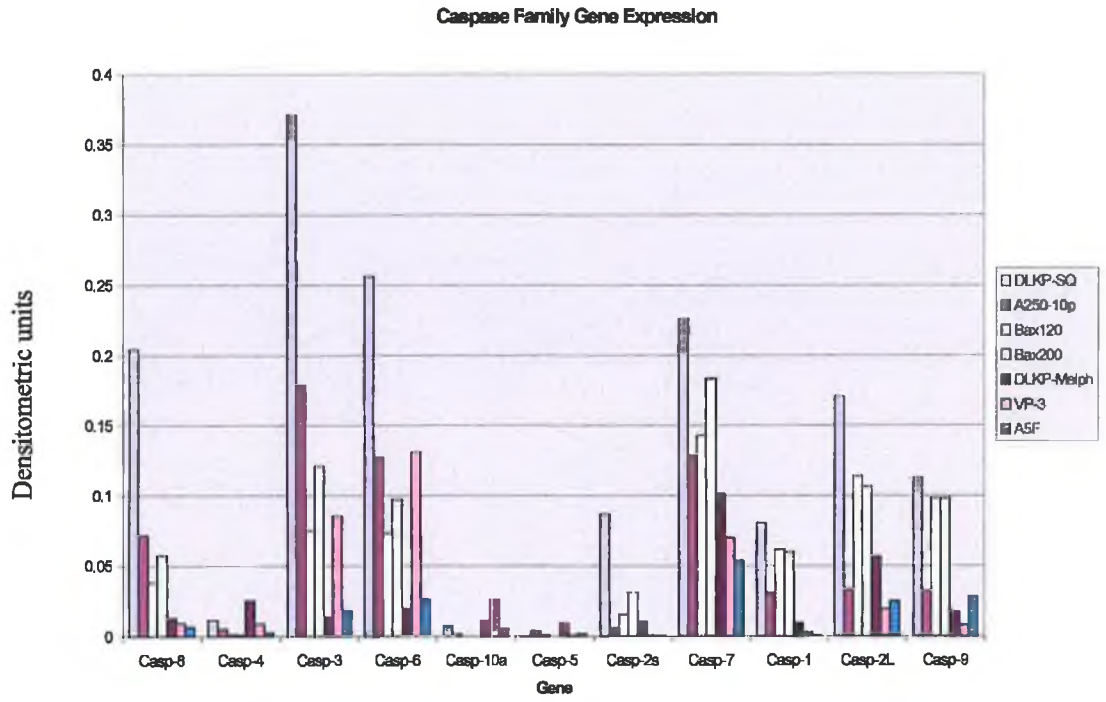
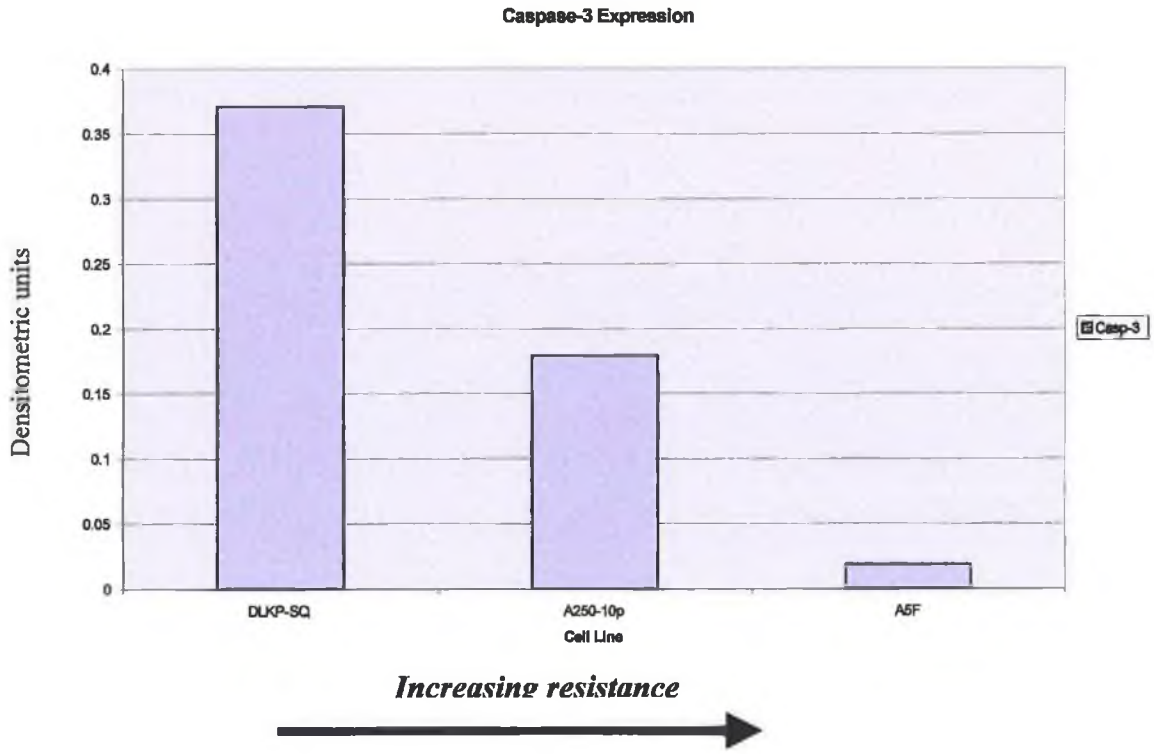


Figure 3.1.15: Graph showing caspase-3 expression in adriamycin-selected DLKP variants.



3.1.7.2: Conclusions on Caspase family RPA work.

The RPA results displayed changes in caspase-3 gene expression in cells with varying levels of resistance to adriamycin. Previous studies in this lab had similar findings when protein analysis was carried out. It was speculated that doxorubicin treatment of these DLKP variants causes apoptosis to proceed via a caspase-independent mechanism (O'Loughlin PhD 1999).

To rule out any possibility that caspase-3 could contribute to this unique apoptotic pathway, a hammerhead ribozyme to human caspase-3 was constructed. This construct was transfected into the most resistant of the cells, namely DLKP-A5F. Despite having the lowest levels of caspase-3, these cells were selected for transfection with the theoretical aim of studying apoptosis in the complete absence of caspase-3. (See section 3.2)

3.2 Caspase-3 Ribozyme.

3.2.1 Design & construction of caspase-3 ribozyme.

Hammerhead ribozymes catalyse the cleavage of mRNA. Ribozymes target GUX sites most efficiently and single stranded loop regions are believed to be most accessible (Gewirtz et al., 1998).

A caspase-3 ribozyme has been constructed targeting rat caspase-3 (Eldadah et al, 2000). However, when human and rat caspase-3 mRNA sequences are aligned, there are no common target sites, therefore human caspase-3 mRNA was analysed for its GUC sites (Clustal W & multiple sequence alignment computer package). There are a total of six GUC sites in the caspase-3 mRNA.

M-fold is a computer program that enables RNA secondary structure prediction. Developed by Michael Zuker and colleagues, it allows the prediction of the minimum free energy or optimal secondary structures of target RNAs (Zuker et al., 1991). As well as examining the optimal secondary structure of caspase-3 mRNA, another nine suboptimal mRNA folding patterns were studied. When the GUC sites were analysed using m-fold structure dot plot analysis for mRNA of human caspase-3 (Zuker, http://mfold2.wustl.edu), it was found that single stranded loop regions were common to all predicted structures around nucleotides 205, 250 and 750.

During target site selection, a number of guidelines were followed:

- Sites selected for target fell within a loop greater than four bases long and were therefore within single stranded regions
- Sites closer to the 5' end of the molecule were preferred as the 5' structure is thought to be more representative of the nascent mRNA molecule
- Ribozyme had short (12-14 bases) antisense arms either side of the cleavage site to enable easy association and dissociation of the ribozyme with target.

These guidelines were based on those of Zhao and Lemke, 1998. The specificity of the ribozyme for caspase-3 was determined by BLAST sequence analysis of all human

sequences currently in the genbank. There was no homology to any other human sequence, so theoretically the ribozyme was specific for caspase-3 alone.

Based on this knowledge, a hammerhead ribozyme was constructed to target caspase-3 at position 205, which is within the p17 subunit of the protein. The ribozyme sequence was cloned into the pTARGET vector (Promega). Figure 3.2.1 details ribozyme design. In addition to ribozyme 1, a non-functioning sequence was cloned into the pTARGET vector. This reversed sequence was complementary to that of the ribozyme and therefore identical to that of the caspase-3 mRNA. This Rz1R acted as a control plasmid.

Figure 3.2.1: The ribozyme primer selections are detailed below. Each was designed according to the following protocol. Restriction enzyme sites were omitted from both ends of the ribozyme as PCR adds on A overhangs. The pTARGET vector facilitated cloning of the PCR products as it has T overhangs at the ends of the insert.

A.

'A----------hammerhead sequence----------A'

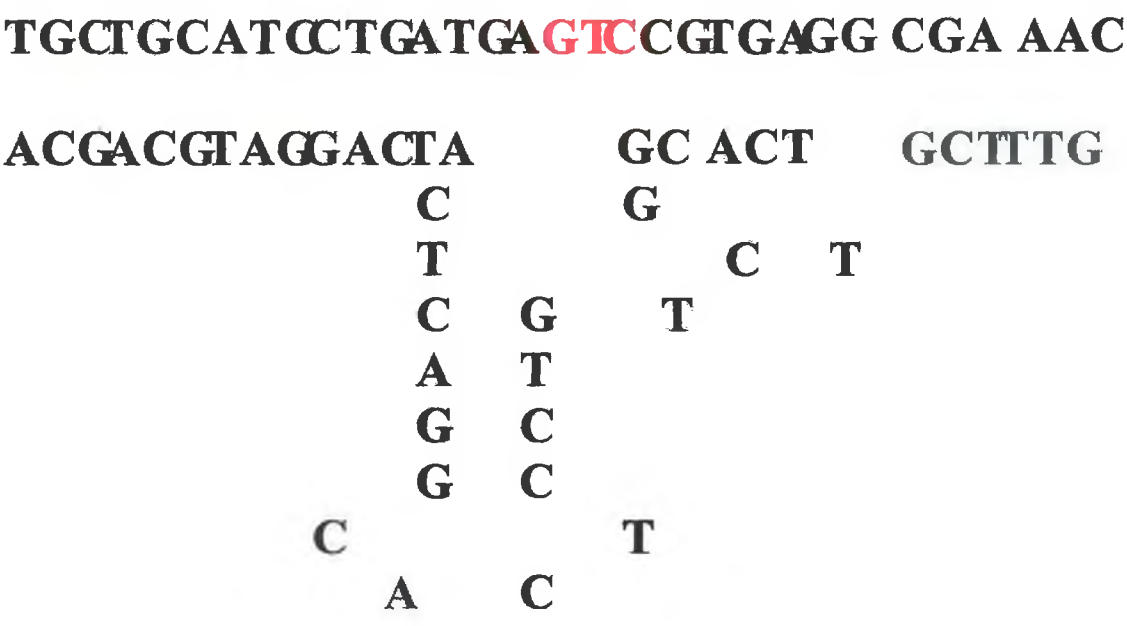
---------- = Caspase-3 sequence.

B. Ribozyme no. 1 Insert Sequence:

5' -TGC TGC ATC CTG ATG AGT CCG TGA GGA CGA AAC ATC TGT CCA A- 3'

5' -TGG ACA GAT GTT TCG TCC TCA CGG ACT CAT CAG GAT GCA GCA A- 3'

Figure 3.2.2: Structure & Sequence of the Caspase-3 Hammerhead Ribozyme (Rz1).



3.2.2: *In Vitro* Cleavage (IVC) analysis of the Caspase-3 Ribozyme.

The ability of the ribozyme to target caspase-3 was initially determined by assessing its ability to cleave caspase-3 mRNA in vitro. Both ribozyme (42 bases) and target sequences (824 bases) had been cloned into pTARGET (Promega) (figure 3.2.3) and pcDNA3.1 (Invitrogen) (figure 3.2.4) vectors, respectively. The T7 polymerase sequence in both vectors facilitates their use in *in vitro* transcription reactions to produce radiolabelled caspase-3 ribozyme and caspase-3 target. The radiolabelled ribozyme and target RNA were then combined in the *in vitro* cleavage reaction and the products were separated on a polyacrylamide gel (The technical details of these experiments are detailed in Section 2.5.8). The sizes of the ribozyme, target and cleavage products were sized using the migrating dye front as a guideline (Sambrook et al., 1982).

Figure 3.2.4: Schematic map of the pcDNA3.1 © Vector.

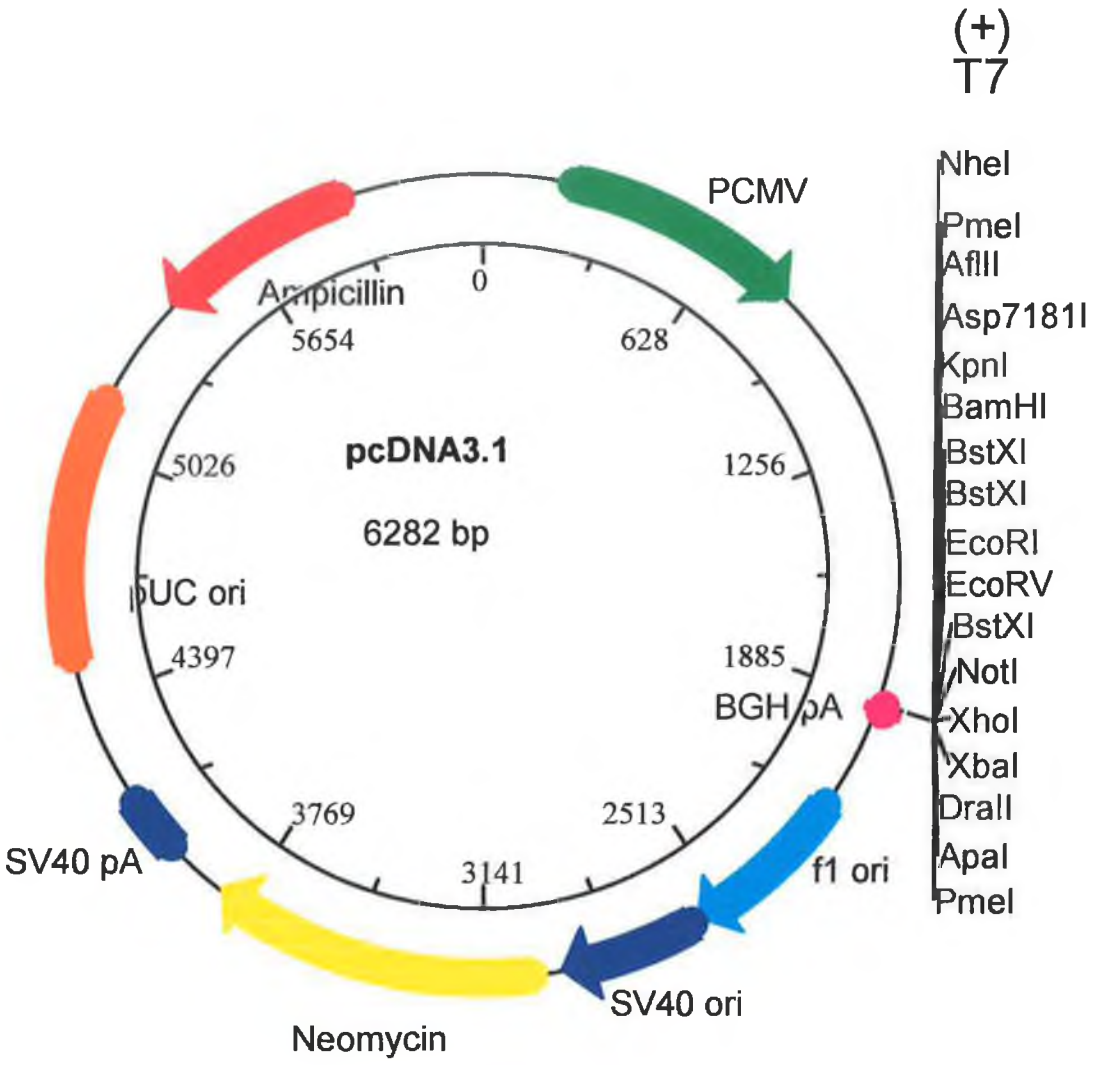


Figure 3.2.4: Schematic map of the pcDNA3.1© Vector, showing location of promoter & polylinker and neomycin and ampicillin-resistant genes. The caspase-3 sequence was cloned into the vector. This was a generous gift from Prof. V. Dixit and is described by Tewari et al, (1995).

3.2.3 $MgCl_2$ Gradient to Optimise *In Vitro* Cleavage Reaction.

$MgCl_2$ initiates the cleavage reaction (Dorai et al., 1997, Chang et al., 1990, Scheid et al., 1998). To optimise the IVC reaction, it was necessary to use an $MgCl_2$ gradient. IVC reactions are commonly performed using magnesium concentrations of between 10 and 20 mM (James & Gibson, 1998). The absolute requirement of the IVC reaction for divalent metal ions is demonstrated by figure 3.2.6. The caspase-3 transcript is unaltered by incubation with caspase-3 Rz1 in the absence of $MgCl_2$. The IVC cleavage of caspase-3 target mRNA proceeds at an optimal concentration of 10mM $MgCl_2$ and at a temperature of 37°C. Time course analysis shows that after 24 hours, substrate concentration decreases and cleavage products appear. It is thought that a certain amount of ribozyme activity will take place at suboptimal conditions and this is evident in figure 3.2.6. which illustrates that at each magnesium concentration after 24 hours a "bleed" of signal appears on the x-ray. This is particularly evident at 5mM and 15 mM $MgCl_2$.

The sizes of the ribozyme, target and cleavage product should be:

824 bases- uncleaved caspase-3 target sequence.

619 bases- first and larger cleavage product.

205 bases- second and smaller cleavage product.

42 bases- caspase-3 ribozyme.

The reactions were separated on a 15% acrylamide gel. At the higher fragment sizes, it was difficult to separate the bound and unbound radioactively-labelled fragments. As a result, these two fragments will be analysed together here as one fragment. As can be seen from the autoradiogram and densitometric analysis in Fig.3.2.6a & b, the amount of the bound CPP-32 target sequence decreases after 24hours, while those of the cleavage bands appear after 24hours. This is entirely consistent with a time-dependent cleavage of the CPP-32 target sequence by the CPP-32 ribozyme. The radioactively labelled ribozyme has run off the gel.

Figure 3.2.5: 5% Acrylamide gel showing radiolabelled target sequence (824 bases) and ribozyme (42 bases). These were added together in the in vitro cleavage reaction in the presence or absence of magnesium. See figure 3.2.6.

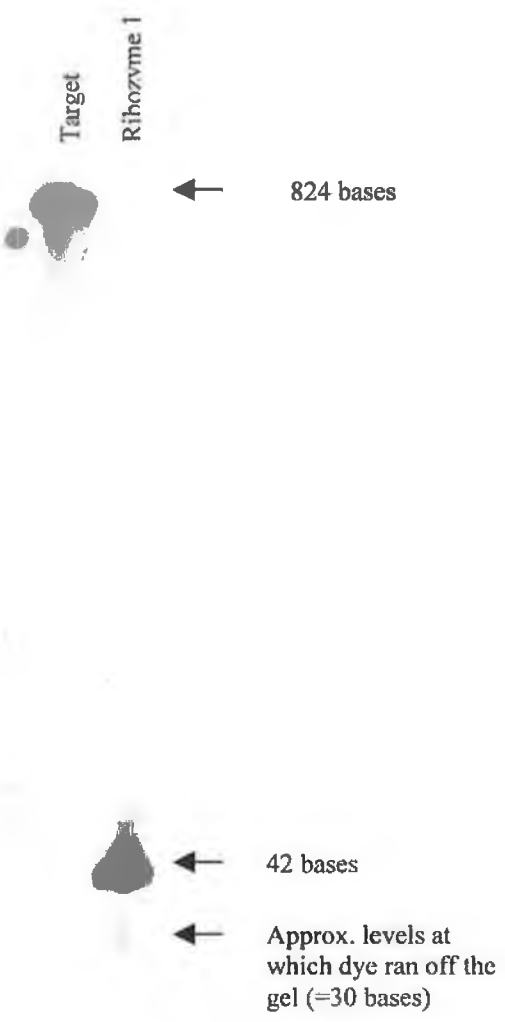


Figure 3.2.6a: IVC Optimisation using Magnesium Gradient (15% Acrylamide gel). Lane 1 contains target caspase-3 and no ribozyme. Each lane following contains increasing concentrations of MgCl2 at 0hr and 24hr. Cleavage products are indicated by arrows at position 619 and 205 base pairs. Full size product is indicated at 824 bases.

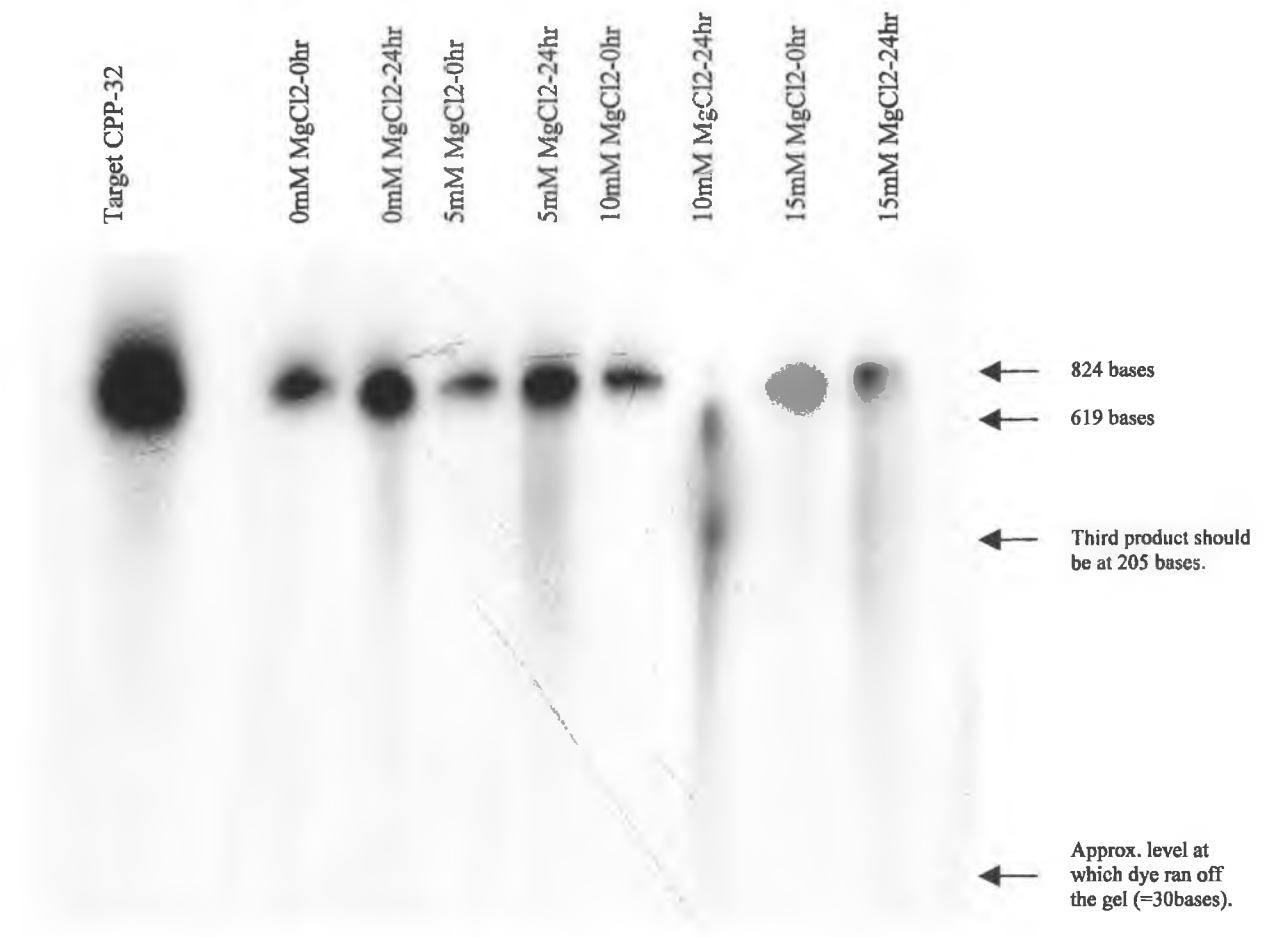
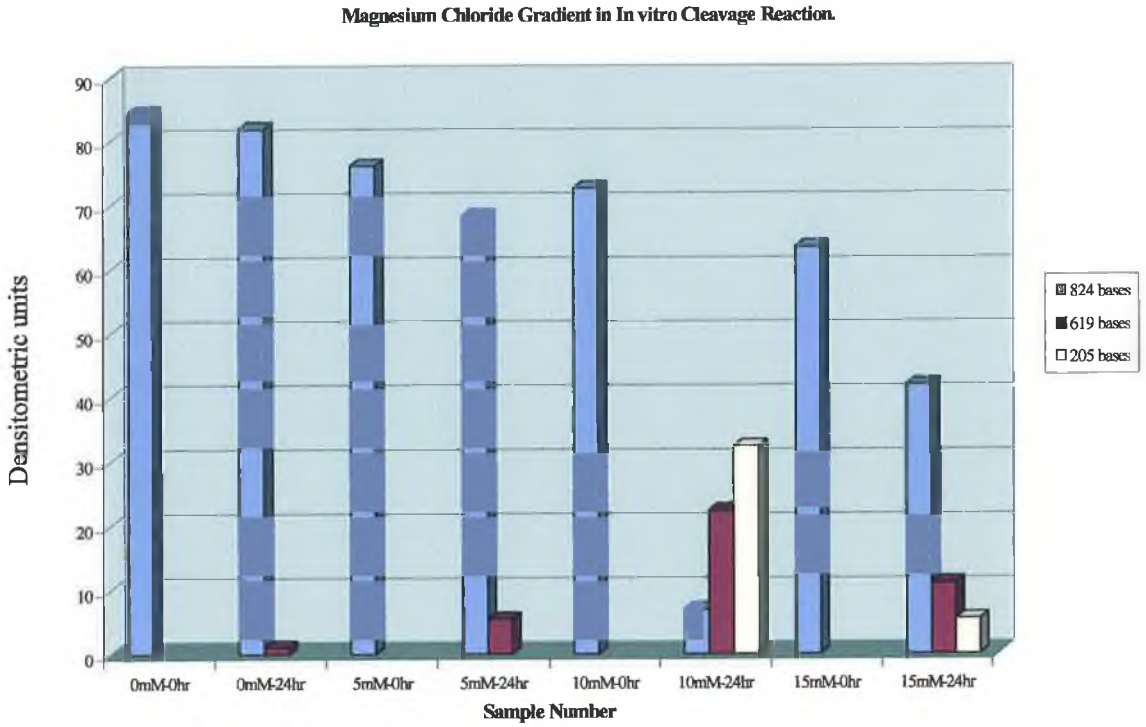


Figure 3.2.6b: Graph of Magnesium Chloride Gradient in IVC Reaction. The purple column represents uncleaved target CPP32 of 824 bases. The wine column represents cleavage product of 619 bases. The yellow column represents the second cleavage product of 205 bases.



3.2.4 Assessment of ribozyme functional activity in cells.

In order to assess the effectiveness of the caspase-3 ribozyme in vivo, a transient transfection assay was optimised using Fugene-6 (Roche) as a transfection reagent. Briefly, cells were transfected as per Section 2.5.5. A β-galactosidase assay was utilised to determine the percentage of transfected cells. A transfection efficiency of 35% was achieved in DLKP cells. This was relatively high as some studies which have used transient transfection reported transfection efficiencies of only 20% in PC12 cells and 0.1-0.3% in cerebellar granule cells (Eldadah et al., 2000). During optimisation, two cell densities were set up to check efficiency. Both had approximately equal levels of transfection. In all experiments shown, cells were set up at $1x\ 10^5$ cells/ml.

Transiently transfected cells were cultured for 24 hours after transfection. This gave cells time to recover from the transfection procedure where the cell wall had been permeabilised allowing for entry of the plasmid and allows expression of the transfected gene. (See Fugene-6 protocol, Roche). Drug treatments or RNA and protein analysis could then proceed.

Figure 3.2.7 shows the pCH110 vector (Pharmacia) containing the β-galactosidase gene and figure 3.2.8 shows transient transfection of the vector into DLKP-A5F cells. The blue cells represent those transfected cells containing the pCH110 vector plus β-galactosidase gene.

Figure 3.2.7: pcH110 Vector showing cloning site, neomycin and ampicillin-resistance genes.

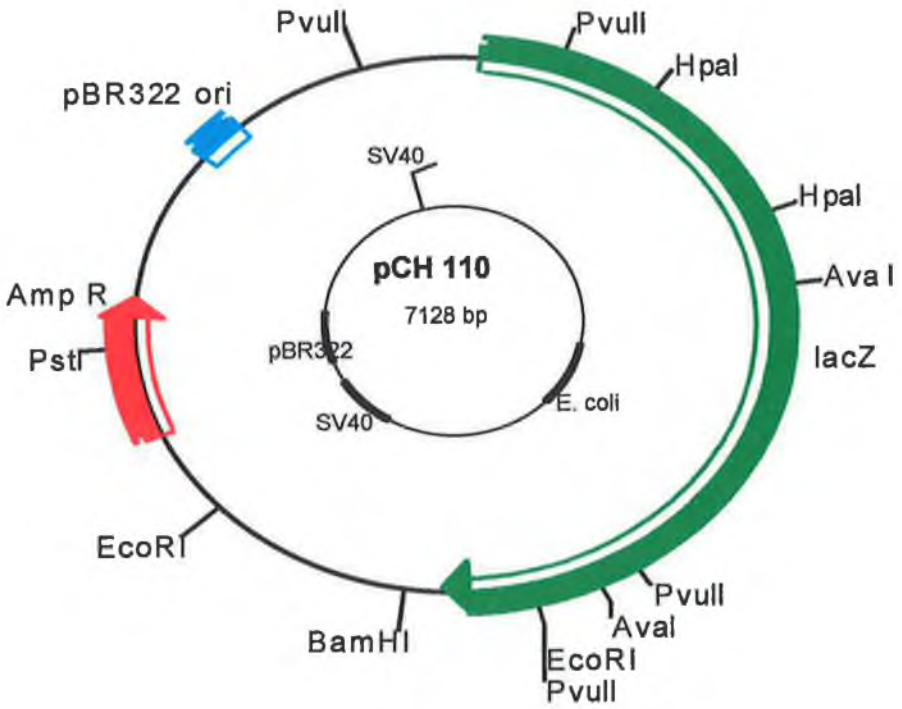


Figure 3.2.8: Transient transfection of pcH110 containing β-galactosidase insert into DLKP cells. Arrows point out blue cells that are transfected with the pCH110- β-galactosidase plasmid.

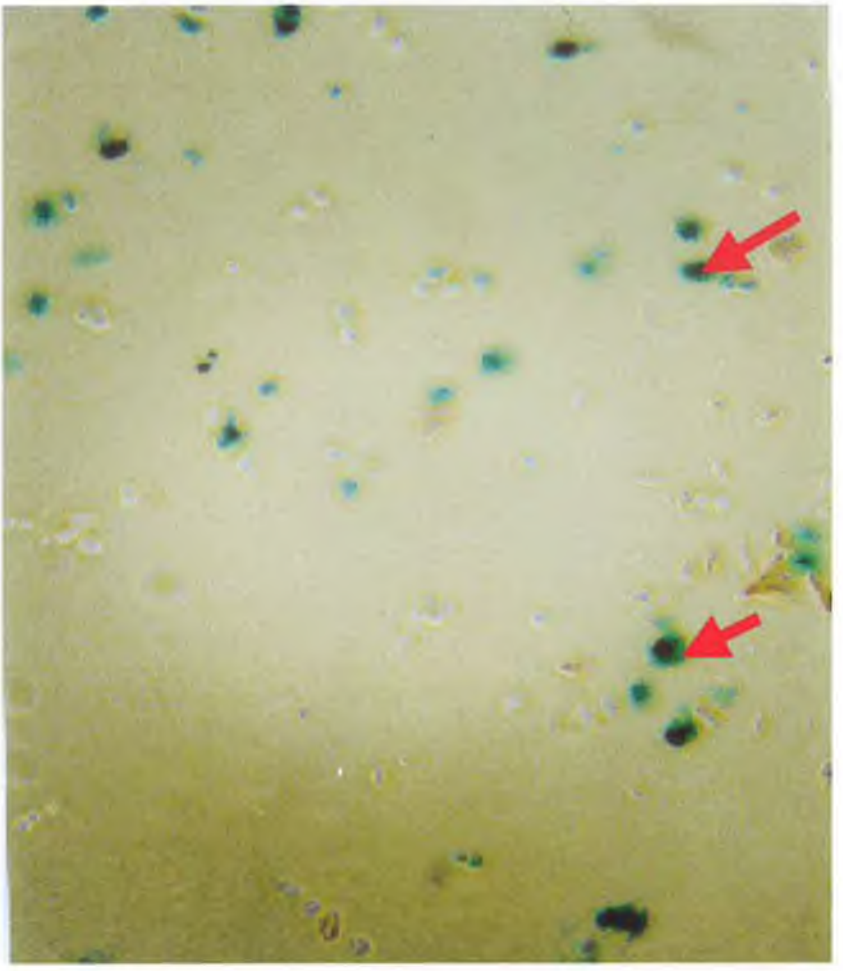


Table 3.2.1: % Transfection Efficiency in DLKP-A5F cells transfected with B-galactosidase.

Cell Density (cells/ml)	6 hours	24 hours
2x10(4)	23%	35.3%
1x10(5)	28%	34.3%

3.2.5 Transient transfection of CPP-32 ribozyme into DLKP-A5F cells.

3.2.5.1: RT-PCR for CPP-32 Expression in transiently transfected A5F cells.

To determine if the cells displayed a reduction in caspase-3 mRNA levels compared to parent or reverse ribozyme transfectants, RT-PCR was carried out on total RNA isolated from each transfectant. The primers used amplified a 313bp fragment of caspase-3 and were designed as previously described in appendix. β-actin was used as an internal control and produced a band at 142 bp. All RT-PCR reactions were repeated.

DLKP-A5F cells were transiently transfected with ribozyme (Rz1) and control reverse ribozyme (Rz1R) to caspase-3 using the optimised transfection protocol. Cells were harvested for RNA extraction at 24 and 48 hours. The expression of caspase-3 mRNA was decreased to very low levels at 24 hours. At 48 hours, caspase-3 expression had increased but was still not back to the levels of the nontransfected or reverse ribozyme transfectants. Results are presented in figure 3.2.9.

3.2.5.2: Western blot analysis of pro-caspase-3 levels in transiently transfected A5F cells.

To confirm that the specific cleavage ability of the ribozyme at targeting caspase-3 RNA could affect protein production, caspase-3 protein levels were analysed. Western blot analysis showed a decrease in pro-caspase-3 protein levels at 24 and to a lesser extent at 48 hours. Results are presented in figure 3.2.10. It was attempted to analyse active caspase-3 levels by Western blot by treating cells with cytotoxic drug, however active caspase-3 protein could not be detected on the Western blot.

Figure 3.2.9: RT-PCR analysis of CPP-32 expression in transiently transfected cells. The molecular weight marker (Mr marker) is "ϕ-X174" Hae III digest (Promega: G1761). Caspase-3 primers amplify a product of 314 bp. Internal control β-actin amplified to yield a 142bp product. Gel shows caspase-3 levels in control, ribozyme 1 and reverse ribozyme transfectants. Graph below shows densitometric analysis.

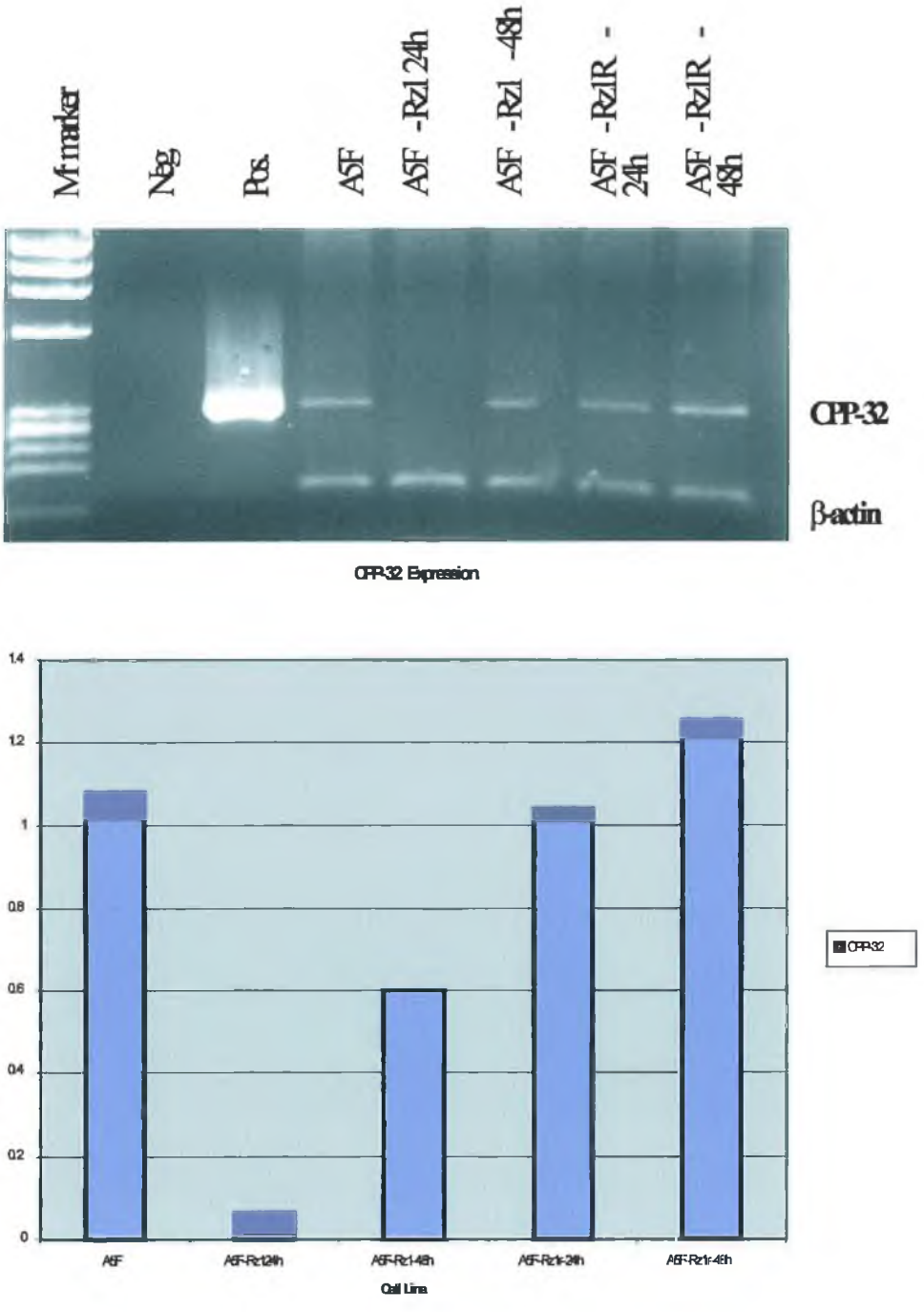
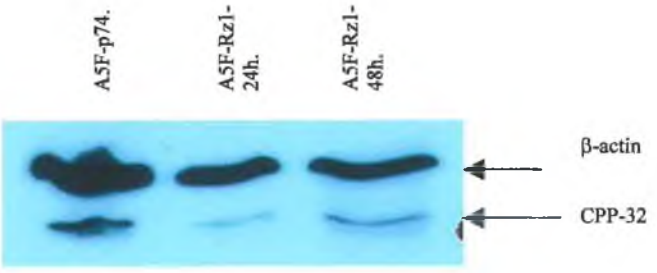
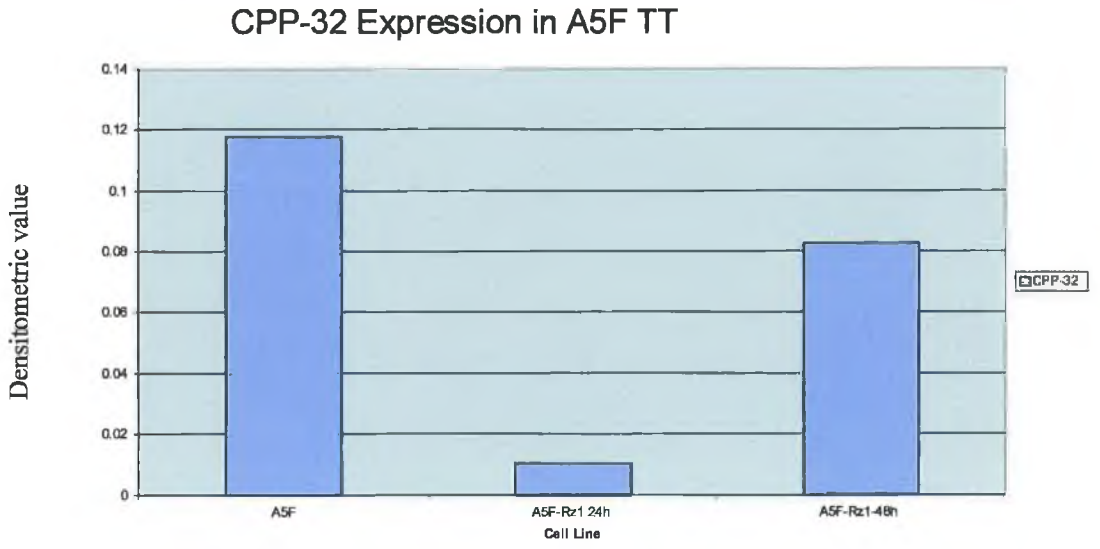


Figure 3.2.10: Western blot analysis displaying pro-caspase-3 levels in control and transfected cells. Pro-caspase-3 is a 32kDa protein. β-actin was used as a control and is a 42 kDa protein. Protein was separated on a 12% SDS-polyacrylamide gel. 25ug of protein was loaded in each case. Graph below shows densitometric analysis.





3.2.5.3: In vitro toxicity analysis of transient transfections.

Efforts were made to analyse the resistance profiles of transiently transfected A5F cells using slightly modified standard toxicity tests. (See Materials & Methods, section 2.3.1) The 96-well plate toxicity assay yielded no consistent change in drug sensitivity in ribozyme transiently transfected cells but trends towards increased sensitivity were observed (results not shown). Transfection efficiency, however, was much lower in 96-well plates (Rasha Linehan, personal communication). It is also possible that the reduction in caspase-3 levels achieved by the ribozyme may have been too low to affect toxicity.

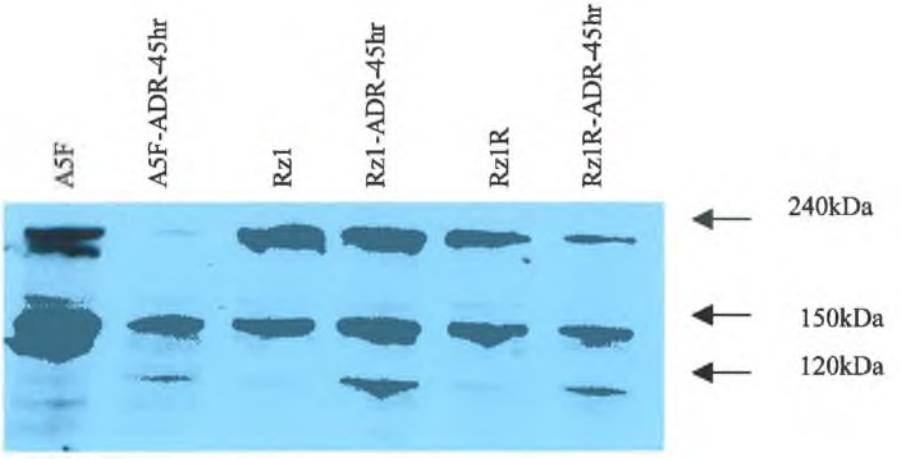
3.2.5.4: α-Fodrin Expression in Transiently Transfected A5F cells

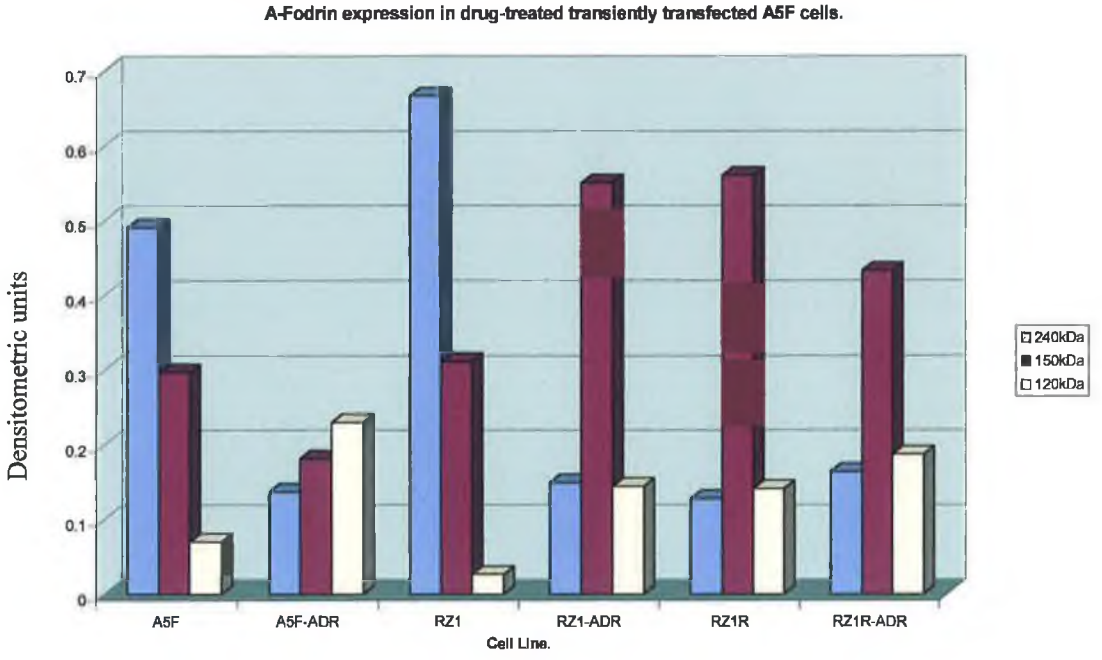
To further our understanding of the effects of the ribozyme expression, an alternative toxicity assay was set up. Cells were treated with adriamycin for 45 hours and then harvested for protein. This assay was based on previous studies in this lab (O'Loughlin, PhD 1999), and it was observed that such treatment induced apoptosis in A5F cells.

A 120 kDa cleavage product of α-fodrin is formed due to caspase-3 activity (Greidinger et al., 1996, Janicke et al., 1998, Martin et al., 1995). Cells were treated with adriamycin for 45 hours and protein was harvested. Levels of the 120kDa cleavage product of fodrin were then examined by Western blot analysis. As the Western blot in figure 3.2.11 shows, there did not appear to be any effect on fodrin cleavage in ribozyme transiently transfected cells.

Calpains are a family of protein kinases whose activity is responsible for the larger cleavage product of fodrin, a 150 kDa product (Wang et al., 1998). The Western blot shows that compared with nontransfected (A5F) and reverse ribozyme (Rz1R) transfectants, there does appear to be an increase in the 150kDa product in the ribozyme transfected cells. This may be due to a compensatory mechanism by the ribozyme transfectants to overcome the reduction in caspase-3 activity.

Figure 3.2.11: α-Fodrin Expression in Transiently Transfected A5F cells. In each case, cells were untreated (lanes 1,3 & 5) or treated with adriamycin for 2 hours and allowed to grow for 45 hours (lanes 2,4 & 6). Graph below shows densitometric analysis.





3.2.5.5: TUNEL Analysis of transiently transfected DLKP-A5F cells.

The TUNEL assay detects DNA fragmentation by staining the 3'-end of the fragments in apoptotic cells. This assay involves the incorporation of dUTP into the 3'-end of fragmented DNA formed during apoptosis. It allows for visual semi-quantitative detection of apoptotic cells.

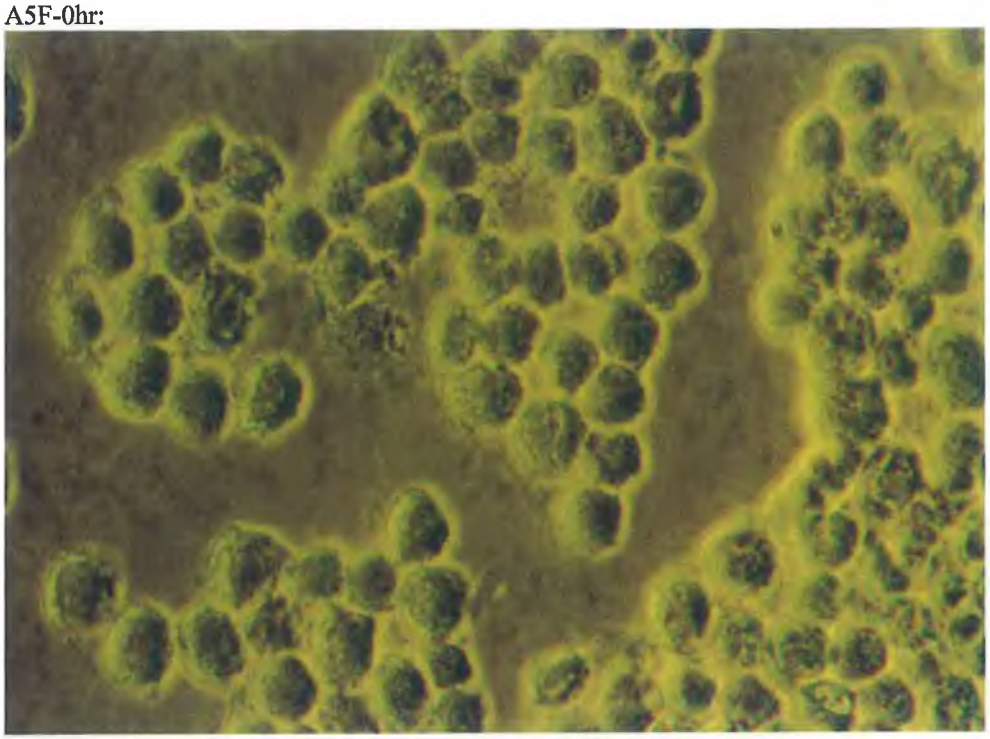
Cells were treated with 100ug/ml adriamycin for 2 hours and harvested 24 and 48 hours post-transfection. The TUNEL staining shows the characteristic condensation of the nucleus and its fragmentation. As can be seen from the photographs, there does not appear to be any change in the number of apoptotic cells at 24 or 48 hours post drug treatment (See figures 3.2.12). There is slightly increased numbers of apoptotic cells in both sets of transfectants (those transfected with ribozyme 1 and those with reverse-ribozyme 1) and since it occurs with both constructs, this increased sensitivity may be due to the transfection procedure.

Each photograph represents a random field of 50-100 cells. For each cell line, cells have been photographed in white and fluorescent light. In each case, untreated cells were photographed at 0hours (green photographs with no cells visible).

Figure 3.2.12: TUNEL analysis of control and transiently transfected cells. Photographs show cells in white light and fluorescent light at 0, 24 and 48 hours for parent A5F cells, control transfectants A5F-Rz1R cells and A5F-Rz1 cells.

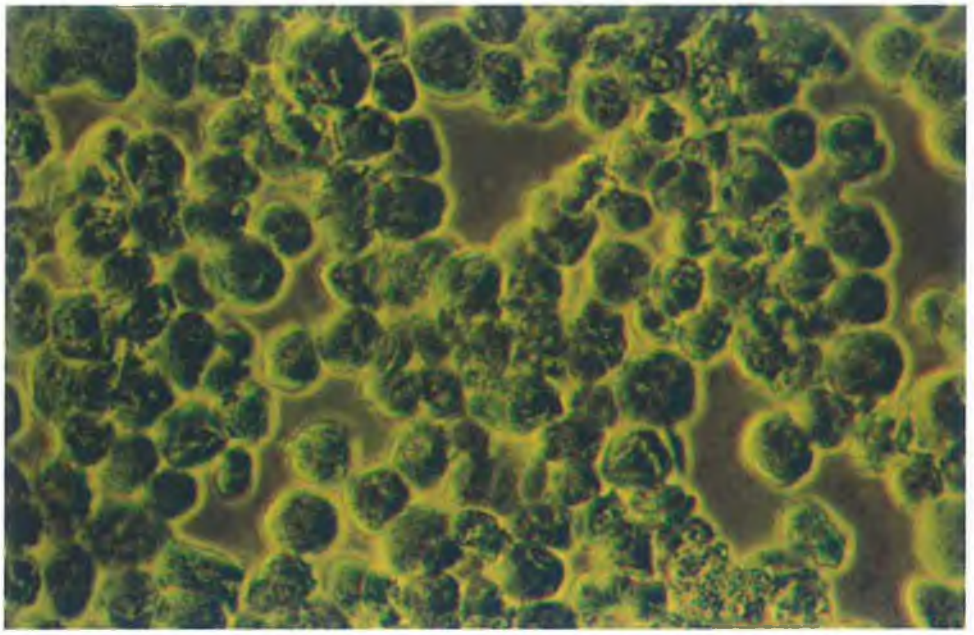
Figure 3.2.12 TUNEL analysis of control and transiently transfected cells.

A5F-0hr:

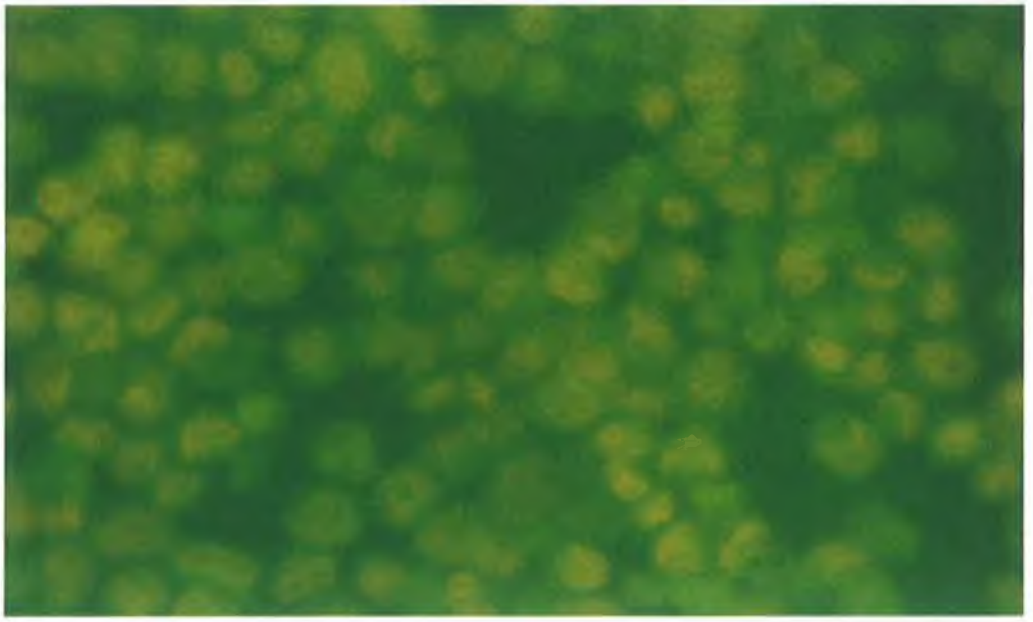


A5F-0hr:

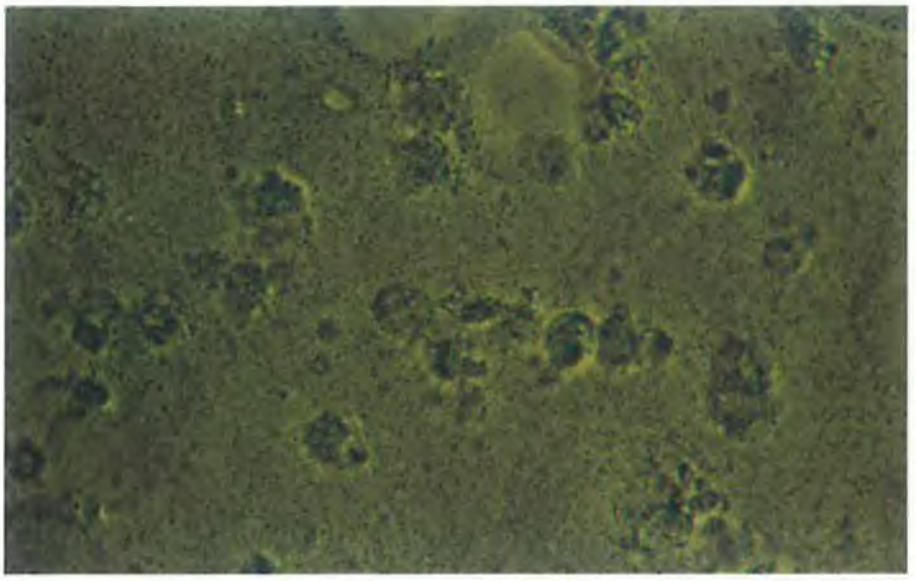
A5F-adr-24hr:



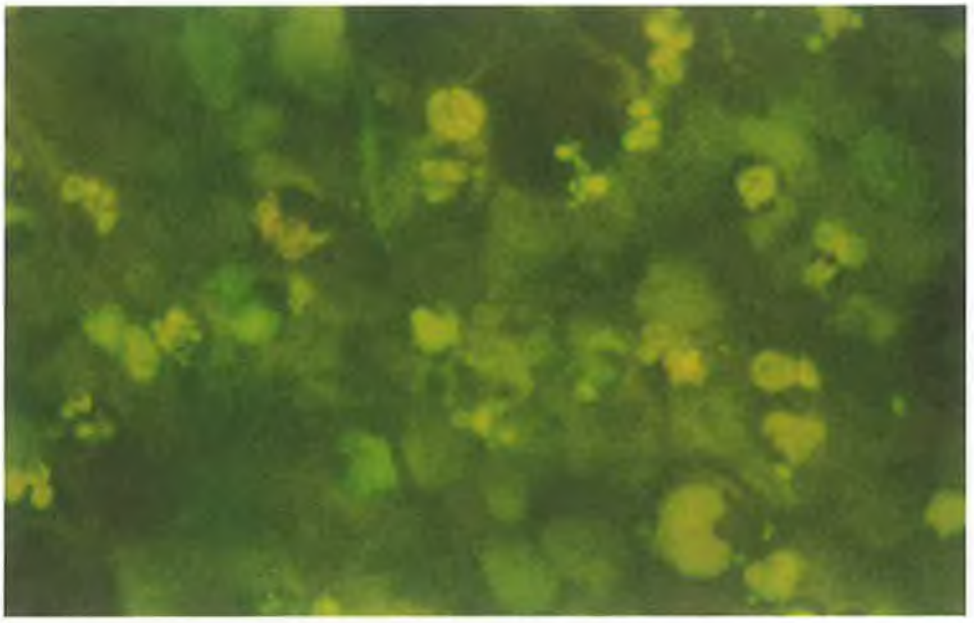
A5F-adr-24hr:



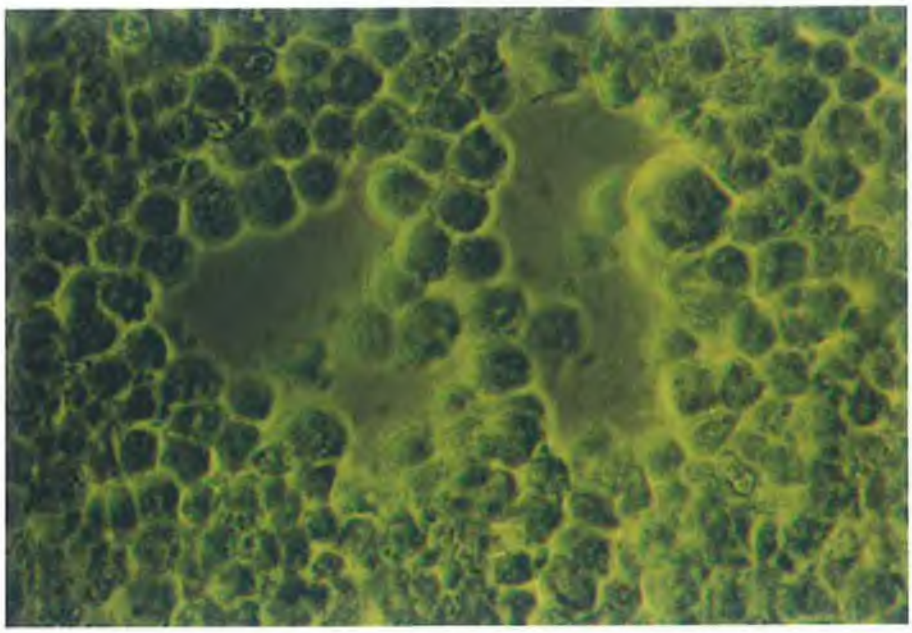
A5F-48hr



A5F-48hr

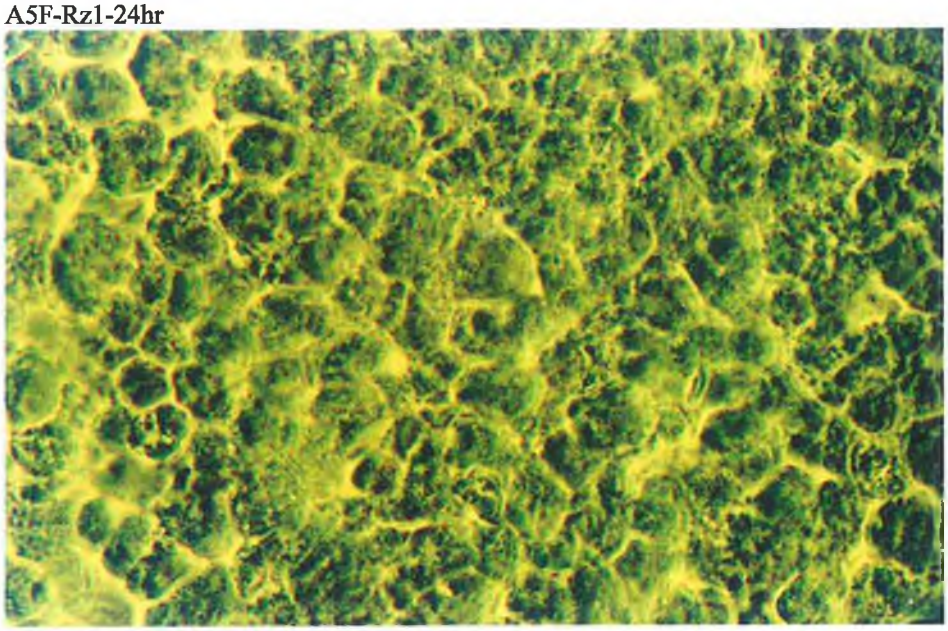


A5F-Rz1-0hr

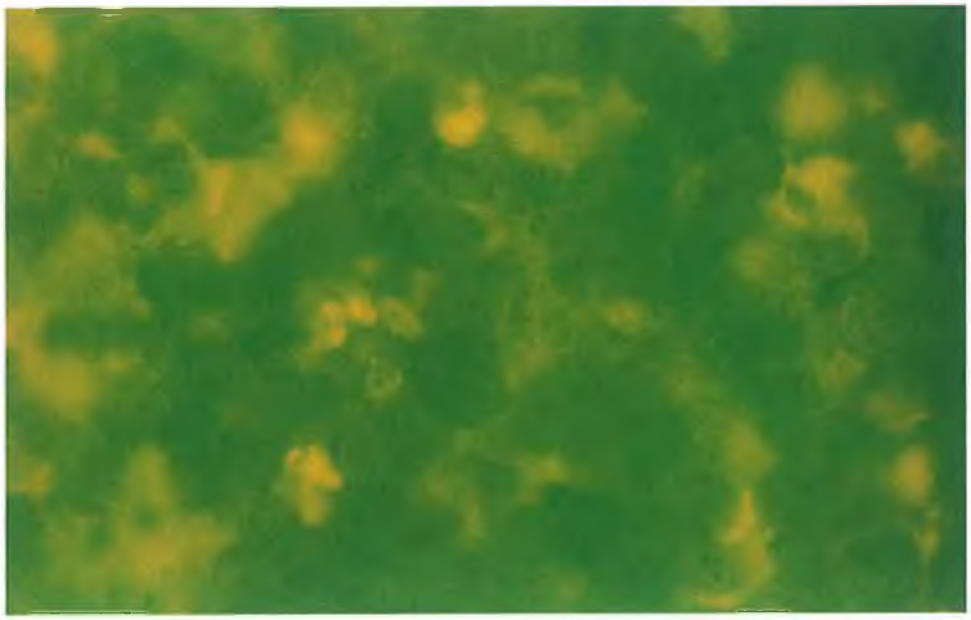


A5F-Rz1-0hr

A5F-Rz1-24hr



A5F-Rz1-24hr



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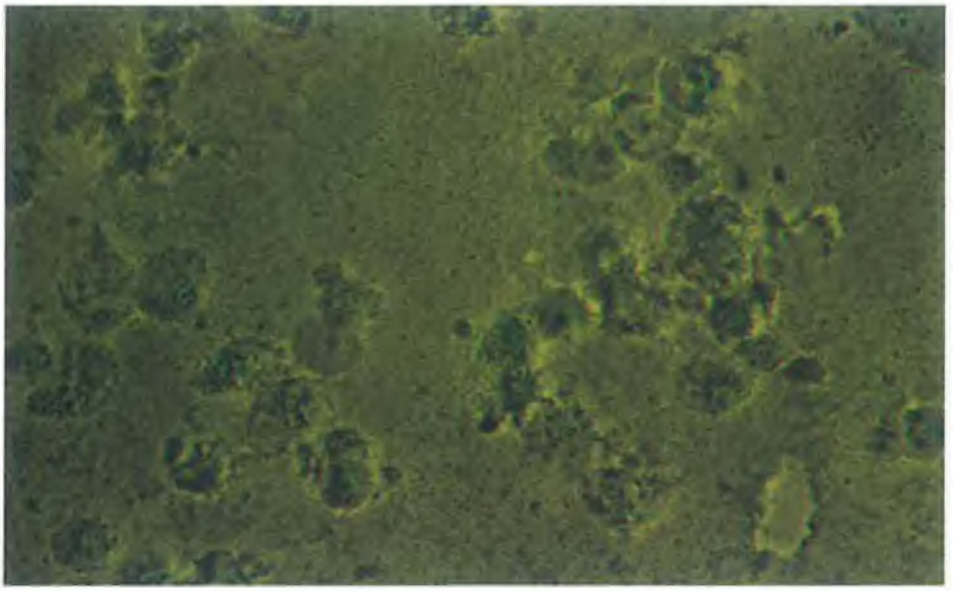

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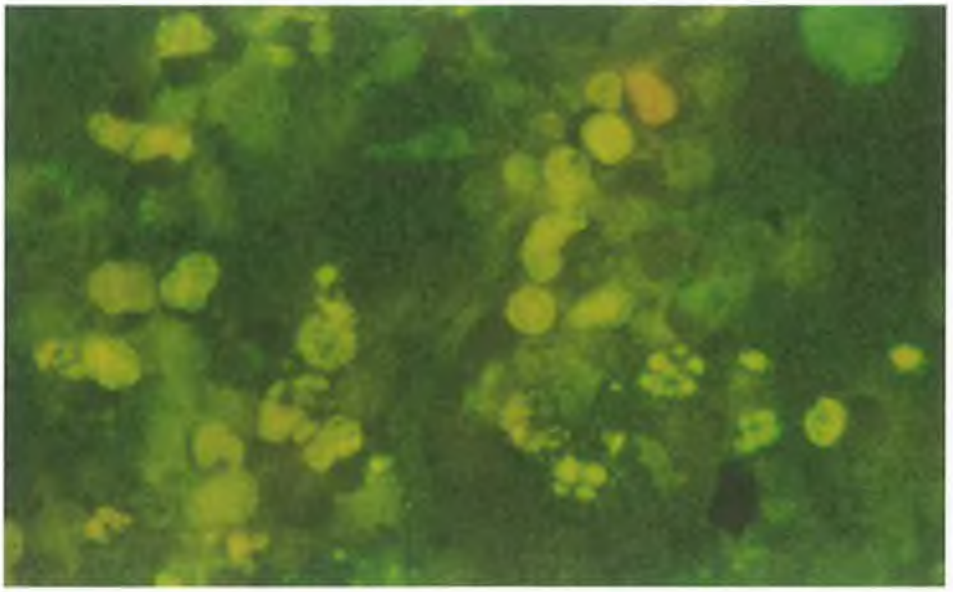
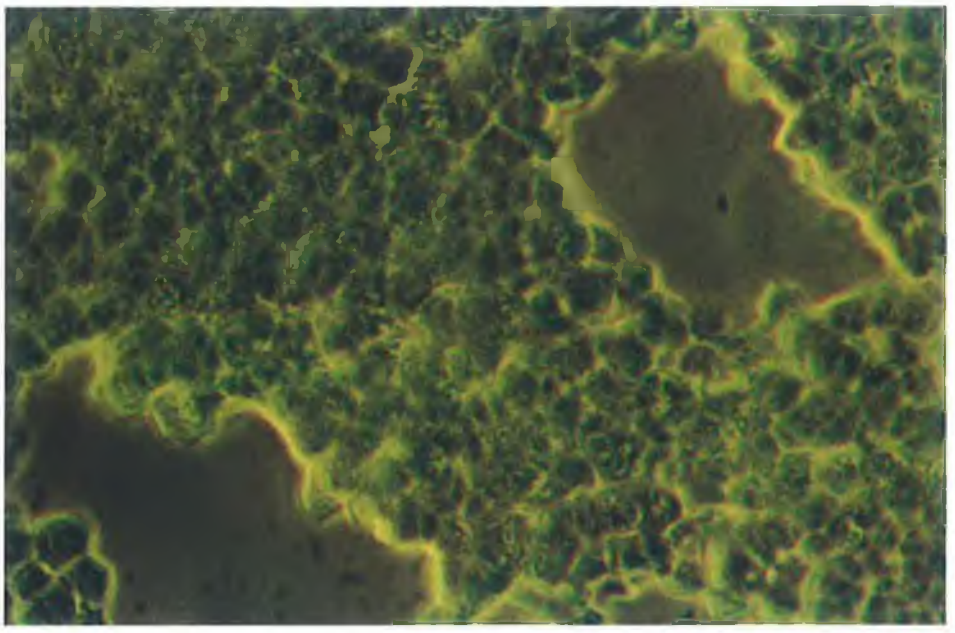
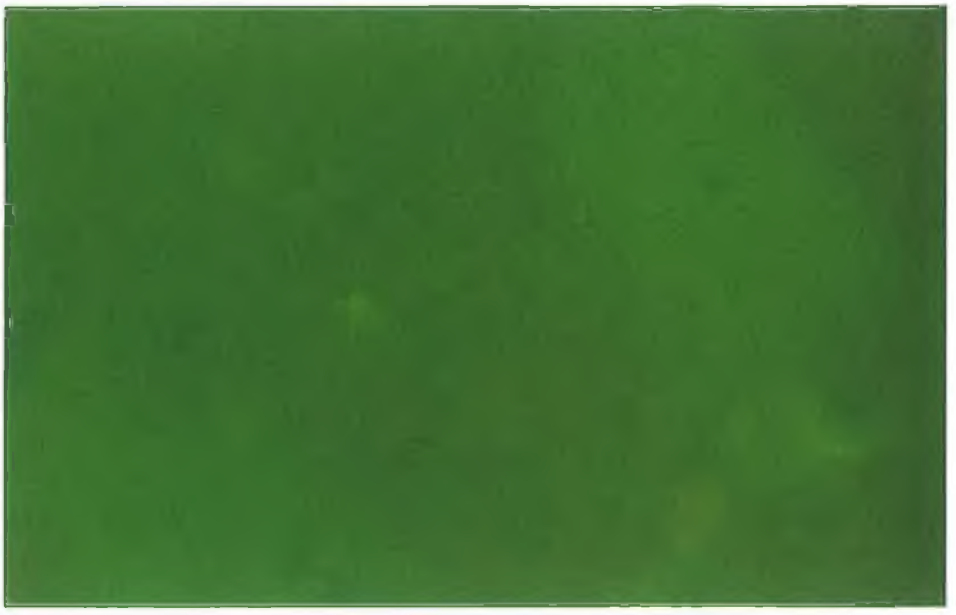


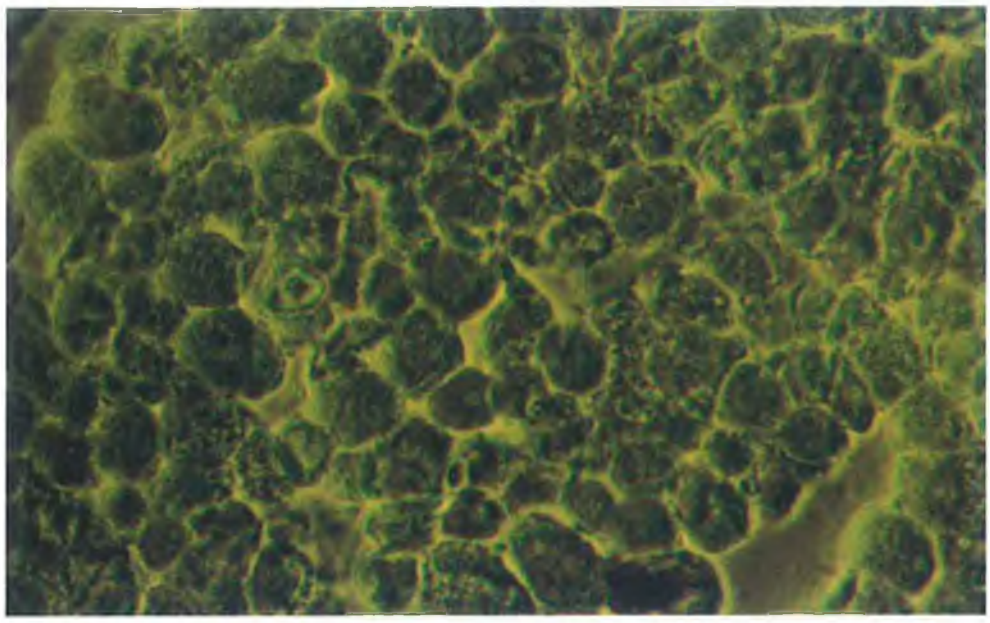
A5F-Rz1R-0hr



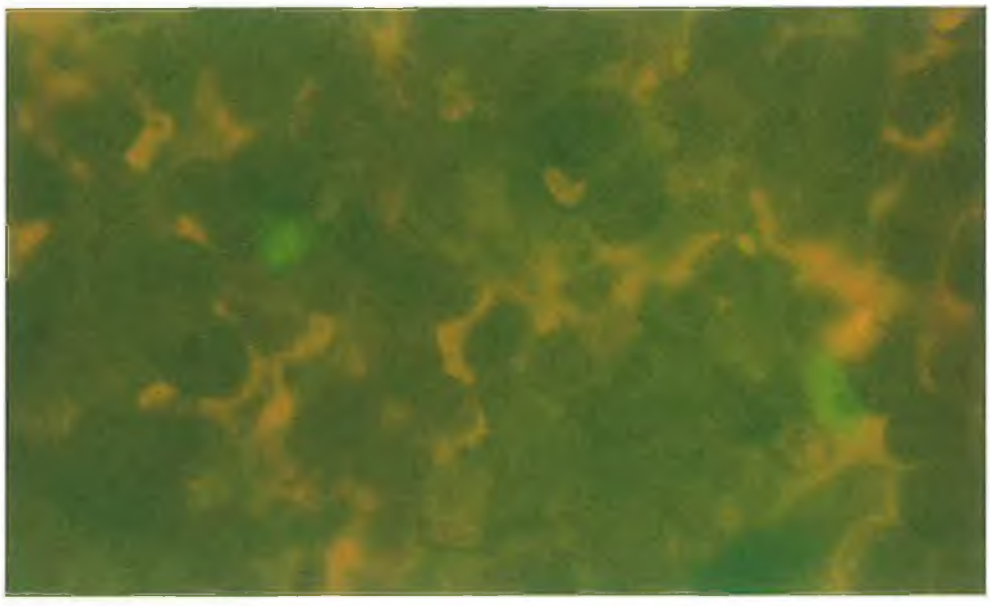
A5F-Rz1R-0hr



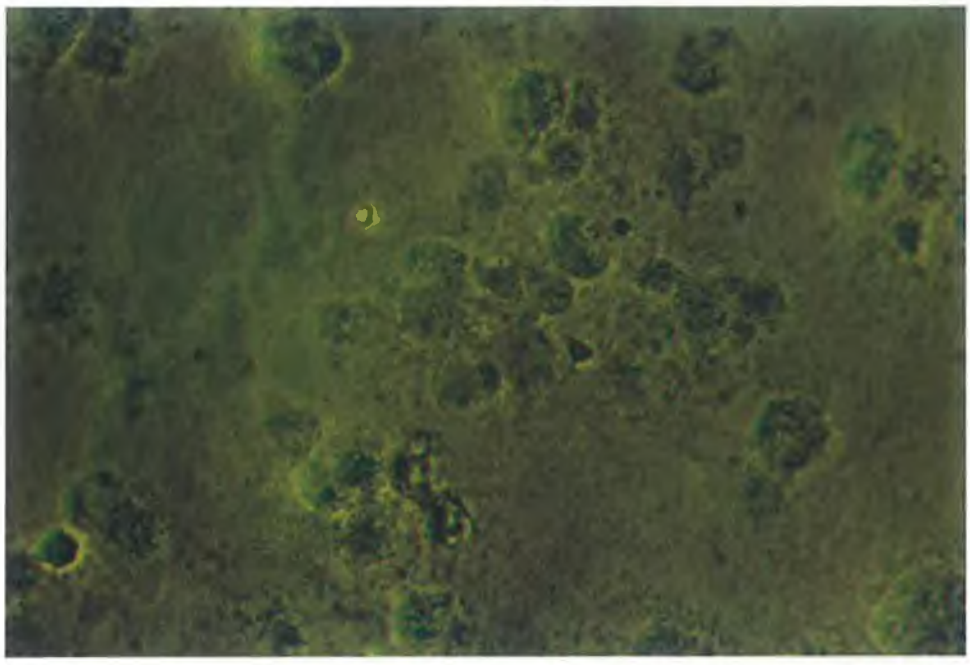
A5F-Rz1R-24hr:



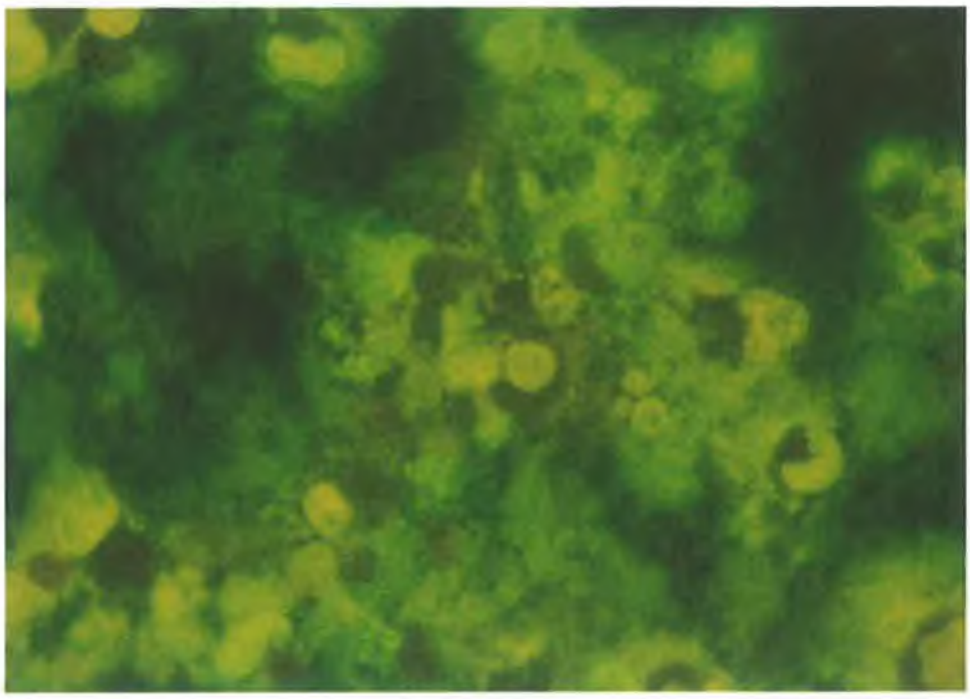
A5F-Rz1R-24hr:



A5F-Rz1R-48hr:



A5F-Rz1R-48hr:



3.2.5.6 Conclusions on *in vitro* and *in vivo* assessment of ribozyme activity.

The in vitro cleavage assay displayed the effectiveness of the ribozyme at targeting caspase-3 RNA at least under in vitro conditions. Controls used also displayed how essential the magnesium concentration is at influencing ribozyme activity. This also gave an indication of how the magnesium concentration in vivo can affect the ribozyme's ability to down-regulate a particular genes expression. This was previously reported by Jen and Gewirtz, 2000.

Thus far the in vivo studies of the ribozyme were centred on the use of transient transfections. RT-PCR and Western blots showed decreases in caspase-3 levels at 24 and 48 hours post-transfection. Efforts to investigate the ribozyme's ability to influence the phenotype of the cells, proved to be somewhat inconclusive. Toxicity assays gave no consistent trends in drug sensitivity. TUNEL and α-fodrin cleavage gave no indication of altered apoptosis or drug sensitivity in ribozyme transfectants compared with controls.

Stable transfectants were therefore generated and the impact of ribozyme activity on their ability to undergo apoptosis was assessed. Surprisingly, cells transiently transfected with ribozyme displayed reduced levels of mdr-1 relative to control transfectants. The impact of this reduction in terms of drug resistance was further analysed in stably transfected clones.

3.2.6 Stable transfection of CPP-32-Rz1 in DLKP-A5F cells.

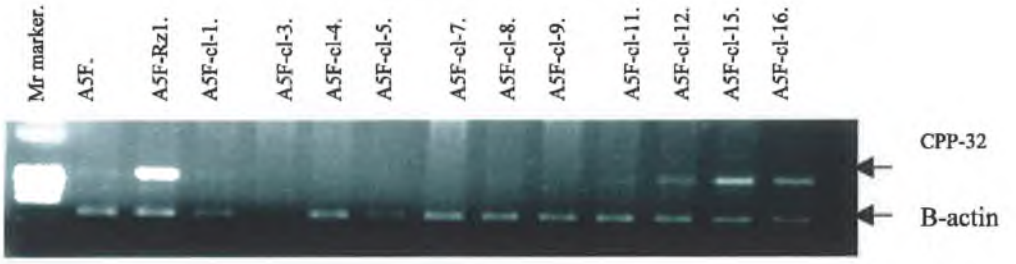
Having confirmed that the ribozyme had the ability to knock down caspase-3 expression in cells, further investigation was necessary to establish its effect on the cells' phenotype. DLKP-A5F cells were therefore transfected with the caspase-3 ribozyme. Stably transfected cells were grown continuously in geneticin at a concentration of 600ug/ml to maintain expression of the pTARGET vector containing ribozyme 1. Clones were isolated from the DLKP-A5F-Rz1 mixed population and cell stocks were frozen in liquid N_2. All clones were analysed for any changes in caspase-3 mRNA by RT-PCR and RNase protection assay, and procaspase-3 protein by Western blotting. Cytotoxicity assays with a number of chemotherapeutic drugs were also carried out. Approximately twenty clones were isolated and based on toxicity tests, five of these were selected for detailed analysis.

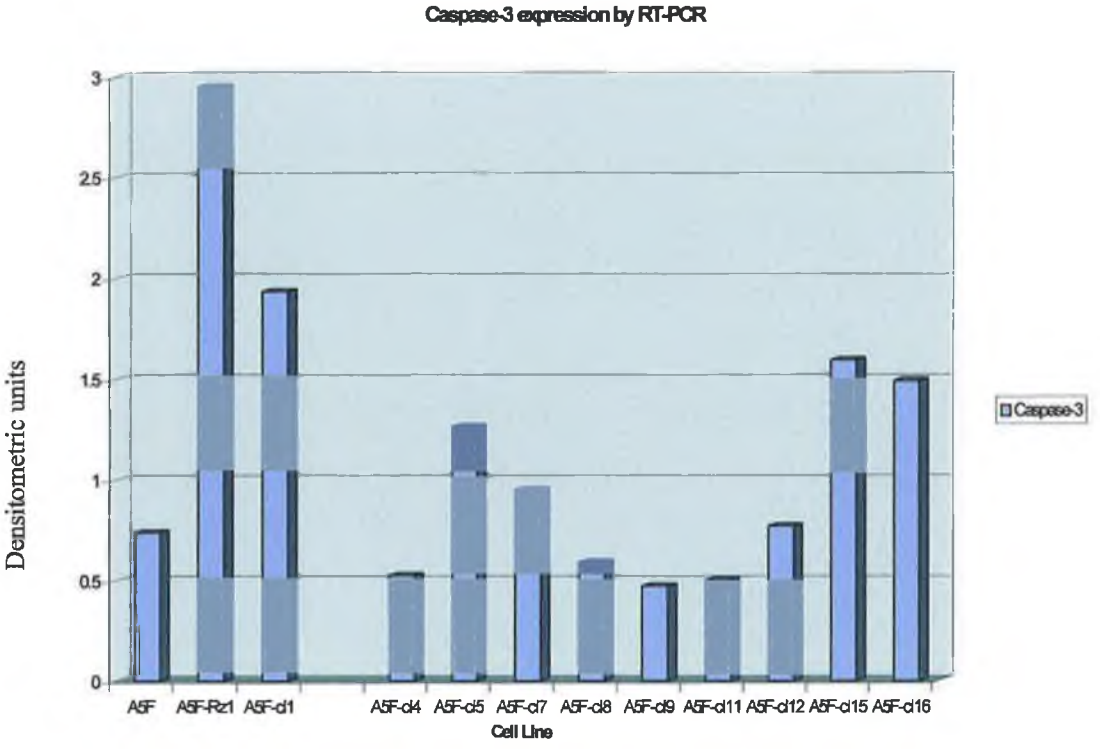
3.2.6.1: RT-PCR analysis of DLKP-A5F cells transfected with CPP-32 ribozyme.

RT-PCR analysis was used to investigate if the expression of the CPP-32 gene was affected by expression of the CPP-32 targeted ribozyme. RNA isolated from clones at early passage showed some reduction of caspase-3 (See figure 3.2.13). However, these results did not hold true for later passages.

Figure 3.2.13 shows down-regulation of caspase-3 mRNA in some clones. The transfected mixed population was designated A5F-Rz1. It is unclear why more caspase-3 shows up in these cells compared with A5F parent cells. This may be due to the parent transfectants heterogeneous population, hence those with decreased caspase-3 may be masked by cells where ribozyme has not downregulated caspase-3. The accompanying graph shows the densitometric analysis of the RT-PCR results.

Figure 3.2.13: RT-PCR analysis of caspase-3 expression in DLKP-A5F clones. PCR amplification of caspase-3 yields a 314bp product. Internal control β-actin amplified yielding a 142bp product. 30 cycles of amplification were used. Graph below shows densitometric analysis.





3.2.6.2: RNase Protection Analysis of caspase family expression in DLKP-A5F-Rz1 clones.

RNase protection assay (Riboquant, Pharmingen) was used to examine the expression levels of caspase-3 and other caspase family genes in the clones. The kit used in this experiment was RiboQuant Multi-Probe RNase Protection Assay System from Pharmingen. hAPO-1c was the probe set used.

As mentioned in section 1, the caspase family comprises initiators and effectors of the apoptotic process. They act in a cascade-like fashion. It was therefore proposed that eliminating one member, caspase-3, could perhaps result in compensation by the cells via upregulation of another caspase protein. As the RPA analysis shows in figure 3.2.14, there did not appear to be any shift in caspase family expression in favour of another executioner caspase, i.e. caspase-2 or caspase-7.

Despite changes detected by RT-PCR, there was no major change in caspase-3 expression in any of the clones was evident using RNase protection analysis. There are two possible explanations for such disagreement. As PCR is a highly sensitive technique, it will detect even the smallest changes in expression levels. Moreover, our PCR primers were designed to target the caspase-3 mRNA surrounding the ribozyme cleavage site (at position 205). Therefore any changes in expression levels were detectable and, due to the sensitive nature of this technique, were illustrated by in some cases complete loss of caspase-3. In contrast to this, the probe for caspase-3 in the Riboquant RNase protection assay targets human caspase-3 between location 911- 1230 (Pharmingen). Therefore, the less sensitive RNase protection technique, may be picking up both cleaved and uncleaved caspase-3. Because of this, changes in caspase-3 levels induced by ribozyme are not evident using the RPA.

Figure 3.2.14: RNase Protection Analysis on clonal populations. Arrows indicate the position of each caspase gene.

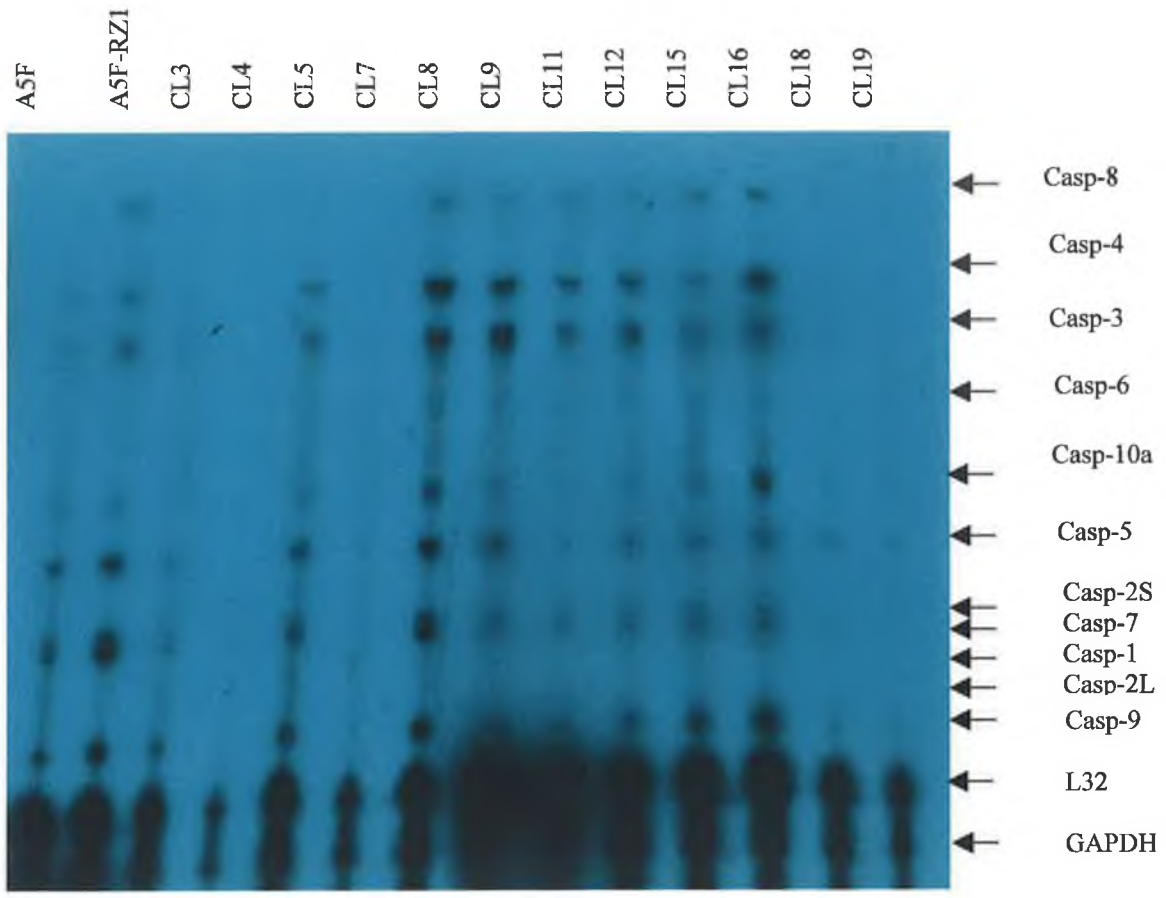


Figure 3.2.15a Graphical representation of RPA showing caspase family gene expression in DLKP-A5F-Rz1 clones.

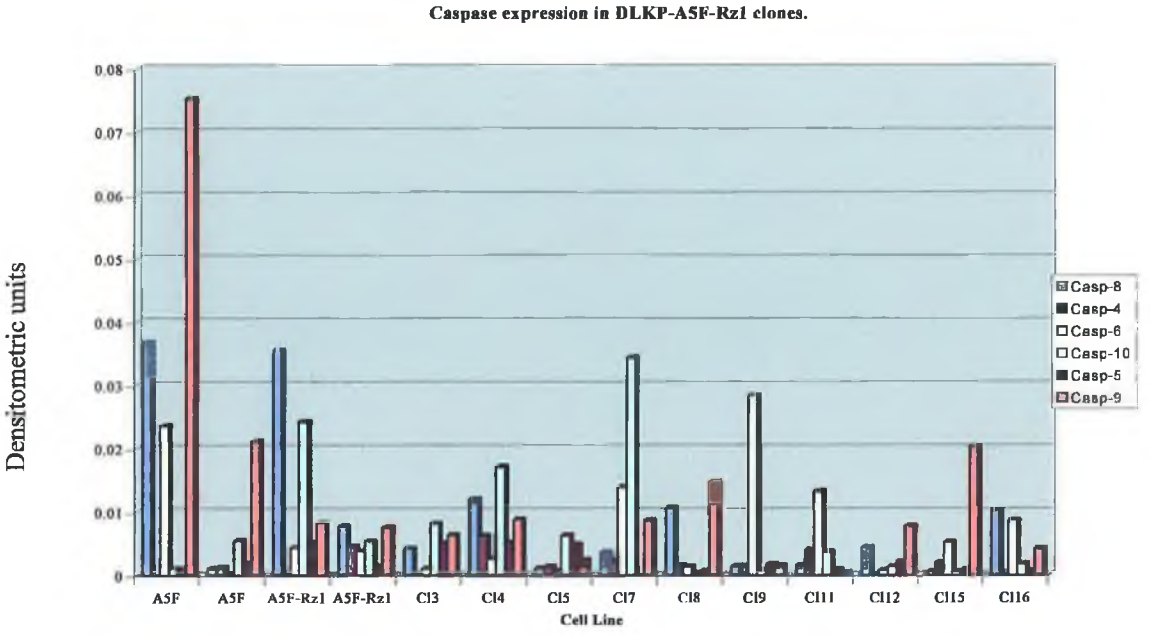
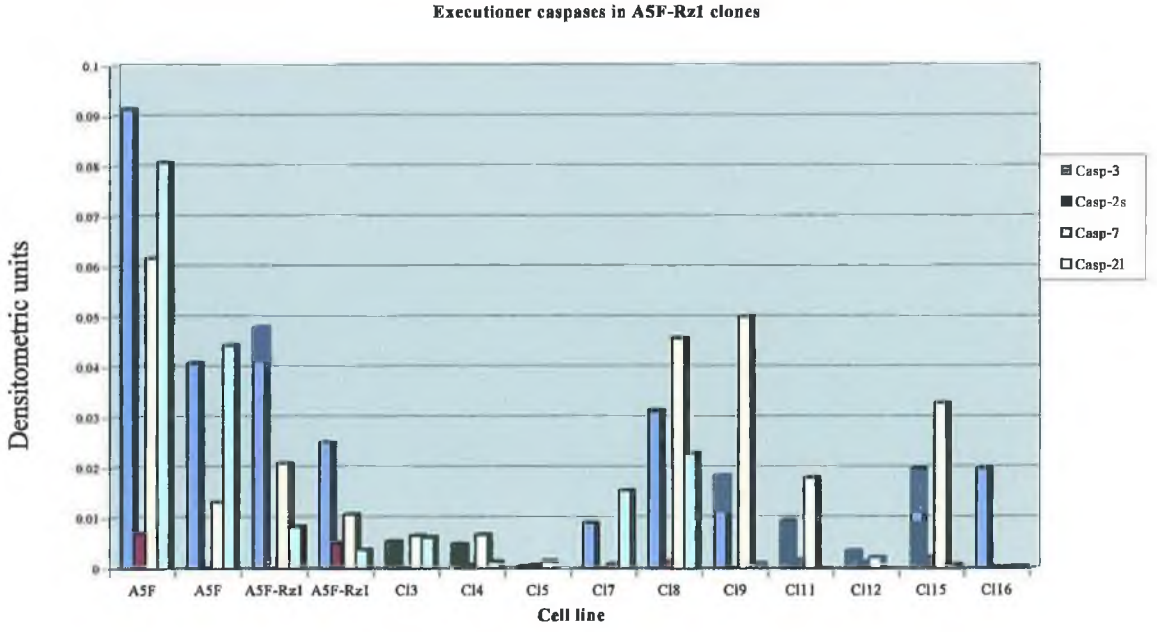


Figure 3.2.15b Graphical representation of RPA showing executioner caspases expression in DLKP-A5F-Rz1 clones.



3.2.6.3: In vitro toxicity analysis of CPP-32 ribozyme-transfected DLKP-A5F clones.

Toxicity assays were utilised to assess what effects decreasing caspase-3 RNA and protein would have on drug resistance profiles. It was thought that ribozyme expression would induce an increase in drug resistance profiles due to cellular levels of a key apoptotic executioner being decreased. Initial results in the mixed population revealed a 4-fold increase in resistance to adriamycin and vincristine, a 2-fold increase in resistance to etoposide, a slight increase in resistance to 5-fluorouracil and a 2-fold sensitivity to cisplatin. (These resistance profiles were also observed in another DLKP variant – A2B-1C7, which had been transfected with CPP-32-Rz1). However, when repeat toxicity assays were carried out, transfected cells gradually reverted back as they were passaged in culture to the resistance levels of the parental cells.

A similar phenomenon was evident upon analysis of the clonal populations. Initially the clones showed increases in resistance levels to etoposide, vincristine, taxol, etc., however, as was found in the transfected parent population, these new resistance profiles could not be obtained consistently. (See tables 3.2.2- 3.2.7). Twenty clones were isolated and five of these were analysed in detail due to their decrease in caspase-3 expression and initial changes in drug resistance profiles. These were considered to be the most interesting of the isolated clones.

Pro-caspase-3 levels in clone 4 at early and late passages showed a decrease when compared with parent cells. (Figure 3.2.16 & 3.2.17) Initial toxicity analysis revealed a 2-fold increase in sensitivity to 5-fluorouracil & VP-16, 5-fold increase in sensitivity to taxol, and 8-fold increase in sensitivity to adriamycin and cisplatin and a 16-fold increase in sensitivity to vincristine. Tests on later passages showed sensitivity to 5-fluorouracil, VP-16 and adriamycin had increased while sensitivity to vincristine had decreased although still increased relative to the parent cells. At initial passages, there was a slight decrease in P-glycoprotein (P-gp), which decreased even further in later passages. The toxicity results therefore may be accounted for by this reduction in P-gp expression. See figure 3.2.18 & 3.2.19.

Clone 6 had reduced pro-caspase-3 at later passages but this was not evident at initial passages. This clone showed an increase in resistance levels on initial analysis to vincristine, 5-fluorouracil, taxol and VP-16. Again, later passages revealed a trend

towards increased sensitivity with respect to adriamycin, vincristine and VP-16. Clone 6 did however show a slight sensitivity to cisplatin. These toxicity results occurred in conjunction with a reduction in P-gp levels at later passages.

Although clone 11 initially displayed no change in caspase-3 levels by Western blot at early and later passages, it had a dramatic change in toxicity levels when compared with parent cells. It displayed 64-fold increase in sensitivity to taxol and a 128-fold increase in sensitivity to adriamycin in early passages. Later passages showed similar trends in sensitivity. Further investigation revealed a loss of P-gp at early and later passages.

Clone 16 displayed an increased sensitivity to all drugs tested. This clone had reduced procaspase-3 levels at either early and later passages. Its P-gp levels were slightly decreased when compared with those of the parent cells.

The final clone selected for detailed analysis was clone 17. Initial results with clone 17 revealed a reduction in pro-caspase-3 levels and this reduction was also seen in later passages. Toxicity results indicated that this clone had become more resistant than the parent cells to the chemotherapeutic drugs tested. It displayed increases in resistance to VP-16, taxol, vincristine with no change to 5-fluorouracil and adriamycin. There was a negligible increase in sensitivity to cisplatin. Later passages showed clone 17 had retained some of its increased resistance to drugs but may have been reverting to the parent levels. Analysis of P-gp levels showed a decrease at later passages, which would account for the observed decrease in resistance levels.

The initial toxicity results (run 1 table 3.2.2 & 3.2.3) indicated that clones 6 and 17 had highest levels of resistance. Assessment of P-gp levels in these clones indicated they had highest levels. Run 3 toxicity assays (table 3.2.6) displayed that 6 & 17 were the most resistant clones. However only clone 16 kept significant levels of P-gp.

Table 3.2.2: IC50 values in A5F caspase-3 ribozyme clones (Run 1).

Cell Line	A5F	A5F-Rz1	CL.2	CL.3	CL.4	CL.6
Drug						
Cispl. (ug/ml)	0.5	0.5	0.5	0.4	0.063	0.5
Adr. (ng/ml)	>32000	32000	>32000	>32000	4000	>32000
Vnc. (ng/ml)	2000	3000	2000	2000	125	>4000
5-FU (ug/ml)	1.5	1.54	1.52	0.76	1.14	>6
Tx (ng/ml)	82	96	96	64	16	190
VP-16 (ng/ml)	9375	18750	12500	18800	3125	20000

Cell Line	CL.11	CL.15	CL.16	CL.17	CL.19
Drug					
Cispl. (ug/ml)	0.25	0.8	0.19	0.4	0.4
Adr. (ng/ml)	190	32000	7000	>32000	24500
Vnc. (ng/ml)	<125	3000	<125	7100	2000
5-FU (ug/ml)	<0.76	1.52	1.2	>1.52	1.2
Tx (ng/ml)	<1	192	24	190	128
VP-16 (ng/ml)	781.25	18750	3125	37500	12500

Table 3.2.3: Fold resistance in first run toxicity assays.

Cell Line	A5F	A5F-Rz1	CL.2	CL.3	CL.4	CL.6
Drug						
Cispl. (ug/ml)	1	1	1	0.8	0.125	1
Adr. (ng/ml)	1	1	1	1	0.125	1
Vnc. (ng/ml)	1	1.5	1	1	0.0625	>2
5-FU (ug/ml)	1	1	1	0.5	0.76	>4
Tx (ng/ml)	1	1.2	1.2	1	0.2	2.3
VP-16 (ng/ml)	1	2	1.3	2	0.5	2.1

Cell Line	CL.11	CL.15	CL.16	CL.17	CL.19
Drug					
Cispl. (ug/ml)	0.5	1.6	0.4	0.8	0.8
Adr. (ng/ml)	0.006	1	0.22	1	0.77
Vnc. (ng/ml)	<0.0625	1.5	<0.0625	3.6	1
5-FU (ug/ml)	<0.5	1	0.8	1	0.8
Tx (ng/ml)	0.012	2.3	0.3	2.3	2
VP-16 (ng/ml)	0.125	2	0.5	4	2

Table 3.2.4: IC50 values in ribozyme transfectants. (Run 2).

Cell Line	A5F	A5F-Rz1	CL.4	CL.6	CL.11	CL.16	CL.17
Drug							
Cispl. (ug/ml)	1.26	1.4	1.3	2	3	1.8	3.2
Adr. (ng/ml)	13850	24224	3765.25	32000	<250	2817.5	32000
Vnc. (ng/ml)	1000	4000	800	4250	<250	800	16000
5-FU (ug/ml)	3.19	1.4	>6	4	2.5	2.84	2.51
Tx (ng/ml)	86.3	76.7	38.6	128	<1	29.6	>128
VP-16 (ng/ml)	15321.33	9920	4000	12500	1150.6	4800	25000

Table 3.2.5: Fold Resistance in run 2 toxicity assays.

Cell Line	A5F	A5F-Rz1	CL.4	CL.6	CL.11	CL.16	CL.17
Drug							
Cispl. (ug/ml)	1	1	1	2	2.38	1	2.5
Adr. (ng/ml)	1	1.7	0.27	2.3	<0.02	0.2	2.3
Vnc. (ng/ml)	1	4	0.8	4.25	<0.25	0.8	16
5-FU (ug/ml)	1	0.5	2	1.25	0.8	0.9	0.8
Tx (ng/ml)	1	0.9	0.5	1.5	0.01	0.3	>2
VP-16 (ng/ml)	1	0.6	0.25	1.22	0.08	0.3	1.6

Table 3.2.6: IC50 values in caspase-3 ribozyme stable transfectants. (Run3)

Cell Line	A5F	A5F-Rz1	CL.4	CL.6	CL.11	CL.16	CL.17
Drug							
Cispl. (ug/ml)	1.5	1.5	0.5	1.5	0.25	0.5	0.75
Adr. (ng/ml)	16000	10000	750	6000	<250	1000	16000
Vnc. (ng/ml)	3000	2000	125	2250	<125	<125	5000
5-FU (ug/ml)	2.3	0.98	1.52	1.5	0.76	1.63	1.52
Tx (ng/ml)	64	64	12	77.5	<1	8	8
VP-16 (ng/ml)	10000	5000	625	8334	78.125	900	7500

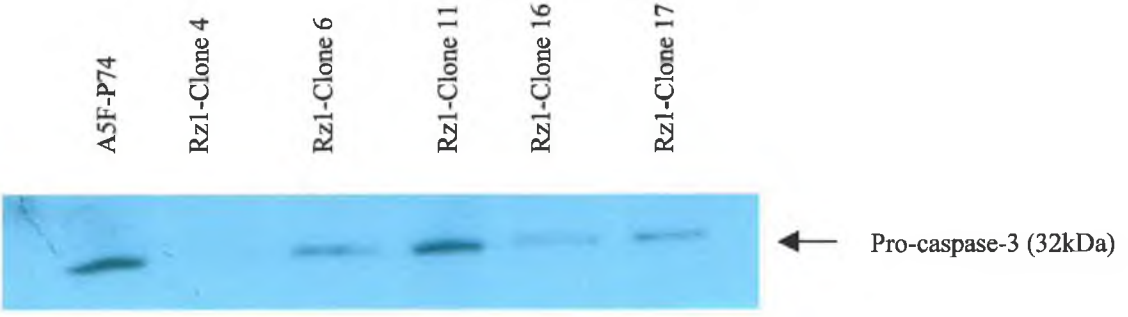
Table 3.2.7: Fold resistance in run 3 toxicity assays.

Cell Line	A5F	A5F-Rz1	CL.4	CL.6	CL.11	CL.16	CL.17
Drug							
Cispl. (ug/ml)	1	1	0.33	1	0.166	0.33	0.5
Adr. (ng/ml)	1	0.625	0.05	0.375	0.02	0.0625	1
Vnc. (ng/ml)	1	0.66	0.042	0.75	0.042	0.042	1.7
5-FU (ug/ml)	1	0.43	0.66	0.65	0.33	0.7	0.66
Tx (ng/ml)	1	1	0.19	1	0.02	0.125	0.125
VP-16 (ng/ml)	1	0.5	0.0625	0.83	0.008	0.09	0.75

3.2.6.4: Examination of pro-caspase-3 levels in DLKP-A5F-CPP-32-Rz1 clones

Western blot analysis was carried out using a monoclonal antibody to procaspase-3 (Section 2.5.1.4.1) on the clones to detect changes in the procaspase-3 protein levels compared to the parental cells. 20ug of protein was loaded in each case. β-actin expression was used as an internal control and densitometric analysis was carried out using this as a loading reference. Figure 3.2.16 illustrates protein levels in early passages of the ribozyme transfectants.

Figure 3.2.16: Pro-caspase-3 expression in CPP-32-Rz1 clones (early passages). 25ug of protein was loaded in each case. Graph below shows densitometric analysis.



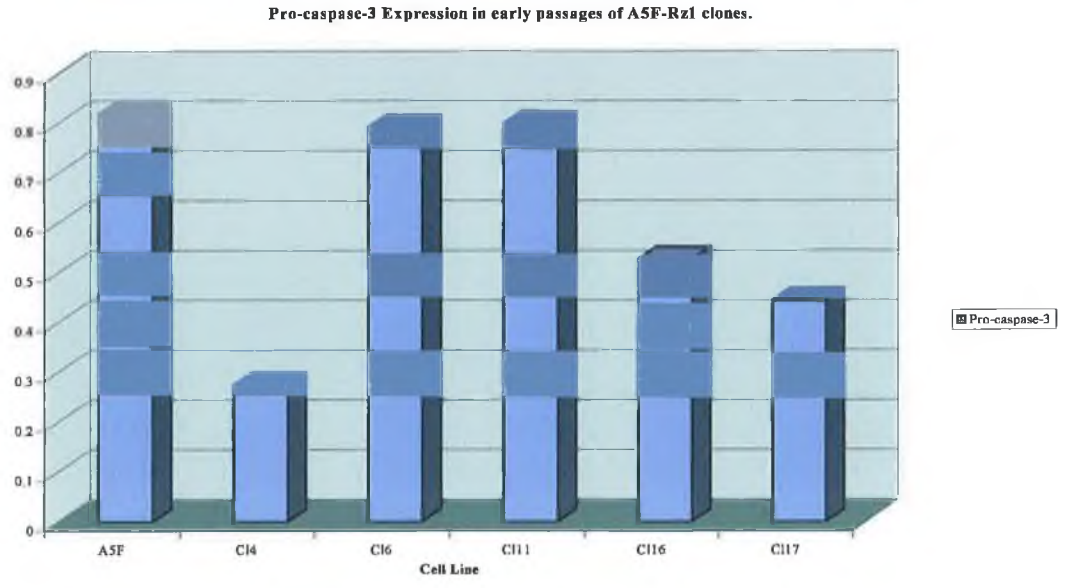
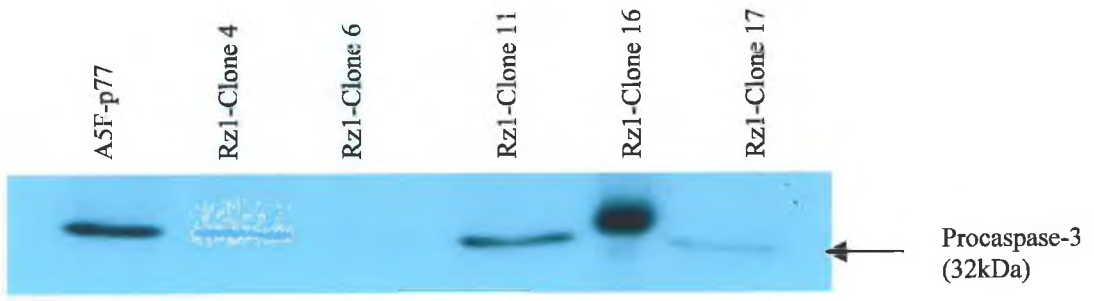
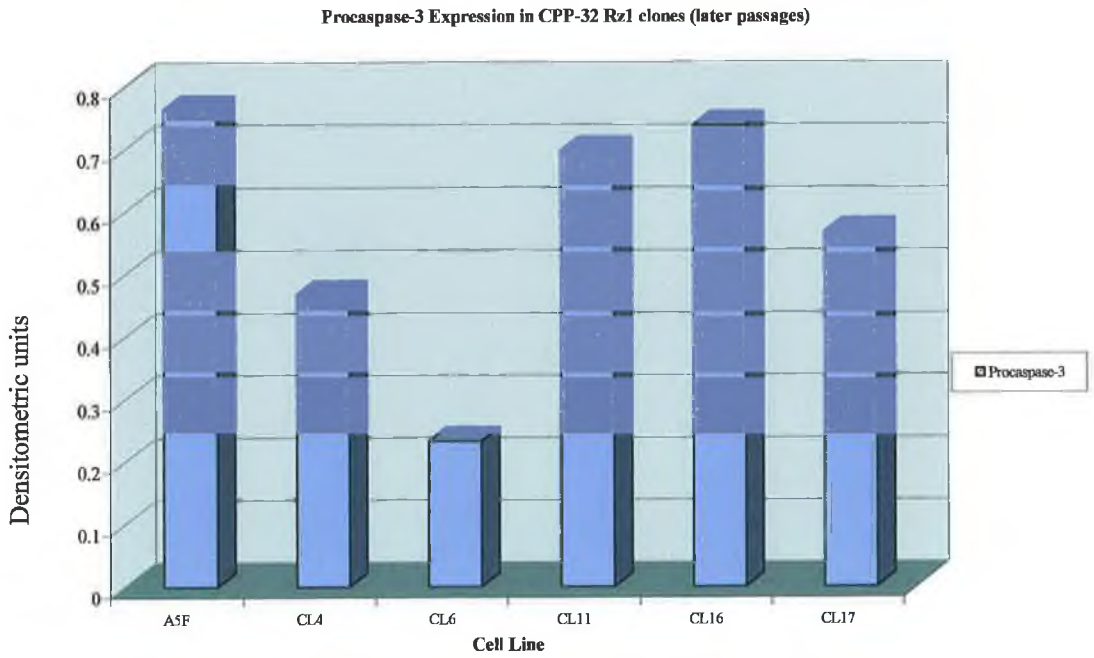


Figure 3.2.17: Pro-caspase-3 expression in CPP-32-Rz1 clones (later passages). 25 ug of protein loaded in each case. Graph below shows densitometric analysis.





3.2.6.5: Analysis of P-glycoprotein levels in CPP-32-Rz1 clones.

It was surprising that two clones (4 & 11) were found to be highly sensitive to all drugs tested except perhaps to cisplatin. Indeed resistance levels had reverted back beyond those of the parental DLKP-A5F cells, to that of DLKP cells (as in the case of clone 11). These findings suggested that perhaps the multi-drug resistance protein, P-gp, had decreased. It has been observed that P-gp expression and caspase-dependent apoptosis are linked (Johnstone et al., 1999).

P-glycoprotein levels were analysed by Western blots. As mentioned in section 3.2.5 due to the changes in resistance levels seen with increasing passage number, P-gp levels were examined in early and late passages by Western blot (See figure 3.2.18- 3.2.19). P-gp levels seemed to decrease with passage in all clones analysed. This may have masked any possible change in resistance levels due to down-regulation of caspase-3.

Figure 3.2.18: Western blot showing P-glycoprotein expression in DLKP-A5F-Rz1 clones (early passages). 25ug of protein was loaded for each sample and samples were separated on 6% SDS-polyacrylamide gels. P-glycoprotein is a 170kDa protein. Graph shows densitometric analysis of P-gp levels after normalisation using β-actin as an internal control.

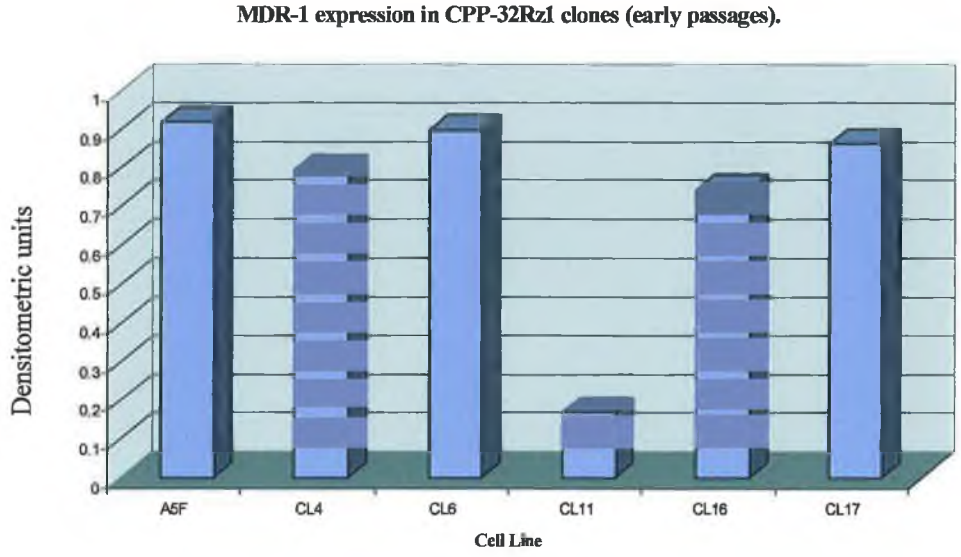
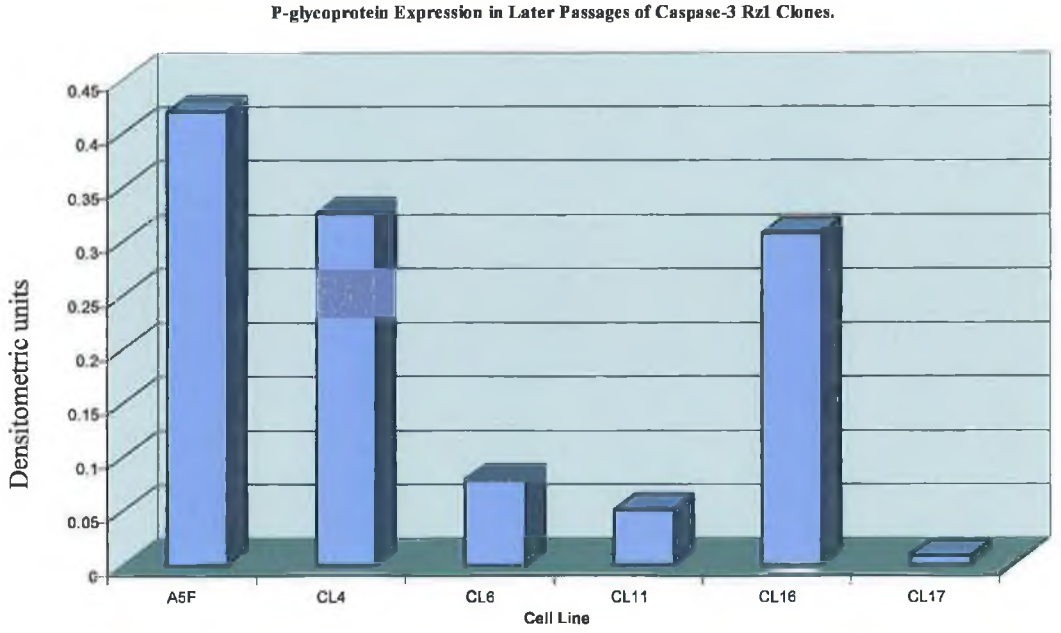


Figure 3.2.19: Western blot showing P-glycoprotein expression in DLKP-A5F-Rz1 clones (later passages). P-gp is a 170 kDa protein. Graph shows P-gp levels after normalisation using β-actin as an internal control.



3.2.6.6 Conclusions on stable transfectants.

Ribozyme 1 was initially transfected into a number of DLKP variants (DLKP, DLKP-A2B-1C7 & DLKP-A5F). Most of the work centred around the DLKP-A5F transfectants. Results for mRNA and protein showed decreases in caspase-3 levels. These were accompanied by changes in toxicity profiles. Mixed population transfectants displayed increases in resistance levels compared to A5F cells. They each displayed increases in resistance to VP-16, vincristine and adriamycin. However in both the A5F transfectants and A2B-1C7 transfectants, resistance levels gradually reverted back to parental levels.

Further analysis of derived clones revealed that mixed population contained populations of cells heterogeneous for their caspase-3 expression. The A5F transfected clones showed down-regulation by RT-PCR and Western blot of caspase-3. Toxicity analysis displayed, in some cases, dramatic changes in resistance profiles. In many cases, cells adopted a more sensitive phenotype. Subsequent investigation revealed down-regulation of P-gp might be involved in such changes for some of the drugs.

To conclude, caspase-3 ribozyme cleaved its target sequence in vitro, shown by IVC assay. The ribozyme down-regulated mRNA and protein in transiently transfected cells. Reductions in mRNA and protein were also seen in stable transfectants. Some initiation of functional change was seen in the stable transfectants initially (via drug resistance levels), but not in the transients. Other changes during subculture altered drug sensitivity of stable transfectants rendering interpretation difficult. A decrease in P-glycoprotein was seen in stably transfected clones.

3.3 Analysis of two drug-resistant variants of DLKP (DLKP-A2B & DLKP-A5F) using DNA Microarray.

3.3.1 Introduction to DNA microarray experiment.

Two clonal populations of DLKP-A cells were analysed using a Clontech DNA microarray. DLKP-A were derived from DLKP cells grown at increasing concentrations of adriamycin. A2B and A5F cells are two derived clonal populations (Heenan et al., 1997). These clonal populations display contrasting drug resistance profiles as well as different invasion characteristics (Dr. Mary Heenan & Dr. Yizheng Liang, personal communication). A2B cells are approximately 30-fold resistant to adriamycin with respect to parent cells, DLKP. They are not very invasive in vitro. In contrast, A5F cells are approximately 300-fold resistant to adriamycin with respect to DLKP cells. They are highly invasive.

A standard DNA array kit has been used in this experiment. (Clontech, 7905-1). This plastic array contains a total of 8327 genes. A complete list of these genes may be obtained from the Clontech website (www.atlas.clontech.com).

In all analyses, A2B (the less resistant & less invasive of the cells) was taken as the control cell line, and A5F cells were compared accordingly. Results from the first and second hybridisation were compared. Separate RNA samples were extracted from each cell line, both of which had been treated identically. Cells were harvested for RNA at approximately 80% confluency. In each experiment, 50ng of RNA was used to generate a probe to hybridise to the array.

Readings were similar for each hybridisation as can be seen in table 3.3.1. In each analysis, screens were exposed to the array membrane for 24 hours and subsequently analysed. Screens were then re-exposed for three days to ensure that the initial exposure had not excluded any genes.

"Up" indicates the gene was not expressed in the control sample. "Down" refers to gene expressed in the control but not the test sample.

Table 3.3.1: Probe readings.

Cell Line	Counts per Minute.
A2B- first hybridisation	9.4×10^6
A5F- first hybridisation	1.2×10^7
A2B- second hybridisation	6.0×10^7
A5F- second hybridisation	5.1×10^7

Figure 3.3.1 Scan of DNA array of A2B and A5F cell lines. Hybridisation took place over 24hours.

Figure 3.3.1a DLKP-A2B:

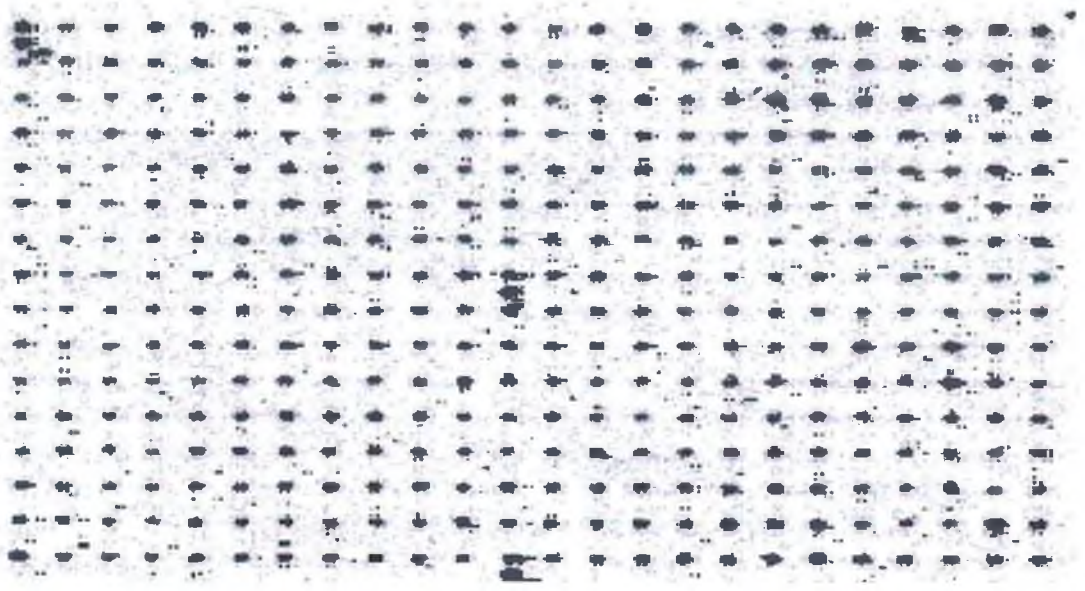
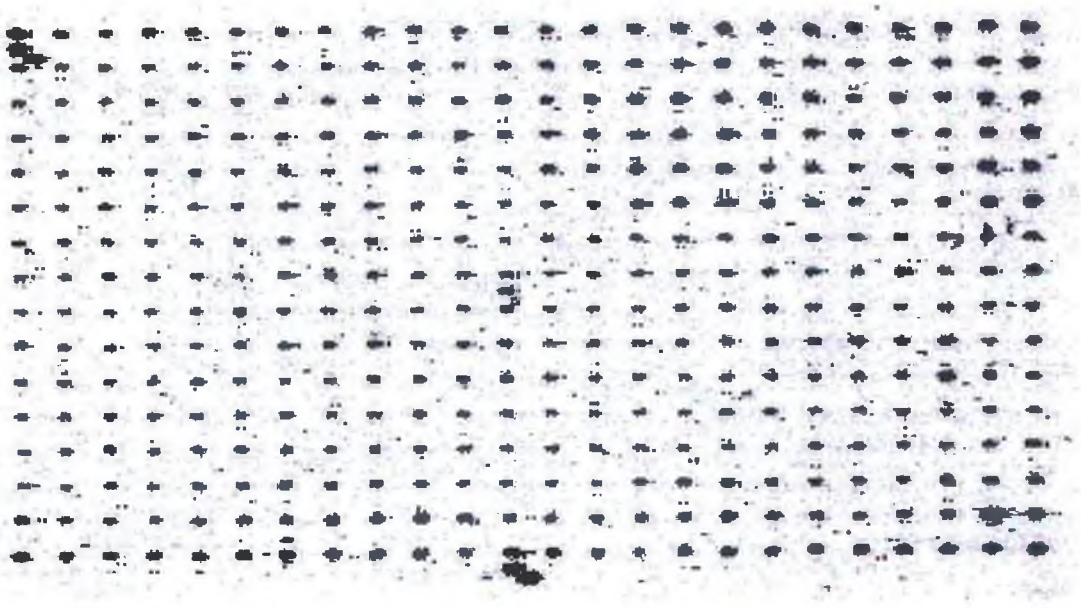


Figure 3.3.1b DLKP-A5F.



3.3.2 Results of DNA microarray experiment.

Overall, there were 80 genes in common when first hybridisation (A2B-1 & A5F-1) was compared with second hybridisation (A2B-2 & A5F-2). Of these, 67 had similar trends of expression, while 13 were conflicting. The genes with matching expression trends are listed in tables 3.3.2 & 3.3.3. An average fold expression between the two arrays is included beside the gene name and the array reference code. Table 3.3.3 illustrates trends in gene expression between A2B and A5F cell lines.

Table 3.3.2 displays genes that had increased expression in the more resistant cell line, A5F, with respect to control A2B cells. Changes of greater than 1.5-fold were noted in the table. In table 3.3.2, fold increases range from 1.5 to 6-fold. There were a number of the ATP-binding cassette proteins with altered expression. An apoptosis-associated tyrosine kinase also displayed increased expression. In addition, there were changes in a number of ribosomal proteins.

Table 3.3.3 shows genes detected in A5F but not A2B cells. Again ribosomal proteins had increased expression, possibly indicating that the cells were actively dividing at the time of RNA extraction. There was increased expression of multi-drug resistance gene, MDR-1, and also the apoptosis-related gene, galectin-1. Genes with increased expression in A5F versus A2B cells are discussed in more depth in section 4.

There was some disagreement between the first hybridisation and repeat hybridisation. Genes that showed opposite patterns of expression between array 1 and 2 are listed in table 3.3.4. There are 11 of these genes in total.

Numerous genes changed expression on array 2 compared with array 1. Table 3.3.5 reports some of the metastases-related genes with altered expression on array 2 only. Table 3.3.6 lists genes expressed in A2B cells only. These include retinoic acid receptor α.

Table 3.3.7 highlights some areas of interest to cancer research. Most of the gene families did not display any changes in gene expression from A2B to A5F cells. There were a number of changes in Rho-related proteins possibly indicative of the changes in invasion and resistance profiles between the two cell lines.

Table 3.3.2 Genes with increased expression in A5F compared with A2B cells.

Gene Code	Array 1	Array 2	Gene/ Protein
A09ab2	2.8	4	arylalkylamine N-acetyltransferase
A15ab2	2	8	ATP-binding cassette, sub-family A (ABC1), member 1
A16ab2	2	10	albumin
A17ab2	2.8	5	ATP-binding cassette, sub-family A (ABC1), member 3
A19ab2	2	2	ATP-binding cassette, sub-family B (MDR/TAP), member 7
A21ab2	2	2	ATP-binding cassette, sub-family A (ABC1), member 4
A21cd1	2.7	2.5	profilin 1
A24ab2	3.4	8.6	aldehyde dehydrogenase 3
B13ab2	2	4	apoptosis-associated tyrosine kinase
C14ab2	2	3	aldolase B, fructose-bisphosphate
D09cd1	2	3	ribosomal protein L12
D16ab2	2	2	ADP-ribosylation factor-like 3
E24ab2	2.3	2.3	adenosine monophosphate deaminase 1 (isoform M)
F09cd1	2	2	ribosomal protein L31
G01cd3	2.3	2.6	ribosomal protein S27 (metallopanstimulin 1)
H06ab2	2	2	arylsulfatase E (chondrodysplasia punctata 1)
H09ab2	2	2.6	myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog); translocated to, 10
H09cd1	2	2	ribosomal protein S7

Table 3.3.2 continued.

Gene Code	Array 1	Array 2	Gene/ Protein
H21cd1	2.5	5	ribosomal protein S15
J21ab2	2.5	2.5	A kinase (PRKA) anchor protein 6
K01ab2	2	3	alcohol dehydrogenase 5 (class III), chi polypeptide
K20ab2	2.4	2.5	amyloid beta (A4) precursor-like protein 1
L03ab2	2	2.8	A kinase (PRKA) anchor protein (yotiao) 9
L21ab2	2	2.7	AND-1 protein
L22ab2	2.4	2.6	ATPase, Na+/K+ transporting, beta 1 polypeptide
M15ab2	2	2	AE-binding protein 1
N13ab8	2	2	parathymosin
N19ab2	3	2.6	nuclear protein, marker for differentiated aortic smooth muscle and down-regulated with vascular injury
N23ab8	3	2.3	protein tyrosine phosphatase, non-receptor type 9
N23cd8	3	2.4	paired immunoglobulin-like receptor beta
O09ab2	2.5	2.5	angiotensin receptor 2
O14ab2	2.8	2.4	aquaporin 8
P06ab2	2	3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e
P07ab2	2.3	2.4	adipose specific 2
P12ef6	2	5	Sp-5
P13ab2	2.1	2.2	amyloid beta precursor protein (cytoplasmic tail)-binding protein 2
P16ab2	2	2.5	ATP synthase, H+ transporting, mitochondrial F1F0, subunit g
P23ab2	2	2.4	ADP-ribosylation factor 4-like

Table 3.3.3 Genes increased expression in A5F cells.

Gene Code	Array 1	Array 2	Gene/ Protein
A16cd1	Up	Up	pancreatic lipase-related protein 1
A23cd1	2	Up	protein geranylgeranyltransferase type I, beta subunit
C12ab6	3.4	Up	ferritin, heavy polypeptide 1
C24cd1	2	Up	protein kinase, AMP-activated, beta 1 non-catalytic subunit
D01cd1	1.6	Up	ribosomal protein L6
D06cd1	2	Up	S100 calcium-binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))
D07ab8	1.6	Up	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform
E18cd6	1.9	Up	myosin regulatory light chain 2, smooth muscle isoform
F03cd1	1.7	Up	ribosomal protein L30
F23ab7	Up	Up	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)
F23ab8	Up	Up	protein kinase C, mu
G18ab2	Up	2.2	ankyrin 1, erythrocytic
H17cd1	2.3	Up	ribosomal protein S12
I01ab8	2.6	Up	ATP-binding cassette, sub-family B (MDR/TAP), member 1
I20ab2	1.6	Up	annexin A6
J09cd1	2	Up	ribosomal protein S23
K22cd2	3.5	Up	SCO (cytochrome oxidase deficient, yeast) homolog 1
N22ab6	3.3	Up	lectin, galactoside-binding, soluble, 1 (galectin 1)
O21ab2	2.2	Up	allograft inflammatory factor 1
O24ef1	1.7	Up	calpain 1, (mu/I) large subunit
P12ef1	1.6	Up	ribosomal protein L13a
P21ab8	2	Up	protein tyrosine phosphatase, receptor type, S
P24ab2	2.3	Up	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58kD, isoform 1

Table 3.3.4 Genes with conflicting expression from Array 1 & Array 2.

Gene Code	Array 1	Array 2	Gene/ Protein
A11ab2	0.6	3.2	alanyl-tRNA synthetase
A11cd1	0.6	Up	poly(rC)-binding protein 2
B18cd1	0.6	Up	ribosomal protein S27a
C18cd1	0.5	Up	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform
C19ab2	0.5	2.5	amiloride-sensitive cation channel 1, neuronal (degenerin)
C22ef5	0.4	Up	prohibitin
D24ab8	Up	Down	retinoic acid receptor responder (tazarotene induced) 1
F21cd1	0.6	Up	ribosomal protein, large, P0
H15ab2	0.6	2.3	anterior gradient 2 (Xenepus laevis) homolog
H16ab5	0.6	Up	FK506-binding protein 4 (59kD)
L16ab2	0.5	2.4	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide

Table 3.3.5 Some of metastases-related genes which changed expression on Array 2 only.

Gene Code	Change	Gene/ Protein
A15cd1	Up	programmed cell death 1
A16ab6	Down	carcinoembryonic antigen-related cell adhesion molecule 3
B20ab2	2.42	ras homolog gene family, member G (rho G)
B22ab2	2.21	Rho GTPase activating protein 1
B24ab2	2.72	Rho GTPase activating protein 6
C01ef6	0.42	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
D02ab2	0.34	Rho GDP dissociation inhibitor (GDI) gamma
D06ab2	2.09	ras homolog gene family, member I
D08ab6	Up	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
E02ef5	Up	non-metastatic cells 1, protein (NM23A) expressed in
E23ab3	Down	B melanoma antigen
G16cd2	Up	tumor protein, translationally-controlled 1
G18cd1	Up	retinoblastoma-binding protein 6
I18cd2	Up	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70-interacting protein)

Table 3.3.6: Genes with increased expression in A2B relative to A5F.

Gene Code	Array 1	Array 2	Gene/ Protein
C10ab7	Up	Up	Interleukin enhancer binding factor 2, 45kD
F11ab2	Up	6.1	A disintegrin and metalloproteinase domain 23
G24cd2	4.1	2.4	Tumour rejection antigen (gp96) 1
G24cd6	Up	Up	Preferentially expressed antigen in melanoma
H12ab4	2.7	2.4	L(-)
H14cd1	Up	Up	Selectin P ligand
I14cd1	Up	Up	Sarcoglycan, gamma (35kD dystrophin-associated glycoprotein)
J21ef4	2.7	2.1	Non-functional folate binding protein
J22cd1	4.0	3.0	TAL1 (SCL) interrupting locus
K11cd1	Up	Up	Pericentrin
K19ab8	Up	Up	Retinoic acid receptor, alpha
L09cd1	Up	2.4	Sex hormone-binding globulin
L20cd1	Up	Up	Solute carrier family 18 (vesicular monoamine), member 1
P15cd1	Up	Up	Ric (Drosophila)-like, expressed in neurons
P19cd1	Up	Up	Regulator of mitotic spindle assembly 1

Table 3.3.7: Some genes of interest in cancer research.

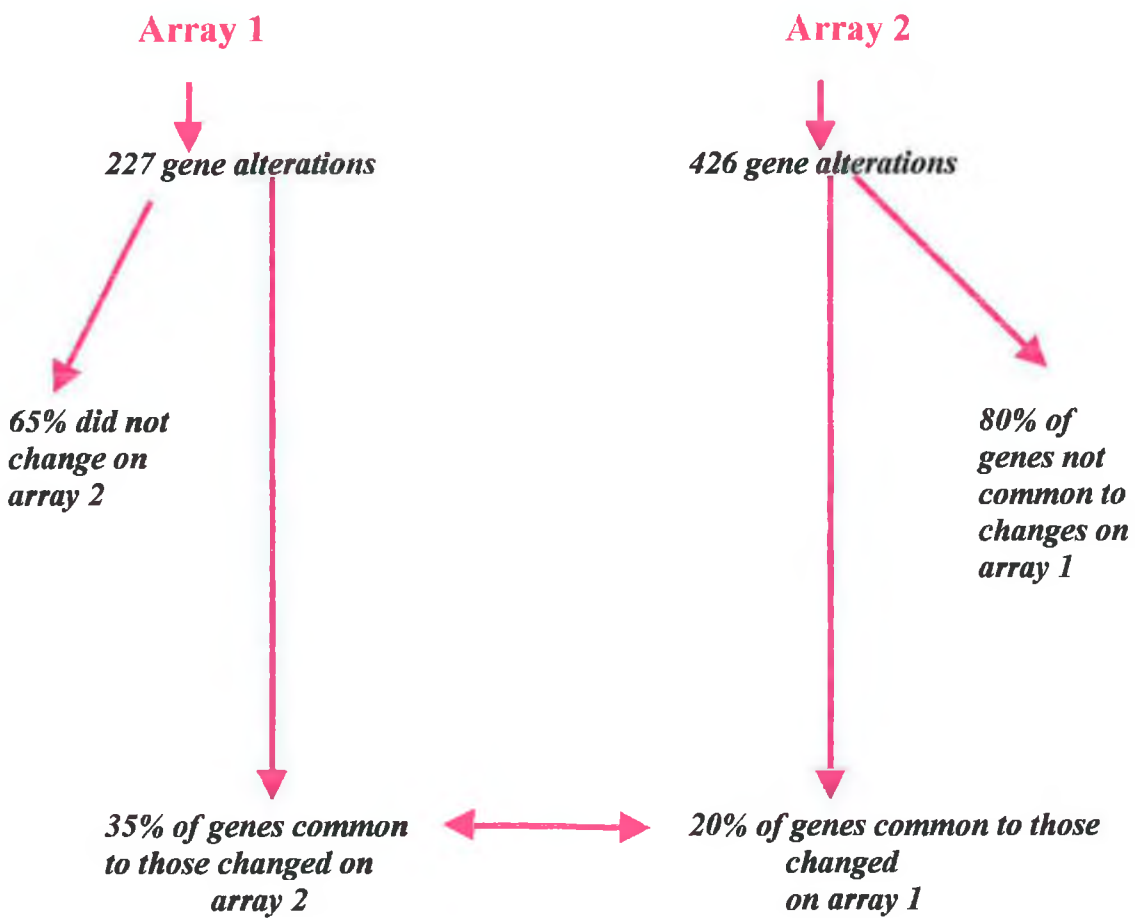
Gene Family/ Group	Change.
Bcl-2 family	No changes detected.
	Galectin-1 had increased expression in A5F cells.
Caspase family	No changes detected.
MDR-associated proteins	Increased expression of MDR-1 in A5F cells.
Matrix metalloproteinases	No changes detected.
Growth factor and chemokine receptors	No changes detected.
Nuclear proteins	No changes detected
Hormone receptors	No changes detected
	Retinoic acid receptor-α decreased in A5F.
DNA replication	No changes detected
c-Fos	No changes detected
c-Myc	No changes detected
Glutathione Peroxidase	No changes detected
Proteins involved in invasion & metastases:	
Src proteins	No changes detected
RHO-related proteins	Rho G increased 2.4-fold in A5F
	Rac-1 decreased 0.4-fold
	Rho GTPase activating protein6 increased 2.7-fold.
	Ras-related c3 botulinum toxin substrate 1 decreased in A5F.
	Rho GDP dissociation inhibitor γ decreased.
	Ras homolog gene family member-1 increased 2-fold.
Metastatic suppressor proteins	NM23A increased in A5F cells.

3.3.3 Reproducibility of the DNA microarray technique.

The array experiment was carried out twice on separate RNA extracts isolated from cells that were treated identically. Despite this, the first and second experiments had large numbers of genes that did not change their expression consistently from one hybridisation to the next. Table 3.3.8 displays the percentage of genes that were common to both hybridisations.

The total number of genes with altered expression in A5F compared to A2B on the first hybridisation was 227 and on the second 426. On first hybridisation, approximately 65% of genes with altered expression did not show altered expression on the second hybridisation. The remaining 35% of genes that changed were also changed on the second hybridisation. On the second hybridisation, approximately 80% of genes were not common to the first hybridisation. Therefore 20% of genes showed the same changes as on the first hybridisation.

Figure 3.3.3 Reproducibility of the DNA array experiments.



The second hybridisation showed an increase in the number of genes expressed. An increase in the number of genes expressed on a repeat hybridisation experiment was also observed by another researcher in this lab using different cell lines (Dr. Carmel Daly, personal communication). It has been noted that a difficulty in achieving "matching" results for RNA samples extracted from the same cell lines from one hybridisation to another can occur in array experiments.

Due to the discrepancies between repeats, the reproducibility of the technique must be questioned. These results clearly illustrate how important it is to repeat the experiment to ensure that accurate and reproducible results are obtained. A third hybridisation could confirm if any of these genes are suitable for further study. However before any subsequent analysis is carried out, results should be validated by another technique such as RT-PCR, Northern blot, etc.

Table 3.3.8 Percentage of genes common to both hybridisations.

First Hybridisation N=227	65% genes not common to 2nd Hybridisation.
Second Hybridisation N=426	80% genes not common to 1st hybridisation

3.3.4 Conclusions on DNA microarray experiments.

From the results presented here, it is apparent that these clones have a large number of genetic changes underlying their phenotypic changes. They display dramatic variation in drug resistance levels (approximately 10-fold increased resistance in A5F cells with respect to A2B cells) and in their tendencies towards invasiveness, with A5F cells being highly invasive when compared with A2B cells.

Previous studies on these cells have shown changes in P-glycoprotein levels and caspase family members. The DNA microarray did not detect any changes in the apoptosis-related genes studied including caspase family and bcl-2 family members. The array did detect changes in other genes including galectin-1, mdr-1, apoptosis-associated protein kinase, etc. Galectin-1 did emerge to have increased expression in the more resistant of the cell lines. Galectins are involved in apoptosis and also play a role in invasion and metastases. The significance of a change in expression of galectin-1 in the context of these cell lines is as yet unclear.

Mdr-1 was also found to have elevated expression in A5F when compared with A2B cells. Undoubtedly, this elevation contributes towards the increased resistance levels of these cells to chemotherapeutic drugs. Mdr-1 ribozyme studies have shown that knocking out levels of mdr-1 in A2B cells, reverted their resistance levels back to those of the parent, DLKP cells (Daly *et al.*, 1996.)

In addition to the above genes, some of the ras family genes and rho family genes had altered expression in the more invasive A5F cells. Approximately 50% of metastatic tumours contain ras mutations (Ward *et al.*, 2001). As these proteins contribute to a cells ability to metastasise, it may be worth investigating, for example by RT-PCR, the levels of expression of these ras and rho-related genes in the cell lines. Such investigation could enhance our understanding of the invasiveness of these cells.

DNA array technology can be used as a guideline to assess gene expression levels in cellular models. In the case of A2B versus A5F cells, the results indicate that several genes may be contributing to the phenotypic differences observed in the cells. As these experiments illustrate however, the arrays should be repeated at least once to ensure

accurate results. These results are representative only and will require confirmation by another method such as RT-PCR or Northern blot at a later date.

3.4.1. Analysis of Gene Expression in Breast Tumour Biopsies.

3.4.1.1a Introduction to the clinical study group.

Patient prognosis is not dependent on the expression of a single protein but on an interplay between a large number of different factors. The emergence of multi-drug resistance in breast cancer patients is one of the key limits to survival and treatment response. Accompanying this drug resistant status is often an imbalance between other proteins. One such group of proteins is represented by the bcl-2 family, a group of heterodimerising proteins that have the ability to influence a cell's tendency to undergo apoptosis.

Many studies suggest that a patient's clinical outcome may depend on the cumulative effect of the expression of anti and pro-apoptotic proteins in breast tumours (Nieves-Neira et al., 1999). The significance of expression of these proteins in breast tumour is as yet unclear, however undoubtedly, apoptosis and a drug's ability to trigger it may play an important role in breast cancer biology, prognosis and therapy.

This study was part of a large survey where a wide number of markers were examined. These clinicopathological factors are listed in tables 3.4.1 & 3.4.2. It therefore provided a unique opportunity to examine the possible prognostic significance of a range of parameters as indicators for response rates and predictive value in breast cancer tissue.

The aim of the clinical study was

- to identify an accurate and reproducible method of RNA extraction from archival human tumours which could be analysed by RT-PCR.

- to establish what links, if any, existed between the expression of apoptosis-related genes, cancer treatment and patient outcome in a panel of invasive breast cancers.

Approximately one hundred and fifty archival breast tumour biopsies were analysed by RT-PCR for their expression of apoptosis related genes. The tumour tissue was very

kindly donated by Dr. Susan Kennedy, Dept. of Pathology, St. Vincent's Hospital, Dublin.

3.4.1.1b Tumour & Patient History.

Table 3.4.3 lists the patient and tumour characteristics that were included in the statistical analyses. Tumour sizes ranged from less than 1cm to up to 9cm. Tumour grade was also recorded. Most patients received a treatment regime of tamoxifen and/ or CMF[1] chemotherapy (1-3 positive nodes). Some received adriamycin in addition to CMF (those with >4 positive lymph nodes). Up to 12 rounds of chemotherapy were given to some patients. One patient received CMF with bleomycin; one received CAF[2] treatment; one received taxol; three received Zoladex and one received Aredia. A small number of patients did not receive any drug treatment. A number of patients received adjuvant radiation therapy. In most of these patients, tumour was contained and lumpectomy surgery was performed.

CMF[1]: Cyclophosphamide, methrotrexate, 5-fluorouracil.
CAF[2]: Cyclophosphamide, adriamycin, 5-fluorouracil.

Table 3.4.1 Markers examined in clinical study group.

Marker of Interest:
Bcl-2 mRNA
Bax mRNA
Bag-1 mRNA
Mcl-1 mRNA
Survivin mRNA
Survivin splice variant 2B mRNA
Survivin splice variant δ3 mRNA
Galectin-3 mRNA
MRP-1 mRNA
MDR-1 protein
MRP-1 protein
Survivin protein

Table 3.4.2 Clinicopathological parameters included in statistical analysis.

Age of patient	< 50 ≥ 50
Estrogen receptor status	ER + or ER –
Tamoxifen treatment	Yes/ No
Chemotherapy	Yes/ No
Size of tumour	<2, 2-5, >5cm
Histological grade of tumour	1,2,3 & 1+2, versus 3.
Lymph node status	Pos or neg
Diagnosis/ Histological type	Ductal Lobular "Special"
Relapse within 5 years	Yes/ No
5 year overall survival	Yes/ No

3.4.1.2 RT-PCR expression results for archival breast tumour biopsies.

3.4.1.3 Nomenclature of tumour biopsies.

To preserve patient confidentiality, tumour samples have been named according to the order in which the RNA extractions were carried out. T1- T134 were extracted by Rasha Linehan and Deirdre Cronin. S1-S38 RNA extractions were carried out by Sharon Glynn. Normal tissue samples were designated N1- N6. N1 also came with a corresponding tumour tissue named T1N1.

3.4.1.4 Statistical Analysis.

The association of these markers with patient and tumour characteristics (tumour size & grade, diagnosis, treatment, ER status, lymph node involvement, age of patients at diagnosis, relapse-free and overall survival) was evaluated using one-degree-of-freedom Chi-squared test. Survival analysis (5 year relapse free and overall survival probabilities) was performed using the Kaplan-Meier method; and the log rank test was carried out to assess the statistical differences between categories of each prognostic factor. Multivariate analysis using Cox's proportional hazards models was applied to evaluate the prognostic power of each variable independently of the others. For all tests a P value of below 0.05 was considered to be statistically significant indicating 95% confidence intervals. These are included in bold type where significant. P values higher than 0.05 but lower than 0.075 are also highlighted as they may be just outside the margins of significance.

3.4.1.5 Bcl-2.

The results of bcl-2 RT-PCRs are detailed in appendix section 7.3.1. Bcl-2 expression was found in five of the six normal tissues. 50% of these were very positive and 33% expressed lower levels of bcl-2. One was negative for bcl-2 expression. The T1N1 sample was positive as was its corresponding normal tissue. Out of a total of 147 RT-PCRs on the tumour tissues, 131 were bcl-2 positive. Bcl-2 expression has been correlated with good prognosis in breast cancer (Bukholm et al., 2002; Reed, 1996; Castiglione et al., 1999; Silvestrini et al., 1996; Shilkaitis et al., 2000; Ahr et al., 2002).

Chi-squared analysis (table 3.4.1.3) revealed that bcl-2 mRNA expression correlated with ER status (P=**0.019**) and bag-1 mRNA expression (P=**0.003**). Kaplein-Meier analysis revealed an association between bcl-2 mRNA expression and a relapse-free survival of five years. P=**0.042**.

Lymph node status failed marginally to be a significant prognostic factor that correlated with bcl-2 mRNA expression as its P value was **0.074**.

The Kaplan-Meier survival curves indicate that the presence of bcl-2 mRNA correlates favourably with both disease (relapse)-free survival (P=0.0273) and overall survival (P=0.042). Graphs are displayed in figure 3.4.1.

Multivariate analysis by Cox regression showed that bcl-2 was independently associated with overall survival but not five year disease-free relapse.

Table 3.4.1.3 Correlation of Bcl-2 mRNA expression with clinicopathological features using Chi-squared test.

Variable	Bcl-2 mRNA neg (%) n=19	Bcl-2 mRNA pos (%) n=69	P value
Diagnosis			
Ductal	78.9%	75.4%	
Lobular	15.8%	20.3%	P=0.901
"Special"	5.3%	4.3%	
Age (yrs.)			
<50	31.6%	20.3%	P=0.298
≥50	68.4%	79.7%	
ER status			
Neg.	55.6% (n=18)	26.5% (n=68)	P= 0.019
Pos.	44.4%	73.5%	
Tumour Size			
<2cm	10.5%	18.8%	P=0.122
2-5cm	78.9%	79.7%	
>5cm	10.5%	1.4%	
Tumour Grade (1&2 v. 3)			
1&2	47.4%	47.8%	P=0.972
3	52.6%	52.2%	
LN status			
No	26.3%	49.3%	P=0.074
Yes	73.7%	50.7%	
Tamoxifen			
No	31.6%	25.8%	P=0.621
Yes	68.4%	74.2%	
Chemotherapy			
No	47.4%	47.5%	P=0.990
Yes	52.6%	52.5%	

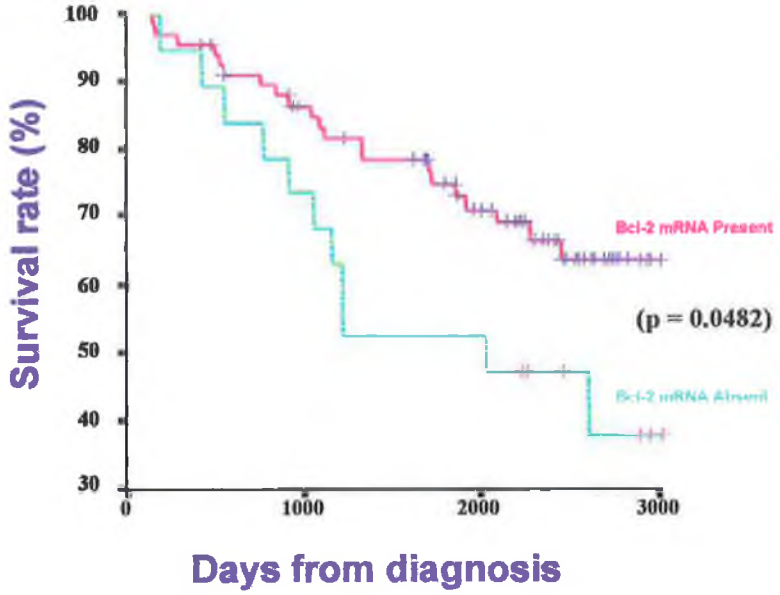
Table 3.4.1.4 Correlation of Bcl-2 mRNA with other apoptosis and drug resistance related genes. (Chi-squared analysis).

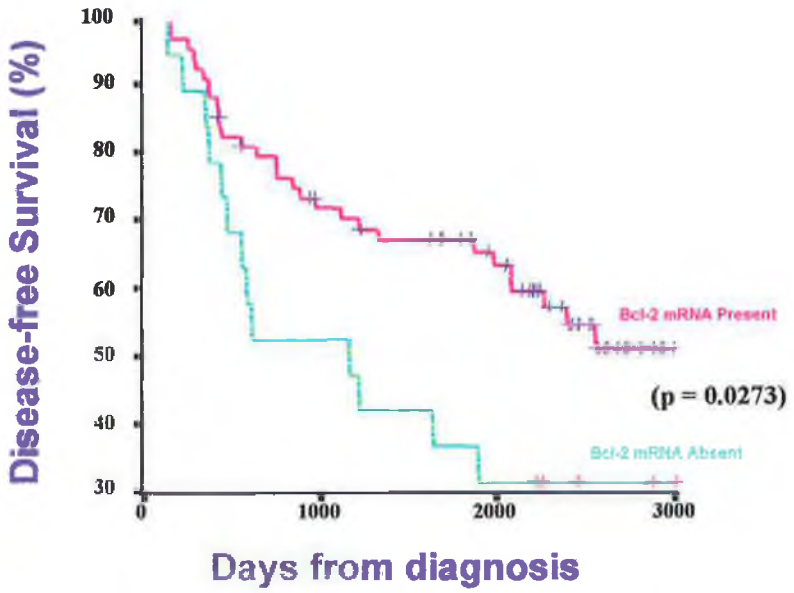
Variable	Bcl-2 mRNA neg (%) n=19	Bcl-2 mRNA pos (%) n=69	P value
Bag-1 mRNA			
Absent	44.4% (n=18)	12.9% (n=62)	P=0.003
Present	55.6%	87.1%	
MRP-1 protein			
Absent	36.8%	40.6%	P=0.768
Present	63.2%	59.4%	

Table 3.4.1.5 Correlation of Bcl-2 mRNA with patient outcome. This analysis, like the earlier tabulated information was done by Chi-Square (x^2).

Variable	Bcl-2 mRNA neg (%) n=19	Bcl-2 mRNA pos (%) n=69	P value
5-year relapse-free survival			
No	36.8%	63.3%	P=0.042
Yes (did not relapse)	63.2%	36.7%	
5-year survival			
No	52.6%	72.4%	P=0.110
Yes (survived 5 years)	47.4%	27.6%	

Figure 3.4.1 Kaplan-Meier Survival Curves showing bcl-2 mRNA correlated favourably with both disease-free survival (P=0.0273) and overall survival (P=0.042).





3.4.1.6 Bax.

The significance of bax expression in breast tumours remains to be elucidated. Most studies report heterogeneous expression of the gene in both normal and tumour specimens (Reed, 1996; Bargou et al., 1996; Sturm et al., 2000). Normal tissue showed five of six samples with some level of bax expressed. Again 50% were strongly positive, and 33% weakly positive with approximately 16% negative. Out of a total of 148 RT-PCR reactions in the tumour samples, 147 showed bax expression.

Using Chi-squared analysis to relate bax mRNA expression with clinicopathological features and gene expression, bax did not appear to have any significant correlations. Kaplan-Meier analysis was carried out to relate the prognostic significance of bax mRNA expression with patient outcome. No significant correlations were found. RT-PCR results for bax expression are detailed in appendix section 7.3.2.

3.4.1.7 Mcl-1.

Of the normal tissue studied, one out of six samples were positive for mcl-1 mRNA expression. 136 out of a total of 143 RT-PCR reactions came up positive for mcl-1 expression in the tumour biopsies. RT-PCR results for mcl-1 expression in tumour biopsies are detailed in appendix section 7.3.3. The significance of mcl-1 expression has yet to be established in a wide variety of tumour types, however some have reported that where bcl-2 expression is high, mcl-1 is low (Soini et al., 1999).

Chi-squared analysis indicated a correlation between mcl-1 mRNA and tumour grade. Mcl-1 negative tumours were of lower grade and mcl-1 positive tumours were higher grade (grade 1,2 & 3, (P= **0.035**) and grade 1 & 2, versus 3 (P=**0.046**)). (See table 3.4.1.6). It marginally failed to be significant when tested for its correlation with lymph node status (P=**0.053**) and for its association with survivin δ3 expression, a splice variant of survivin (Rasha Linehan, personal communication).

Table 3.4.1.6 Statistical Analysis of Mcl-1 expression in breast tumours.

Variable	Mcl-1 mRNA neg (%) n=6	Mcl-1 mRNA pos (%) n=89	P value
Diagnosis			
Ductal	83.3%	80.9%	
Lobular	16.7%	13.5%	P=0.826
"Special"		5.6%	
Age (yrs.)			
<50	50% (n=6)	28.1% (n=89)	P=0.255
≥50	50%	71.9%	
ER status			
Neg.	50%	34.1%	P= 0.431
Pos.	50%	65.9%	
Tumour Size			
<2cm		18% (n=89)	P=0.449
2-5cm	100% (n=6)	78.7%	
>5cm		3.4%	
Grade			
1		10.1% (n=89)	
2	83.3% (n=6)	31.5%	P=0.035
3	16.7%	58.4%	
Tumour Grade (1&2 v. 3)			
1&2	83.3% (n=6)	41.6% (n=89)	P=0.046
3	16.7%	58.4%	
LN status			
No	83.3% (n=6)	42.7% (n=89)	P=0.053
Yes	16.7%	57.3%	
Tamoxifen			
No	50% (n=6)	29.1% (n=79)	P=0.284
Yes	50%	70.9%	
Chemotherapy			
No	66.7% (n=6)	45% (n=80)	P=0.305
Yes	33.3%	55%	

Table 3.4.1.7 Correlation of Mcl-1 mRNA expression with other markers. (Chi-squared analysis).

Variable	Mcl-1 mRNA neg (%) n=6	Mcl-1 mRNA pos (%) n=89	P value
Bag-1 mRNA			
Absent		19.4% (n=72)	P=0.276
Present	100% (n=5)	80.6%	
Bcl-2 mRNA			
Absent		20% (n=70)	P=0.225
Present	100% (n=6)	80%	
MRP Protein			
Absent	16.7%	45.5%	P=0.169
Present	83.3%	54.5%	

Table 3.4.1.8 Mcl-1 mRNA related to patient outcome. (Chi-square analysis).

Variable	Mcl-1 mRNA neg (%) n=	Mcl-1 mRNA pos (%) n=	P value
5-year relapse free survival	80%	51.9%	P=0.222
No	20%	48.1%	
Yes (did not relapse)			
5-year survival			
No	80%	64.9%	P=0.491
Yes (survived 5 years)	20%	35.1%	

3.4.1.9 Bag-1

Bag-1 was expressed in 50% of normal tissues. The level of expression was quite low in these normal samples compared with tumour samples. The tumour corresponding to normal sample one (T1N1) was positive. Bag-1 was expressed in 128 of 143 tumour specimens. RT-PCR results are displayed in appendix section 7.3.4.

Chi-squared analysis revealed that bcl-2 mRNA expression had significant correlation with bag-1 mRNA (P=**0.003**). Previous reports have proposed that bag-1 binds and enhances the function of bcl-2 and bcl-xL. It also binds the oestrogen, androgen and glucocorticoid receptors, however it did not associate with ER status in this study (P=0.779) (Tang et al., 1999, Brimmell et al., 1999).

3.4.1.8 Summary of overall results from clinical study.

Table 3.4.1.9 displays the markers that had significant associations with disease free and overall survival. The survivin protein and its subcellular (nuclear) location was a significant marker of good prognosis. Bcl-2 and ER status were also indicators of favourable prognosis. Lymph node status and tumour grade were indicators of unfavourable outcome.

Table 3.4.1.9 Summary table of Kaplan-Meier analysis on breast tumour biopsies.

Parameter	**Disease free survival Log Rank (p value)**	**Overall Survival Log Rank (p value)**
Bcl-2 mRNA	**0.0273 (G)**	**0.0482 (G)**
Bax mRNA		
Bag-1 mRNA	0.3188	0.4717
Mcl-1 mRNA	0.8988	0.8261
Age	0.6640	0.7592
ER	**0.0020 (G)**	**0.0069 (G)**
Tamoxifen	0.8182	0.7463
Chemotherapy	0.1432	0.2387
Size (<2, 2-5, >5)	0.1034	0.0651
Grade (1,2,3)	**0.0001 (B)**	**0.0001 (B)**
Grade (1+2 v. 3)	**<0.0001(B)**	**<0.0001(B)**
LN status	**0.0002 (B)**	**0.0035 (B)**
Parameter	Disease free survival Log Rank (p value)	Overall Survival Log Rank (p value)
MRP-1 protein +/-	0.4899	0.2467
MDR-1 protein	0.7699	0.9817

Note: (G) = good/ favourable prognosis

(B) = bad/ poor prognosis DFS = disease-free survival = relapse-free survival

3.4.1.10 Conclusion.

The objective of this study was to evaluate the significance of a number of clinicopathological factors with the expression of apoptosis and multi-drug resistance associated parameters. In many studies carried out to date, the significance of such cancer-related markers has only been assessed using immunohistochemistry. Here we present results using RNA extracts from a series of archival breast tumour biopsies which were used for RT-PCR reactions assessing the expression of a number of bcl-2 family genes.

Statistical analyses revealed that bcl-2 correlated with survival, and bag-1 mRNA expression in the breast tumours. Bcl-2 was expressed in 89% of the tumour biopsies (n=147).

Bax did not correlate with any other marker tested. It was expressed in almost all tumours assessed (99%, n=148).

The mcl-1 gene transcript was found in 95% of tumours (n=143). Of the apoptosis-related genes tested, it displayed most associations with the other markers investigated. It correlated with tumour grade (no mcl-1 associated with lower grade) and was borderline significant when tested for association with lymph node status, survivin-δ3 mRNA and MRP-1 protein expression. Mcl-1 protein expression is currently being assessed.

Bag-1 correlated with bcl-2 expression. Bag-1 was expressed in 90% of tumours (n=143). It did not prove to associate with any other marker tested.

This study is unique in that it is the first time that such a wide range of markers have been tested on such a large number of archival breast tumour biopsies.

Section 4.0: Discussion.

4.1 Analysis of apoptosis-related gene expression in DLKP variants.

Apoptosis is a tightly regulated physiological mechanism governing the selection of cells to die. Cancer cells develop mutations enabling them to evade the tendency to die (See section 1.2 & 1.4). This first became apparent when the bcl-2 gene was identified in B-cell follicular lymphoma cells (Vaux et al., 1988). It enabled cells to escape death in response to stimuli that would normally induce cell death. Since then, a whole panel of homologous genes have been identified and classified according to whether they promote or protect against apoptosis. Together with the caspase family genes, the initiators and executioners of apoptosis, an intricate web of interactions forms, determining the fate of the cell.

The expression of these genes, or at least an imbalance of their expression, predisposes the cells to tumorigenic conversion and resistance to chemotherapeutic drugs (Roy et al., 2000, Schmitt et al., 1997). The focus of this project was to identify such genetic changes that underlie drug resistance and to investigate the impact of increasing or decreasing their expression in a panel of drug resistant and sensitive cell lines.

4.1.1 Analysis of bcl family genes using RNase protection assay.

The RNase protection assay allows for the simultaneous detection of several genes, thus allowing multiple determinations from a single RNA sample. The technique allows comparative analysis of different mRNA species within samples that can be compared to housekeeping gene transcripts. In this way, the level of individual mRNA species can be compared between samples.

As a technique, RPA has been used in a wide variety of studies in conjunction with RT-PCR, Northern and Western blotting. In this study, RPA was used as an initial evaluation of the expression of apoptosis related mRNA species in a panel of drug resistant and sensitive variants of DLKP cells and in panels of bcl-xL and caspase-3 ribozyme transfectants. Many reports in the literature have demonstrated that the tendency of a cell towards apoptosis is modulated by the ratio of anti-apoptotic versus pro-apoptotic gene expression (Section 1.4). The DLKP cell lines analysed displayed phenotypic variation with regard to drug resistance and RPA revealed some of the underlying genotypic changes. These initial studies revealed that of pro-apoptotic genes,

bax had highest expression. Mcl-1 had highest expression of the anti-apoptotic genes. It is possible that some association may exist between these genes as a study by Borner et al., found that the expression of mcl-1 and bax correlated in 49 NSCLCs (Borner et al., 1999). Indeed, the two proteins, bax and mcl-1, are thought to heterodimerise and neutralise each other (Sato et al., 1994, Zhou et al., 1997).

The advantages of the RPA lie in its ability to display a spectrum of genes from a very low amount of RNA. In addition, multiple RNA samples can be analysed at once. As a predecessor to DNA microarray technology, it is a less expensive, alternative approach to pinpoint gene expression in a number of related gene families. However, literature searches have indicated that few researchers use it as an alternative to RT-PCR or Northern blotting. Using RPA it proved difficult to obtain consistent trends in gene expression as the results in the sensitive and resistant variants of DLKP proved.

This technique was a useful tool at displaying gene expression trends but should only be considered an initial approach to identifying genes of interest to cancer biology. In retrospect from my experience gained in the course of this thesis, I would recommend RT-PCR rather than RPA for surveying mRNA expression profiles. The advent of DNA microarray technology, while still in its infancy in research labs, will probably be used in preference to RPA as an indicator of the expression of large groups of genes.

4.1.2 Bax transfection into DLKP-SQ cells.

The bax gene is associated with increasing the tendency of cells towards apoptosis and is often upregulated when cells are stimulated to die (Schmitt et al., 1997, Xiang et al., 2000, Pastorino et al., 1998, Sugimoto et al., 1999, Weinmann et al., 1997). Surprisingly, results from previous studies in this lab, had identified bax upregulation in an adriamycin-pulse-selected variant of DLKP-SQ, namely A250-10p cells (NicAmhlaoibh, PhD 1997). Bcl-xL was also upregulated in these resistant cells. To attempt to establish the significance of this increase in bax expression, bax cDNA was transfected into DLKP-SQ cells.

Two concentrations of selection agent (hygromycin) were used in the transfectants, based on a study by Richard Bertrand's group where increasing the selection agent resulted in a higher level of expression of the induced gene (Schmitt et al., 1997). Despite the differences in selection agent used, no consistent change in bax mRNA and protein expression could be detected in either set of transfectants.

Studies by other research groups indicated that increasing bax levels increased the tendency of cells to die (Sugimoto et al., 1999, Pastorino et al., 1998). This was seen where cells were treated with cytotoxic drug (bax transfection in IMC-3 cells resulted in increased sensitivity to cisplatin in cisplatin-resistant cells – Sugimoto et al., 1999) and also where MCF-7 cells transfected with bax had restored sensitivity to serum starvation and fas-ligand triggered apoptosis (Bargou et al., 1996). Bax is a pore-forming protein. It is thought to mediate apoptosis via caspase activation. Transfection of bax into COS-7 cells caused the activation of caspase-3-like proteases (Kitanaka et al., 1997). In the current study, when bax transfectants (Bax120 and Bax200) were analysed by RPA for their expression of caspase family genes, there did not appear to be any change in levels of the caspase-3-like proteases, i.e. caspase-2 and caspase-7. This, presumably, was due to no consistent increases in bax RNA and protein in either mixed population.

As mentioned, results from the bcl-xL ribozyme studies in this lab showed no down-regulation in bcl-xL RNA expression detected by RT-PCR or protein expression detected by Western blot but dramatic changes in resistance profiles or growth rates (Dr. Carmel Daly, personal communication). Therefore, despite the absence of changes in RNA or protein expression in the bax transfectants, toxicity assays and TUNEL assays

were carried out to determine any phenotypic effects. Neither of the bax transfectants displayed a change in resistance to chemotherapeutic drugs tested. In addition, there was no increased apoptosis observed when cells were analysed by TUNEL assay.

A study by Dubrez et al. (2001) reported that caffeine caused bax to translocate from the cytosol to the mitochondria and trigger apoptosis. Both sets of bax transfectants were treated with caffeine and then analysed by standard toxicity tests. Caffeine is a methylxanthine – a group of compounds that increase the sensitivity of cells to DNA damage (Busse et al., 1978). It prevents cells from arresting at the G2/M phase of the cell cycle, which normally enables them to repair damaged DNA. Caffeine treatment resulted in increased apoptosis in not only the bax transfectants but also the parent, DLKP-SQ cells.

The heterogeneous nature of the mixed populations of both sets of bax transfectants could have masked the emergence of interesting clones. Weinmann and coworkers isolated twelve clones from BH-41 cells transfected with bax. They found variation in the levels of bax expressed. The bax expression levels were directly proportional to the percentage of apoptosis of each clone induced by treatment with IgM (Weinmann et al., 1997). This paper highlighted the differential expression levels of an induced gene in mixed populations. However, due to no phenotypic changes in both Bax120 and Bax200, no clones were isolated and no further analysis was carried out.

4.1.3 Mcl-1 transfection into DLKP cells.

Most studies on mcl-1 to date have centred on its role in apoptosis resistance in hematopoietic cell lines. The gene was originally identified from myeloid leukaemia cells undergoing differentiation (Kozopas et al., 1993). The short half-life of the protein of approximately 30 minutes to 3 hours is thought to confer on the cells an ability to respond rapidly to an apoptotic stimulus, until those with longer half-lives, such as bcl-2 (T ½ = 10 hours) can be recruited. DNA damaging agents such as UV irradiation or cytotoxic drugs induce bcl-2 to decrease in the first few hours following exposure, whereas mcl-1 mRNA levels are rapidly but transiently increased (Johnson, 1999). As a result of its short half-life, mcl-1 has been used in many transient transfections investigating its role in drug resistance (Schubert et al., 2001, Kuo et al., 2001, Moulding et al., 2000, Katoh et al., 1998, Leuenroth et al., 2000, Kozopas et al., 1993).

In our experiments, transient transfection of mcl-1 into DLKP cells revealed increased RNA at 24 and 48 hours. The phenotypic effects of this increase however could not be established and stable transfectants were set up. The stable transfectants revealed an increase in mcl-1 protein that was accompanied by an increase in resistance to adriamycin and etoposide, in mixed populations. Indeed, some of the drug resistant variants of DLKP illustrated increases in mcl-1 levels when analysed by RPA.

Such an increase in resistance to etoposide, as was seen in DLKP-Mcl-1, has been widely reported in cells transfected with mcl-1 (Zhou et al., 1997, Moulding et al., 2000, Wang et al., 1999b). A study by Zhou et al. (1997) reported that cells continuously exposed to etoposide had decreased viability when no mcl-1 was induced, however, the decrease was reduced in cells induced by dexamethasone to express mcl-1. In Zhou's study, transfectants expressing mcl-1 constitutively exhibited decreased cell death in response to etoposide and pulse exposure to etoposide resulted in mcl-1 affording partial protection from apoptosis. The integrity of the potassium channels appears to be essential to the ability of mcl-1 to prevent apoptosis as in mcl-1 transfected cells, suppressing the potassium channels in membranes led to decreased viability after VP-16 treatment (Wang et al., 1999a). It has also been reported that mcl-1 expression induced by treating leukaemia cells with etoposide resulted in decreased caspase-3 activity (Katoh et al., 1998).

Mcl-1 has been found to protect cells against apoptosis induced by other drugs. Wei and coworkers reported that cells that overexpressed mcl-1 had increased resistance to cisplatin and when the same cells were treated with antisense to mcl-1, they displayed increased sensitivity to adriamycin treatment (Wei et al., 2001). As yet, there is no evidence that mcl-1 is post-translationally phosphorylated, as is the case with the bad protein, bcl-2 and bcl-xL by treatment with cytotoxic drugs, such as taxol (Johnson, 1999).

Further analysis of the stably transfected DLKP-Mcl-1 mixed populations, revealed an increase in P-glycoprotein expression. This increase may account for the increased resistance of transfectants to adriamycin and etoposide. There did not appear to be a change in resistance levels to vincristine. If the increases in resistance levels to other drugs are attributable to increased P-gp expression, it is unusual to see no change in vincristine resistance levels. Previous studies in this and other labs where mdr-1 was decreased due to ribozyme transfection, saw decreases in resistance levels to adriamycin and vincristine, with less decreases in resistance to etoposide (Daly et al., 1996, Scanlon et al., 1994, Bertram et al., 1995). Studies involving transfection of mdr-1 cDNA into cells have also indicated that resistance levels increased to daunorubicin and vinblastine (drugs related to adriamycin and vincristine respectively) (Warren et al., 1995). To determine if P-gp is conferring this increased resistance on the cells, combination toxicity assays should be carried out with P-gp inhibitors such as verapamil or cyclosporin, or perhaps using non-P-gp substrates such as staurosporine to induce cell death.

As previously mentioned with regard to the bax transfectants, it is possible that the mixed population of DLKP-Mcl-1 contained a heterogeneous population of clones with varying levels of the transfected gene. However, despite numerous attempts, it was not possible to isolate clones from the DLKP-Mcl-1 transfectants. This may have been due to the mycoplasma infection that prevented any further analysis of the cells.

4.1.4 Bcl-xL ribozyme studies.

Previous research in this laboratory involved transfection of both DLKP-SQ cells and their resistant variant, A250-10p cells with a ribozyme targeted to bcl-xL. The resistant cells had displayed increased levels of bcl-xL and bax. No changes in bcl-xL RNA levels assayed by RT-PCR or protein levels by Western blot were evident in cells transfected with ribozyme, however phenotypic changes in both sets of transfectants were evident. Clones of A250-10p cells displayed dramatic reversals in resistance rates while those of DLKP-SQ had reductions in growth rate. Northern blot analysis revealed the emergence of a lower 0.9kb band in ribozyme-transfected clones as well as the 3kb band representing bcl-xL. The identity of this extra band could not be established.

The RPA probe protected the 5' end of the bcl-xL RNA, from bases 185-548 (Pharmingen). It was speculated that the Northern blot "extra band" was a cleavage product of bcl-xL generated by ribozyme activity. This band was thought to represent the 3' end of the bcl-xL gene due to its size (0.3kb) and the position of the ribozyme cleavage sites (Rz1 cleaved at 548 and Rz1 at 617).

Previous studies have illustrated that bcl-xL (and bcl-2) cleavage by caspases (caspase-3) generated stable, pro-apoptotic fragments. In general, the cleavage of bcl-2 and bcl-xL proteins produced a pro-apoptotic fragment which when transfected into cells, induced apoptosis (Fujito et al., 1998a, Uhlmann et al., 1996). In many cases, stable transfectants expressing the caspase-cleaved products of bcl-xL and bcl-2 could not be established, perhaps due to their lethality in the cells or cell line selection. Therefore, if the extra band seen by Northern blot in our panel of cells did represent a cleaved fragment of bcl-xL it could account for the reduction in resistance levels in the A250-10p clones and the reduction in growth rate in the DLKP-SQ clones.

No other studies have indicated the emergence of a proposed RNA cleavage product in response to transfection with ribozyme. However, Clem et al (1998) did mention the production of an expected 16kDa protein but also of a smaller unidentified fragment when radiolabelled bcl-xL protein was incubated with apoptotic cell extracts. They postulated that this fragment could have been the product of another caspase enzyme's activity from the apoptotic cell extracts and their experiments did not distinguish between caspase-3 and DEVD-sensitive enzymes (See section 1.5).

RPA analysis was carried out on the transfectants to eliminate the possibility that this "extra band" was another known bcl-2 family member. Because the bcl family promote or prevent apoptosis through a series of dimerisation reactions, it was entirely possible that reducing one member, in this case bcl-xL, could result in a compensatory mechanism where another bcl family member was increased.

In this thesis, the possibility that the pattern of expression of bcl family members was altered in the bcl-xL ribozyme transfectants was investigated by RPA. The reduction in bcl-xL levels seen by RPA in A250-10p clones suggested that the balance of bcl family members had shifted towards increased expression of mcl-1, bcl-2 and bax, and decreased expression of bad. In contrast, in DLKP-SQ clones, bcl-xL expression appeared to be increased as did bcl-2 and bad, while mcl-1 expression was decreased. It has been demonstrated that bad and bcl-xL heterodimerise with each other more strongly than bad and bcl-2 (Yang et al., 1995). Mcl-1 and bad have been reported to bind to each other (Bae et al., 2001, Leo et al., 1999). Such a dimerisation has reportedly enabled bax homodimers to form and induce apoptosis (Yang et al., 1995). It is via the BH3 domains that pro-apoptotic proteins such as bax and bak dimerise with bcl-2 and mcl-1, and depending on the ratios of each, can overcome the anti-apoptotic effects of bcl-2 expression (See section 1.4). Reports have also indicated that where bcl-2 expression is high, mcl-1 expression is low (Soini et al., 1999). In the DLKP-SQ clones, mcl-1 was decreased relative to parent whereas bcl-2 and bcl-xL had increased. The pro-apoptotic protein bad was also increased. The indications are that the introduction into cells of ribozyme to bcl-xL upset the balance of bcl family members in the cells, perhaps favouring the formation of homodimers of bax or another pro-apoptotic protein and facilitating increased sensitivity in the A250-10p clones or retarding growth rate in the DLKP-SQ clones.

Subsequent work has established tetracycline-inducible clones expressing the bcl-xL ribozymes and the emergence of the extra band by Northern blot is currently being investigated (Isabella Bray, personal communication). Given the suspected identity of the 0.9kb band, a more specific RNase protection assay could possibly be fashioned using probes to full-length bcl-xL, the 5' region of the gene (up to the Rz1 cleavage site) and the 3' region thought to be represented by the 0.9kb fragment (from Rz1 cleavage site to 3' end). These probes could be generated by PCR and subsequently

used in RPA. Such a technique would specifically target the proposed fragments of the bcl-xL gene and perhaps establish the identity of the extra band. Dorai and coworkers utilised a similar technique to assess the ability of a ribozyme to bcl-2 to cleave a number of bcl-2 mRNAs of various sizes (Dorai et al., 1997).

The RPA studies did reveal some interesting findings as mcl-1 and bad had opposing expression trends in the DLKP-SQ and A250-10p clones. The significance of these expression patterns is not known but presumably contributes to the phenotypic changes seen in both sets of cell lines. It may be worth investigating mcl-1 and bad expression in the tetracycline-inducible clones to examine if their levels are affected by ribozyme transfection.

4.1.5 Caspase Family expression in DLKP variants.

Levels of caspase family genes were screened using the RPA to select for a suitable cell line for ribozyme transfection. RPA analysis of caspase family expression in DLKP variants revealed an inverse relationship between adriamycin resistance and caspase-3 levels. This correlated with previous findings where caspase-3 levels were analysed using Western blot (Colette O'Loughlin, PhD 1999). The cell lines involved DLKP-SQ, A250-10p and DLKP-A5F were approximately 1, 10 and 300-fold resistant to adriamycin respectively. Other researchers have also reported that caspase-3 expression was decreased in P-gp-overexpressing cell lines and in aggressive tumours (O'Loughlin, PhD 1999, Ruefli et al., 2000, Johnstone et al., 1999).

As already mentioned, analysis of the bax transfectants using RPA did not display changes in the expression levels of caspase family genes. The other resistant variants studied, DLKP-Melph and VP-3 had decreased levels of all caspases, presumably due to their increased drug resistance. This was also reported by other researchers studying drug resistant cell lines (Fulda et al., 1998, Ruefli et al., 2000, Johnstone et al., 1999).

4.2 Analysis of caspase-3 ribozyme and transfectants.

4.2.1 Cell line selection.

The objective of the current study was to investigate the role of caspase-3 in doxorubicin-induced apoptosis. We observed a decrease in caspase-3 expression in cells selected with doxorubicin, with lowest levels of caspase-3 found in the most resistant cells, DLKP-A5F. These cells are a clonal population of DLKP-A cells, (DLKP cells made resistant by continuous selection in adriamycin – see appendix 7.1 for details) (Heenan et al., 1997).

The molecular mechanisms underlying Pgp-mediated inhibition of caspase-3 activation are not known. In an attempt to eliminate completely the expression of caspase-3 from cells, the DLKP variant with lowest levels of caspase-3, DLKP-A5F, was chosen. In this way, any changes in drug resistance observed could not, in theory, be overcome by any underlying caspase-3 activity. Because of the low levels of caspase-3 expressed in DLKP-A5F, it would be easier to eliminate caspase-3 expression completely. The MDR-1 ribozyme transfectant, DLKP-A2B-1C7, which has complete loss of MDR-1 expression due to mdr-1 ribozyme transfection was also chosen for transfection (Daly et al., 1996).

There are many studies that document a down-regulation of caspase-3 in Pgp overexpressing cells and more aggressive cancers (O'Loughlin, 1999, Johnstone et al., 1999, Ruefli et al., 2000). Drug resistant cervical cancer cells had reduced levels of caspase-3 (Ding et al., 2000). Procaspase-3 expression has been correlated with decreased incidence of lymph node metastases and longer median survival in patients with non-small cell lung cancer (Mow et al., 2001). Ruefli and coworkers found that Pgp+ cells were less sensitive to those nuclear apoptotic events that occur following caspase activation (Ruefli et al., 2000).

It is suspected that in our Pgp+ cell lines, apoptosis occurs via caspase-independent mechanisms (O'Loughlin, 1999). Transfecting a ribozyme targeting caspase-3 mRNA therefore aimed at confirming such caspase-independent cell death in our cell lines. We constructed a ribozyme to caspase-3 to generate a loss of function phenotype and thus to elucidate the role of the gene in drug resistance in our cell model.

4.2.2 Ribozyme selection & design.

A website search revealed two other groups had previously used a caspase-3 ribozyme, both in neurological studies. Eldadah and coworkers constructed a hammerhead ribozyme to rat caspase-3. They found that ribozyme expression conferred protection against apoptosis at 24hours post-transfection (Eldadah et al., 2000). Xu and coworkers also used a ribozyme to rat caspase-3. Ribozyme transfected PC12 cells had increased protection induced by low levels of 6-hydroxydopamine when compared with non-transfected cells (Xu et al., 2001).

We assessed whether or not either of these ribozymes could be used to target human caspase-3. When rat and human mRNA sequences were aligned however, it was found that the rat ribozyme did not cleave at a target site common to human caspase-3. Therefore, we designed our own caspase-3 ribozyme. We chose to use a hammerhead ribozyme to target caspase-3.

To eliminate the possibility that our ribozyme was targeting another caspase, all human caspase sequences were aligned and studied. Using BLAST, (Blast computer program, NCBI homepage), it was found that the ribozyme should exclusively cleave caspase-3. In addition, RNase protection assay detected no shift in the balance of caspases when transfected cells were compared with parent cells. Therefore in vivo, the ribozyme does not appear to target another caspase enzyme.

When choosing a ribozyme target site, it is desirable to choose a site where the RNA is looped as this should allow the ribozyme, once in the cell, to access and cleave the single stranded RNA. GUC target sites are believed to be the best target (See section 1.7.1). There are a total of six GUC sites in the caspase-3 mRNA. When these sites were analysed using structure dot plot analysis for mRNA of human caspase-3 (Zuker m-fold program website), it was found that loops occur in all predicted structures around nucleotides 200, 250 and 750.

Four suitable ribozyme target sites were selected at positions 192, 205, 252 and 743. During activation, the caspase-3 protein is cleaved into p12 and p20 subunits and then p20 is further cleaved into p17. Cleavage occurs initially at the IETDS at amino acid

residue 172. The removal of the short prodomain follows, the function of which is to prevent spontaneous activation of the inactive zymogen in mammalian cells. This removal occurs at ESMDS residues at position 25 (Section 1.5). Ribozyme 1 was chosen to target position 205 of human caspase-3. This area lies within the potential p17 subunit of the protein.

The ribozyme had 12-14 base sequences flanking either side of its catalytic site. This ensured that they were long enough to ensure specific interaction with target but short enough so as to ensure a catalytically efficient reaction. Once ribozymes have cleaved their target sequence, they should be able to dissociate and then bind to another target site, thus continuing the cleavage reaction (James & Gibson, 1998).

4.2.3 The caspase-3 ribozyme was capable of cleaving CPP-32 *in vitro*.

Measurements of ribozyme activity described in this thesis (Section 3.2.3) have proved that this ribozyme targets and cleaves human caspase-3. An *in vitro* cleavage assay demonstrated that the purified ribozyme was capable of cleaving the target sequence *in vitro*. The cleavage assay is technically very challenging as the ribozyme activity is dependent on optimal conditions. In addition, a ribozyme that appears to work *in vitro* may not necessarily cleave its target *in vivo*. Hammerhead ribozymes depend on divalent cations for binding and cleavage of their substrate (Gewirtz et al., 1998). *In vitro*, they work most efficiently at concentrations of 10 to 20 mM. However, the intracellular environment has a much lower magnesium concentration of approximately 800uM (James and Gibson, 1998). It is likely that this lowered concentration of magnesium, as well as the intracellular environment, reduces the effectiveness of the ribozyme's activity. The magnesium gradient in Section 3.2.3, illustrates exactly how the ribozyme is affected by magnesium concentration. Each cleavage reaction at different magnesium concentrations showed a bleed of signal at 24hours. This was probably due to ribozyme activity taking place at suboptimal conditions. *In vitro* cleavage of CPP-32 target cDNA proceeded at an optimal concentration of 10mM MgCl-2 – consistent with the report by James and Gibson (1998).

4.2.4 Ribozyme 1 transiently down-regulates caspase-3 mRNA and protein.

The effectiveness *in vivo* (i.e. with living cells) of our ribozyme was illustrated by developing a transient transfection assay. The use of transient transfection, particularly in apoptosis research, has been widely reported (Wrone-Smith et al., 2001, Los et al., 1997, Katoh et al., 1998, Townsend et al., 1999, Kuo et al., 2001, Friedrich et al., 2001). It is a useful tool to illustrate the short-term effects of induced gene expression and has become a fast and effective method in our lab for investigating gene function.

To achieve high expression efficiency, most studies use viral vectors thereby obtaining very high viral replication and hence induced gene expression (Friedrich et al., 2001). By optimising transient transfection, a high level of transfection efficiency was achieved.

Many researchers have also used mammalian expression vectors in their transient transfections. In cells such as Hela cells, transfection efficiencies of up to 40% were achieved (results not shown). The DLKP variants were also readily manipulated via transfection and a transfection efficiency of approximately 35% was achieved in these cells. This level of transfection enabled sufficient expression of ribozyme to decreases in caspase-3 at both the mRNA and protein levels in transiently transfected cells. This assay had advantages over an *in vitro* cleavage assay because as mentioned previously, IVC is technically quite difficult to work and may not always be representative of the ribozyme's effectiveness *in vivo*. Other studies where cDNA and ribozyme to rat caspase-3 were investigated using transient transfection to illustrate the ribozyme's efficacy (Eldadah et al., 2000, Wrone-Smith et al., 2001, Xu et al., 2001).

Numerous attempts were made to elucidate the phenotypic effects of reducing caspase-3 levels in cells. A standard toxicity assay was first used to assess the effects of ribozyme on drug resistance. Drug addition was staggered, i.e. drug added 24, 48 & 72 hours post transfection, to attempt to target the window period of down-regulated caspase-3. Despite numerous experiments, a consistent IC50 value could not be obtained, although the results did show a trend towards increased sensitivity. In two separate experiments, cells showed a dramatic reversal in drug resistance, almost to that of parent DLKP cells.

Technically, the toxicity assay was quite difficult to optimise especially as cells were being monitored over seven days when presumably, caspase-3 levels were reverting back to that of parental cells. There is a study by Los et al. (1997) where transient transfection was used together with short-term (36hour) selection in geneticin. Further studies in this lab will employ a similar technique. Such a method may facilitate drug resistance investigation as the effects of transfection could perhaps be monitored by the standard toxicity assay.

4.2.5 Analysis of α-fodrin cleavage in transiently transfected cells.

α-fodrin is a major component of the cytoskeleton of most eukaryotic cells. It has the ability to bind actin, calmodulin and the microtubules. It has been well documented that during apoptosis, a wide variety of substrates are cleaved including α-fodrin, PARP, Rb, PAK-2, DNA-PKcs, gelsolin and DFF-45 (Section 1.6.1). Caspase-3 is essential for DNA fragmentation and morphological changes in apoptosis; i.e. cell blebbing and formation of apoptotic bodies (Janicke et al., 1998). During apoptosis, fodrin is cleaved in a biphasic manner. This was displayed in Jurkat cells induced to die by fas ligation where at 30 minutes a 150kDa product appeared followed by a 120kDa product at 50 minutes (Greidinger et al., 1996). The former product is a consequence of calpain (a serine protease that acts independently of caspase enzymes), as well as other caspase enzyme activation (Martin et al., 1995).

Caspase-3 is responsible for α-fodrin cleavage creating the 120kDa product specifically. Caspase-3 cleaves at a precise and characteristic sequences in α-fodrin (Cryns et al., 1996). The MCF-7 breast carcinoma cell line is naturally caspase-3 negative. Studies investigating the contribution of caspase-3 to apoptosis have found that when these cells are induced to die using TNF-α or staurosporine, only cells transfected with cDNA to caspase-3 are capable of producing the 120kDa cleavage product of α-fodrin (Cryns et al., 1996, Greidinger et al., 1996, Janicke et al., 1998a, Martin et al., 1995, Wang et al., 1998). In addition, Wang et al. utilised recombinant caspase enzymes to digest cell lysates. They found that all caspases generated the 150kDa product, but only caspase-3 produced significant levels of 120kDa (Wang et al., 1998).

Previous studies in this lab by Dr. Colette O'Loughlin, showed that DLKP-A5F cells undergo apoptosis without PARP cleavage, however they did display fodrin cleavage (O'Loughlin, PhD 1999). Cells were treated with adriamycin and then harvested 45 hours post-treatment. Results showed that adriamycin did in fact induce cleavage of α-fodrin, indicated by an increase in the production of the 120kDa band. It therefore appeared that caspase-3 was active in these cells.

In ribozyme transfectants, there did not appear to be any difference in levels of the 120kDa cleavage product compared with reversed ribozyme or non-transfected cells. This result was surprising as α-fodrin is specifically cleaved by caspase-3 and a

reduction in caspase-3 due to ribozyme should, in theory, lead to a reduction in the levels of the 120kDa cleavage product of α-fodrin. This result is interesting but difficult to explain and reasons as to why this occurred can only be speculated. It could indicate that the reduced caspase-3 levels were not sufficiently decreased to display changes in α-fodrin cleavage levels. It is also possible that ribozyme activity decreased pro-caspase-3 levels thereby increasing the concentration of active caspase-3, in which case enough active caspase-3 would still be cleaving α-fodrin. However, there was an increase in the production of the 150kDa cleavage product - perhaps a compensatory action by the cell to enable similar levels of apoptosis to take place in response to drug treatment as this is due to the activity of other caspases and calpains (Wang et al., 1998). This could explain why in the TUNEL photographs, there does not appear to be any appreciable decrease in the number of apoptotic cells between control transfectants and ribozyme transfectants (See section 4.2.6).

Many of the studies investigating the role of caspase-3 in apoptosis have used caspase-3-specific inhibitors (Section 1.6.2). One such study in neuronal cells found that even in the absence of caspase-3, apoptosis could still occur although its morphological hallmarks were not observed (Tanabe et al., 1999). In this study, it was concluded that death occurred via non-apoptotic means. It is possible that adaptation from one type of death pathway, due to knockout of a key protein, may render cells more susceptible to alternative death pathways. Again, the increased 150kDa product of α-fodrin may be due to death occurring via an alternative pathway, in this case one involving an increase in calpain activity. The possibility that another protease, yet to be discovered, is being activated by caspase-3 down-regulation producing the 150kDa product cannot be ruled out either.

4.2.7 Analysis of stably transfected caspase-3 ribozyme clones.

Our studies on mRNA levels have shown decreased caspase-3 by RT-PCR and protein in both transient transfection and in some of the stably transfected clones. One surprising result was that RT-PCR results did not agree with the levels of caspase-3 seen by RNase protection assay.

There are two possible explanations for such disagreement. As PCR is a highly sensitive technique, it will detect even the smallest changes in expression levels. Moreover, our PCR primers were designed to target the caspase-3 mRNA surrounding the ribozyme cleavage site. Therefore any changes in expression levels were detectable and, due to the sensitive nature of this technique, were illustrated by, in some cases, complete loss of caspase-3. In contrast to this, the probe for caspase-3 in the Riboquant RNase protection assay targets human caspase-3 between location 911- 1230 (Pharmingen). Therefore, the less sensitive RNase protection technique may be picking up both cleaved and uncleaved caspase-3. Because of this, changes in caspase-3 levels induced by ribozyme are not evident on the RPA. Again, this suggested that RT-PCR should be used in preference to RPA when analysing gene expression profiles.

The RNase protection assay was used to estimate whether or not the down-regulation of caspase-3 seen by RT-PCR was compensated for by the cells, via a shift in the balance of caspase activity, i.e. by increasing levels of other group two caspases such as caspase-2 or caspase-7, for example (Greidinger et al., 1996). As the RPA graphs showed, this was not observed to be the case. It appears that no compensatory caspase activation occurred in the clones.

4.2.8 Stable transfectants illustrated a reversal in their resistance to cytotoxic drugs.

Western blots on protein extractions from later passages, as displayed in the results section, did not reveal a down-regulation of caspase-3. There are a number of possible reasons for this. It has been documented that certain antibiotics interfere with the effectiveness of hammerhead ribozymes. Antibiotics disrupt the intracellular magnesium concentration, essential to the ability of the ribozyme to act efficiently (James & Gibson, 1998, Clouet-d'Orval et al., 1995, Stage et al., 1995, Rogers et al., 1996, Olive et al., 1995, Murray & Arnold, 1996, Hermann & Westhof, 1998).

As the caspase-3 ribozyme was subcloned into the pTARGET vector, which contains a neomycin B resistance gene, cells were selected using 600ug/ml of geneticin (G-418, Gibco). They were continuously cultivated in selection agent to maintain a cellular population containing the expression vector. It is possible however, that this continued cultivation in the presence of antibiotic, did in fact interfere with ribozyme activity, and may have played a part in the reversal of resistance levels to cytotoxic drugs seen in the parent and clonal populations. However, it cannot be solely responsible for reversal of drug resistance because later passages of stably transfected clones maintained reduced levels of caspase-3, attributable to the ribozyme's activity.

It is also possible that the cells' regulatory mechanism increased caspase-3 transcription to compensate for ribozyme activity - RT-PCR results on later passages did not display any significant reduction in caspase-3 levels. It could be interesting in the future to determine the active caspase-3 protein levels in the clones as this could indicate how the ribozyme affected the activity of the enzyme.

4.2.9 Does down-regulation of caspase-3 change the drug resistance profiles of stably transfected clones?

One would expect that down-regulating CPP-32 expression, a key executioner, would increase the resistance of cells to apoptosis. For this reason, caspase-3 has been the target of much research particularly into therapeutics of neurological diseases. CPP-32-knockout mice were smaller than their littermates, had neurological abnormalities and visible masses on their heads. These mice only survived 4-5 weeks. CPP-32 negative cells had increased resistance to CD-95 and CD-3 induced cell death (Kuida et al., 1996). In addition, neutrophils, negative for CPP-32, were resistant to apoptosis stimulated by treatment with cyclohexamide (Woo et al., 1998).

Hepatocytes negative for CPP-32 had altered morphology (Zheng et al., 1998). During fas-mediated apoptosis, there was no cytoplasmic blebbing, no detectable nuclear fragmentation, irregular chromatin clumping and slower breakdown of the DNA. Similar findings were seen in CPP-32 negative thymocytes.

Los and coworkers used antisense to caspase-1 & -3 in a panel of sensitive and resistant cell lines. They report that transfectants with antisense to caspase-1 were able to proliferate but mitotic divisions were infrequently observed in transfectants with antisense to caspase-3. They did observe that expression of the constructs promoted cell survival. Interestingly, when P-gp expression was assessed, highest levels were seen in sensitive cell lines (Los et al., 1997).

In all these studies, despite the reduced levels of CPP-32, apoptosis could still proceed, due possibly to other caspases compensating for the lack of CPP-32 or perhaps via a mechanism where the caspases are redundant in function.

Despite initial findings whereby caspase-3 ribozyme-transfected cells had increased resistance levels to vincristine, adriamycin & etoposide, without changes in cisplatin resistance, these results could not be repeated. Similar resistance patterns were observed in DLKP variant clone, A2B-1C7-Rz1 (results not shown). However, again, cells seemed to overcome down-regulation of caspase-3 indicated by a loss of changes in resistance levels, once experiments were repeated. (Experiments in both sets of cell lines were set up independently and on separate weeks).

This suggested that the cells developed a mechanism to compensate for the previously decreased levels of caspase-3. However, the recurrence of caspase-3 expression accompanied a reversal in toxicity levels, back to those of the parent cells, which indicated that initial increases in resistance profiles were in fact due to decreases in caspase-3 levels.

It is possible that any change in toxicity due to caspase-3 reduction may be contributing to the decreased resistance seen in clones. As previously discussed with respect to transient transfection, an explanation for this may be that ribozyme is in fact only targeting pro-caspase-3 rather than active caspase-3. Since caspase-3 is thought to be involved in an amplification loop (Slee & Martin, 1998), whereby it multiplies the impact of a given death stimulus, reducing the caspase-3 zymogen could increase the ratio of inactive versus active caspase-3. The intracellular level of procaspase-3 could play a key role in the impact of apoptotic stimuli.

4.2.10 P-gp levels in stable transfectants.

Further investigation as to why resistance profiles were reversed revealed that mdr-1 levels were decreased in the cells. This was shown by Western blots in early and later passages. In clones of A5F-Rz1 cells, we observed a decrease in mdr-1 levels and an enhanced sensitivity of cells to chemotherapeutic drugs. The cause of this decrease in mdr-1 is as yet unknown. In clones that reverted back to resistance levels of the parent, perhaps a decrease in mdr-1 levels led in some way to increased caspase-3. Johnstone and coworkers reported that the inhibition of Pgp function restored caspase-3 activation upon crosslinking of cell-surface Fas. They found that treatment of Pgp+ and Pgp- cells with soluble, recombinant FasL also resulted in caspase-dependent apoptosis of Pgp- cells, whereas Pgp+ cells were relatively resistant (Johnstone et al., 1999). Such results suggest a direct relationship between mdr-1 and caspase-3. In such a case, it is possible that ribozyme-induced down-regulation of caspase-3 could be overcome and masked by an increase in caspase-3 due to decreased mdr-1. This is contrary to initial findings in this laboratory that drug resistant cells (DLKP-SQ-A250-10p, DLKP-A2B & DLKP-A5F) where those with highest mdr-1 had lowest caspase-3 (O'Loughlin, PhD 1999).

There is a report of transient transfection of bcl-2 cDNA into MCF-10A cells where the bax:bcl-2 ratio was increased causing an increase in caspase-3 activation. Therefore more apoptotic cells appeared. This study speculated that bcl-2 overexpression led to the stabilisation of bax, thus enabling bax to initiate apoptosis (Kim et al., 1999). In the same way, it is possible that transfection with caspase-3 ribozyme or cDNA disrupted the balance between it and mdr-1. If a positive correlation exists between the two genes, then perhaps disrupting levels of one results in an imbalance in the other also.

4.2.11 Do P-glycoprotein and caspase-3 regulate each other's expression and/or activity?

The aim of the caspase-3 ribozyme project was to investigate the changes in drug resistance when caspase-3 was eliminated from cells. Previous studies on these drug resistant cells suggested an inverse relationship between caspase-3 and P-gp (Colette O'Loughlin, PhD 1999). The decrease in P-gp in the ribozyme transfectants was unexpected. Initial investigations centred on ensuring that the caspase-3 ribozyme did not also target P-gp. Cross checking this against other known sequences (via the NCBI database) revealed that ribozyme specifically cleaved human caspase-3.

The importance of Pgp has been illustrated in its associations with drug resistance, chloride channel activity, cholesterol metabolism, immune cell function, regulating differentiation, proliferation and survival. The ion channel activity of P-gp results in an increase in intracellular pH and a decrease in plasma membrane potential (Johnstone et al., 2001). Therefore, its expression affects a wide range of cellular functions.

P-gp has been associated with apoptosis regulation also where it is thought to protect cells against caspase-dependent apoptosis mediated by fas receptor and TNF receptor ligation, serum starvation and UV irradiation (Ruefli et al., 2002; Johnstone et al., 2000a & b). Pgp does not protect against apoptosis induced by pore-forming proteins or granzyme B, as this cell death proceeds via a caspase-independent mechanism (Ruefli et al., 2002, Johnstone et al., 2001). Functionally, P-gp is thought to efflux drugs and to inhibit caspase-dependent tumour cell apoptosis (Smyth et al., 1998, Johnstone et al., 2000 a&b, Ruefli et al., 2000). Given this information, it is possible that P-gp functions to downregulate caspase-3 or other caspase-3-like caspases (i.e. caspase-2, -7, -8, etc). The initial results in our cell model, where caspase-3 was decreased in cell lines with increasing drug resistance and hence increased P-gp levels, along with the results of Johnstone et al. (1999), Smyth et al. (1998) and Ruefli et al. (2002), could be indicative of this effect.

A study by Gollapud and Gupta (2001) used an anti-Pgp monoclonal antibody on mononuclear cells that were activated to die using anti-CD3mAb. This decrease in Pgp sparked an increase in active caspase-3 leading to apoptosis. P-gp overexpression was therefore protecting the cells from death. In the same way, reduced levels of caspase-3

in our caspase-3 ribozyme transfectants could be reversed due to loss of P-gp expression. Thus any change in toxicity due to caspase-3 down-regulation is perhaps so subtle that it is masked by the loss of P-gp.

It seems as though by a compensatory mechanism to overcome the decrease in caspase-3 due to ribozyme, P-gp was gradually decreased which accounts for the reversal of drug resistance seen in later passages of the caspase-3 ribozyme clones. This could possibly be a mechanism to maintain homeostasis in the cells.

One could speculate that some kind of interaction between caspase-3 and P-gp overcame the initial decrease in caspase-3 expression seen in ribozyme clones and led to caspase-3 levels increasing at later passages – the reverse of what has been reported in cells that overexpress P-gp and which was previously observed in our own cells (Ruefli et al., 2002, Smyth et al., 1998, Johnstone et al., 1999, O'Loughlin, PhD, 1999). Johnstone and coworkers found that P-gp overexpression resulted in decreased production of active caspase-3. They also reported that decreasing P-gp restored caspase-3 activation (Johnstone et al., 1999). Our results indicated that caspase-3 levels in later passages had increased somewhat (relative to earlier passages) but that P-gp levels in some clones had decreased. This suggested that P-gp could be regulating cell survival.

Other reports have emerged where proteins contained natural caspase-3 cleavage sites and studies showed that they were cleaved by caspase-3. Such natural substrates for caspase-3 include filamin and PMCA4b (plasma membrane Ca2+ ATPase isoform 4b) (Paszty et al., 2001, Browne et al., 2000). Analysis of the amino acid sequence of P-gp revealed two possible cleavage sites for caspase-3 (and caspase-2, -6, -7 & -8). As mentioned in section 1, caspase-3 prefers to cleave substrates with DxxD sequences, where x is any amino acid. There are two DxxD sites in P-gp – at position 97-100 is a DIND sequence, and at 164-167 is DVHD. Although speculative, the possibility that caspase-3 cleaves P-gp at the DxxD sites cannot be ruled out without analysis of the P-gp protein's folding structure. It is possible that a mechanism of survival in P-gp-overexpressing cells is to down-regulate caspase-3, thereby preventing its own possible cleavage.

Our initial aim was to eliminate completely the expression of caspase-3 in the P-gp-overexpressing cell line, DLKP-A5F, and hence investigate caspase-independent apoptosis. Together with the evidence from Ruefli's group and that of Gollapud & Gupta, one could speculate a link between caspase-3 and P-gp. However, further investigation will be needed to examine the impact of ribozyme transfection on sensitive DLKP variants (i.e. non-P-gp-overexpressing cells) as well as an unrelated cell line, perhaps a sensitive and resistant variant of HL-60 cells (HL-60 & HL-60-Adr), to ensure that the effects seen on P-gp are not specific to DLKP cells. Transfection of ribozyme into other DLKP variants could also indicate whether the changes in P-gp seen in DLKP variants was due to clonal variation or due to transfection with the caspase-3 ribozyme and cDNA.

4.2.12 The importance of investigating P-gp expression in transfection studies.

The unexpected change in P-gp expression in the caspase-3 ribozyme transfectants has been difficult to explain. It has also compromised our efforts to assess the phenotypic effects resulting from ribozyme transfection. However, the interference with P-gp expression was not unique to this transfection. In fact, it has been observed on two separate transfections in two different cell lines.

In conjunction with the caspase-3 ribozyme work, researchers Dr. Mary Heenan and Mr. John Cahill transfected caspase-3 cDNA into a DLKP variant (DLKPA2B-1C7) already transfected with ribozyme to mdr-1 (Daly et al., 1996) (approximately 300-fold more sensitive to adriamycin than A5F cells as the mdr-1 ribozyme reduced mdr-1 mRNA levels (See appendix)). They observed a marked increase in caspase-3 expression in the transfected mixed population. Despite numerous attempts, clones could not be isolated. Coincidently, this increase in caspase-3 was accompanied by an increase in mdr-1 mRNA, shown by real-time RT-PCR and semi-quantitative RT-PCR. This was not observed in control transfectants. In addition, there was no change in drug resistance profiles. It is unclear what relationship, if any, exists in these cell lines between caspase-3 and P-gp.

As mentioned, this phenomenon was also observed in an independent transfection where the anti-apoptotic gene, mcl-1, was transfected into the sensitive cell line, DLKP.

In the DLKP-Mcl-1 transfectants, P-gp levels were increased. Again, changes in drug resistance profiles due to mcl-1 overexpression could not be determined due to the P-gp increase.

Our observations indicate the importance of assessing P-gp levels in transfection studies, especially where drug resistance levels are subsequently being monitored. Control transfectants from previous studies indicated no change in P-gp or caspase-3 levels. Therefore one could conclude that the affects on P-gp were not a transfection phenomenon. In addition, different selection agents were used in both the cDNA and ribozyme transfections, (hygromycin B & geneticin, and geneticin respectively) so the effects on P-gp were probably not due to the selection procedure.

One could speculate that this effect on P-gp is perhaps specific to our cell line. However, there is some evidence to suggest otherwise. Three different variants of DLKP cells (i.e. DLKP, DLKP-A2B-1C7 and DLKP-A5F) were transfected. In each case, a knock-on effect of transfection was a change in P-gp levels. Both DLKP-Mcl-1 and DLKP-A2B-1C7-CPP-32 had increased P-gp while the DLKP-A5F cells transfected with caspase-3 ribozyme had decreased P-gp. Given that three different cell lines were transfected, this P-gp interference is probably not a result of clonal variation in the cells.

My experience with these transfection studies has certainly highlighted how much drug resistance proteins interfere with the ability of cancer cells to evade apoptosis induction. It has also stressed how important it is to investigate their expression levels before any changes in resistance levels can be attributed to transfected genes. A recommendation to other researchers, based on work carried out in this thesis, is to always check for altered P-gp levels (and possibly other multi-drug resistance proteins) in transfected cell populations.

4.2.13 Conclusions of the caspase-3 ribozyme project.

This study involved the design and construction of the first ribozyme to human caspase-3. It is also the first study where a ribozyme to caspase-3 was used to investigate drug resistance in cancer cells. Previous studies involved the use of a ribozyme to rat caspase-3 and focused on its role in neuronal cell apoptosis (Eldadah et al., 2000, Xu et al., 2001).

A transient transfection assay was optimised and utilised to assess ribozyme function *in vivo*. This ribozyme functions both *in vitro* and *in vivo*, as illustrated by *in vitro* cleavage, RT-PCR, Western blot and toxicity analysis. The ribozyme proved effective at decreasing RNA and protein with some effects on drug resistance levels. The phenotypical changes due to ribozyme transfection were also studied by analysing the downstream effects on α-fodrin and by TUNEL assay. Stably transfected cells displayed changes in both caspase-3 expression and mdr-1 expression. A combination of these factors could be held responsible for changes seen in drug resistance profiles. Alternatively, it is possible that knockout of mdr-1 caused an increase in caspase-3 activity which would counteract its loss due to ribozyme transfection. These stably transfected cells were consistently maintained in geneticin (600ug/ml) to prevent loss of expression vector.

Future work will serve to establish stably-transfected clones which will be used to investigate caspase-3-independent apoptosis in our cell model and to examine whether or not it could proceed despite the elimination of caspase-3. Exactly what proteins are recruited and how apoptosis can proceed in the absence of caspase involvement will also be investigated. Transfection of ribozyme and cDNA into other cell lines could also indicate whether the change in mdr-1 expression levels was exclusive to DLKP variants and that these cell lines have a unique relationship between caspase-3 and P-gp.

In addition, drugs that are not P-gp substrates but which still induce caspase-dependent cell death will be utilised to display apoptosis induction without interference of P-gp.

The expression of other caspase proteins between early and later passages also needs to be addressed. It is possible that other caspase proteins are compensating for loss of caspase-3 in the stably transfected clones.

4.3 Analysis of gene expression in two clonal populations of DLKP-A using DNA microarray.

4.3.1 Introduction to DNA microarray technology.

The advent of DNA array technology promises to revolutionise biotechnology and cancer biology. Such tools facilitate the simultaneous analysis of a large number of mRNAs from a single sample (Cuzin, 2001, Kashiwagi et al., 2000). Although a relatively new technique, already more accurate, more automated and more cost effective DNA microarrays are emerging. No longer a tool exclusive to large biotechnology firms, arrays are now accessible to academic research labs.

The scope of the DNA microarray is immense such that new insights into disease mechanisms and treatment targets can potentially be pinpointed. Eventually, perhaps even cancer therapy could be individualised based on a molecular profile of each patient's tumour.

4.3.2 Cell line selection.

Resistance to adriamycin is associated with alterations in gene expression (NicAmhlaoibh et al., 1999, Daly et al., 1996, Watts et al., 2001, Kudoh et al., 2000, Chang et al., 2002). The clonal populations of DLKP-A, A2B and A5F, were chosen for analysis by DNA microarray. Phenotypically, they have altered drug resistance profiles (Heenan et al., 1997).

A2B cells are 30-fold resistant to adriamycin while A5F cells are 300-fold resistant to adriamycin when compared with DLKP cells (Heenan et al., 1997). Western blotting analysis indicated that concentrations of P-gp in the cells increased with increasing drug resistance. Thus P-gp expression was reflective of their drug-resistance profiles.

In addition to their increased resistance levels, A5F cells were more invasive in vitro than DLKP parental cells or DLKP-A2B (Dr. Yizheng Liang, personal communication). To date, no metastasis-related gene or protein expression has been analysed in these cells.

A wealth of information about variation in gene and protein expression had already been uncovered in previous studies in this lab. Table 4.3.1 and 4.3.2 display findings on gene and protein expression in these two cell lines. Table 4.3.1 lists RT-PCR results illustrating variation in oncogenes and bcl family genes between the cell lines (Roisin NicAmhlaoibh, PhD 1997). Table 4.3.2 lists differences in protein expression in caspase and cell cycle related proteins (Colette O'Loughlin, PhD 1999).

Table 4.3.1 Gene Expression in A2B and A5F cells where A2B are taken as control reference cells.

Gene	A5F cells.
Bcl-2α	Down
c-fos	Up
Bcl-xL	Up
Baxαβ	Down
c-erbB2	Up
c-Ha ras1	Up

Table 4.3.2 Protein Expression in A2B and A5F cells where A2B is reference control.

Protein	A5F cells
Caspase-2	Up
Caspase-3	Down
Caspase-7	Same
Caspase-6	Same
Caspase-8	Down
Caspase-4	Same
Caspase-9	Same
P-glycoprotein	Up
STAT proteins	Same
CD95 receptor	Same
Cyclin B	Same
Cdk-1	Up
Cdc25B	Same
Cdc25C	Same

The objective of most DNA array studies is to elucidate the profile of gene expression underlying phenotypic variation between samples. The use of DNA array technology provided an opportunity

- to detect known changes in gene expression in DLKP-A2B versus DLKP-A5F and,
- to identify changes in gene expression previously not examined in this lab.

4.3.3 The Clontech human 8k DNA plastic array.

A microrarray with the potential to detect over 8000 genes was selected for analysis of the cell lines. The scope of the array detection extended far beyond the limits of known cancer and apoptosis-related genes and could detect genes encoding proteins related to transcription, translation, post-translational modification, cytoskeletal proteins, cell cycle regulators, cell ligands and receptors, signal transduction related genes, metabolic regulators, etc. (Clontech, www.clontech.com).

To date, DNA chips have been mounted on glass, plastic, nitrocellulose and nylon membranes. Glass microarrays can be used in conjunction with fluorescent probes. Glass microarrays can hold more DNA chips than the other membranes. In addition, RNA from test and reference samples can be compared on one slide (Clarke et al., 2001). Glass microarrays allow for only a single use and therefore have high initial cost. Because of this, nylon, nitrocellulose and more recently, plastic membranes have become available. The plastic membrane was chosen for use in this research. These membranes are used in conjunction with radiolabelling. Unlike glass arrays, parallel processing of test and reference samples occurs on separate arrays (Clarke et al., 2001). Such membranes have brought array technology into academic research labs as they can be used up to four times in the case of plastic arrays (www.clontech.com).

The plastic arrays used in this study were easily stripped and reused on separate RNA samples extracted from the same cell lines. As much as possible, the condition of the cells was standardised. Cells were harvested for RNA at approximately 80% confluency. However, as for all biological systems, biological noise and natural variation can replicate RNA extracts (Clarke et al., 2001). P33 instead of P32 labelling was used during the hybridisation process. Previous studies in this laboratory found that P32 labelled probes too strongly and resulted in difficulties in distinguishing differences in gene expression.

4.3.4 Analysis of A2B and A5F mRNA by DNA array.

Numerous studies have been published on the use of arrays with regard to gene expression in various types of cancers and cancer cell lines (van't Veer et al., 2002, Luo et al., 2002, Umar et al., 2001, Oh et al., 2001, Fejzo et al., 2001, Sakamoto et al., 2001, Watts et al., 2001). Many such studies involve gene induction due to drug treatment or changes in gene expression across sensitive and resistant cell lines. Comparisons of cell lines with varying resistance levels have displayed changes in genes encoding transcription factors, those involved in proteolysis (ubiquitin-associated factors), apoptosis-related proteins (cytochrome c) and multidrug-resistance-associated protein (Kudoh et al., 2000, Watts et al., 2001, Sakamoto et al., 2001).

Overall, there were 67 genes that displayed similar trends in gene expression in our experiments between A2B and A5F cells. These included genes encoding mdr-1, galectin-1, calpain-1, retinoic acid receptor, apoptosis-associated tyrosine kinase. Some genes of potential interest are discussed individually below.

4.3.4.1 ABC transporter proteins.

There were a number of ATP-binding cassette (ABC) proteins with altered expression. These included ABC-1-member 1, ABC-1-member 3, MDR/TAP-member 7, ABC-1-member 4 and MDR/TAP-member-1. All were increased in A5F when compared with A2B cells. These genes encode active transmembrane pumps that transport substrates, ranging from simple sugars to large proteins either into or out of the cell via an inner-membrane complex against a concentration gradient (Armstrong et al., 1998, Bakos et al., 1997). This function is fuelled through ATP processing.

The ABC transporters are of biomedical importance as this protein family includes members such as the multidrug resistance (MDR-1) protein, MRP-1 and the cystic fibrosis-related transmembrane conductance regulatory protein (CFTR) (Armstrong et al., 1998).

4.3.4.2 MDR-1.

Overexpression of the multidrug resistance gene contributes to a tumour cell's ability to evade a cytotoxic stimulus via its ATP-efflux pump (Schurr et al., 1989). As expected, the first array experiment indicated that both cell lines expressed mdr-1. However, there was a 2.6-fold increase in expression in the more resistant of the cell lines, A5F. Analysis in the second array was consistent that A5F had increased levels of mdr-1 with respect to A2B cells. Previous studies, using Western blotting, also indicated an increase in P-gp levels in A5F cells relative to A2B cells (Heenan et al., 1997).

Other studies using microarrays have illustrated this increase in mdr-1 in response to adriamycin treatment (Kudoh et al., 2000, Zembutsu et al., 2002). Studies investigating gene expression underlying cisplatin-induced resistance by microarray, have identified GST-π associated with increased resistance (Sakamoto et al., 2001). In addition, a study investigating cisplatin-induced resistance in MCF-7 cells found 10% of genetic alterations overlapped with those changes due to adriamycin-induced resistance (Kudoh et al., 2000). This illustrated the variation in resistance-related pathways.

4.3.4.3 Galectin-1.

Despite previous findings illustrating changes in apoptosis-related genes in the two cell lines, only galectin-1 appeared to be upregulated in A5F cells. Galectins are a family of lectin proteins capable of binding β-galactosides (Gabius et al., 2002). Galectins are involved in regulating apoptosis resistance and metastasis and are thought to regulate immune cell homeostasis (Rabinovich et al., 2002). In the analysis of these drug resistant cell lines, alterations in apoptosis-related genes occurred. Galectin-1 was 3.3-fold increased in A5F cells compared with A2B cells on the first array. The second array showed that A5F cells had increased expression of galectin-1 but it was not detected in A2B cells. A study by Zembutsu and coworkers reported increased expression of another galectin, galectin-4 and mdr-1 in human cancer xenografts when analysed by DNA array (Zembutsu et al., 2002).

Galectin-1 localises to skeletal/ smooth muscle, motor/ sensory neurons, kidney, placenta and thymus during embryogenesis. However, subcellular localization is

modified by external stimuli, such as tumour suppressor genes, etc. (Cytokine bulletin, R & D systems). Galectin-1 is thought to have proinflammatory functions and its role in inflammatory cells has been the focus of much research (Almkvist et al., 2002, Delbrouck et al., 2002).

The galectins function in growth regulation (Gabius et al., 2002). Galectin-1 is thought to be mitogenic at low concentrations but at higher concentrations it inhibits cell proliferation and growth. Its expression has been correlated with the proliferative activity of tumour cells (Gabius et al., 2002). It is thought to trigger apoptosis in lymphoid cells but the exact mechanism involved is not yet known (Fajka-Boja et al., 2002). Such an induction of apoptosis in T-cells due to galectin-1 expression is accompanied by caspase activation and bcl-2 down-regulation (Rabinovich et al., 2002).

The role galectin-1 plays in the DLKP variants examined by DNA array could contribute to the increased invasiveness seen in A5F cells with respect to A2B cells. Galectins have the ability to cross-link glycoproteins of the cell and extracellular matrix and at high concentrations can cause a loss of adhesive potential of the cells (Cytokine bulletin, R & D systems). Galectin-1 is thought to enhance the migratory potential and hence aggressiveness of tumours. This was illustrated where galectin-1 had higher levels of expression in invasive areas of glioblastoma xenografts from nude mice (Camby et al., 2002). In addition, Camby's study found increases in small GTPase RhoA expression. The second array experiment displayed increases in Rho-related genes in A5F cells that may be related to the increased expression of galectin-1.

4.3.4.4 Calpain-1 (large subunit).

A5F cells had 1.7-fold increased expression of calpain-1 by first array and showed increased expression by second array analysis. Calpain-2 correlates positively with calpain-1 expression (Ueyama et al., 1998). A study by Carragher et al (2002) examined the oncogenic transformation of cells due to v-src expression. They reported that v-src increased synthesis of calpain-2 leading to the down-regulation of its endogenous inhibitor calpastatin, possibly playing a role in v-src-induced cell transformation.

The role calpain-1 has in A5F cells is unclear as calpains-1 and -2 are involved in apoptosis execution (Iizuka et al., 1991). Overactivation of calpains-1 and 2 has been linked to neurological diseases (Huang et al., 2001). Calpain-1 can activate TGF-β (Abe et al., 1998). They are also thought to play a role in inflammation. A report by Sasaki and coworkers suggested that the large subunit within calpain-1 had chemotactic activity for neutrophils (Sasaki et al., 1991). They speculated that such subunits could participate in migration and accumulation of neutrophils in inflammation.

4.3.4.5 Retinoic acid receptor-α.

Retinoic acid receptor-α expression was increased in A2B cells when compared with A5F in both arrays. Retinoic acid receptors are a family of steroid hormone nuclear receptors (Giguere et al., 1987). There are two classes of retinoic acid receptors – RARs (retinoic acid receptor) and RXRs (retinoid X receptors) (Xu et al., 1997). There are three subfamilies of each α, β and γ. The receptors are thought to mark a cell for response to retinoic acid treatment, the active metabolite of vitamin A. Retinoic acid has been administered in the treatment of certain cancers, such as promyelocytic leukaemia with a relatively high success rate however during remission, expression of the RAR-α appears to be a predictor of clinical relapse (Tallman, 1998). Mutations in the RAR-α gene confer resistance to retinoic acid treatment (Imaizumi et al., 1998). Treatment of colon carcinoma cells (HT-29) with all-trans retinoic acid inhibited anchorage-independent growth mediated by RAR-α (Nicke et al., 1999).

In breast cancer studies, differentiated, estrogen-dependent ER-positive cells have been found to express high levels of RAR-α and are responsive to retinoic acid treatment

(Schneider et al., 2000). Retinoic acid treatment activated protein kinase C in B16 mouse melanoma cells and resulted in increased RAR-α expression (Boskovic et al., 2002).

The increase in RAR-α in the context of A2B cells compared with A5F cells could perhaps be partially explained by evidence from a study by Stromskaya and coworkers. Transfection of melanoma and hepatoma cells with cDNA to RAR- α resulted in differentiation of the cells which was accompanied by an increase in the amounts of MDR-1 mRNA but no increase in P-gp activity (Stromskaya et al., 1998). The report by Stromskaya suggested that signalling mediated by the RAR-α up-regulates mdr-1 gene expression.

4.3.4.6 Apoptosis associated tyrosine kinase (AATYK).

Apoptosis and differentiation in neuronal cells has been shown to be associated with the expression of AATYK. This protein was originally isolated from 32D mouse myeloid cells undergoing differentiation (Raghunath et al., 2000). It has a tyrosine kinase domain and src homology domain binding sites. AATYK induces differentiaion in neuronal cells in response to agents such as IGF-1 or retinoic acid (Raghunath et al., 2000). Its expression is increased during apoptosis of myeloid cells as well as neuronal cells (Tomomura et al., 2001). This is mediated via its tyrosine kinase activity.

Results in the A2B and A5F cells lines displayed a 2-fold increase on first array and 4-fold increase on second in A5F cells. The significance of this up-regulated expression in the context of these cells is unknown. Again, confirmation of results by validation techniques such as RT-PCR, Western blots, etc. is necessary before any further emphasis can be placed on examining this gene.

4.3.4.7 Metastasis-related genes.

Some of ras-related genes had altered expression on the second array experiment. Follow-up work will be necessary in order to validate these findings. Among the increased ras-related genes in A5F mRNA were rhoG, rho GTPase activating protein 1 & 6, ras homologue gene family member I. Genes decreased in A5F relative to A2B included small GTP binding protein Rac 1 and rho GDP dissociation inhibitor γ.

Invasiveness and metastatic potential correlates with the expression of ras related oncogenes in tumour cells. It has been reported that approximately 50% of metastatic tumours contain ras mutations (Ward et al., 2001). Transfection studies have indicated that ras-related proteins enhance the metastatic behaviour of tumour cells (Brunner et al., 1989).

The NM23A (non-metastatic) gene was increased in A5F cells as displayed by the second array experiment. This result was unexpected due to the increased invasiveness of the cells. Presumably, other genes such as the ras related genes contribute to the increased invasiveness of the cells.

Adhesion molecules are essential for the interaction of tumour cells with surrounding endothelial cells during invasion. The expression of integrin-α-3 in A5F cells was increased on the second array experiment. This could be significant in the cells' ability to invade. Integrin-α-3 has been found to mediate adhesion and enable fibrosarcoma cells (HT1080) to invade transendothelial cells (Doi et al., 2002,Okada et al., 1994).

In addition to the genes discussed above, changes in the expression of transcription factors occurred between the two cell lines. A5F cells had increased expression of SP-5, myocyte enhancer factor 2A and AE-binding protein 1.

There were also alterations in the expression of other transport-related proteins. Aquaporin-8 expression was increased in A5F cells. Aquaporins are membrane water channels that regulate the water contents of cells. Several diseases (congenital cataracts and nephrogenic diabetes insipidus) are connected to the impaired function of these channels. They form tetramers in the cell membrane, and facilitate the transport of water and, in some cases, other small solutes across the membrane (Tajkhorshid et al., 2002).

4.3.5 Reproducibility of the DNA array experiments.

Despite so much evidence from previous studies on gene and protein expression in the two cell lines, the DNA array did not display agreement with most of the previous findings by RT-PCR and Western blot. This was displayed by very low percentages of gene trends repeating from one array to the second array. In addition, greater numbers of genes had changed in expression on the second array where 426 genes altered as compared with 227 on the first array. From those listed in table 4.3.1 and 4.3.2, only mdr-1 was detected.

Very few papers cite problems with the reproducibility of the technique. A handful of papers suggest conflict between array results and validation techniques such as PCR, RPA, Western and Northern blots. Heiskanen and coworkers suggest that the complexity of the probe and small sizes of arrayed target cDNAs place high demands on the sensitivity of the system (Heiskanen et al., 2000). In their study, to enhance signal amplification, they used a tyramide-based fluorescent labelling system and reported gene amplification of up to 1000-fold.

A report by Kudoh et al. (2000) comparing adriamycin-induced and adriamycin-resistance gene expression in MCF-7 cells found that changes in gene expression detected by DNA microarray matched 58% of those displaying changes in subsequent PCR validation reactions. In contrast to this, another study where adriamycin-sensitive RPMI-8226 cells and resistant RPMI-8226/Dox6 and RPMI-8226/Dox40 cells were compared found 90% agreement when array results were validated with subsequent Northern blotting experiments (Watts et al., 2001).

Chang and coworkers examined senescent and proliferating adriamycin-treated cells originating from the same colon carcinoma cell line (Chang et al., 2002). RT-PCR analysis revealed 90% of downregulated and 82% of upregulated genes agreed with the trends seen by microarray analysis. However, they reported that the differences in gene expression by RT-PCR were much higher than the values indicated by the array.

The use of plastic-membrane arrays is still in its infancy. As it is a relatively new membrane, it is possible that the stripping and rehybridisation process warps the membrane in some way and that this contributed to the disagreement between array

experiments in this study. Presumably, this together with natural fluctuations in biological systems, added to such discrepancies.

4.3.6 DNA arrays and the future of cancer biology.

DNA array technology lends itself not only to disease-related studies, but to applications in the agricultural, pharmaceutical and other industrial disciplines. Its application in cancer biology could advance the understanding of cancer initiation, progression and underlying signalling pathways. The birth of array technology has been accompanied with the emergence of preliminary findings from the human genome project. Combining the two will increase the numbers of genes available for analysis by microarray.

Many papers have cited that the potential exists to customise cancer treatment and prognosis based on a patient's molecular signature from a DNA microarray analysis (Clarke et al., 2001, Aitman, 2001, Cooper, 2001, Perou et al., 2000). To date, methods of RNA extraction from tumours have not accounted for their heterogeneous subpopulations (Zembutsu et al., 2002). The development of techniques such as laser capture microdissection facilitates exclusive selection of tumour cells from surrounding cells (Clarke et al., 2001). This, combined with DNA microarray, enables the molecular blueprint of an individual tumour to be typed. In this way, a more accurate assessment of cancer-related gene expression could be uncovered. Studies in this lab will now focus on the analysis of gene expression in the archival breast tumour biopsies previously analysed by RT-PCR. Gene expression profiles will be correlated with treatment regimens and disease outcome and also with results from similar studies on breast cancer (Sotiriou et al., 2002, Unger et al., 2001, Ahr et al., 2002).

In cancer biology, the use of DNA arrays stretches from the identification of gene alterations in drug-treated cell lines, to perhaps even potentially lending itself as a tool in cancer screening in the future. Given the discrepancies in repeatability between experiments in this study, further refinement and optimisation are necessary before it can be relied upon as a routine tool. However, as with any new scientific technique, already improvements have been made on first generation microarrays (Aitman, 2001, Clarke et al., 2001, Blohm & Guiseppi-Elie, 2001, Brown & Botstein, 1999). Further refinement, may lead to cancer treatment moving from the current non-selective cytotoxic drug therapy to a more specialised treatment, targeting a selective molecular abnormality that contributes to neoplastic transformation.

4.4 The expression of Bcl-2 family genes in a panel of invasive breast tumour biopsies.

4.4.1 Introduction.

The identification of important functional markers which play a role in the development and progression of human tumours or which are targets of anticancer agents is essential to the development of individualized cancer treatment. The pretreatment detection of such markers that could influence clinical tumour responsiveness may be used to select chemical and physical agents and to modulate their activity in individual patients.

When studying cancer, we are constantly reminded of the alarming statistics that one in three people suffer cancer during their lifetime and one in four people die from it. Over 1700 people are diagnosed with breast cancer annually and approximately 16 cases occur in men each year (National Cancer Registry, Ireland, www.cancer.ie). In 2000, a breast cancer-screening program was set up in Ireland called "Breastcheck". This invites women between the ages of 50-65 for a free mammogram every two years. Early screening can prevent the development of advanced, untreatable disease. However, the challenge remains to identify markers that could indicate prognosis or response to therapy. This study provided a unique opportunity to examine the possible prognostic significance of a range of genes and proteins as markers for response rates and disease outcome in breast cancer tissues.

Breast carcinoma is a heterogeneous disease displaying an imbalance of a wide number of genes and proteins. Neoplastic growth does not depend exclusively on uncontrolled proliferation but also on cell loss. It has been widely documented that the dysregulation of apoptosis contributes to the pathogenesis of breast cancer at least in part due to an imbalance between anti-apoptotic genes and apoptosis-promoting genes. It is likely that patient prognosis is not dependent on the expression of a single apoptosis-related protein but on an interplay between the different factors.

4.4.2 Treatment of breast cancer – identification of predictive markers.

The anthracyclines have traditionally been recognized as the most effective treatment for breast cancer. Drugs such as adriamycin are often combined with CMF treatment. While such drugs are successful at treating cancer, they do not discriminate between normal and neoplastic cells. Severely debilitating side-effects are experienced by patients and treatment often causes the emergence of a multi-drug resistant secondary tumour, more aggressive than the primary. To estimate the prognosis for a patient is extremely important as it strongly influences the therapeutic strategy – this is highlighted where ER+ patients are selected for tamoxifen therapy. No single prognostic marker currently available can give unequivocal information on prognosis and subsequent therapy. However, the identification of tumour markers has the potential to lead to better-individualized selection of adjuvant therapy for breast cancer patients.

In this study, patients had been administered CMF with or without adriamycin. Tumours from these patients were examined for their expression of a wide range of markers to estimate their implications for prognosis and outcome. Many of the patients had ER+ tumours and were given tamoxifen, a drug that has improved survival and decreased tumour recurrence in many women with invasive breast cancer. Other studies have reported that patients whose tumours express ER and c-erb-B2 have displayed a trend whereby tamoxifen therapy can become ineffective. Patients who are c-erb-B2 positive are being assessed for the efficacy of treating them with herceptin, a monoclonal antibody that targets c-erb-B2. Using these two markers is an illustration of how important it is to establish exactly what proteins are being expressed in malignant cells. In this way, clinical treatment can become customized so that each patient will get the most effective therapy.

A factor that can be important when attempting to evaluate the "predictive" markers of a tumour is the heterogeneity of the tumour's cellular population. Techniques such as fine-needle biopsy can only obtain information from a limited cellular population (Ciocca & Elledge, 2000). Immunohistochemistry is a choice method for identifying the various cellular populations contained within a tumour, providing a picture of where the markers are located. Part of this study involved the use of IHC to investigate the expression of mdr-1, mrp-1 and survivin protein expression. In addition, RNA was extracted from tumours to

examine a cross-section of the heterogeneous tumorigenic population. It was hoped that information on gene and protein expression could be correlated with clinicopathological parameters and perhaps a unique predictive marker could be identified.

4.4.3 RNA extraction from a panel of archival breast tumour biopsies.

One of the challenges during this study was to extract high quality RNA that provided for accurate and reproducible analysis by RT-PCR. Based on a method optimised by Dr. Padraig Doolan, yields of up to 100ug of RNA were obtained from some tumour samples (Padraig Doolan, PhD 2001). As is displayed in the results section, this allowed for generation of high quality RT-PCR results.

4.4.4 Bcl-2 expression in archival tumour biopsies.

Bcl-2 was originally identified in human follicular B-cell lymphomas at t-14;18 chromosomal translocation (Tsujimoto & Croce, 1986). This placed the bcl-2 gene adjacent to the enhancer region of the immunoglobulin heavy chain gene locus that in B-cells is actively transcribed. Therefore, the bcl-2 gene was actively transcribed also.

Bcl-2 is normally expressed in the ductal epithelia of the breast (Binder et al., 1995). During early pregnancy, bcl-2 is expressed but its expression reduces as pregnancy progresses (Schorr et al., 1999). In normal breast tissue, bcl-2 acts as a regulator of the cell renewal system (Lee et al., 1996). Most studies report that high bcl-2 expression in breast cancer is indicative of favorable disease outcome (Bukholm et al., 2002, Lipponen et al., 1995, Krajewski et al., 1997, Shikaitis et al., 2000, Rochaix et al., 1999, Maciarowski et al., 2000, Teixeira et al., 1995, Castiglione et al., 1999, Ciocca & Elledge, 2000). It is thought that this strong expression, similar to the normal breast, is related to normally functioning bcl-2 protein (Lipponen et al., 1995). During the development of malignant disease, a deregulation of bax and bcl-2 synthesis and function occurs at late, premalignant and malignant stages. This shifts the balance of apoptotic proteins, which ultimately leads to disease progression.

Analysis of the tumour biopsies for bcl-2 expression indicated that 131 out of a total of 147 of the tumours expressed bcl-2, while 5 out of 6 normal samples were bcl-2 positive. Statistical analysis revealed that bcl-2 mRNA correlated with ER status (P=0.019) and bag-1 mRNA expression (P=0.003). Lymph node status failed marginally to be a significant prognostic factor that correlated with bcl-2 mRNA expression (P=0.974). Bcl-2 expression

was also associated with both relapse-free survival (P=0.0273) and with overall survival (P=0.042). However, multivariate analysis showed that bcl-2 was independently associated with overall survival but not with five year disease-free relapse.

Our results indicate that bcl-2 mRNA expression correlated with estrogen receptor (ER) positivity. This association has been observed in a large number of breast cancer studies (Bukholm et al., 2002, Lipponen et al., 1995, Krajewski et al., 1997, Shikaitis et al., 2000, Rochaix et al., 1999, Maciarowski et al., 2000, Teixeira et al., 1995, Castiglione et al., 1999, Ciocca & Elledge, 2000). Estrogen promotes the survival of estrogen-dependent ER+ breast cancer cells. Since bcl-2 is an estrogen-regulated protein, estrogen administration increases bcl-2 mRNA and protein and produces downregulation of bcl-xL, an effect that is blocked by tamoxifen. For this reason, patients with ER+ tumours are usually bcl-2+ and benefit from tamoxifen treatment. Tamoxifen increases apoptosis and decreases bcl-2 in breast cancers (Ciocca & Elledge, 2000). Although not examined in this study, a study by Maciarowski et al. (2000) reported that bcl-2 expression correlated also with PR (progesterone receptor) expression, as well as ER in 63 fine-needle biopsies of primary breast tumours.

Another study on 240 resectable cancers showed that those patients with low cell proliferation, PR positivity and bcl-2 overexpression were low risk (Silvestrini et al., 1996). In our study, bcl-2 expression proved an independent prognostic factor and correlated positively with overall survival in our study. It was also a marker of five year relapse-free survival. Other studies have also found a positive correlation between bcl-2 expression and relapse free survival. Castiglione investigated 138 operable carcinomas for their expression of ER, bcl-2 and disease outcome. Similar to our study, bcl-2 was found to associate with steroid hormone receptors, good relapse free and overall survival (Castiglione et al., 1999). These findings were also reported by Charpin's group where positive bcl-2 immunohistochemistry indicated favourable disease-free and local recurrence free survival, and found it to be an independent, weakly prognostic indicator in breast carcinomas (Charpin et al., 1998).

Lymph node metastases may be associated with altered rate of apoptosis as well as several genetic alterations, including adhesion proteins, cell motility and angiogenesis (Bukholm et

al., 2002). As greater numbers of lymph nodes become involved in a cancer, the chances of a favourable prognostic outcome decrease due to an increased likelihood that the cancer will metastasise. Our results indicated that bcl-2 mRNA expression failed marginally to be a significant prognostic factor that correlated with lymph node status with increased percentages of bcl-2 negative patients displaying lymph node positivity. This borderline significance has been debated in the literature with some reports suggesting an association between bcl-2 and lymph node status and others concluding no association. Bukholm and coworkers (2002) reported that decreased levels of bcl-2 and bax were associated with lymph node metastases. However, a study by Krajewski's group on 149 patients with invasive ductal carcinomas that had not received chemo- or radiation- therapy reported that bcl-2 was not predictive of outcome in node-negative patients (Krajewski et al., 1997).

Other studies have correlated bcl-2 expression with less aggressive tumours. Kapucuoglu report bcl-2 positivity was associated with well differentiated DCIS and tumour grade IIC, whereas bax positivity was found in poorly differentiated DCIS and more aggressive tumours (Kapucuoglu et al., 1997). Consistent with our results, it has also been reported that loss of bcl-2 expression is associated with loss of hormonal regulatability, increased dedifferentiation and deregulated proliferation (Binder et al., 1995). In our study, bcl-2 mRNA expression did not correlate with diagnostic features, tumour size or grade.

Despite all the evidence that bcl-2 expression is an indicator of good prognosis, it cannot be relied upon as a definite predictive marker as some studies have observed a poorer outcome bcl-2 positive breast cancer. Wu and coworkers investigated gene expression in 91 breast cancer patients. They found that increased bcl-2 and low or absence of bax was associated with high apoptotic index, high tumour grade, lymph node involvement, post-operative recurrence and metastasis. Bcl-2 associated with short relapse free and overall survival. It was therefore a marker of poor prognosis (Wu et al., 2000). Yang and coworkers used HDRA (histoculture drug response assay) to assess drug resistance of tumours. They found that bcl-2 negative cells were more sensitive to drug than bcl-2 positive cells and therefore concluded that bcl-2 was a marker of poor prognosis (Yang et al., 2000).

Bcl-2 shows a paradoxical behaviour in breast cancer compared with other neoplasms. In the majority of breast cancer studies, its expression has been correlated with favourable

biological prognostic factors and better clinical outcome. However, bcl-2 expression is associated with drug resistance as it provides a background of prolonged cell survival enabling mutations to form in cancer cells. In addition to B-cell lymphoma, bcl-2 is overexpressed in a large number of malignancies including solid tumours such as those of the prostate, lung, thyroid, gastric and breast. Its expression is often associated with disease outcome. In oligodendrogliomas, overexpression of bcl-2 is correlated with progression of the disease (Deininger et al., 1999). In small cell lung carcinomas, bcl-2 is associated with increasing tumour mass through inhibition of apoptosis, thus increasing the pathogenesis of disease (Jiang et al., 1995). Its role in non-small cell lung cancer has yet to be elucidated (Borner et al., 1999, Fontanini et al., 1995). In thymomas, bcl-2 expression correlates with increased tumour aggressiveness (Chen et al., 1996).

This evidence suggests that bcl-2, while a possible indicator of good prognosis in breast cancer, cannot be thought of as a foolproof diagnostic marker. The majority of studies, including this study, suggest that tumours that overexpress bcl-2 have potentially good prognosis. However as discussed, this is not always true. Presumably, it is the interplay of markers that determine the aggressiveness of a tumour.

4.4.5 Bax mRNA expression in archival breast tumour biopsies.

Bax is a homologue of bcl-2 that promotes cell death. Its expression is predominantly located in epithelial cells (Krajewski et al., 1994a & 1997). Bax-α is over-expressed in high amounts in normal breast epithelia but has been reported decreased or absent in malignant breast tissue (Bargou et al., 1996 & 1999). Deregulation of bax occurs at late, premalignant and malignant stages of the neoplastic process (Shikaitis et al., 2000).

Our results showed that bax was expressed in 99% (147 of 148) of tumour tissue examined. However, its expression was not found to correlate with any of the other clinicopathological markers investigated. These results are reflected in the literature where other studies display similar findings. The previously mentioned study by Maciorowski and coworkers (2000) investigating 63 primary breast tumours found that bax did not correlate with any marker investigated. Reports by Yang et al. (2000) also revealed that bax did not correlate with any other markers in 177 breast carcinomas investigated.

It has been widely reported that the dysregulation of apoptosis contributes to the pathogenesis of breast cancer. Many studies detail a correlation between bcl-2 and bax expression. Krajewski et al. (1995a) investigated bax expression by IHC in primary invasive breast tumours. Bax expression correlated with bcl-2 expression. A decrease in bax was found to associate with faster time to progression and shorter survival. This loss of bax was associated with poor response to therapy. As previously mentioned, Kapucuoglu (1997) reported higher bax expression in poorly differentiated DCIS and more aggressive tumours in breast cancer.

There have, however, been a number of studies reporting that bax expression was lost in breast cancer tissues. This loss was accompanied by high apoptotic index, high tumour grade, lymph node involution, post-operative recurrence and metastasis (Wu et al., 2000). The effects of decreased bax in malignancy have been associated with shorter time to progression and overall survival and also decreased sensitivity to cytotoxic drugs (Sjostrom et al., 1998, Ikeguchi et al., 2001, Sturm et al., 2000, Friedrich et al., 2001). A study by Knudson and coworkers (1995) claimed that bax null mice had selective hyperplasia of lymphoid tissues, enlarged spleen and an increase in the number of thymocytes. This study

indicated that loss of bax expression was associated with uncontrolled cellular proliferation such as would occur in neoplastic disease (Knudson et al., 1995).

It has been reported that breast and ovarian cancers display common hereditary evidence. As a result of which women with breast cancer are susceptible to the risk of ovarian cancer (Giannios and Ioannidou-Mouzaka et al., 1997). In ovarian cancers, bax associated with tumour differentiation and improved prognosis. It also correlated with smaller amounts of residual disease after surgery. Bax was found to correlate with bcl-xL but not mcl-1 or p53 (Baekelandt et al., 2000).

Unlike bcl-2, bax is not affected by estrogen and does not correlate with ER or PR status (Teixeira et al., 1995, Binder et al., 1995). Binder, however, reported an inverse correlation between bax with ER and PR status (Binder et al., 1995). Bax expression has been correlated with c-erb-B2 expression (Krajewski et al., 1997) and c-erb-B-1, tumour grade and proliferation activation, especially in tumours that do not express bcl-2 (Binder et al., 1996).

Future work will examine the expression of erb family receptors by RT-PCR. Results will be statistically analysed for their associations with all other parameters. It may also be worth investigating the association of bax and p53 in these tumour tissues. P53 is a transcriptional activator of bax (Miyashita and Reed, 1995; Heermeier et al., 1996). Sturm and coworkers discovered a subset of breast cancer cells expressing mutated p53 had no bax expression (Sturm et al., 2000). However, Krajewski et al did not find any association between bax regulation and p53 status in breast cancers (Krajewski et al., 1997).

Results from this and other studies suggest that a role as a predictive marker in malignant tissue cannot be determined for bax. Bax deficiency or mutation seems to promote oncogenic transformation. Its expression therefore could be a determinant of chemosensitivity. Mutations that decrease bax in tumours contribute to drug resistance (McCurrach et al., 1997). Further studies are necessary to determine its role in breast cancer progression.

4.4.6 Mcl-1 expression in archival breast tumour biopsies.

Analsysis of mcl-1 mRNA levels in the breast tumour biopsies revealed that 136 out of 143 tumours were positive for mcl-1 expression. Of the normal samples, 1 of 6 expressed mcl-1. Our study showed that mcl-1 mRNA expression correlated with tumour grade (grade 1,2 &3, P=0.035 and grade 1 & 2, versus 3 P=0.046). Its correlations with lymph node status (P=0.053) was borderline significant. Mcl-1 mRNA also had borderline significance with a splice variant of survivin, survivin-δ3 mRNA (Rasha Linehan, personal communication).

It is as yet unclear what impact mcl-1 expression has on the progression of malignant disease. Higher levels of mcl-1 confer resistance in hematologic malignancies (Baekelandt et al., 2000). To date, many of the studies on mcl-1 have focused on hematopoietic cells but the role mcl-1 plays in the progression of solid tumours is currently under debate (Johnson, 1999 & references therein).

Mcl-1 has a more widespread distribution than bcl-2. It is found in mitochondrial and non-mitochondrial compartments and localises to the nucleus and cytoplasm to membrane-bound organelles (Leuenroth et al., 2000). It is also found on light intracellular membranes where bcl-2 is not (Moulding et al., 2000). The expression of mcl-1, and other bcl family proteins, has been examined in a number of tumour and tissue types.

As previously mentioned, it has been noted that where bcl-2 expression is low, mcl-1 expression is usually high (Soini et al., 1999, Ando et al., 1998). A study by Krajewski et al. (1994b) looked at the intracellular distribution of mcl-1 and bcl-2 proteins by immunohistochemistry in normal and neoplastic lymph node biopsies. Intracellular mcl-1 localised to the cytosol in a punctate pattern and was also seen in association with the nuclear envelope. Bcl-2 resided on the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum. While bcl-2 expression was highest in mantle zone lymphocytes and absent from most germinal centre lymphocytes, the mcl-1 expression pattern was the direct opposite. Mcl-1 was also found in some interfollicular lymphocytes, especially those that appeared to be activated. These results suggest that mcl-1 and bcl-2 are differentially regulated in normal and neoplastic cells and nodes. Each protein may have a different role in the control of cell death in these tissues.

95% of tumours tested by RT-PCR in this study expressed mcl-1. Mcl-1 mRNA correlated positively with tumour grade and lymph node involvement. These results suggest that mcl-1 was expressed in more invasive and advanced breast tumours. The association of mcl-1 with more advanced tumours was also seen in a study by Baekelandt et al. (2000) who looked at 185 patients with stage 3 ovarian cancer. (Ovarian cancer is often secondary to breast cancer). 53% of tumours were found to be mcl-1 positive. Mcl-1 expression was associated with poorer prognosis and shorter survival. Lymph node involvement or tumour grade was not specified in this study. None of the bcl family markers correlated with response to chemotherapy (Baekelandt et al., 2000). A study by Nieves-Neira, examined the expression of various enzymes (protein kinase c) as well as bcl family genes in human breast cancer cell lines. It was concluded from their work that the cumulative expression of bcl-2, mcl-1 and bcl-xL were correlated with apoptotic response (Nieves-Neira et al., 1999). These findings were mirrored in prostate cancers, where expression of mcl-1, bcl-2 and bcl-xL increase during progression of the cancer, which may have contributed to the hormone-insensitive, metastatic phenotype of most advanced adenocarcinomas of the prostate (Krajewska et al., 1996). From these studies, it could be speculated that mcl-1 expression contributes to poor prognosis in more advanced cancers – such as was seen in our study.

However, there are also many studies where mcl-1 has displayed no correlations with any markers investigated. Another study on ovarian carcinomas could not associate mcl-1 with any other marker (Wehrli et al., 1998).

To date, studies on breast cancers have not elucidated the significance of mcl-1's expression. Rochaix et al investigated 110 pretreatment invasive breast carcinomas by immunocytochemistry. They found that bcl-2, bcl-x, mcl-1, bax, bak, ER and p53 were expressed in 62, 75, 68, 75, 60, 68 and 26 per cent of cases. Mcl-1 was not found to correlate with apoptotic index or any particular tumour marker (Rochaix et al., 1999). Zapata and coworkers (1998) also found that mcl-1 did not associate with any markers investigated in breast cancer cell lines and primary tumours. Most investigations have been on pretreatment biopsies in contrast with our post-treatment tumours. It is likely that treatment of the breast tumours in our study with cytotoxic drug has contributed to the

expression of the genes and proteins investigated such as has been reported in cell line studies (Schubert et al., 2001, Katoh et al., 1998). This may therefore influence the association of genes such as mcl-1 with other markers.

Some research has observed that mcl-1 correlated with poorer prognosis in other types of neoplastic disease. In pulmonary lymphangioleiomyomatosis (LAM), a disease in women, where abnormal proliferation of smooth muscle cells occurs, bcl-2 and mcl-1 were found in malignant tissue rather than in normal tissue (Usuki et al., 1998). In gallbladder carcinomas, mcl-1 was expressed in 87% of cases, bax was expressed in all cases and bcl-2 was expressed in only 10% of tumours (Turunen et al., 2000). Up to 90% of pancreatic cancers were found to express mcl-1 and bcl-xL (Miyamoto et al., 1999). Studies such as the above suggest that mcl-1 is a marker of poor prognosis.

Our study displayed that mcl-1 expression correlated with the expression of survivin δ3 mRNA (Rasha Linehan, personal communication). Survivin is a member of the inhibitors of apoptosis (IAP) family. Through alternative splicing, it is transcribed into three variants – survivin, survivin-δ3 and survivin-b. The former encode anti-apoptotic proteins while the latter is pro-apoptotic (Mahotka et al., 1999). Survivin-δ3 has a deletion of 118bp in exon 3. Survivin and its variants have displayed expression in foetal and neoplastic cells, but have not been found in adult tissues. They are thought to associate with centromeres, microtubules of the mitotic spindle and cdk4 and are thereby involved in the regulation of mitosis (Krieg et al., 2002).

A study by Krieg and coworkers investigated the expression of survivin variants by RT-PCR in gastric carcinomas. They did not report any change in expression of the two anti-apoptotic variants between premalignant and malignant stages of the disease, but did report that the pro-apoptotic form decreased in advanced disease. This study indicated that the interaction of the three variants was essential in the fine-tuning of apoptosis control in gastric cancer.

There only appears to be one other study that examined mcl-1 and survivin. It investigated the protective effect of phorbol esters on Fas-mediated apoptosis in T-cells. Results

revealed an inverse association where mcl-1 (and bcl-xL) expression increased following PMA treatment but survivin decreased (Herrant et al., 2002).

The significance of association between mcl-1 and survivin-δ3 in our panel of breast carcinomas is not known. As both encode anti-apoptotic proteins, it is possible that a cumulative effect of their expression aids in disease progression and therefore adverse disease outcome.

A general pattern for mcl-1 expression in cancerous tissue cannot be recognized. Clinical studies suggest it is a negative prognostic factor. As mentioned, there are very few studies relating mcl-1 expression to breast tumours. Two published studies show that mcl-1 expression is not related to clinical outcome however, these were on pre-treatment cancers. This study is unique in that it has examined bcl family expression in a panel of post-treatment biopsies, where clinical follow-up information is available. At the time of writing, mcl-1 protein expression in our panel of breast tumours is being investigated and results are pending.

4.4.7 The expression of Bag-1 in archival breast tumours.

Bag-1 is an anti-apoptotic protein, thought to cooperate with bcl-2 and aid the suppression of apoptosis. It interacts with steroid hormone receptors (estrogen, androgen and glucocorticoid receptors) and with retinoid receptors and thereby influences transcriptional activation and apoptosis induced by steroid hormones and retinoids. It binds growth factors (tyrosine kinase growth factor receptor, hepatocyte growth factor receptor and platelet-derived growth factor receptor) enhancing their ability to inhibit apoptosis. It can also interact with and activate raf-1 serine/ threonine kinase and heat shock proteins (hsp/ hsc 70).

Bag-1 isoforms display cytoplasmic and nuclear distribution in normal cells. Their expression has been reported to be very positive in luminal cells, less intense or absent in myoepithelial cells and undetectable in surrounding lymphocytes and stromal cells (Brimmell et al., 1999).

Bag-1 mRNA was expressed at very low levels in 50% of the normal tissues investigated. In the panel of archival breast tumours investigated, its expression was positive in 128 of 143 (90%) tumour tissues. Bag-1 mRNA expression was not found to correlate with any of the clinicopathological factors investigated. In addition, it could not be associated with disease outcome. It did however display a positive correlation with bcl-2 mRNA expression. This was not surprising as these genes have been found co-expressed in a large number of cancers, including breast cancer. As mentioned in section 1, there are up to six human isoforms of bag-1 generated through alternative translation initiation. A study by Yang and coworkers examined their expression in a panel of breast primary tumours showed that those isoforms that localise to the cytoplasm, p46 and p33, were highly expressed. Bag-1 correlated positively with bcl-2 and bcl-xl in breast normal and carcinoma cell lines. However, in invasive breast carcinomas, bag-1 expression was increased but bcl-2 was decreased (Yang et al., 1999).

Many studies have reported that approximately two thirds of breast cancers have cytoplasmic bag-1 expression (Cutress et al., 2002). The significance of bag-1 expression in breast cancer progression and prognosis is still under investigation. Statistical analysis in

our study concluded that bag-1 did not correlate with ER status. Examination of MCF-7 cells in the presence or absence of estrogen revealed that bag-1 expression appeared independent of estrogen and was not coordinately regulated with bcl-2. By contrast, Brimmell reported that 90% of ER positive tumours were positive for bag-1 expression. In some cases, bag-1 stained stronger in tumour cells relative to uninvolved adjacent epithelial cells. Bag-1 was less readily detectable in ER-negative cases. Bag-1 and bcl-2 were highly expressed in ER positive cells. This indicated that although bag-1 and bcl-2 were often co-expressed in ER positive breast cancers, the steady state expression and subcellular location of bag-1 appeared independent of estrogens.

Another study found that an increase in bag-1 expression in invasive breast carcinoma tissues was accompanied by a decrease in bcl-2 expression (Tang et al., 1999). Tang et al. examined 140 paraffin-embedded breast carcinomas by immunohistochemistry. The majority of breast cancer specimens expressed bag-1 but levels of expression varied between samples. Similar to our findings, bag-1 expression did not correlate with conventional prognostic factors (i.e. tumour size, stage & grade, histology and receptor status). They speculated that it could perhaps serve as an independent predictive factor in breast cancer prognosis.

It may be useful to examine bag-1 expression by IHC in our panel of tumours as studies have shown that tumours expressing bag-1, especially nuclear bag-1, had shorter disease-free and overall survival. Because the antiapoptotic effect of bag-1 depends on bcl-2, overexpression of bag-1 without increase in bcl-2 may result in deregulation of apoptosis of breast cancer cells and hence a worse prognosis (Tang et al., 1999). In addition, examination of the bag-1 isoforms may reveal correlations with disease outcome.

In keeping with its association with bcl-2, studies have found that well-differentiated tumours have also displayed bag-1 positivity. Turner and coworkers found that bag-1 expression correlated with longer disease free and overall survival and cytoplasmic expression correlated with bcl-2 expression. Its expression was also associated with node negative patients (Turner et al., 2001).

From these reports there is evidence to suggest that the role of bag-1 in breast cancer should be further investigated. Due to its association with bcl-2 in this study, and the confusion surrounding its association with ER status in other studies, it may have some role as a therapeutic target in breast cancer. One could speculate that, if bag-1 is expressed in tumours that also express bcl-2, then could it be considered a marker of good prognosis?

4.4.8 Conclusions.

The great advantage tumour cells possess over normal cells is their uncanny ability to mutate and proliferate thus overcoming the body's immune surveillance system. The effectiveness of early diagnosis of cancer cannot be underestimated. Breast screening programs have been established to identify the early signs of malignancy. Such a process aims to delay and where possible, prevent the tumorigenic conversion of normal cells.

Despite the identification of markers that are often changed in malignant tissue, the implications such markers have on therapy and prognosis is under constant scrutiny. Studies such as this, have aimed to identify predictive markers – those that have displayed indications on how a tumour is going to behave in response to various cytotoxic treatments. The challenge remains for oncologists to provide tailor-made therapy to each individual patient. Therefore molecular markers have been proposed to play an important role in breast cancer biology, prognosis and therapy.

The importance of apoptosis-related genes in breast cancer has been seen especially in the inverse association of bcl-2 expression with disease outcome. There are many studies that detail this relationship, however few investigate other parameters such as mcl-1, bad and bag-1. This study has provided the first opportunity to examine a panel of post-treatment archival breast tumours. Overall, our results indicated that bcl-2 expression in breast tumours was indicative of favourable outcome. Bag-1 associated with bcl-2 but no other markers and the significance of this association therefore could only be speculated. Bax did not prove to be an important predictive marker in these tumours, however low bax expression in breast cancer has been associated with higher resistance to chemotherapy and poorer prognosis (Soini et al., 1999). Mcl-1 displayed most associations with other parameters investigated. This was surprising as previous reports had not suggested any correlation between its expression and breast cancer development. It related to tumour grade and lymph node involvement and with the anti-apoptotic splice variant of survivin, survivin-δ3. This may suggest that mcl-1 is a marker of more advanced disease. It will be interesting to correlate mcl-1 protein expression with markers already examined in this study.

As no one marker has emerged as a predictor of disease outcome, our results must be pooled with those of other researchers. In this way, trends of gene expression in advanced cancers will be identified and subsequently indicators of disease outcome may be identified. Because the bcl-2 family proteins are a family of heterodimerising proteins, it is possible that clinical outcome may depend on a cumulative effect of the expression of anti-apoptotic proteins in breast and other neoplasms.

Ideally the significance of gene expression in each individual tumour type needs to be examined and only then can correlations between expression levels and prognosis be assessed. Studies such as this strive to unite the research findings with the clinic with the eventual aim of improved therapies. With the advent of array technology and more efficient methods of PCR, this goal is attainable.

Section 5.0: Summary, Conclusions and Future work.

5.0 Summary and Conclusions.

A major objective of this work was to investigate the expression of apoptosis-related genes in panels of drug sensitive and resistant cell lines. Resistant variants of the non-small cell lung carcinoma cell line, DLKP, were generated by continuous growth in drug or by pulse-selection with drug. Previous studies in this and other labs had indicated that drug treatment alters the expression of bcl and caspase family genes. Some members of the bcl family and caspase family were selected for further analysis.

1. The RNase protection assay was used initially to examine bcl and caspase family genes and indicated that the expression of bax (pro-apoptotic) and mcl-1 (anti-apoptotic) were highest in the cell lines investigated. Both were selected for further investigation via transfection studies. Due to previous reports in this laboratory indicating increased bax expression in adriamycin-resistant DLKP variants, sensitive cells, DLKP-SQ were transfected with bax cDNA. No consistent changes in mRNA or protein expression were, however, observed.

2. Evidence from the literature indicated that mcl-1 could be responsible for increases in resistance to some cytotoxic drugs, such as etoposide. Transfection of mcl-1 into sensitive DLKP cells resulted in increases in mcl-1 protein expression, accompanied by increased resistance to adriamycin and etoposide, without changes in resistance to any of the other drugs (vincristine, cisplatin and 5-fluorouracil) investigated. Because both drugs are substrates for P-glycoprotein, the levels of P-gp were analysed in the transfectants by Western blot. Results indicated an increase in P-gp expression in transfectants relative to parent cells which was possibly responsible for the increases in resistance levels. It was unusual that resistance to vincristine was not significantly increased given that it is associated with P-gp overexpression. The transfectants became infected by mycoplasma and were not analysed any further. This work highlighted the importance of investigating the expression of multi-drug resistance associated proteins, such as P-gp, mrp, GST-π, etc. in transfection studies where drug resistance levels are being monitored.

3. The RPA was also used to determine the expression of bcl family members in bcl-xL ribozyme transfectants. Originally this work served to rule out the

possibility that the bcl family members were compensating for changes in bcl-xL expression. Clones of the more resistant cell line, A250-10p transfected with bcl-xL ribozyme, indicated decreases in bcl-xL mRNA levels whereas those of the sensitive cell line, DLKP-SQ, indicated no change or increases in bcl-xL levels by RPA analysis. While clones of the A250-10p cell line generally displayed trends of increases in mcl-1 expression and decreased bad expression, those of the DLKP-SQ cell line indicated the opposite trends.

4. The caspase family RPA investigated the expression of caspase family genes in a number of drug sensitive and resistant cell lines. This, along with previous results, indicated that caspase-3 expression was decreased in cells with increased resistance levels, and increased levels of P-gp. To further investigate the possibility that the down-regulation of caspase-3 was contributing to drug resistance in these cells, a ribozyme to human caspase-3 was designed and transfected into cells.

   The ribozyme to human caspase-3 was shown to target and cleave caspase-3 both by *in vitro* cleavage and in transfection studies. An *in vitro* cleavage assay was optimised and ribozyme-induced cleavage products of caspase-3 were generated. Further investigation involved transfection of the ribozyme into DLKP-A5F cells. A transient transfection assay was optimised and revealed reduced levels of mRNA and protein in ribozyme-transfected cells. Unfortunately, changes in the phenotype of transient ribozyme-transfectants could not be identified as transfection efficiency in the 96-well plates used for toxicity assays was very low. There was, however, a suggestion of increased sensitivity in the transfectants relative to control (reverse ribozyme) transfectants and parent cells. Analysis of α-fodrin did not indicate any changes in fodrin cleavage. TUNEL assay indicated that both control and ribozyme transfectants had increased sensitivity when cells were treated with drug possibly due to the transfection procedure. RT-PCR analysis revealed a slight reduction in mdr-1 levels in ribozyme transfectants relative to the control and parent cells. This indicated a possible role for caspase-3 in downregulation of mdr-1.

5. To elucidate the phenotypic changes in cells due to the caspase-3 ribozyme, stable transfectants were established. Analysis of the clones revealed down-

regulated mRNA and protein in some clones. Initial toxicity analysis indicated increased resistance in some clones, with dramatically increased sensitivity in others. Subsequent toxicity analysis saw a reversal of resistance levels back to, in some cases, those of parental DLKP cells. P-gp levels in early and later passages of clones were examined. They revealed that P-gp levels had also been down-regulated in the cells. Taken together with the transient transfection results, these findings suggested that caspase-3 and P-gp could be linked in some way.

6. The mcl-1 and caspase-3 ribozyme transfection projects revealed some unexpected alterations in P-gp expression. In the mcl-1 transfections, P-gp had increased and could be responsible for increases in drug resistance levels in the cells. In the caspase-3 ribozyme transfectants, P-gp had decreased and could possibly have masked changes in drug resistance due to down-regulation of caspase-3. Both sets of results highlighted the importance of investigating the expression of multi-drug resistance associated proteins, such as P-gp in transfection studies where drug resistance levels are being monitored.

7. Further analysis of adriamycin-resistant variants, DLKP-A2B and DLKP-A5F, was carried out using a DNA microarray. The array revealed increased levels in a number of the ABC transporter genes, including mdr-1, in some apoptosis-related genes, including galectin-1 and apoptosis-associated tyrosine kinase, as well as a number of other genes. Results revealed quite low levels of repeatability between one array experiment and the next.

8. Analysis of the breast tumour biopsies revealed that mRNA could be extracted from archival tumour samples and used to generate reliable and reproducible RT-PCR results. Results indicated that bcl-2, bag-1 and mcl-1 could be important parameters in determining response to therapy and clinical outcome. None of these genes, however, could be taken individually as a diagnostic or prognostic tool. Bcl-2 mRNA correlated with bag-1 mRNA, ER status, LN status and five year relapse-free survival. Mcl-1 mRNA correlated with tumour grade and had borderline significance with lymph node status and survivin δ3 mRNA.

5.1 Future Work.

1. Future work using the caspase-3 ribozyme could involve transient transfection with short-term selection in geneticin in order to optimise a cytoxicity assay, as transfection efficiency in our 96-well plates was so low, <0.5% (Rasha Linehan, personal communication). Such a system was used by Los and coworkers (1997) and involved adding geneticin 8 hours post-transfection for 36 hours, then treating cells with cytotoxic drug for a further 30 hours. Cell viability was monitored by trypan blue exclusion after 14 days. A system like this would aim to identify the phenotypic effects of ribozyme expression in cells.

2. To determine if the decreased P-gp expression is neutralising the increased drug resistance of the caspase-3 ribozyme clones, apoptosis induction using non-Pgp substrates such as staurosporine or anti-fas antibody could be carried out. This could indicate the effects of down-regulating caspase-3 without interference from P-gp. Subsequent analysis of fodrin cleavage in ribozyme-transfected clones in response to treatment with non-P-gp substrates could be a route to investigating the phenotypic effects of the ribozyme transfection without the interference of P-gp. Apoptosis, induced by non-P-gp drugs should be confirmed by TUNEL assay.

3. Future work could investigate the proposed interaction between caspase-3 and P-glycoprotein. Firstly, transfecting caspase-3 ribozyme into other non-DLKP cell lines would investigate how apoptosis proceeds in the cells in the absence of caspase-3. Suitable cells for transfection of the ribozyme would be a pair of HL-60 cell lines i.e. drug sensitive and P-gp-overexpressing variants, as these cells also express caspase-3 (Mansson et al., 2001, Dr. Mary Heenan, personal communication). In addition, active caspase-3 has previously been analysed by Western blot in HL-60 cells, therefore the effects of ribozyme expression on the active enzyme as well as pro-caspase-3 could be investigated. Secondly, transfection into a cell line, other than DLKP, could establish whether or not the

down-regulation of P-gp after transfection with ribozyme to caspase-3 was exclusive to DLKP cells.

4. To determine whether or not the fluctuations in P-gp expression were due to clonal variation in DLKP-A5F cells, it may be useful to clone out the DLKP-A5F cells.

5. Further investigation of caspase-3 and P-gp may involve transfecting DLKP-A5F cells with cDNA to caspase-3. This could investigate if the increase in caspase-3 causes an increase in P-gp levels – the reverse of what happened in the ribozyme transfectants. Subsequent changes in drug resistance profiles would be monitored.

6. The DNA microarray analysis indicated changes in some genes between the drug resistant and sensitive cell lines. These could be validated by RT-PCR and Western blot. This could identify genes influencing drug resistance and perhaps pinpoint genes suitable for further analysis via transfection studies.

7. Future work may investigate drug-induced gene expression by transient treatment of the sensitive and resistant DLKP variants with drug. Analysing extracts from these cells using DNA arrays could identify any induced gene expression changes due to drug treatment.

8. Based on the low level repeatability of the DNA microarray technique, future work could focus on its further optimisation. This could involve analysing an identical RNA sample on two separate arrays and gene expression analysis. Comparison of results should indicate the reproducibility of the technique. Subsequent to this, an identical RNA sample could also be analysed on the same microarray twice, so as to establish if re-using the arrays affects the accuracy of the technique.

9. When the DNA microarray technique has been optimised satisfactorily, some of the breast tumours could be analysed for gene expression. Because tumours are heterogeneous in nature, it is possible that the original RNA extractions included RNA from surrounding non-tumorigenic tissue. For this reason, laser capture

microscopy may be used in future investigations on the breast tumours. This technique would ensure that tumour cells were exclusively targeted for analysis and could establish exactly what genes are exclusive to tumour cells compared with normal cells. Once such methods have been optimised and become more accessible in terms of budget, they could in the future, become tools for routine analysis in both research and diagnostic labs.

10. Tetracycline-repressible clones of SKOV-3Tet-off cells containing the bcl-xL ribozyme have been established in our laboratory. This tetracycline-repressible system allows the transfected gene expression to be switched on or off in response to tetracycline. In this case, ribozyme-transfected cells are sensitive to tetracycline and ribozyme expression is switched off in the presence of the antibiotic. Expression of the other bcl family members could be investigated in these cell lines with particular emphasis on mcl-1 and bad, due to their changes in the DLKP-SQ and A250-10p clones.

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Section 7.0: Appendices.

7.1 Appendix A: Primer sequences & conditions used in this study.

7.1.1 Primers used for RT-PCR gene expression analysis.

Table 7.1.1 Primer sequences used for RT-PCR analysis.

Gene	Length (bases)	Tm (C)	Size (bp)	Sequence
β-actin (large)	29	55	383	GAA ATC GTG GCT GAC ATT AAG GAG AAG CT
				TCA GGA GGA GCA ATG ATC TTG A
β-actin (small)	23	55	142	TGG ACA TCC GCA AAG ACC TGT AC
				TCA GGA GGA GCA ATG ATC TTG A
Bag-1	20	52	251	TCC AGC TGG TTA GCT ATC TT
	20			AGC AGT GAA CCA GTT GTC CA
Baxα	21	50	230	GAC GAA CTG GAC AGT AAC ATG
	20			AGG AAG TCC AAT GTC CAG CC
Bcl-2α	22	50	306	TCA TGT GTG TGG AGA GCG TCA A
	24			CTA CTG CTT TAG TGA ACC TTT TGC
Caspase-3	18	49	314	GAA TGA CAT CTC GGT CTG
	18			ACG GCA GGC CTG AAT AAT
Mcl-1 (large)	18	41	523	TCTCTCGGTACCTTCGGG
	18			GCACTTACAGTAAGGCTATC
Mcl-1 (small)	18	53	217	TCT CTC GGT ACC TTC GGG
	18			CTA TCT TAT TAG ATA TGC

Table 7.2 IC50 values in DLKP cell lines.

Cell Line	IC50 Adriamycin
DLKP[1]	42.8 ± 25 ng/ml
DLKP-I[2]	10.0 ± 2.4 ng/ml
DLKP-M[5]	14.9 + 4.8 ng/ml
DLKP-A10[3]	11545 + 781 ng/ml
DLKP-A[1]	10879.3 ± 5112 ng/ml
DLKP-A2B[1]	1574 ± 682.8 ng/ml
DLKP-A6B[1]	4048.3 ± 1397 ng/ml
DLKP-A5F[1]	14152 ± 2933 ng/ml
DLKP-A11B[1]	3590 ± 534 ng/ml
DLKP-A2B-1C7*	46 ± 31ng/ml
DLKP-SQ[2]	13.9 ± 3.5 ng/ml
A250-10p[2]	139.2 ± 58.8 ng/ml
DLKP-Melph-ST[4]	115.9 ± 14.8 ng/ml
DLKP-Melph-LT[4]	142.8 ± 13.3 ng/ml
SQ-fos-Rz[2]	18.6 ± 12.5ng/ml
DLKP-VP-3[1]	3857 ± 567
DLKP-VP-8[1]	3923.3 ± 1630
T1-10[1]	18 ± 0.4 ng/ml
T2-10[1]	290 ng/ml

[1] Mary Heenan, PhD 1994.

[2] Roisin NicAmhlaoibh, PhD 1997.

[3] Irene Cleary, PhD 1995.

[4] Yizheng Liang, PhD 1999.

[5] Shirley McBride, PhD 1995.

* Daly et al., 1996.

7.3.1 RT-PCR results for Bcl-2 in breast tumour biopsies. All graphs display densitometric analysis of gene expression levels. Units are densitometric arbitrary units.

Figure 7.3.1.1a: N1-N6.

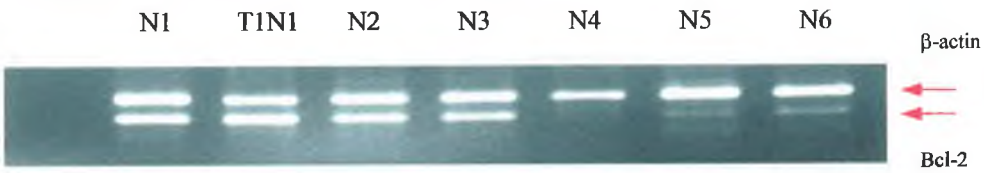


Figure 7.3.1.1b:

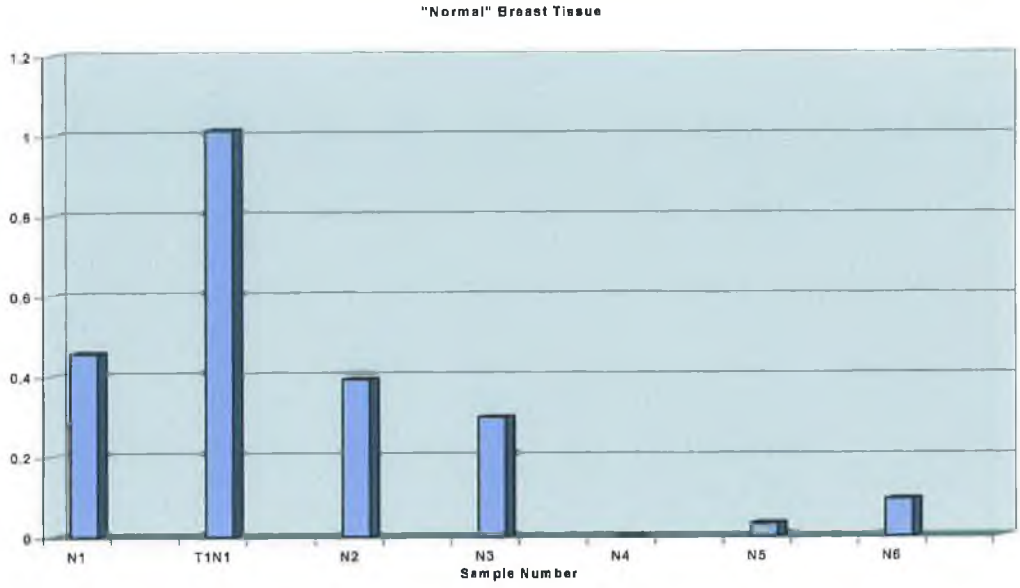
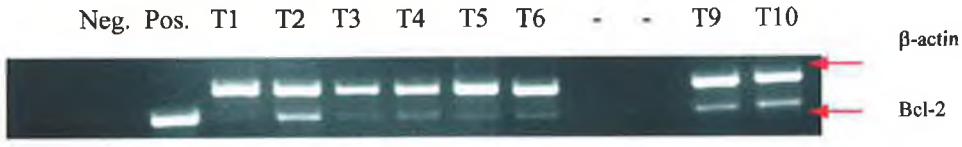


Figure 7.3.1.2a: T1-T10.



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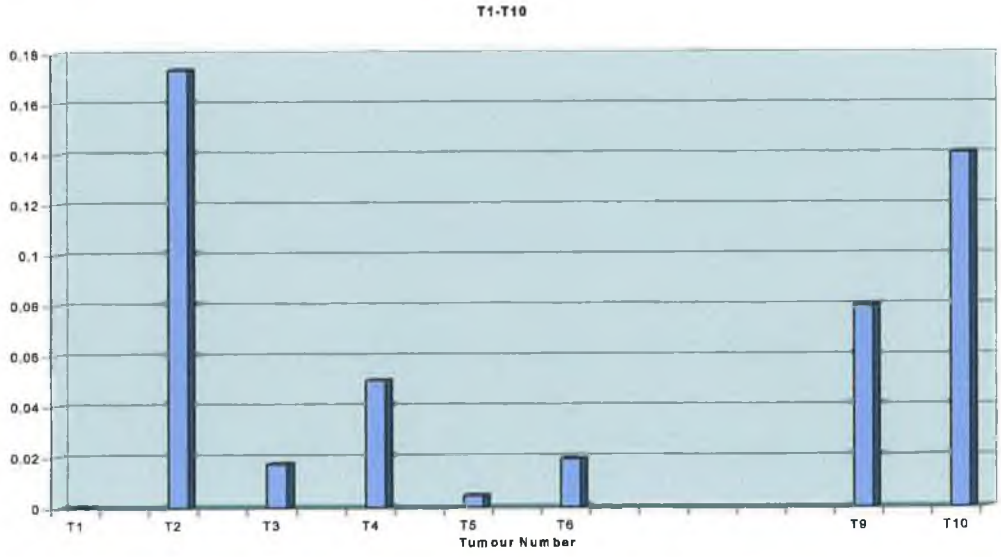


Figure 7.3.1.3a: T11-T20.

Figure 7.3.1.3b:

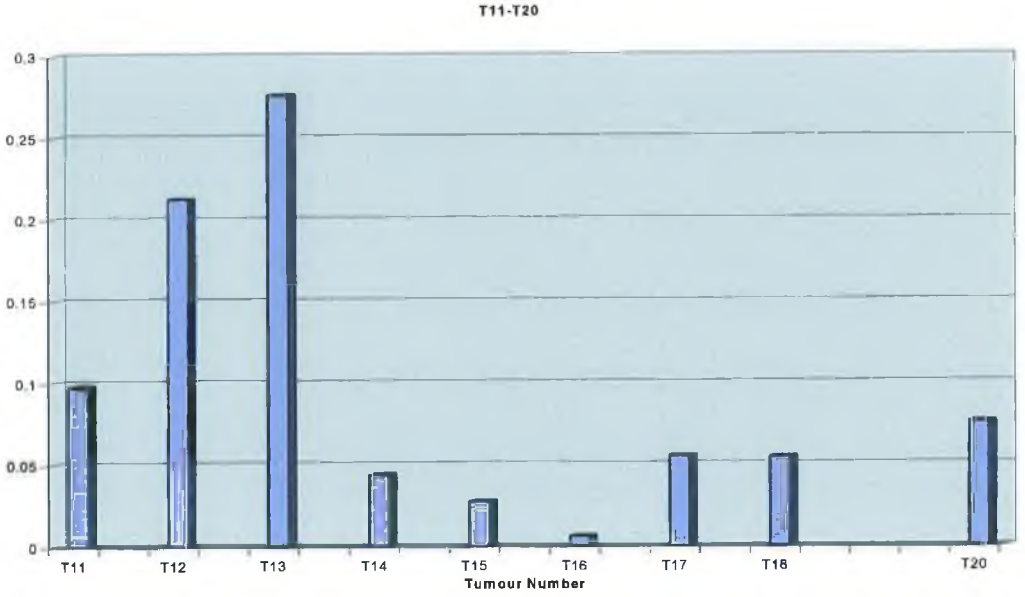


Figure 7.3.1.4a: T21-T30.

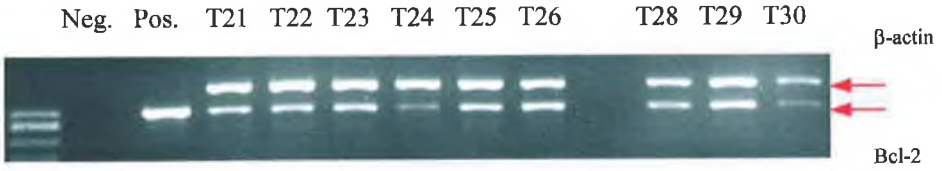


Figure 7.3.1.4b

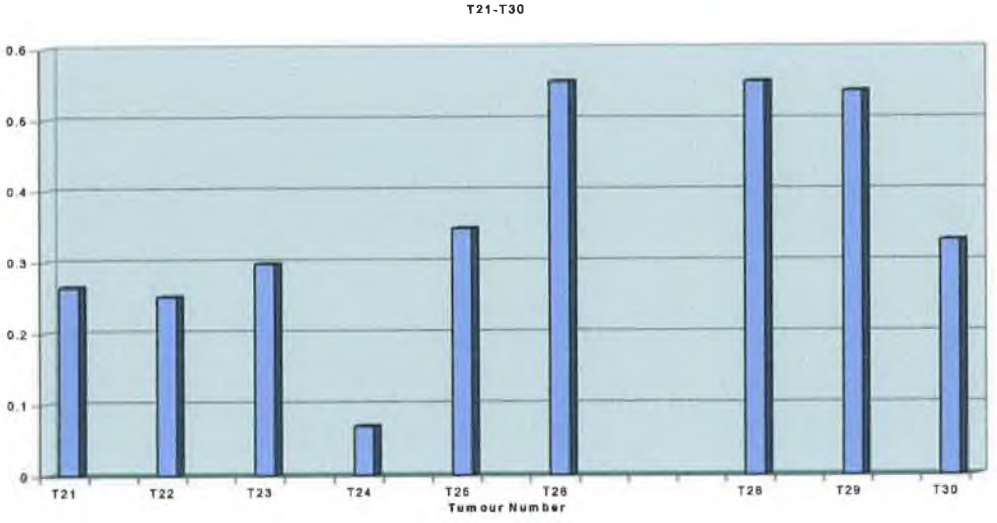


Figure 7.3.1.5a: T31-T39.

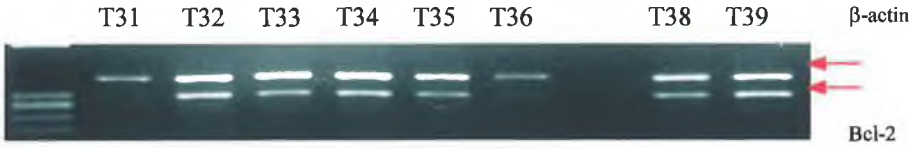


Figure 7.3.1.5b

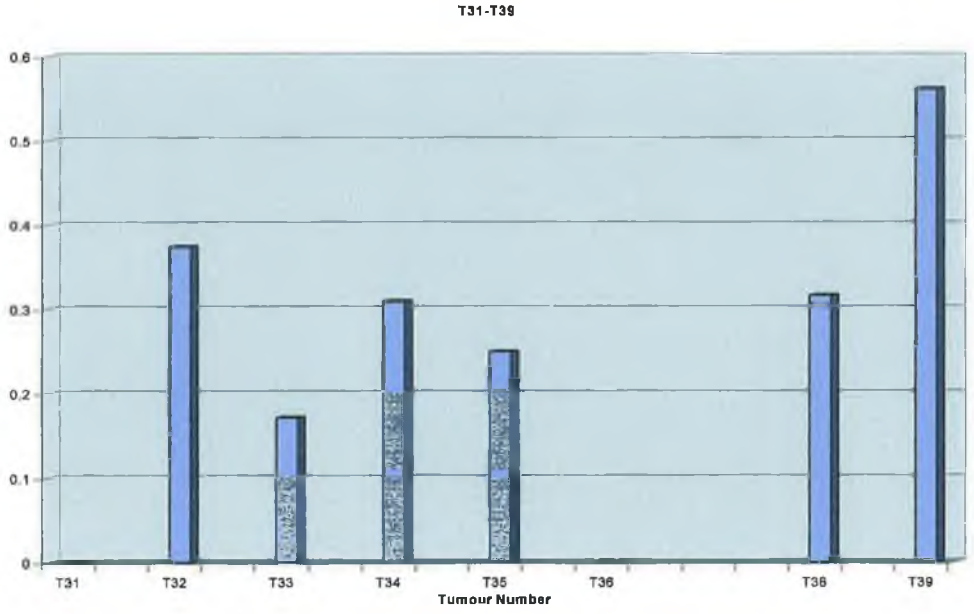


Figure 7.3.1.6a: T41-T50.

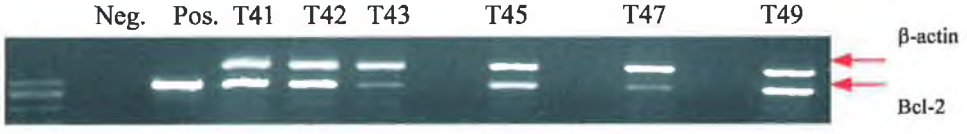


Figure 7.3.1.6b:

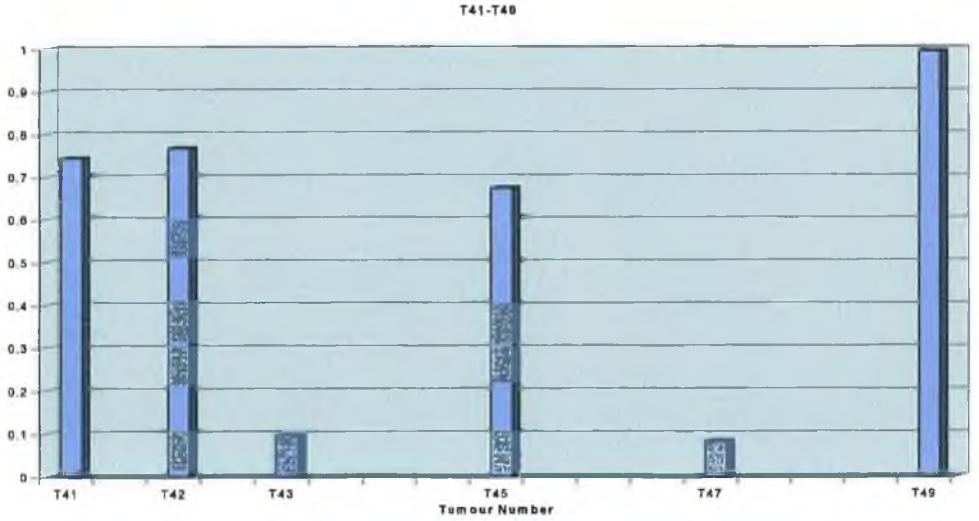


Figure 7.3.1.7a T51-T60.

Figure 7.3.1.7b

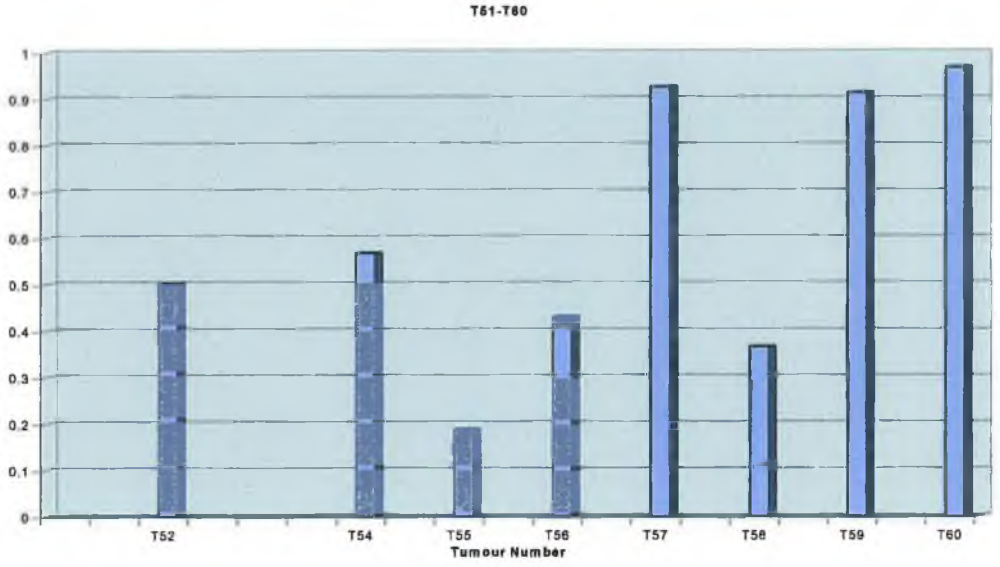


Figure 7.3.1.8a: T61-T69.

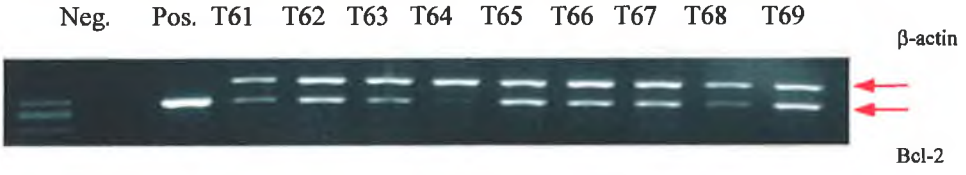


Figure 7.3.1.8b

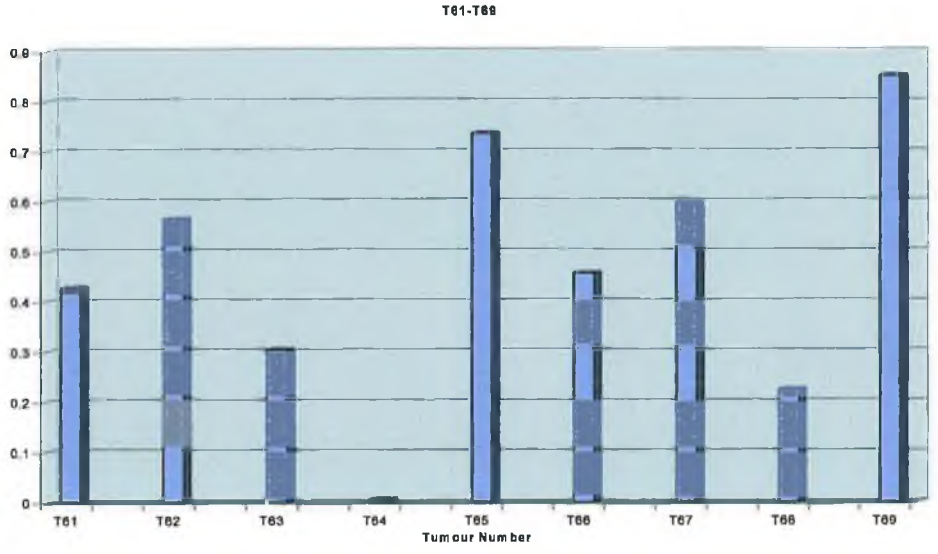
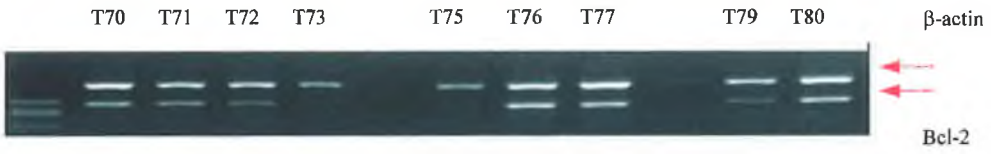


Figure 7.3.1.9a: T70-T80.



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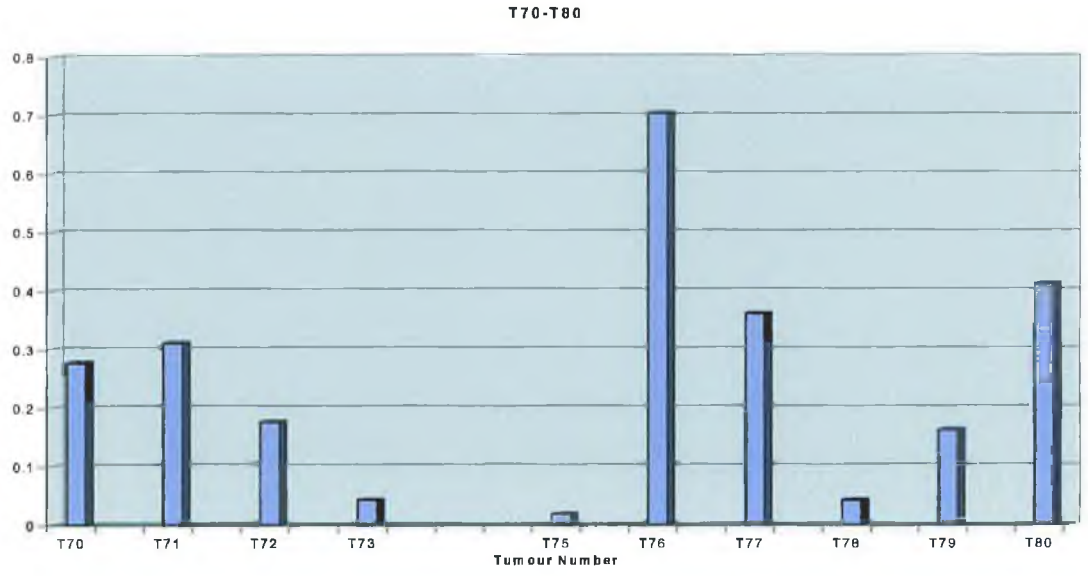


Figure 7.3.1.10a: T81-T90.

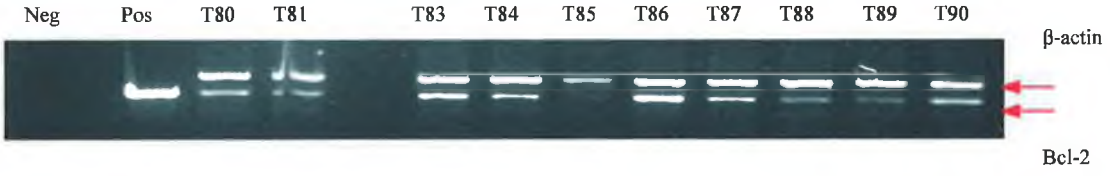


Figure 7.3.1.10b

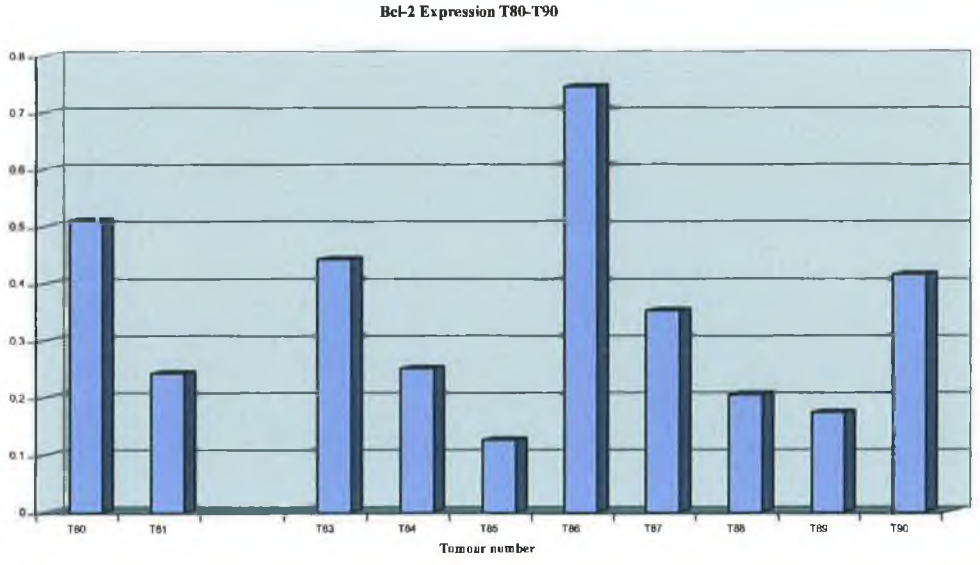


Figure 7.3.1.11a: T91-T100

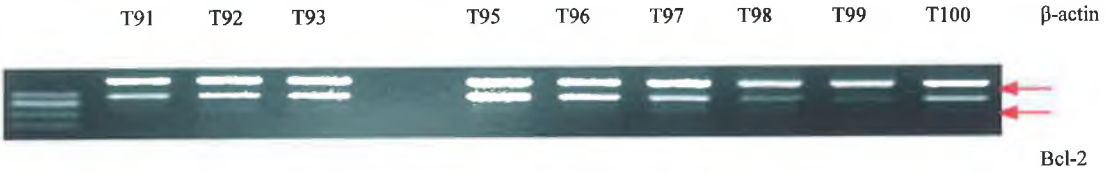


Figure 7.3.1.11b

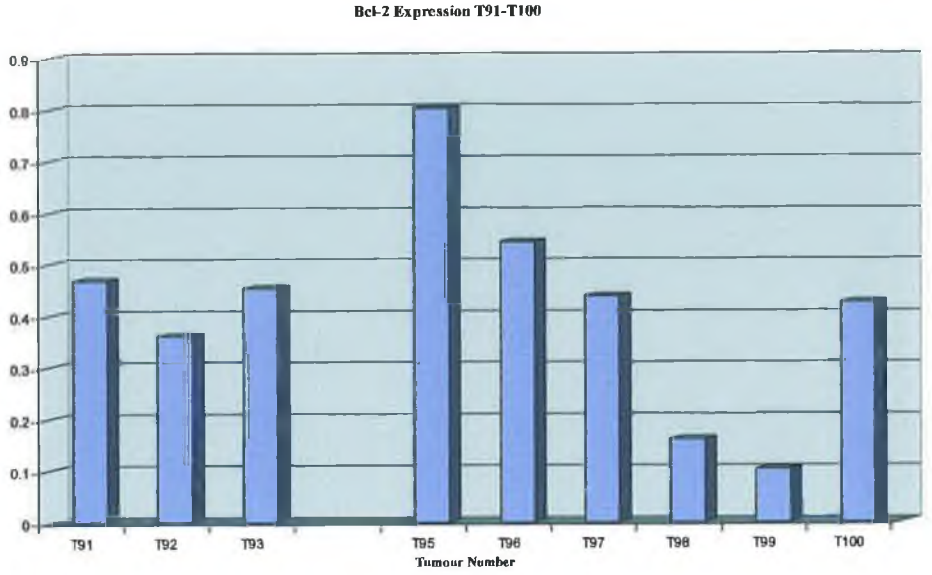
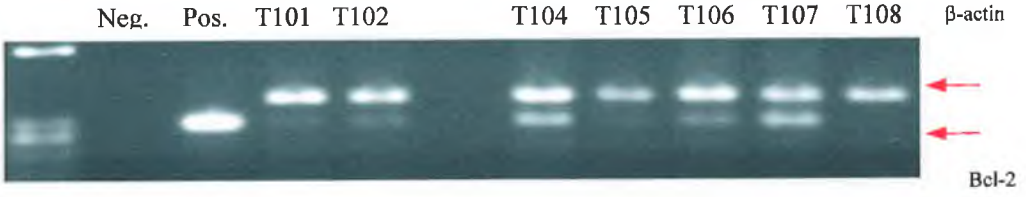


Figure 7.3.1.12a: T101-T108.



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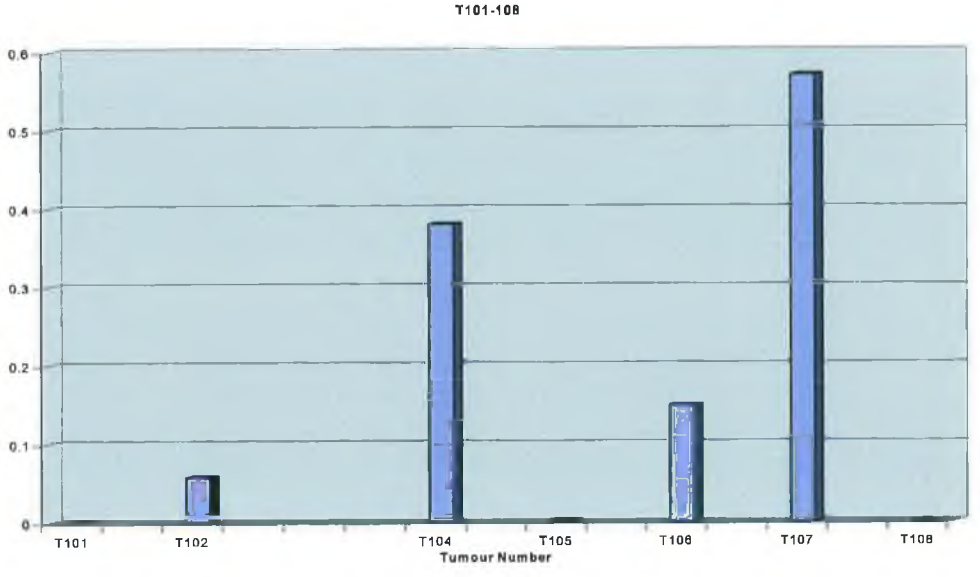
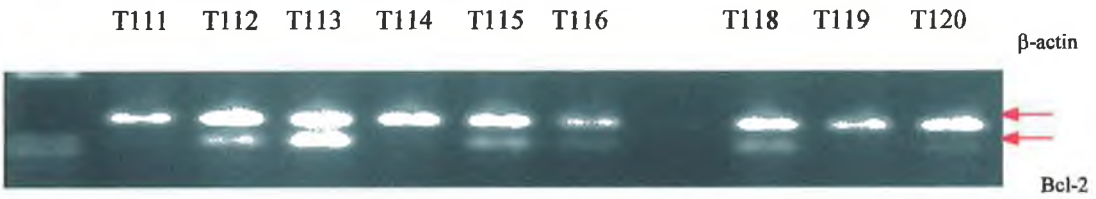


Figure 7.3.1.13a: T111-T120.



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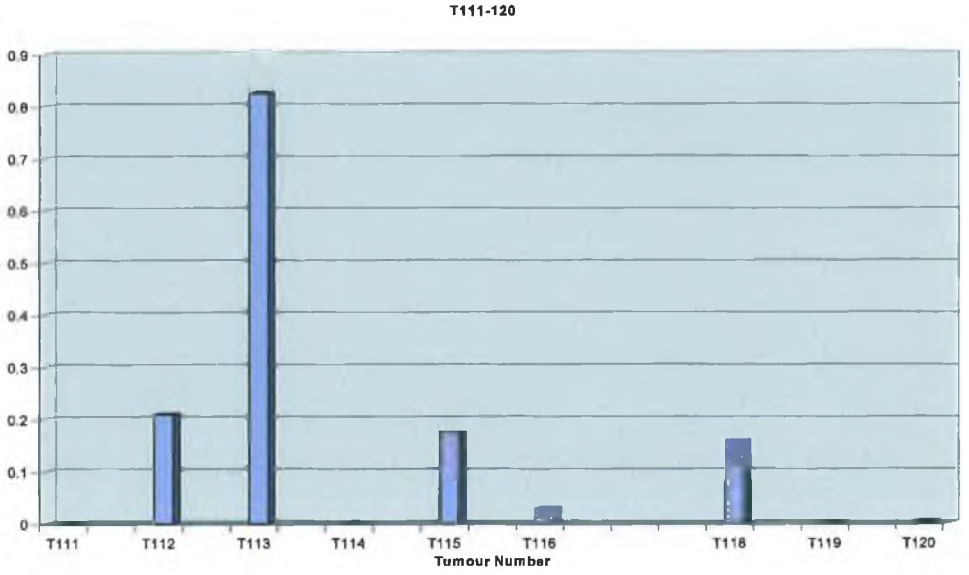


Figure 7.3.1.14a: T121-T129.

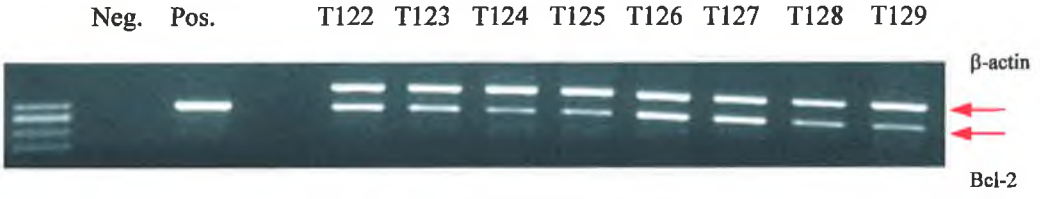


Figure 7.3.1.14b:

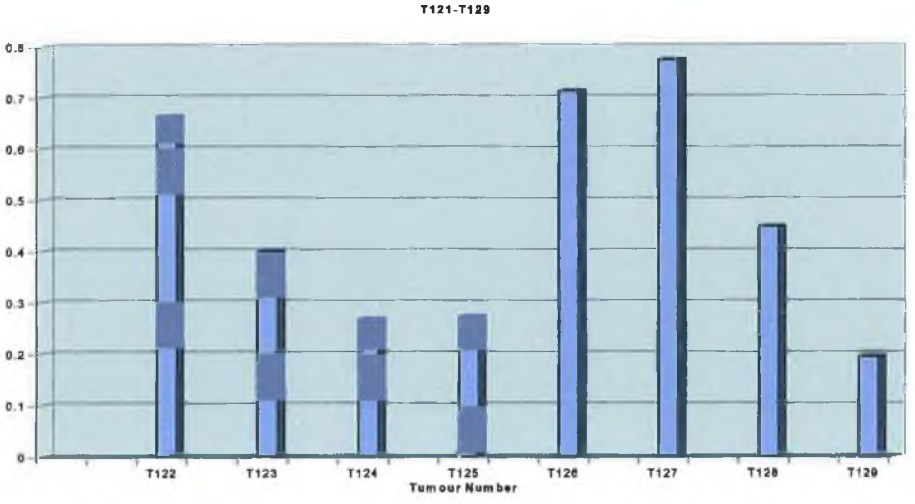


Figure 7.3.1.15a: T131-T134.

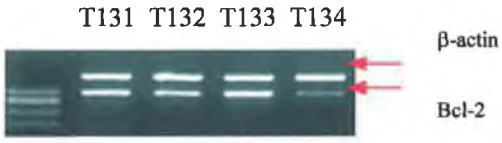


Figure 7.3.1.15b

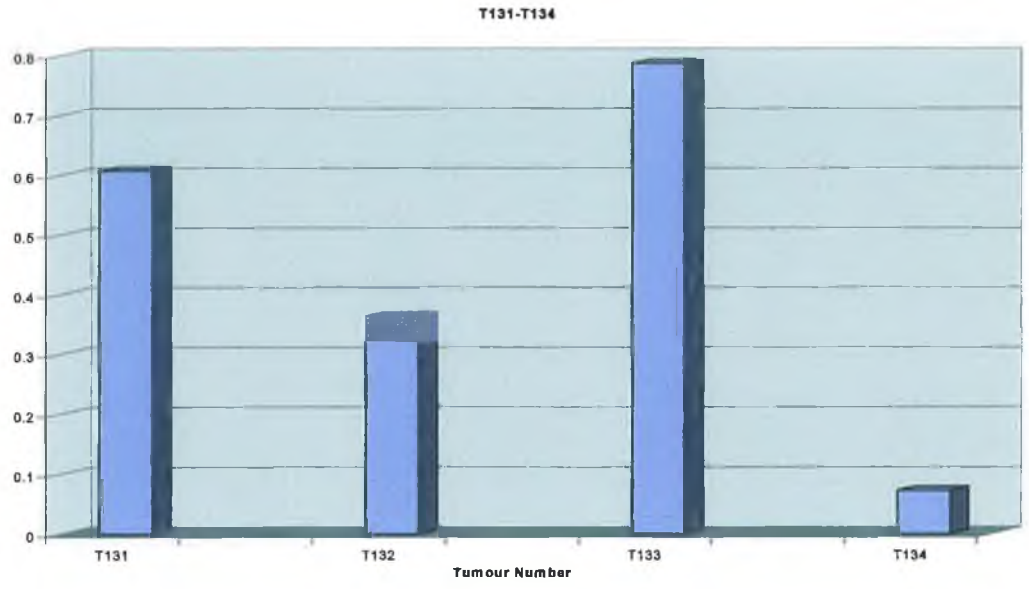


Figure 7.3.1.16a: S1-S10.

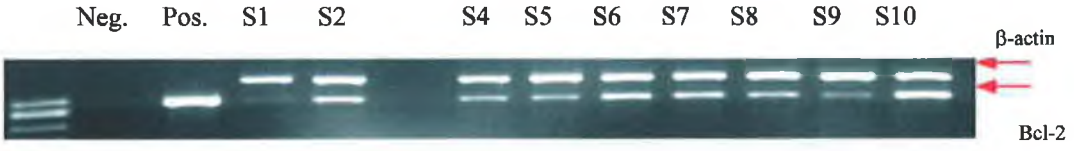


Figure 7.3.1.16b:

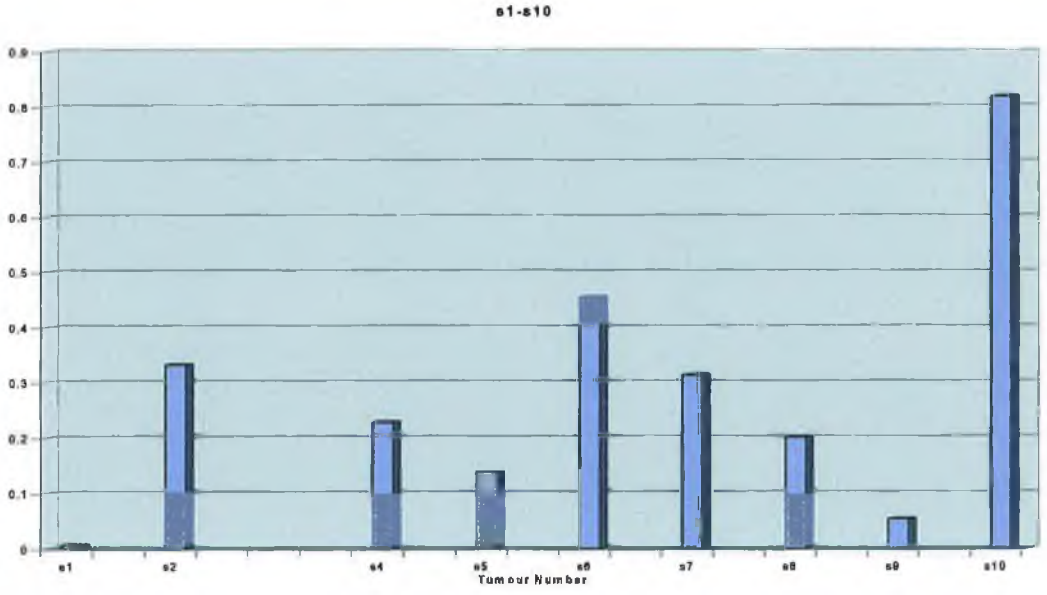


Figure 7.3.1.17a: S11-S20.

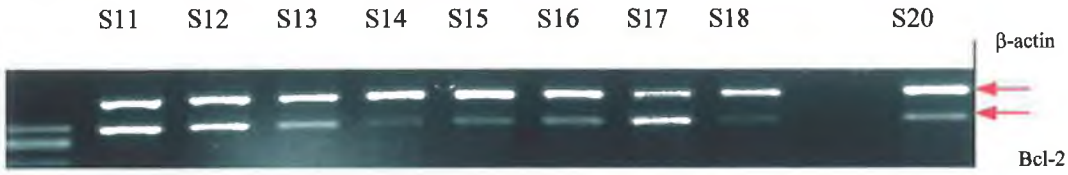


Figure 7.3.1.17b:

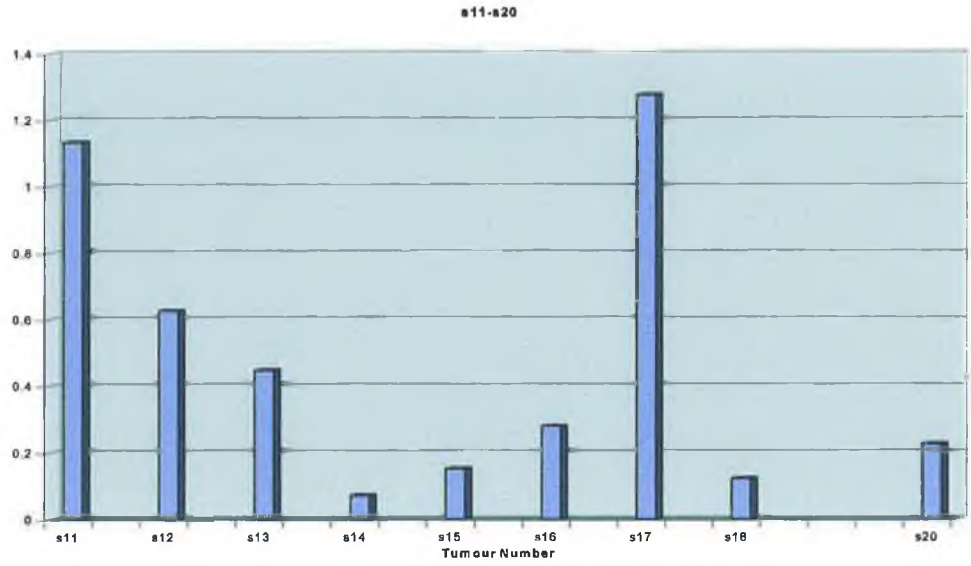
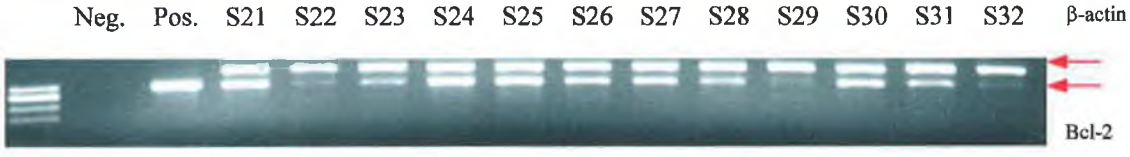


Figure 7.3.1.18a: S21-S32.



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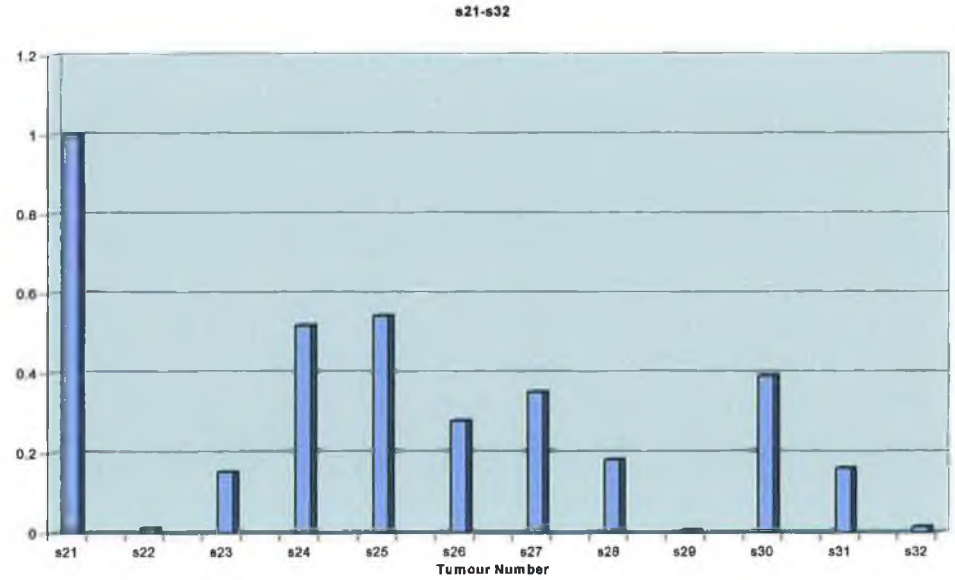


Figure 7.3.1.19a: S33-S37.

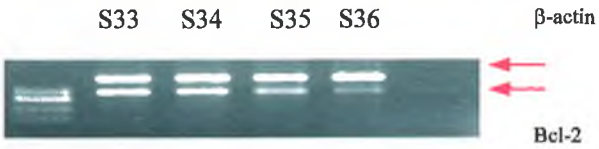
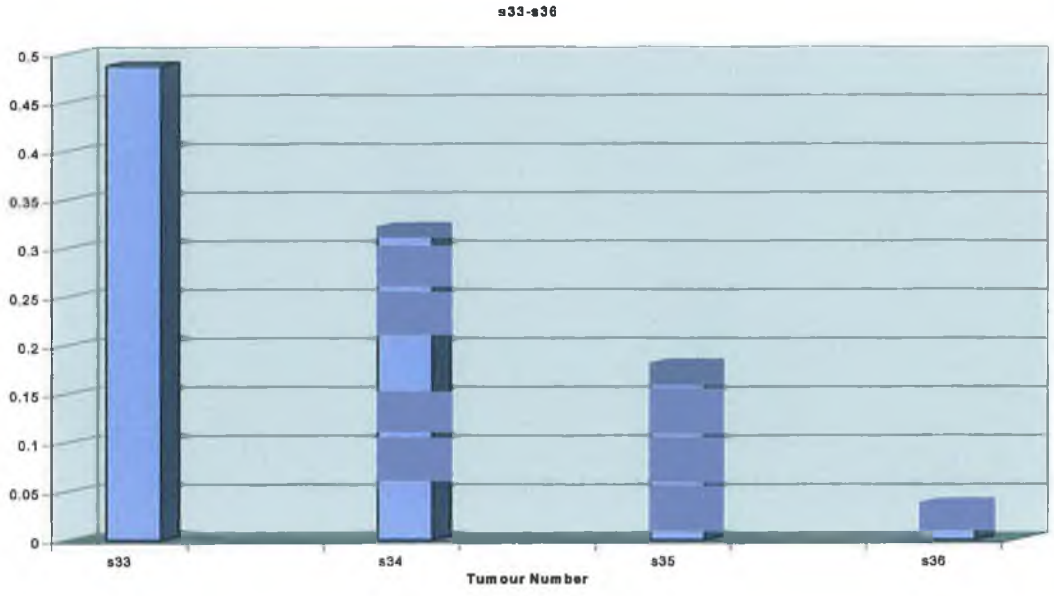
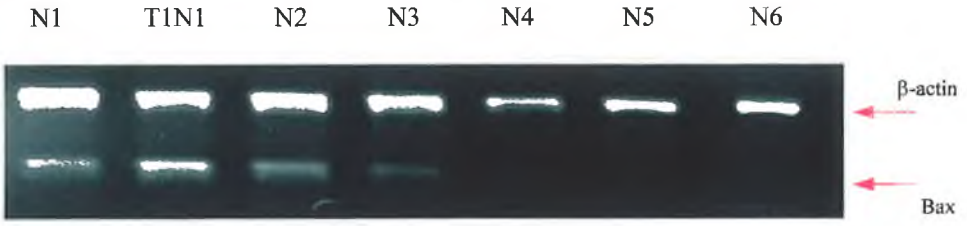


Figure 7.3.1.19b:



7.3.2 RT-PCR results for bax expression in breast tumour biopsies. Graphs display densitometric analysis of gene expression. Units are densitometric arbitrary units.

Figure 7.3.2.1a: N1-N6.



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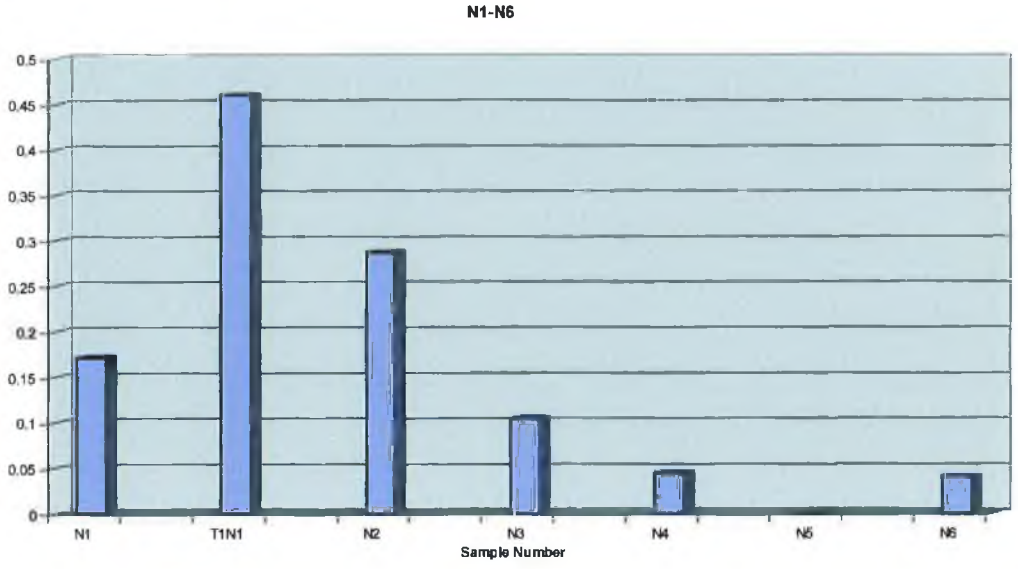
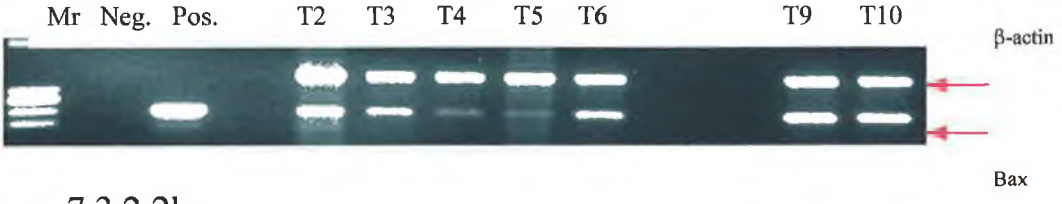


Figure 7.3.2.2a: T1-T10.



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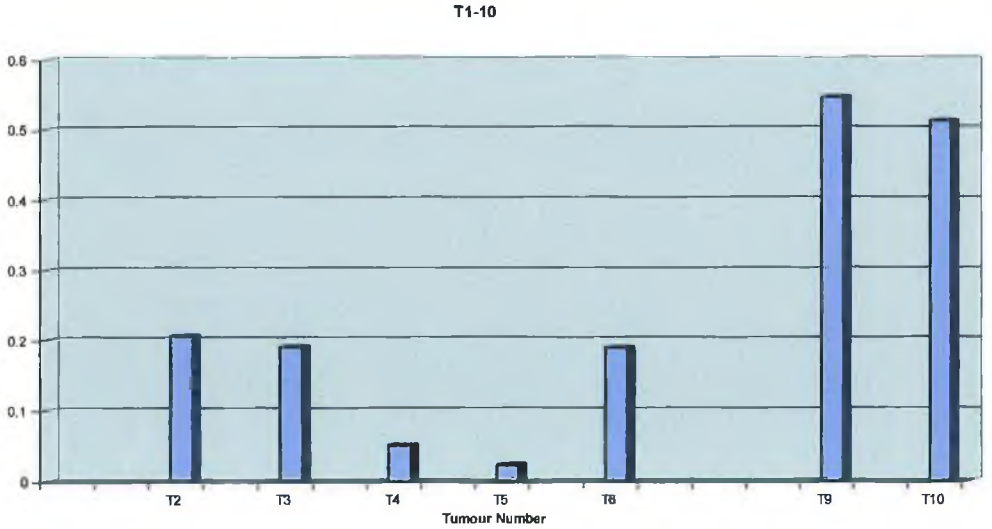


Figure 7.3.2.3a: T11-T20.

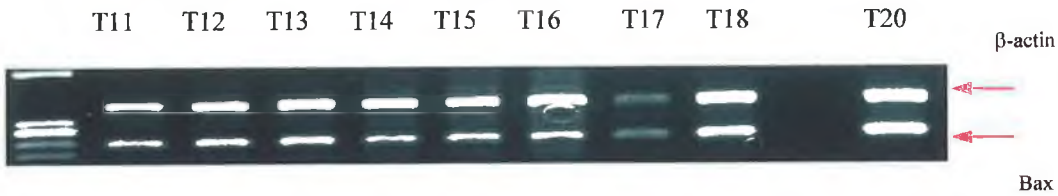


Figure 7.3.2.3b:

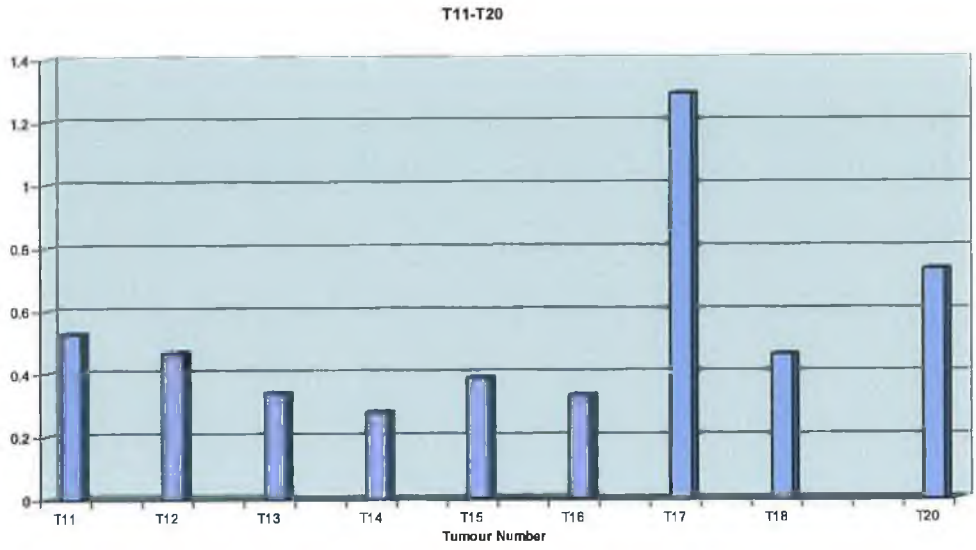


Figure 7.3.2.4a : T21-T30.

Figure 7.3.2.4b:

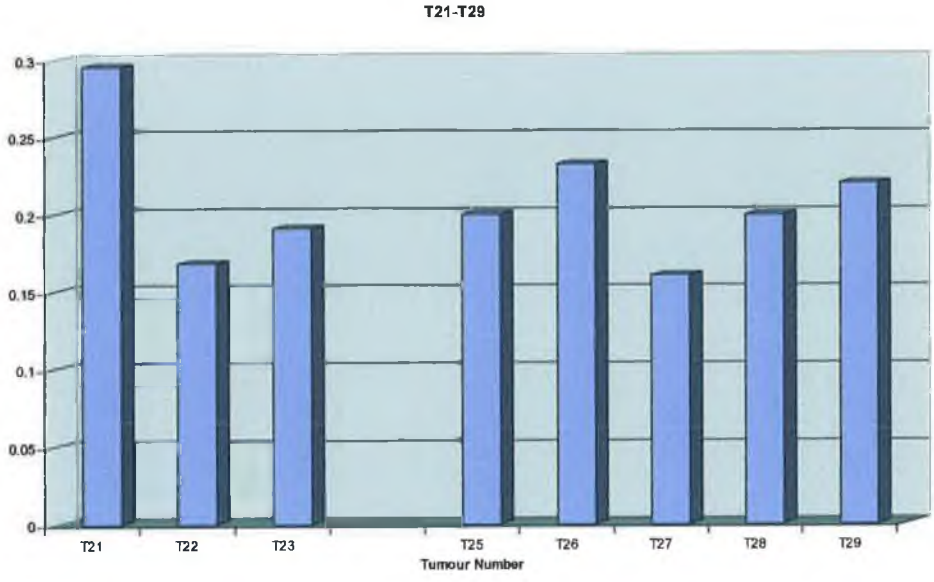


Figure 7.3.2.5a T31-T40.

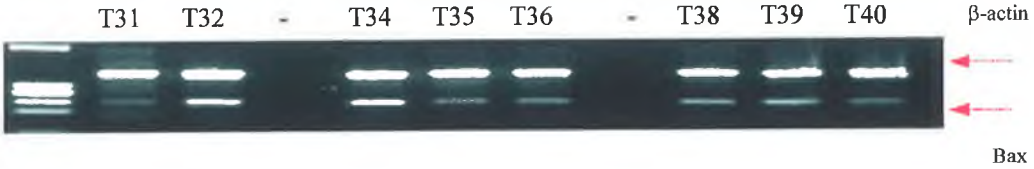


Figure 7.3.2.5b:

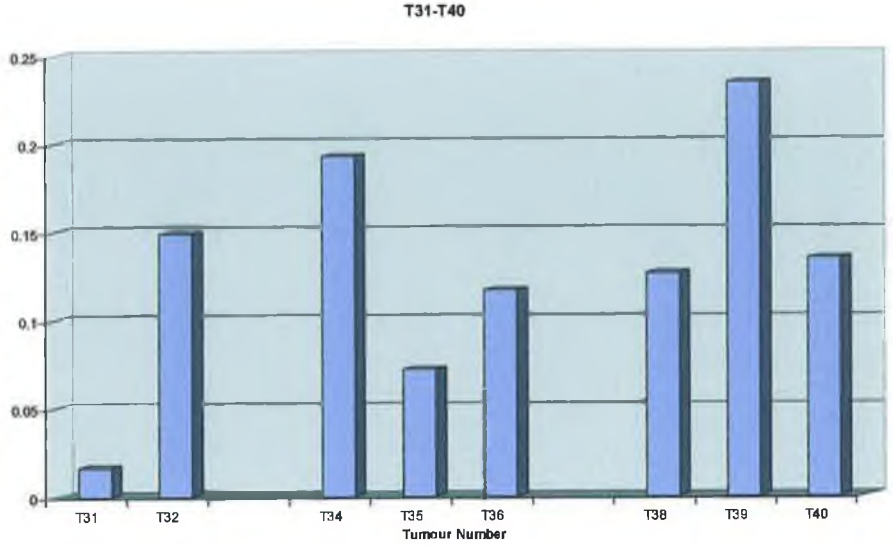


Figure 7.3.2.6a: T40-T50.

Figure 7.3.2.6b:

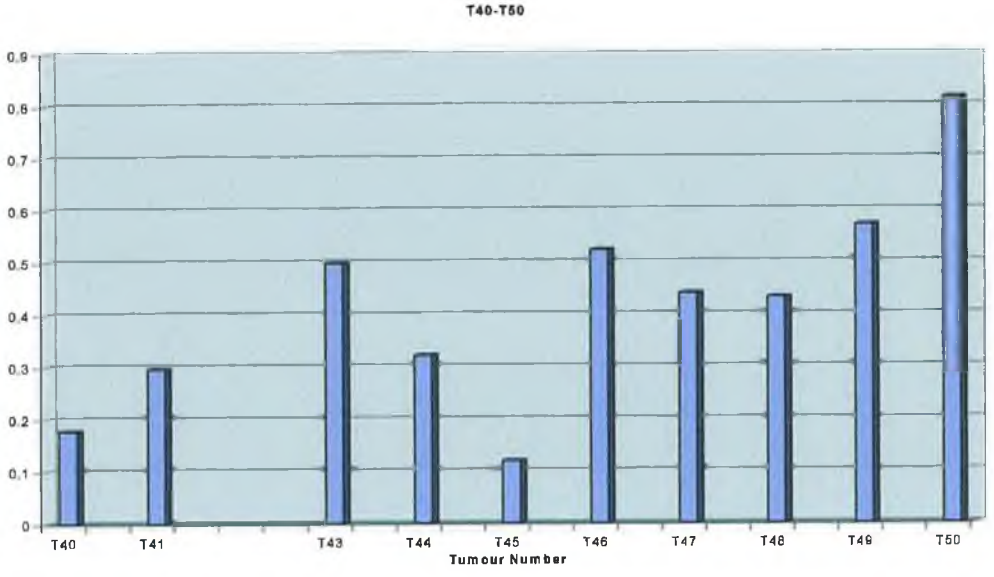


Figure 7.3.2.7a: T51-T60.

Figure 7.3.2.7b:

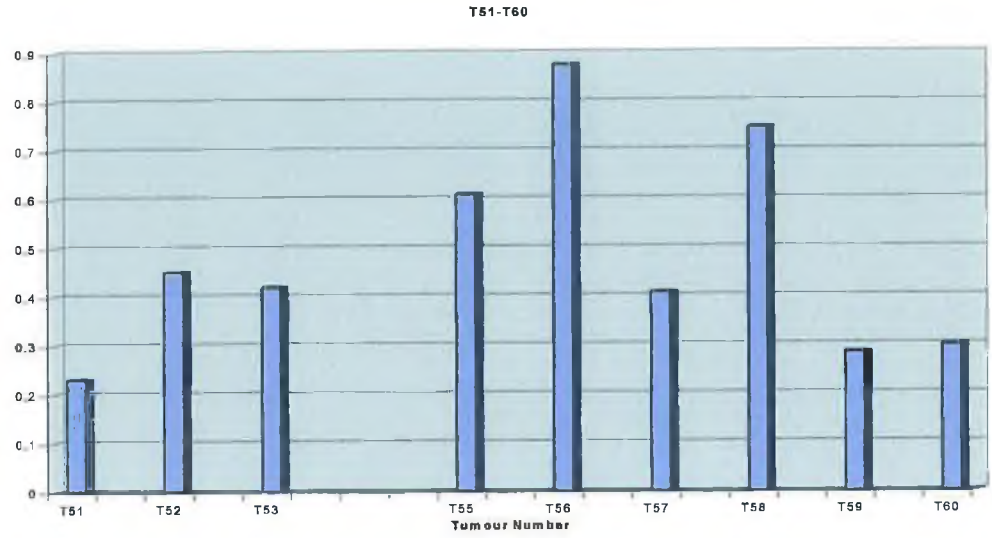


Figure 7.3.2.8a: T61-T70.

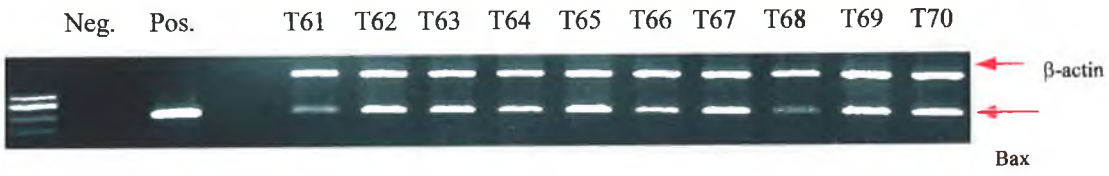


Figure 7.3.2.8b:

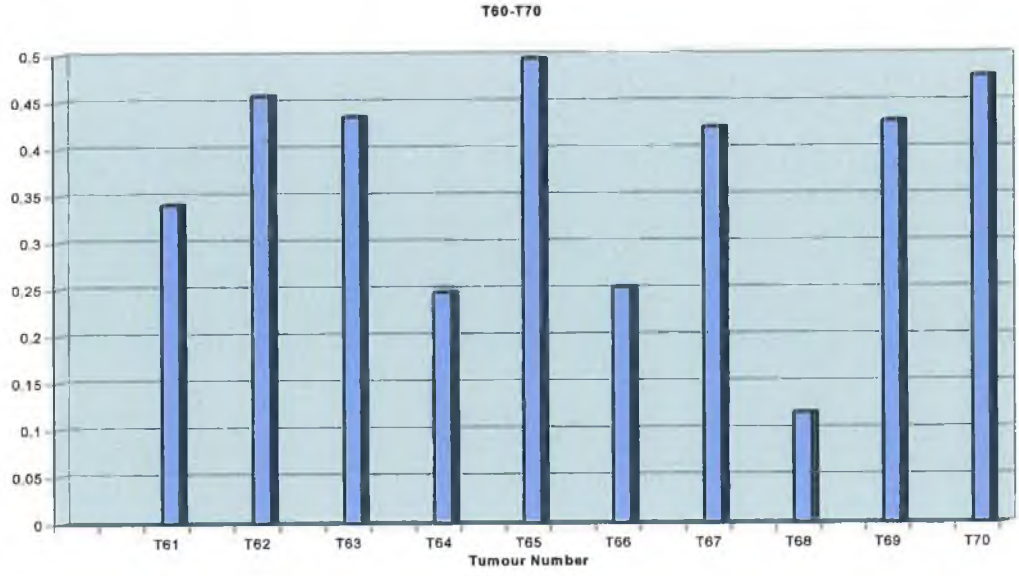


Figure 7.3.2.9a : T71-T80.

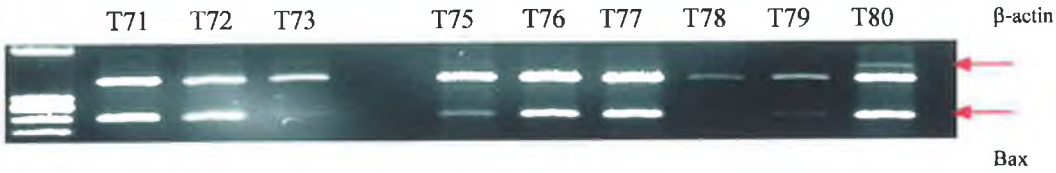


Figure 7.3.2.9b:

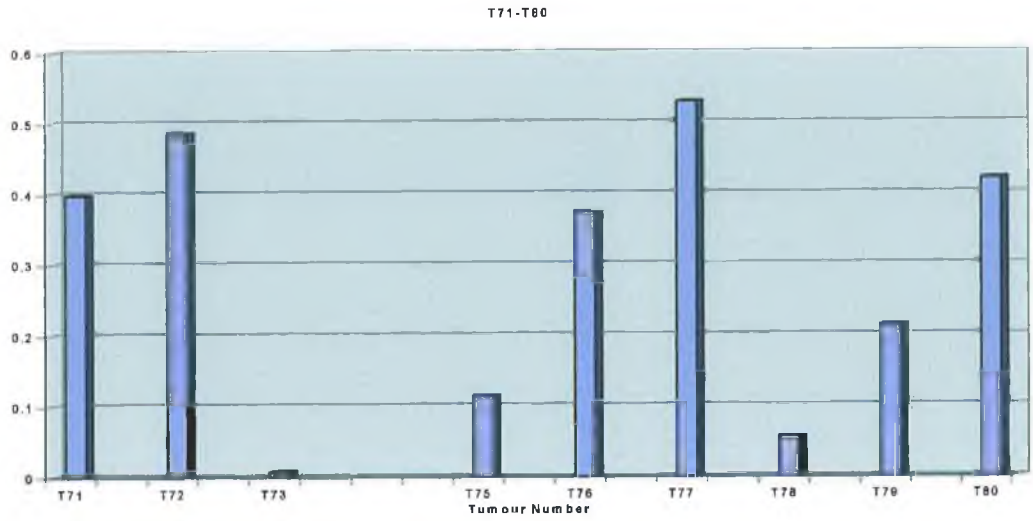


Figure 7.3.2.10a : T81-T90.

Figure 7.3.2.10b:

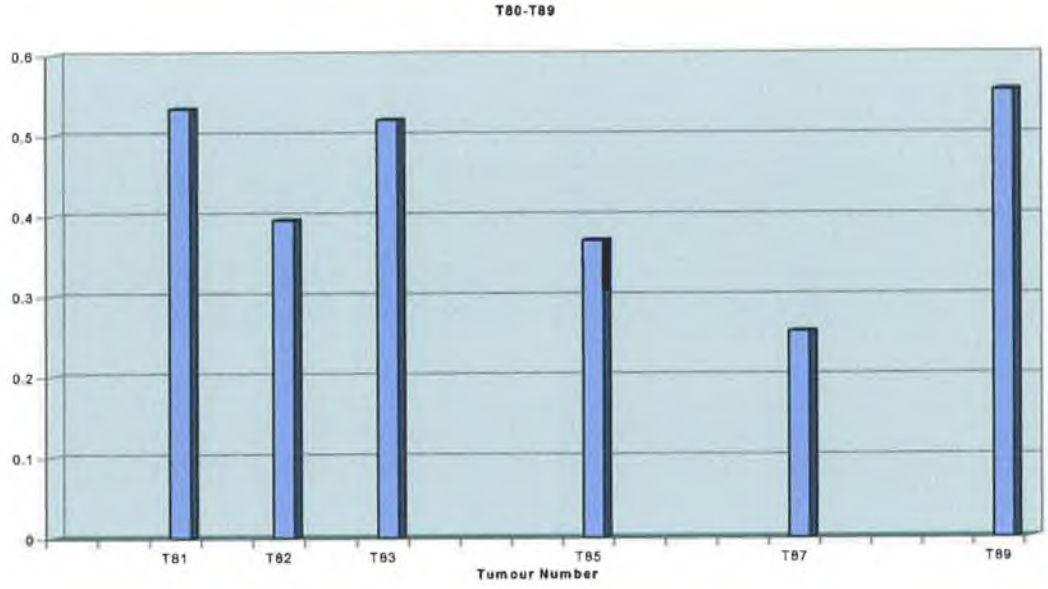


Figure 7.3.2.11a: T91-T100.

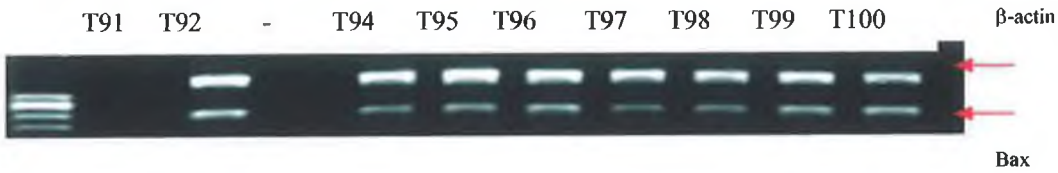


Figure 7.3.2.11b:

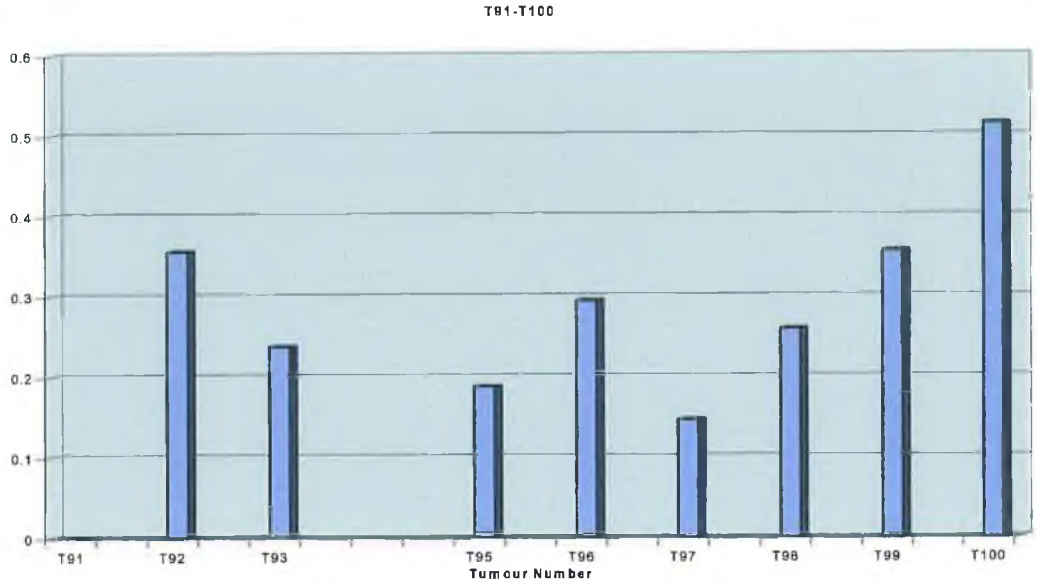


Figure 7.3.2.12a: T101-T108.

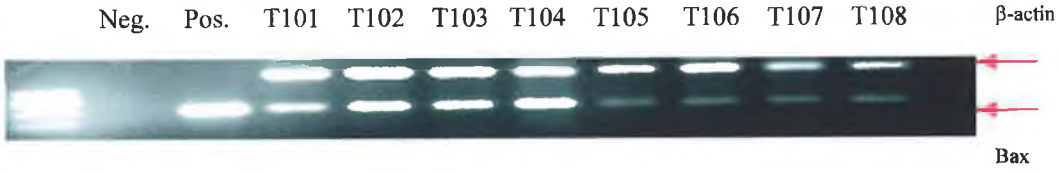


Figure 7.3.2.12b:

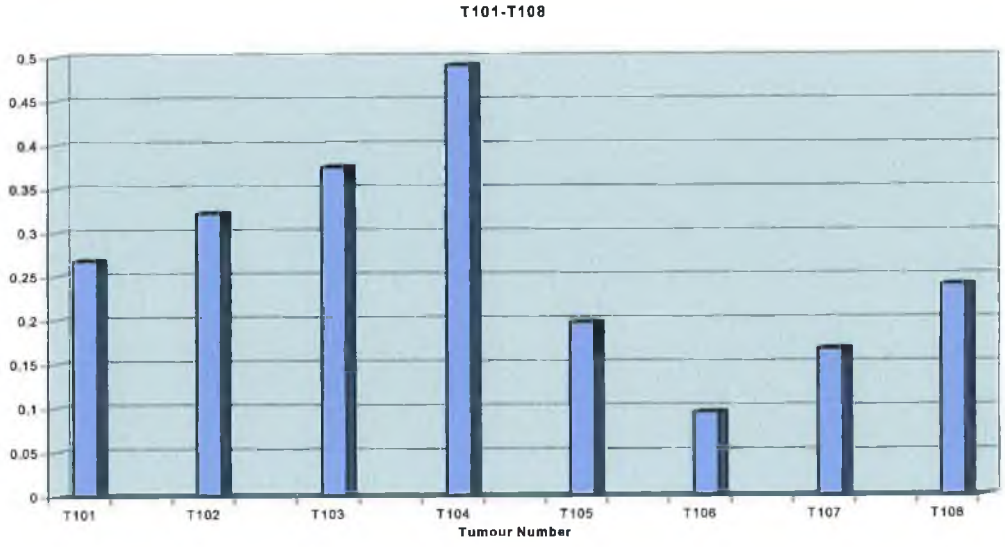


Figure 7.3.2.13a: T111-T120.

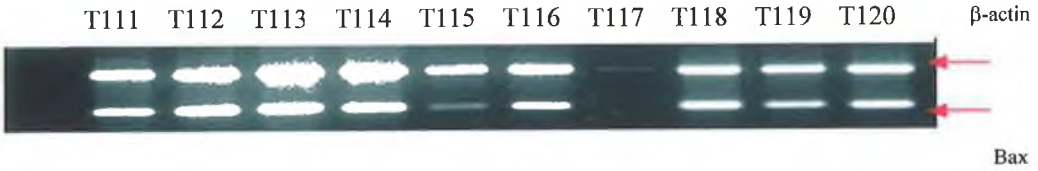


Figure 7.3.2.13b:

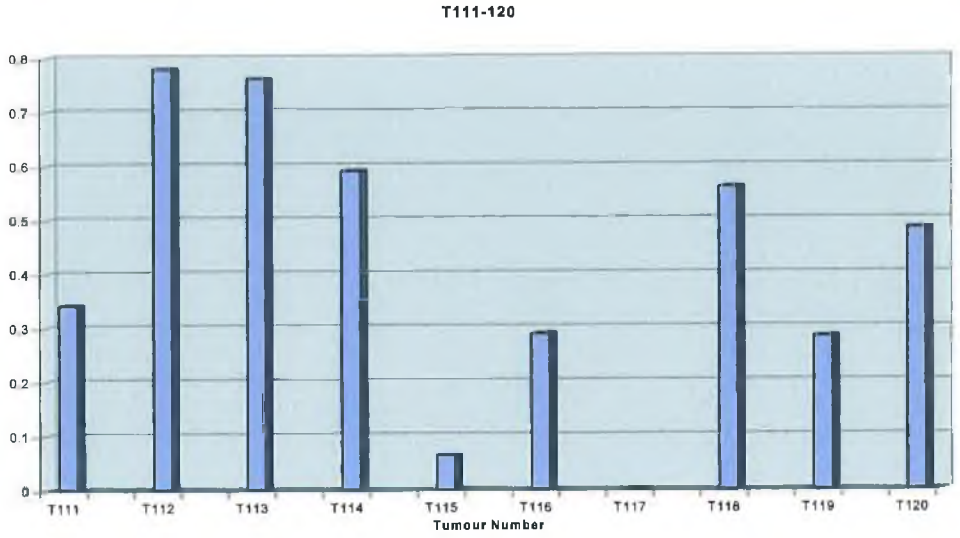


Figure 7.3.2.14a: T121-T130.

Figure 7.3.2.14b:

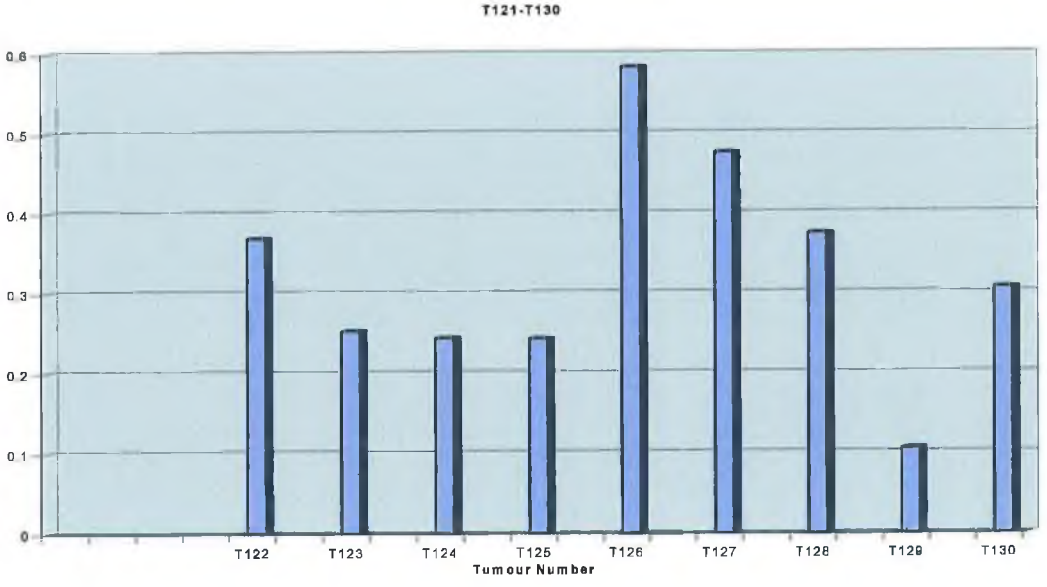


Figure 7.3.2.15a: T131-T134.

Figure 7.3.2.15b:

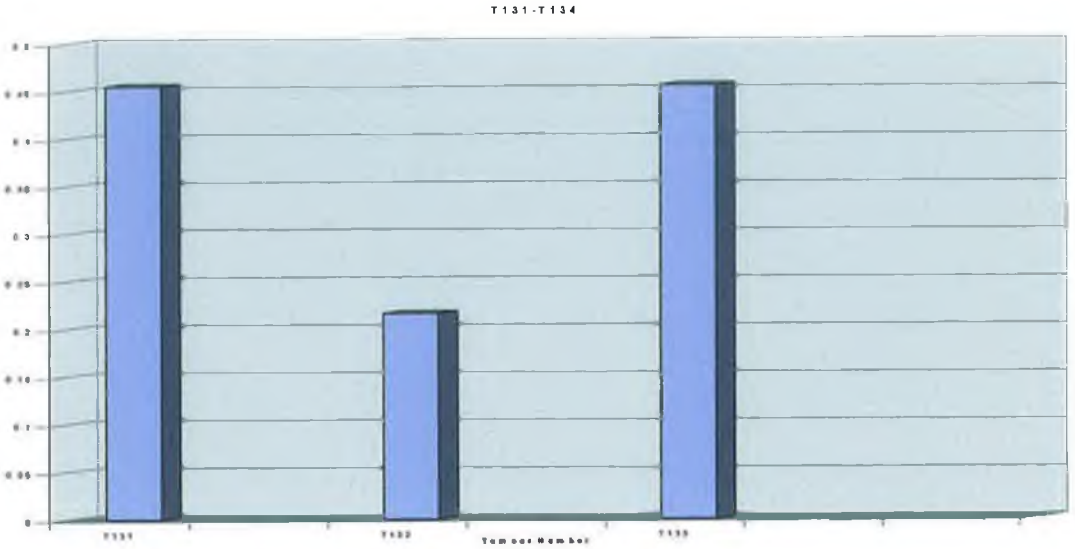


Figure 7.3.2.16a: S1-S10.

Figure 7.3.2.16b:

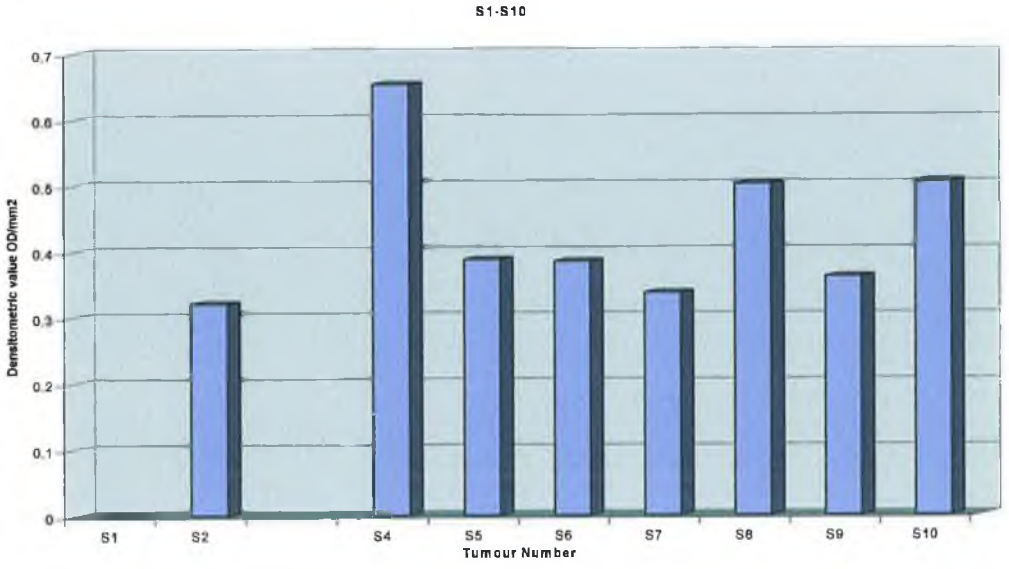


Figure 7.3.2.17a: S11-S20.

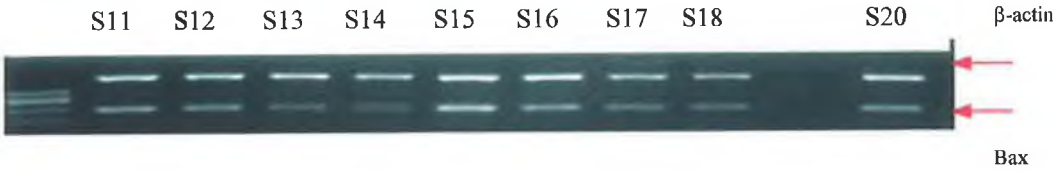


Figure 7.3.2.17b:

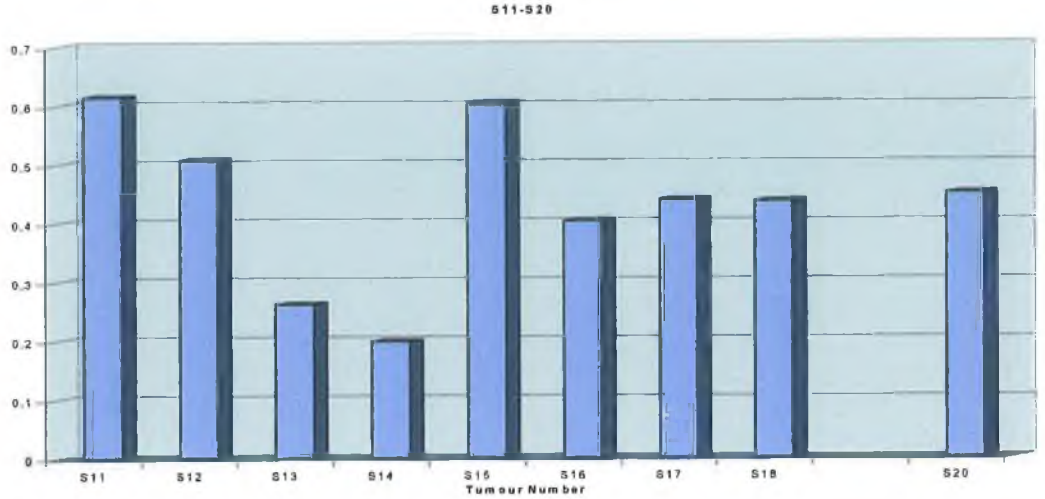


Figure 7.3.2.18a: S21-S30.

Figure 7.3.2.18b:

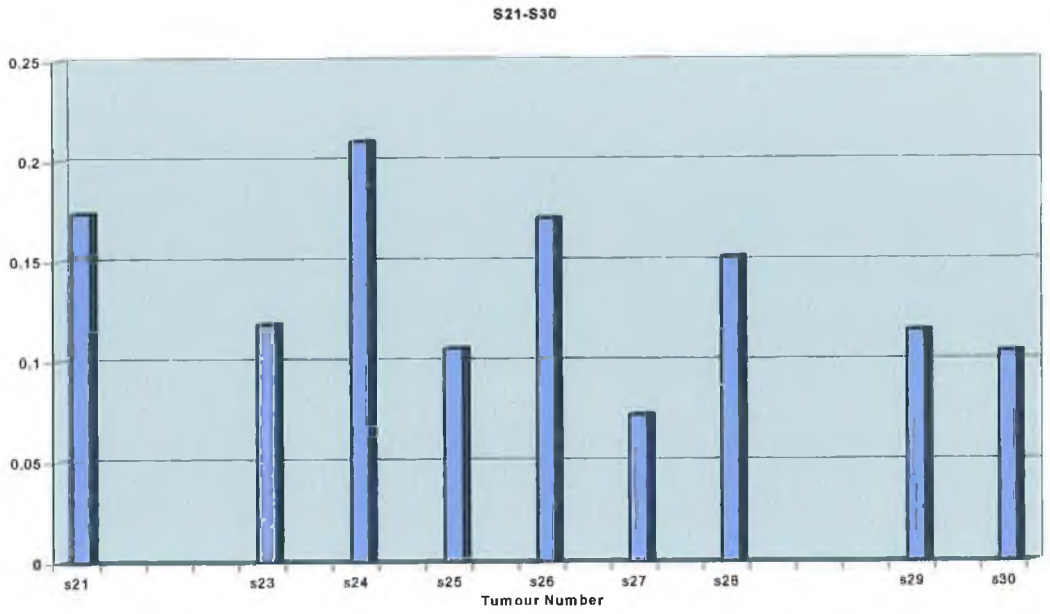
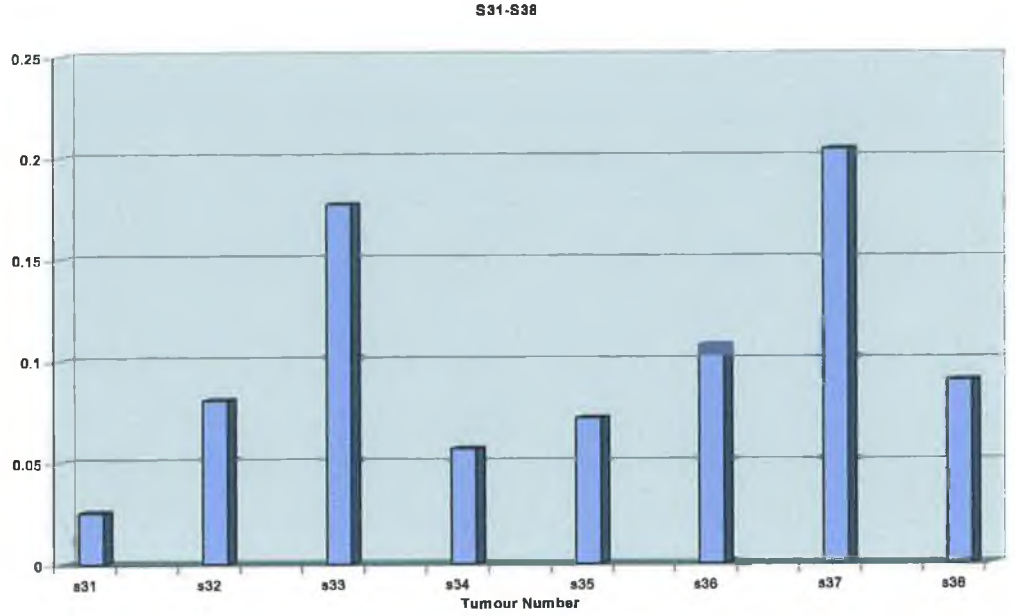


Figure 7.3.2.19a: S31-S38.

Figure 7.3.2.19b:



7.3.3 RT-PCR results for Mcl-1 expression in breast tumour biopsies. Graphs show densitometric analysis of gene expression. Units are densitometric arbitrary units.

Figure 7.3.3.1a N1-N6.

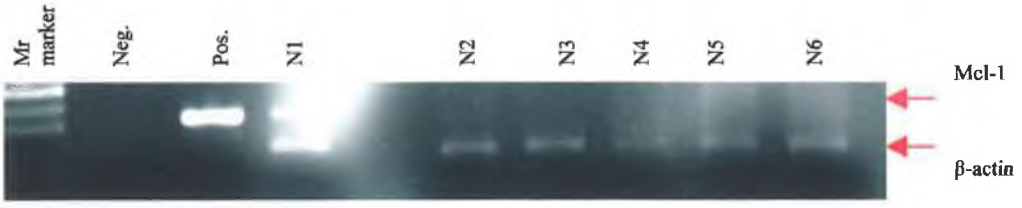


Figure 7.3.3.1b.

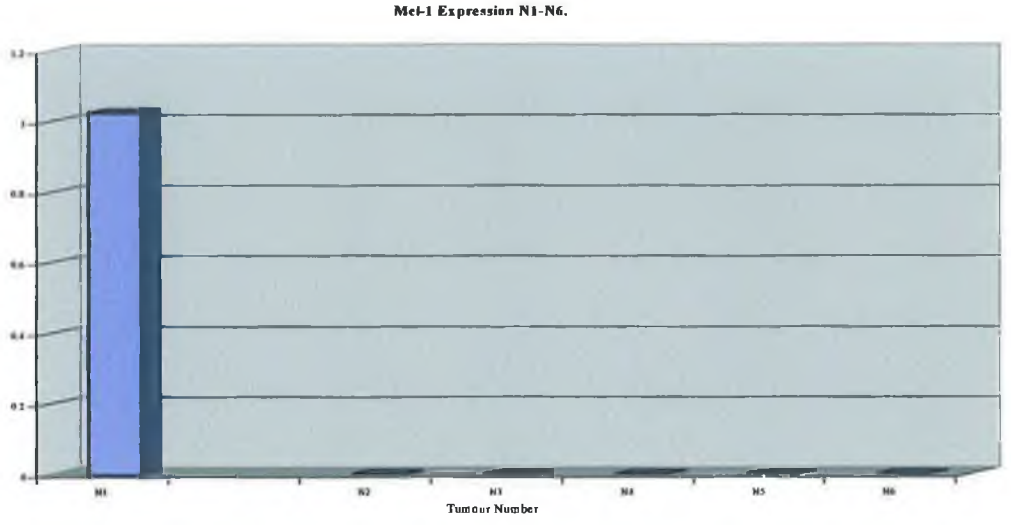


Figure 7.3.3.2a T1-T10.

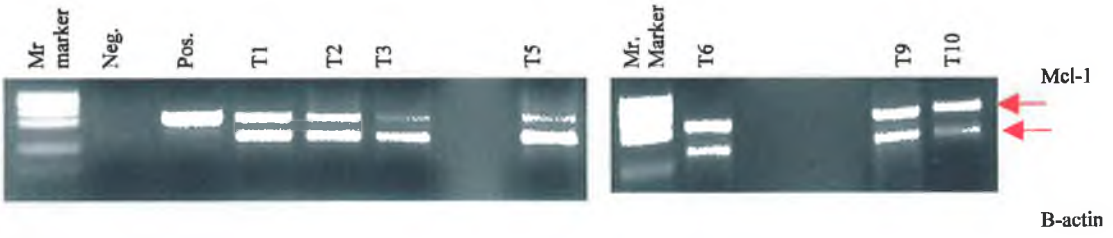


Figure 7.3.3.2b

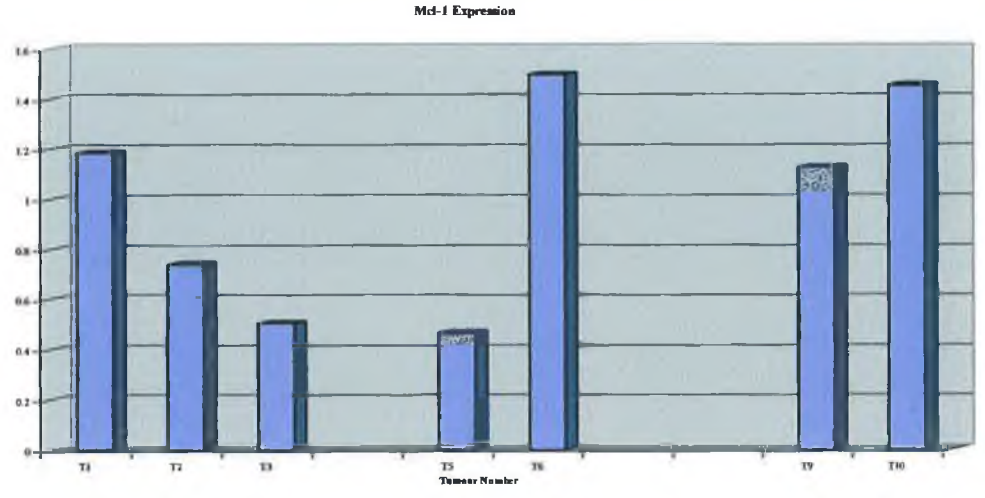


Figure 7.3.3.4a : T21-T30.

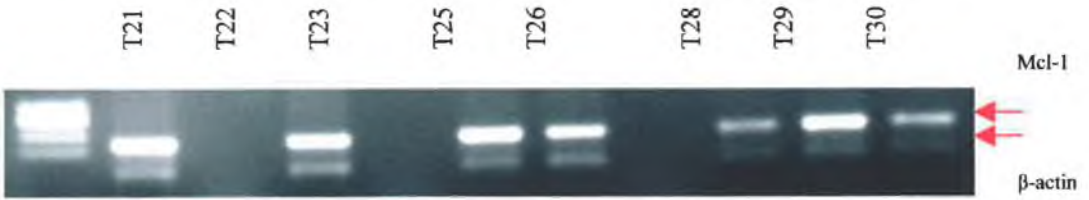


Figure 7.3.3.4b

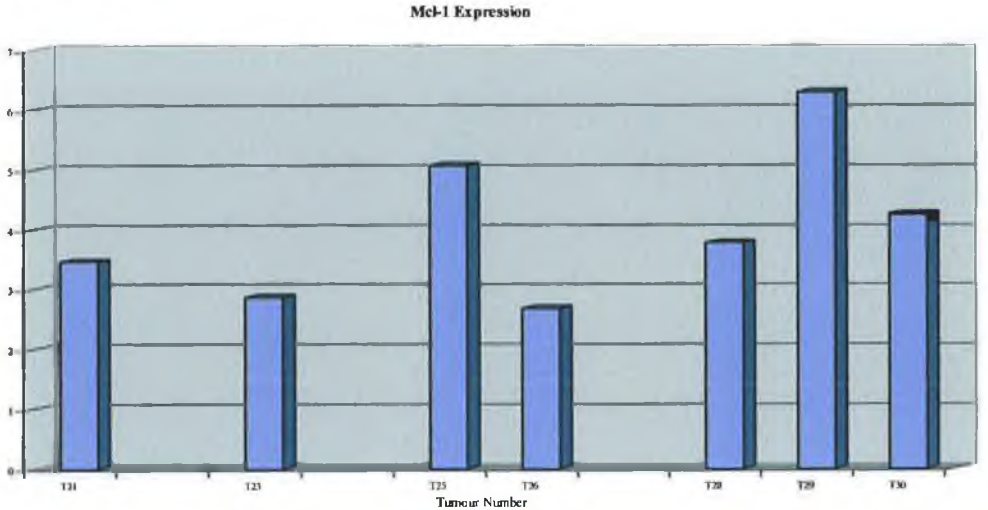


Figure 7.3.3.3a: T11-T19.

Figure 7.3.3.3b

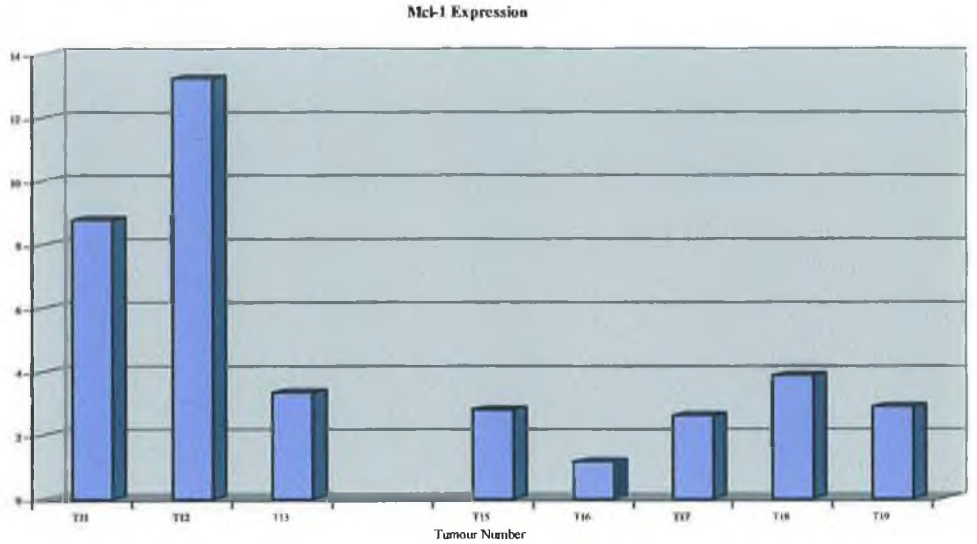


Figure 7.3.3.5a : T31-T39.

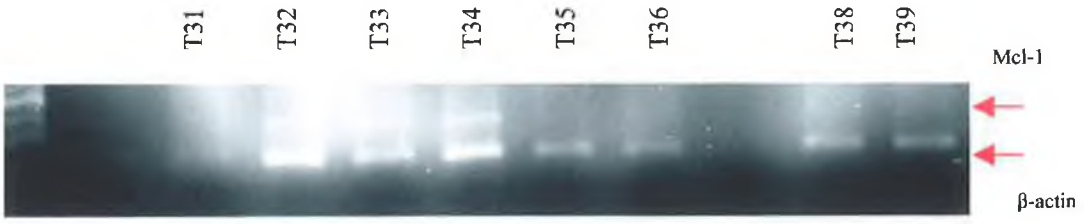


Figure 7.3.3.5b

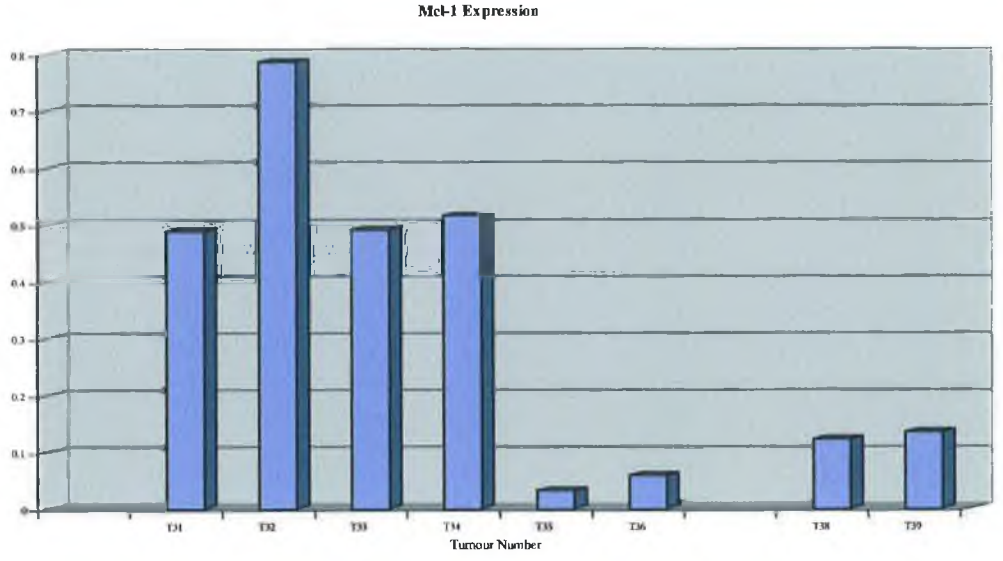


Figure 7.3.3.6a : T40-T51

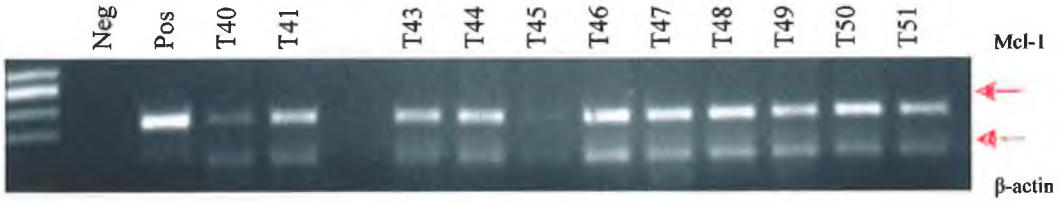


Figure 7.3.3.6b

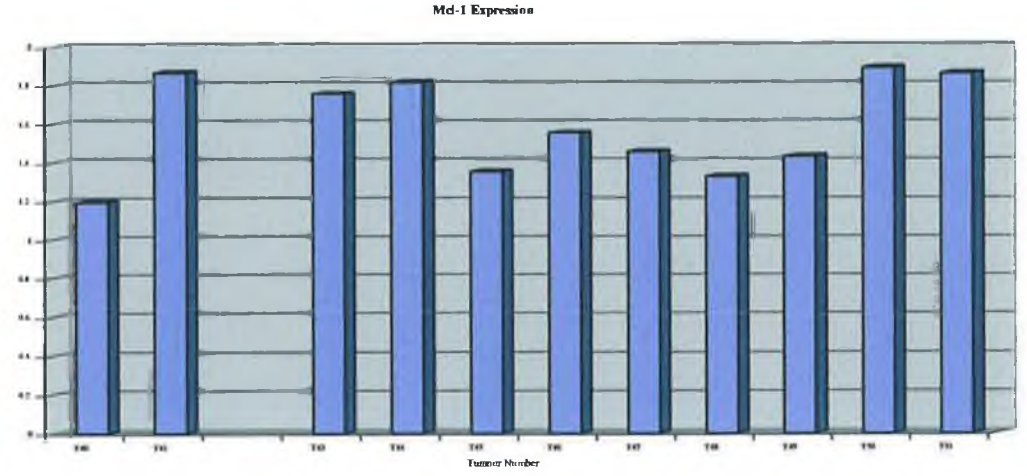


Figure 7.3.3.7a : T52-T60.

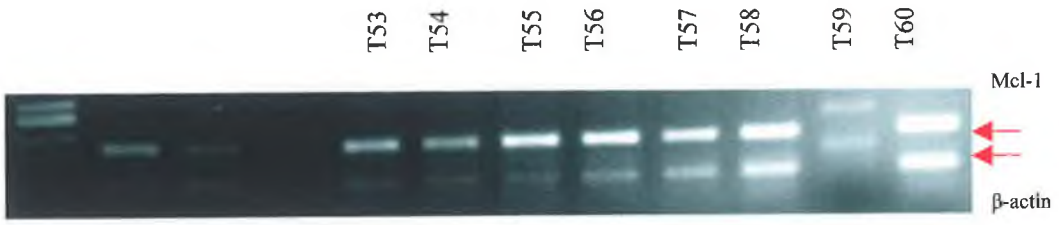


Figure 7.3.3.7b

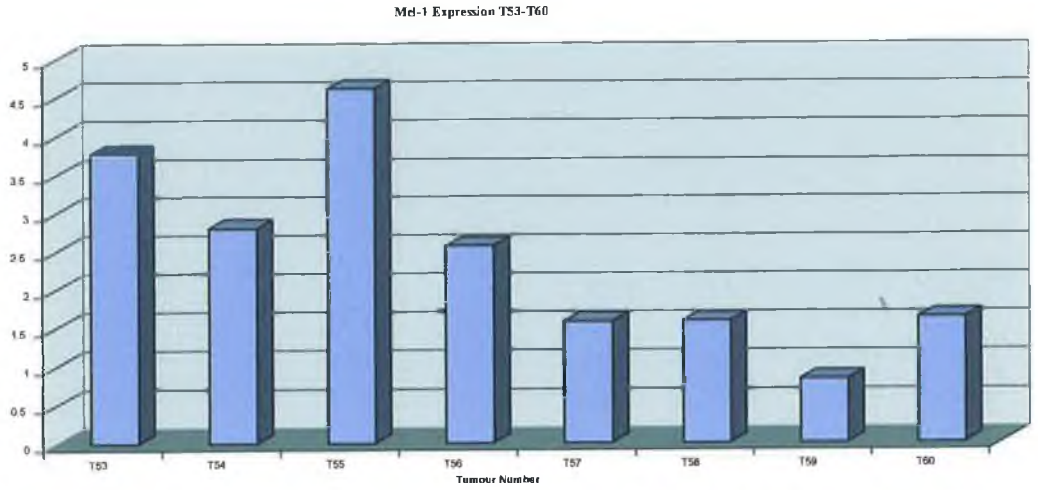


Figure 7.3.3.8a : T61-T70

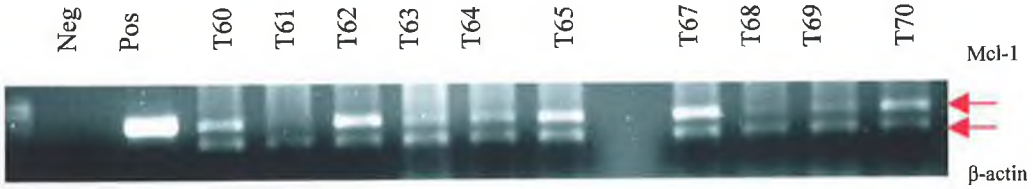


Figure 7.3.3.8b

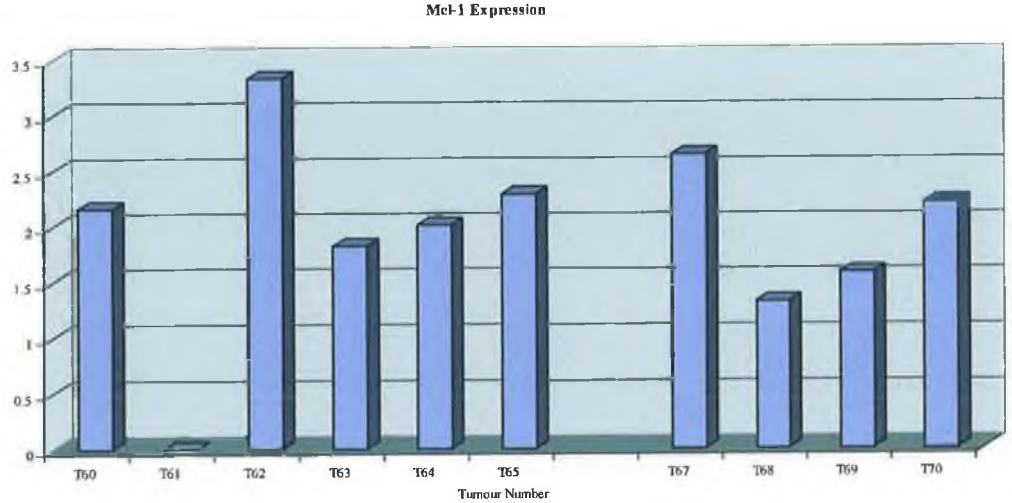


Figure 7.3.3.9a : T71-T83

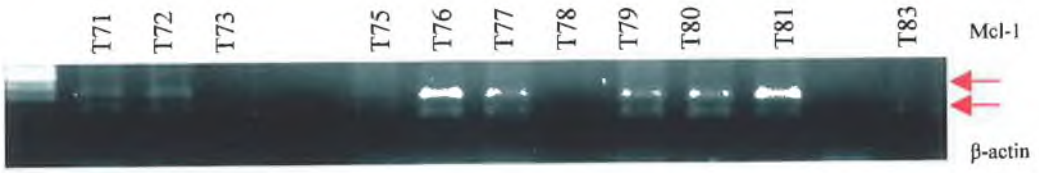


Figure 7.3.3.9b

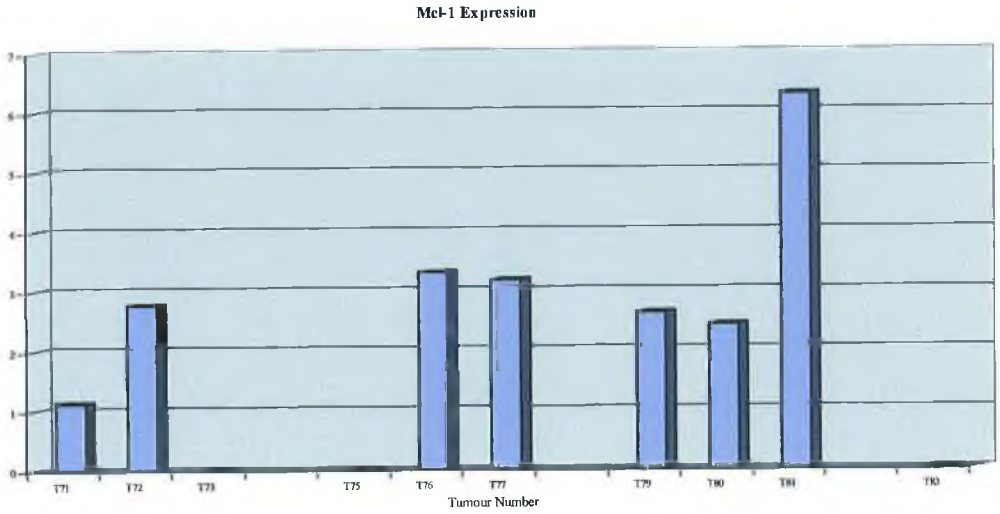


Figure 7.3.3.10a : T85-T99

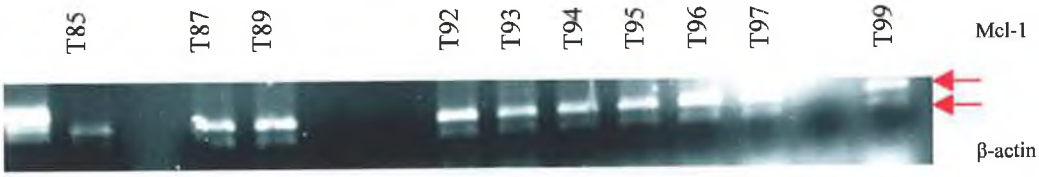


Figure 7.3.3.10b

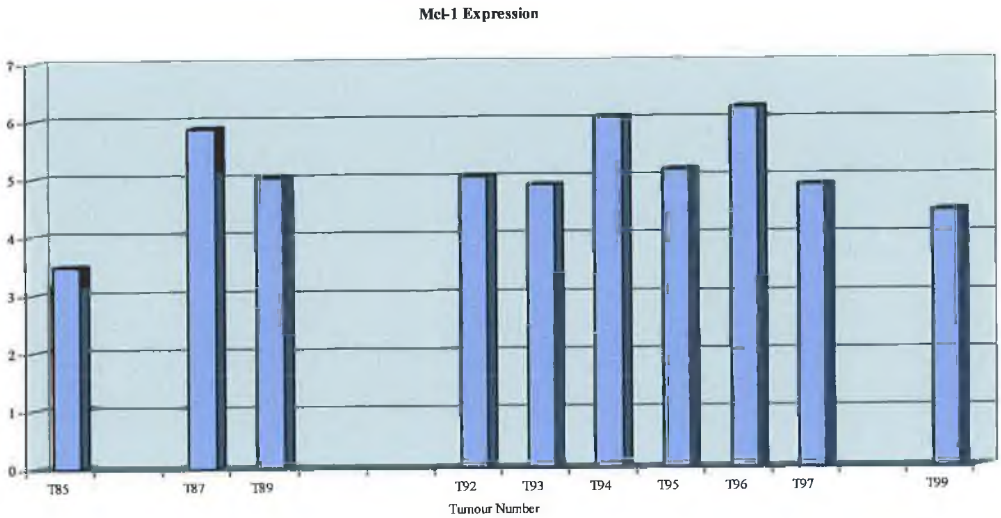


Figure 7.3.3.12a : T111-T120

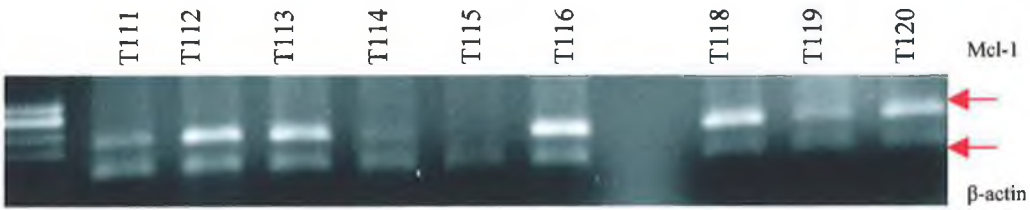


Figure 7.3.3.12b

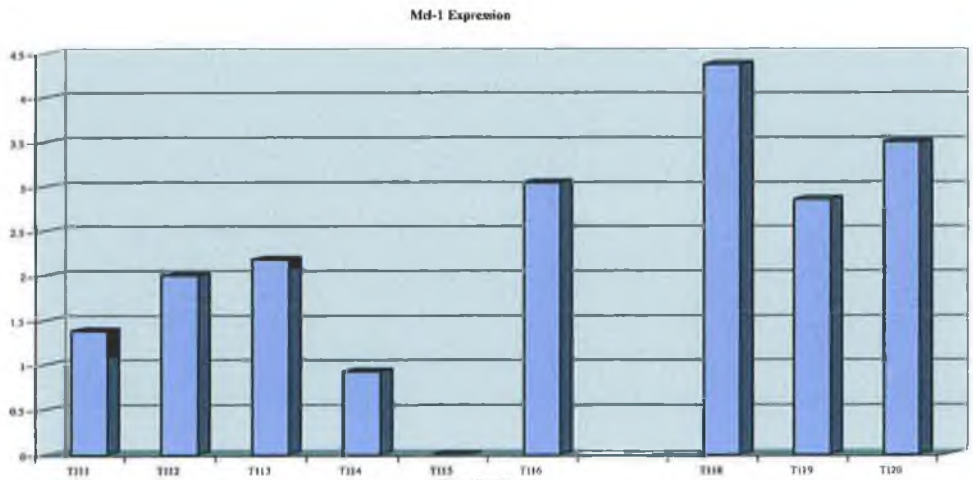


Figure 7.3.3.11a T100-T107

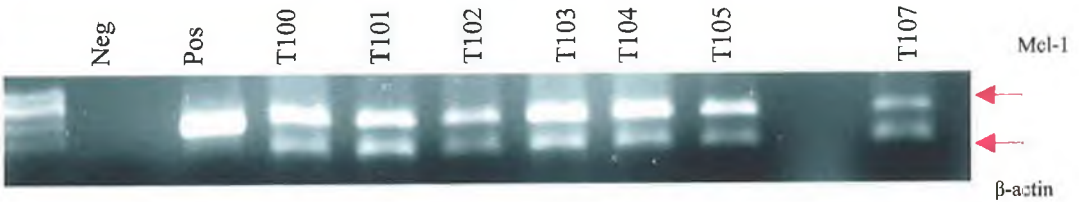


Figure 7.3.3.11b

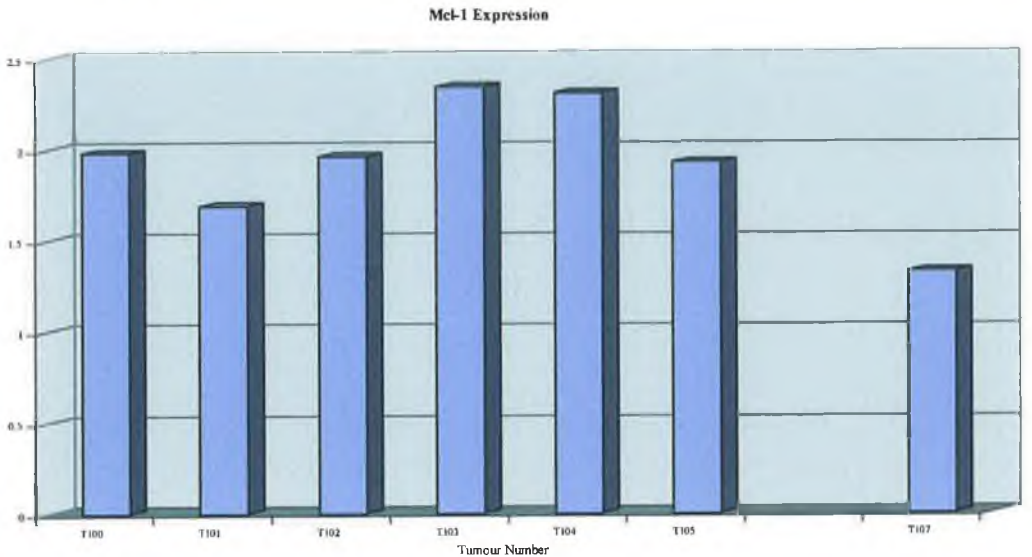


Figure 7.3.3.13a : T122-T134

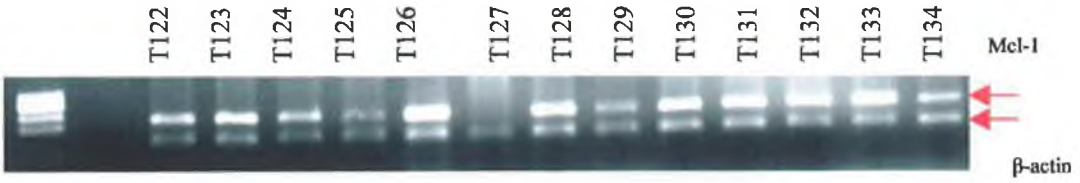


Figure 7.3.3.13b

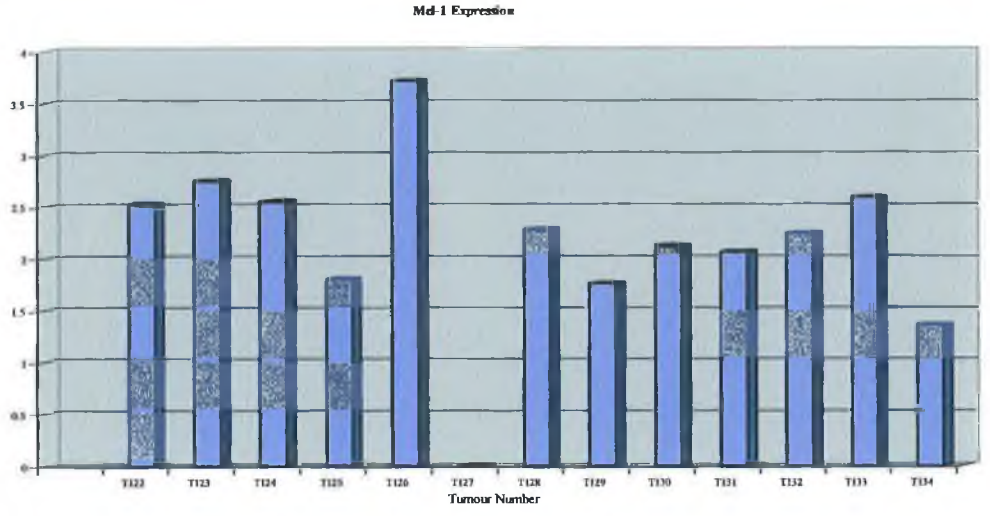


Figure 7.3.3.14a : S1-S12

Figure 7.3.3.14b

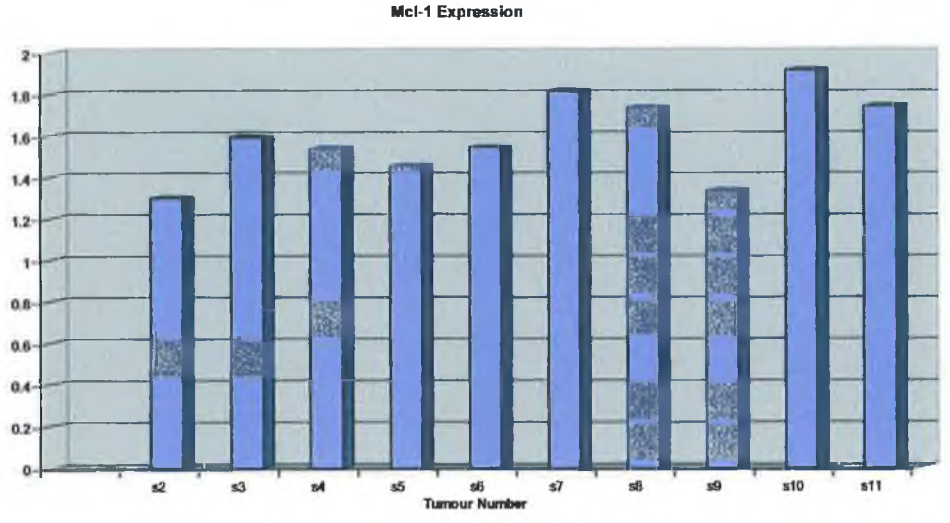


Figure 7.3.3.15a: S13-S26

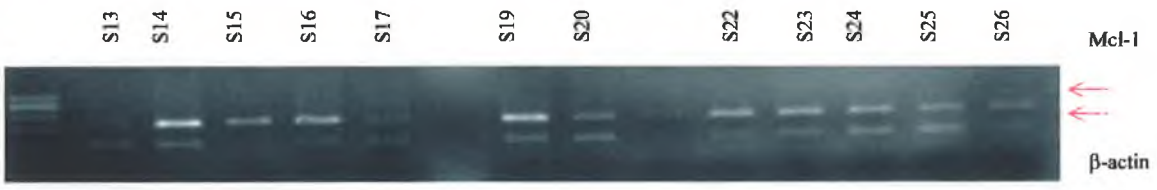


Figure 7.3.3.15b

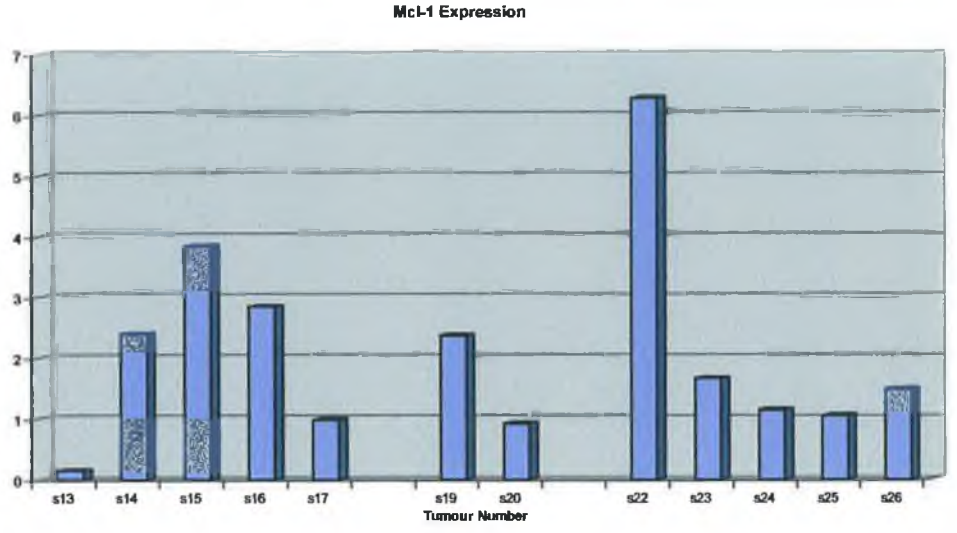


Figure 7.3.3.16a: S28-S39

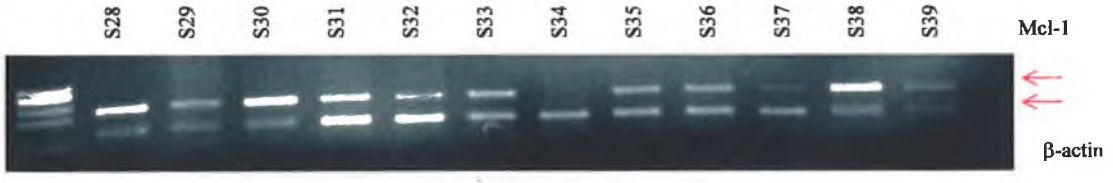
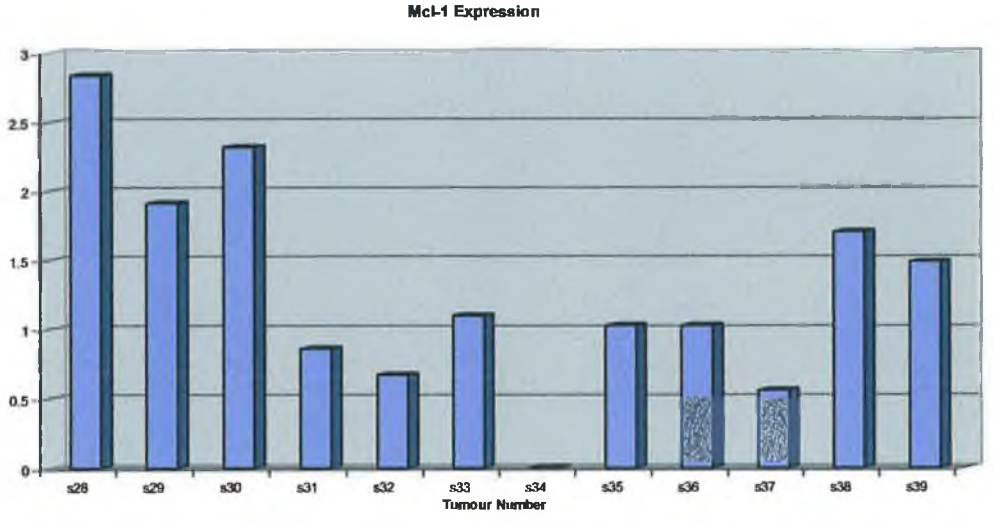


Figure 7.3.3.16b



7.3.4 RT-PCR results for Bag-1 expression in breast tumour biopsies. Graphs show densitometric analysis of gene expression. Units are densitometric arbitrary units.

Figure 7.3.4.1a: N1-N6.

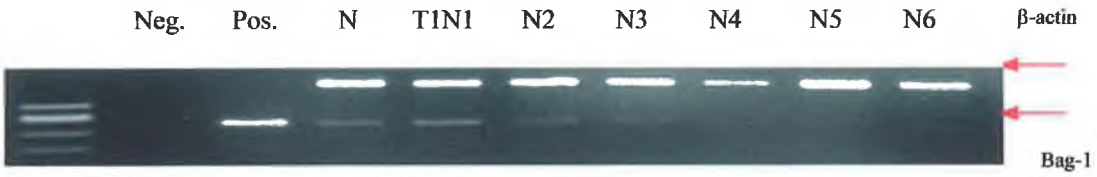


Figure 7.3.4.1b:

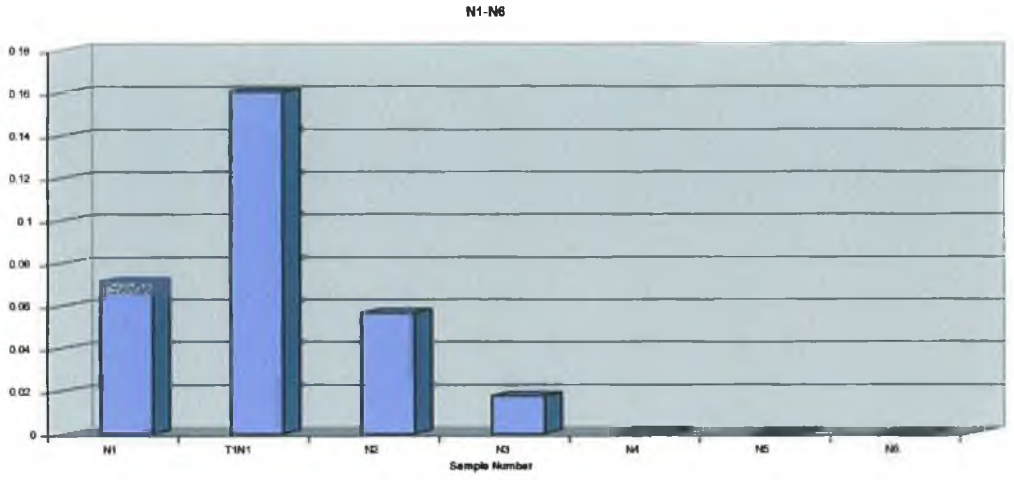


Figure 7.3.4.2a: T1-T10.

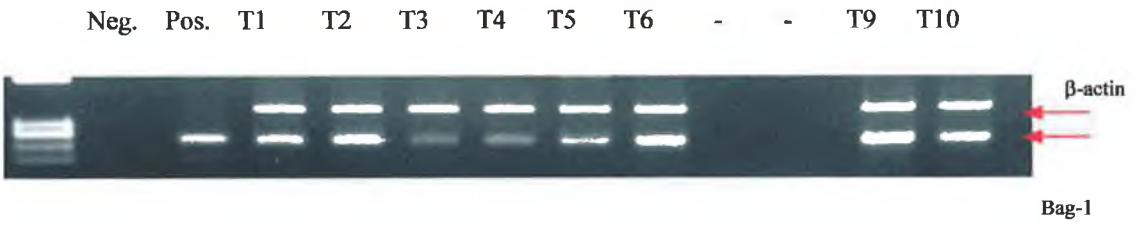


Figure 7.3.4.2b:

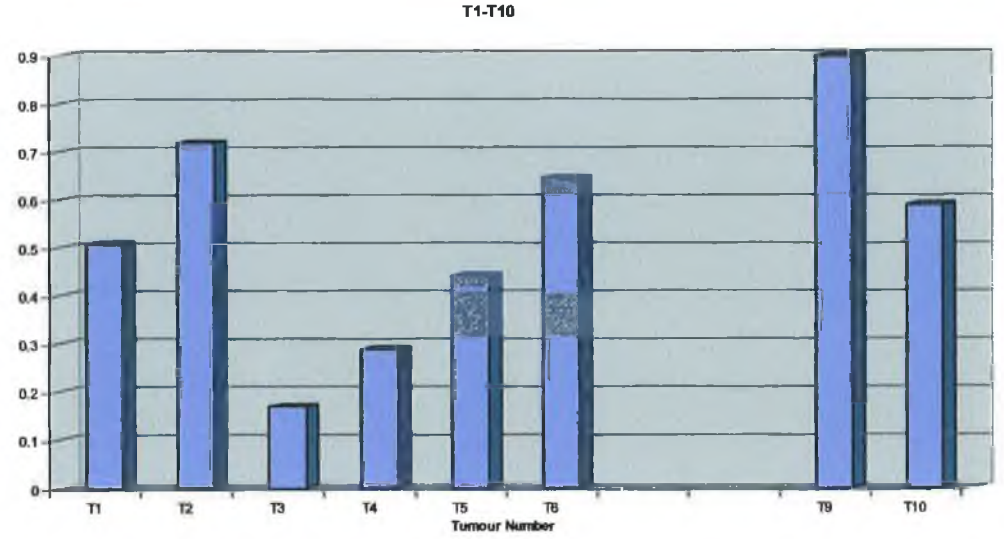


Figure 7.3.4.3a T11-T20.

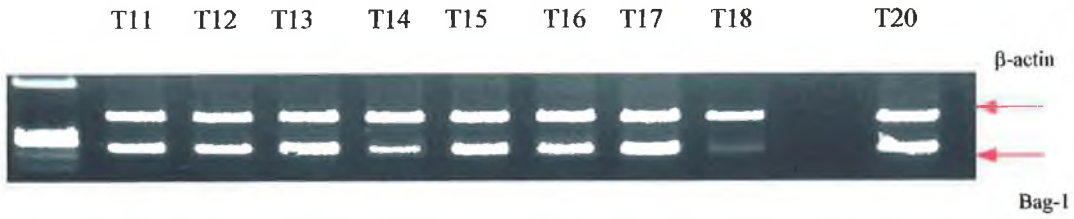


Figure 7.3.4.3b:

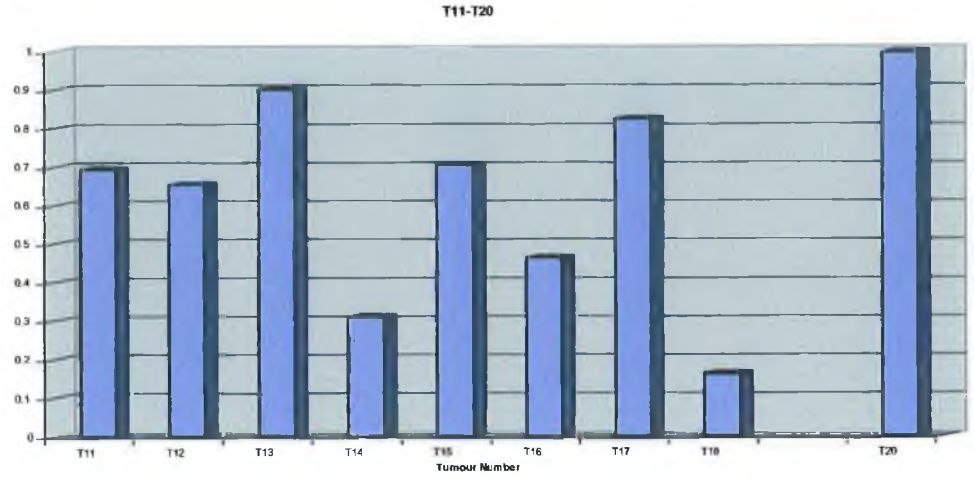


Figure 7.3.4.4a: T21-T30.

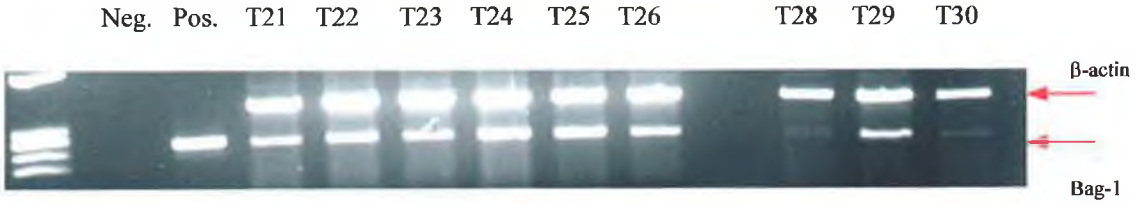


Figure 7.3.4.4b:

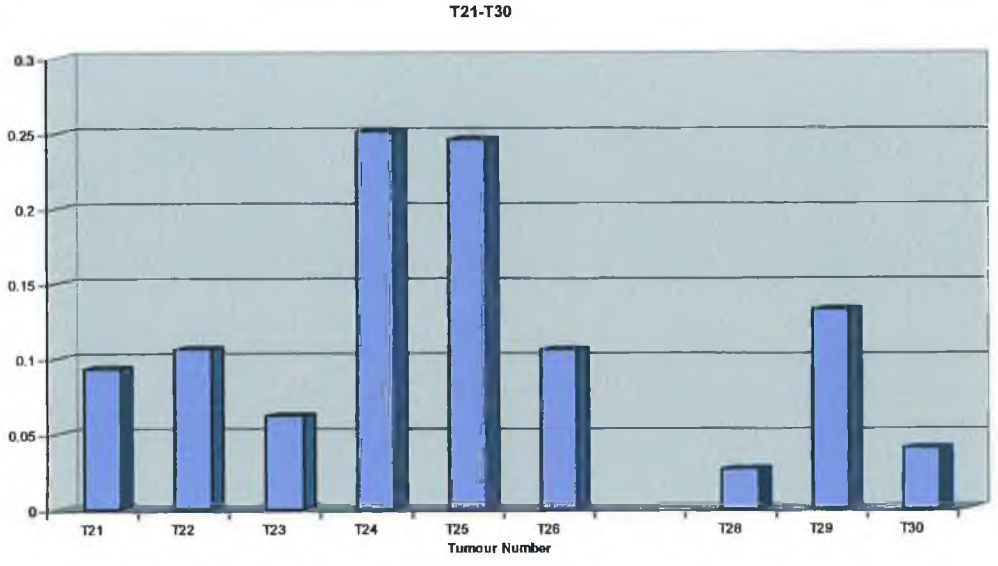


Figure 7.3.4.5a: T31-T40.

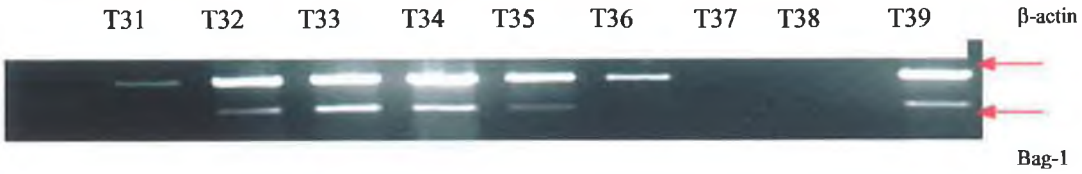


Figure 7.3.4.5b:

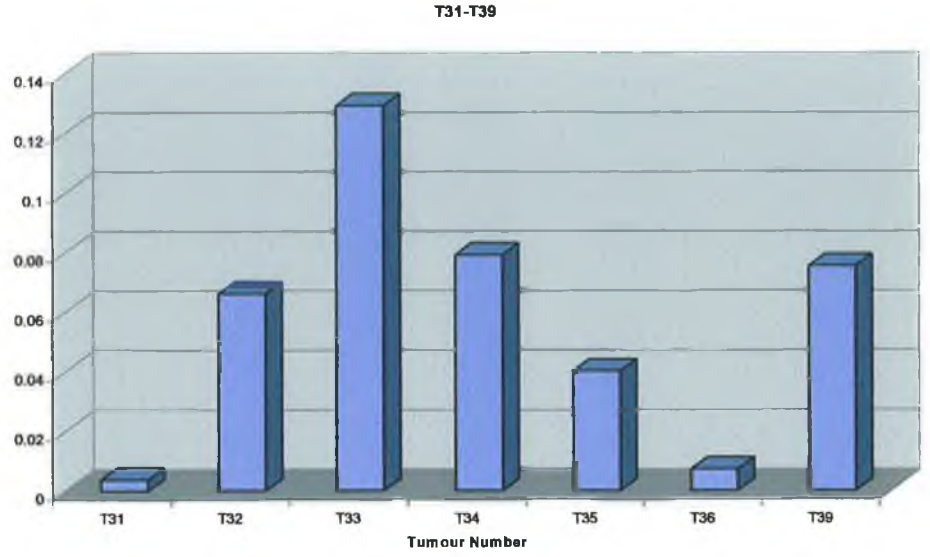


Figure 7.3.4.6a: T41-T50.

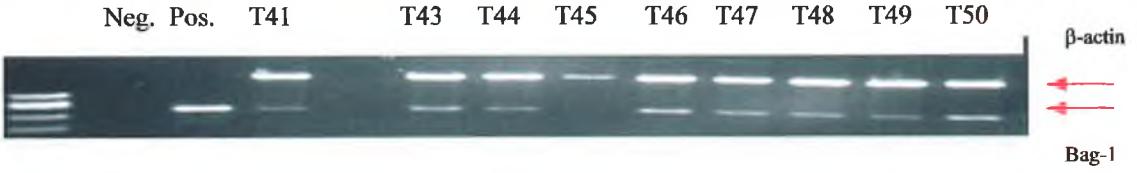


Figure 7.3.4.6b:

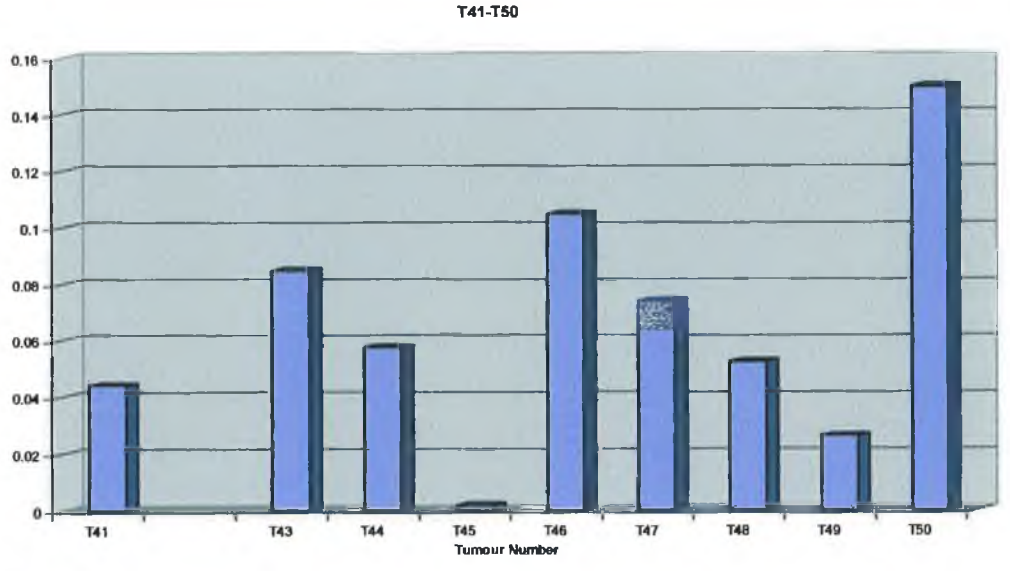


Figure 7.3.4.7a: T51-T60.

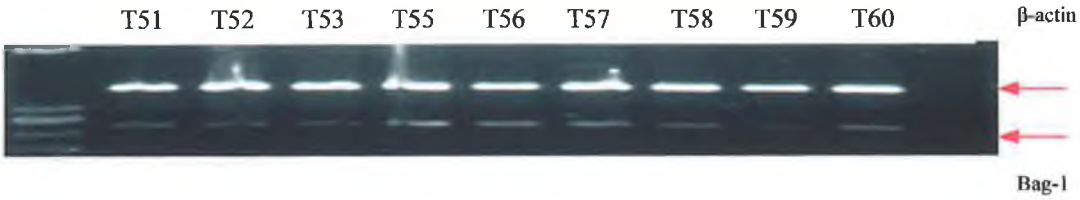


Figure 7.3.4.7b:

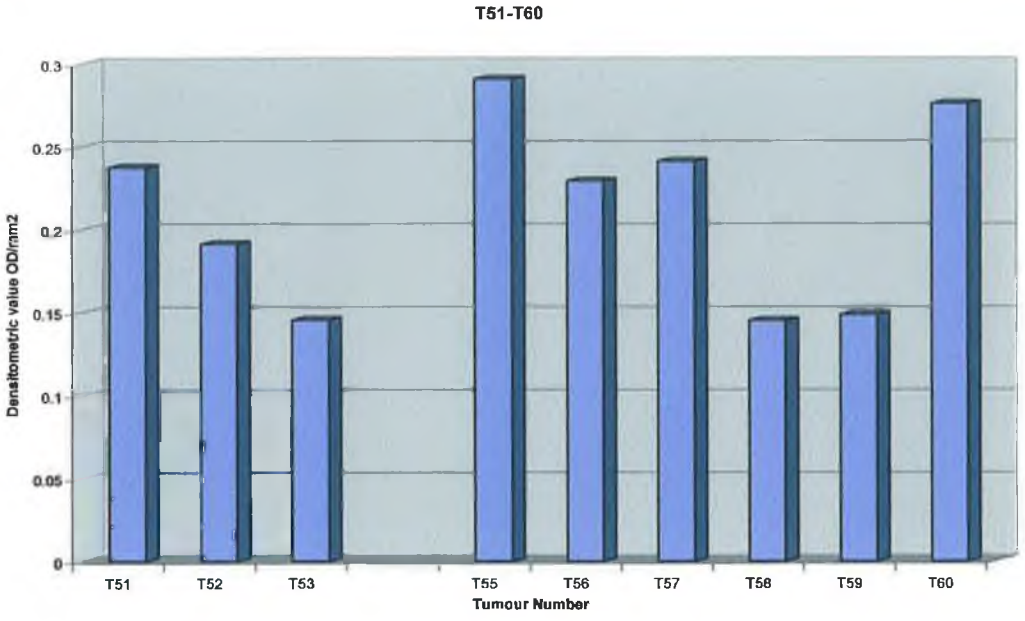


Figure 7.3.4.8a: T61-T70.

Figure 7.3.4.8b:

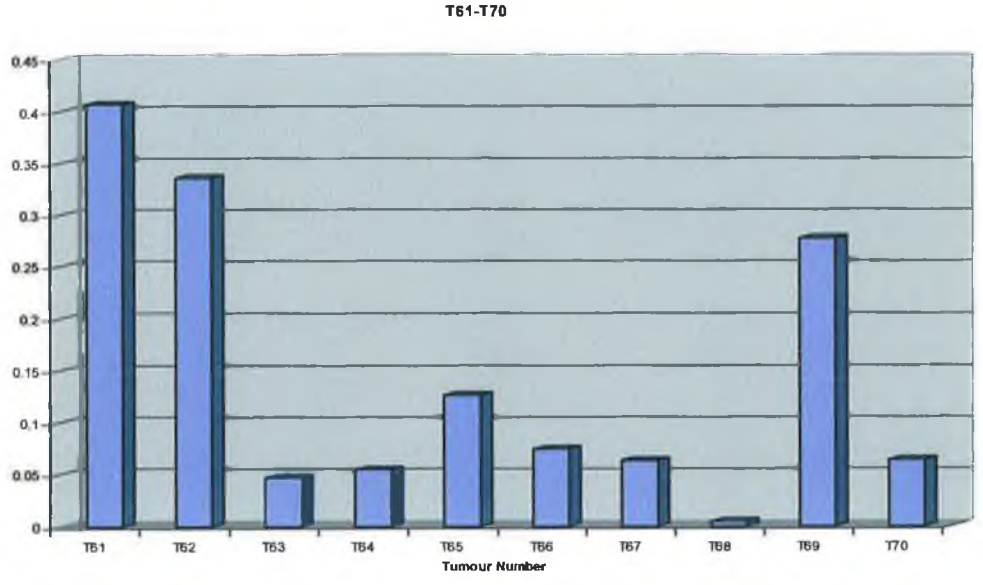


Figure 7.3.4.9a: T71-T80.

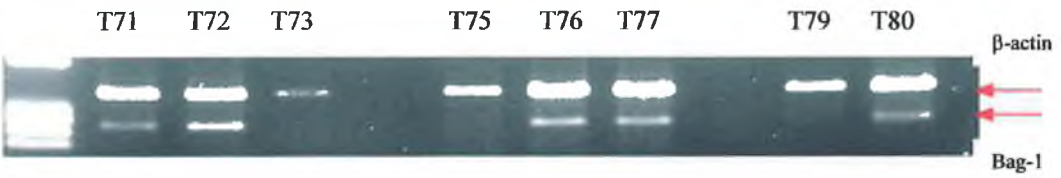


Figure 7.3.4.9b:

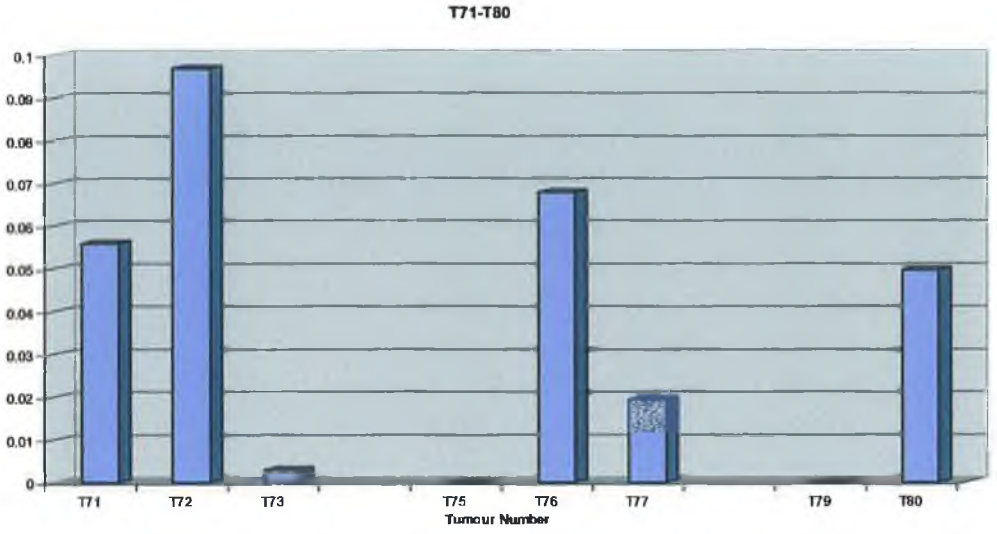


Figure 7.3.4.10a: T81-T89.

Figure 7.3.4.10b:

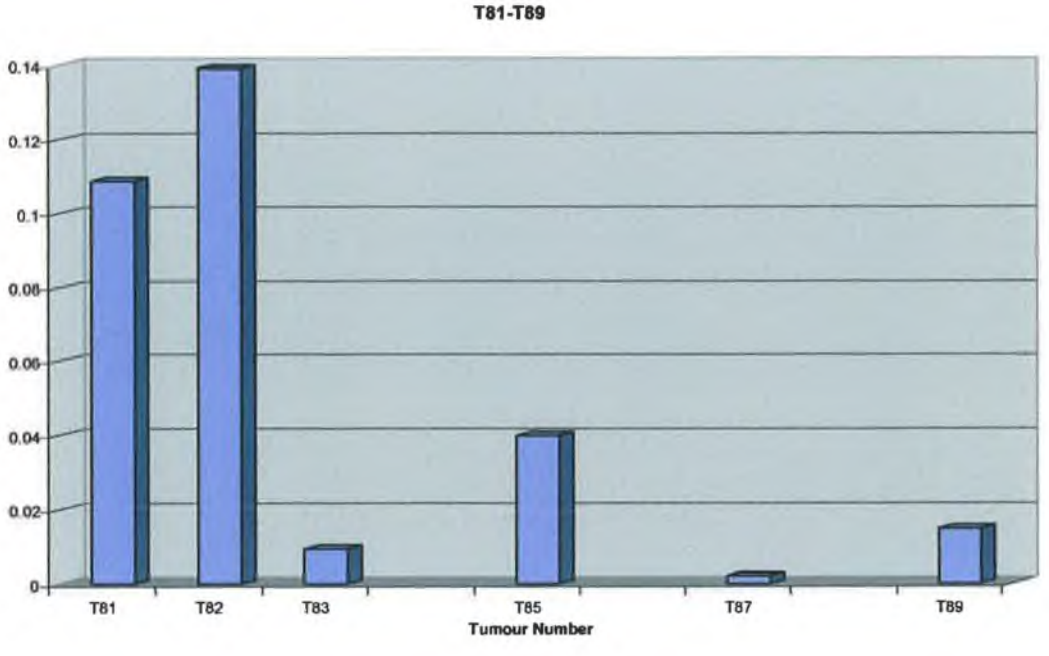


Figure 7.3.4.11a: T91-T100.

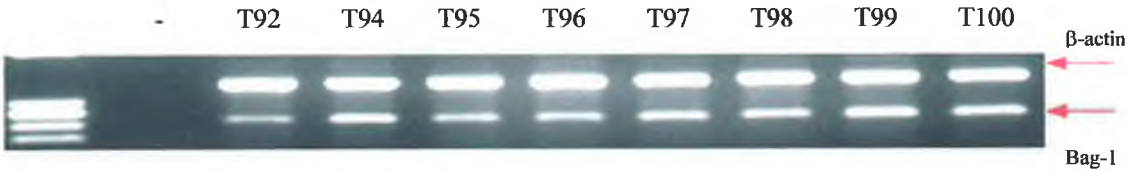


Figure 7.3.4.11b:

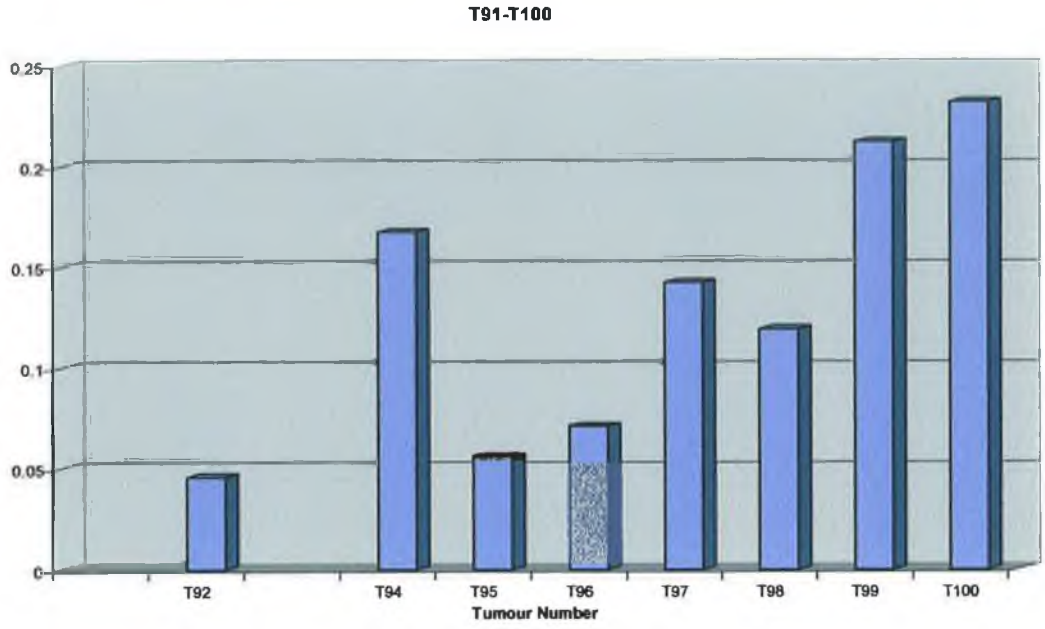


Figure 7.3.4.12a: T101-T109.

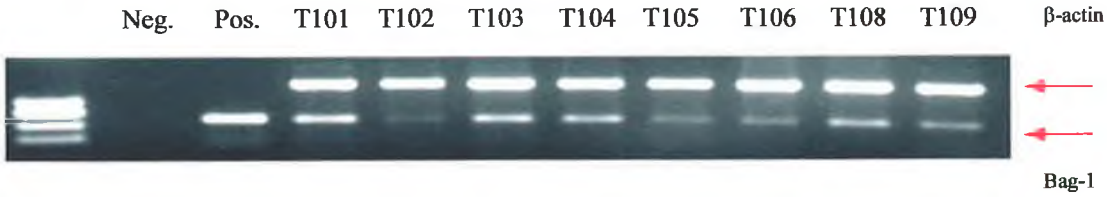


Figure 7.3.4.12b:

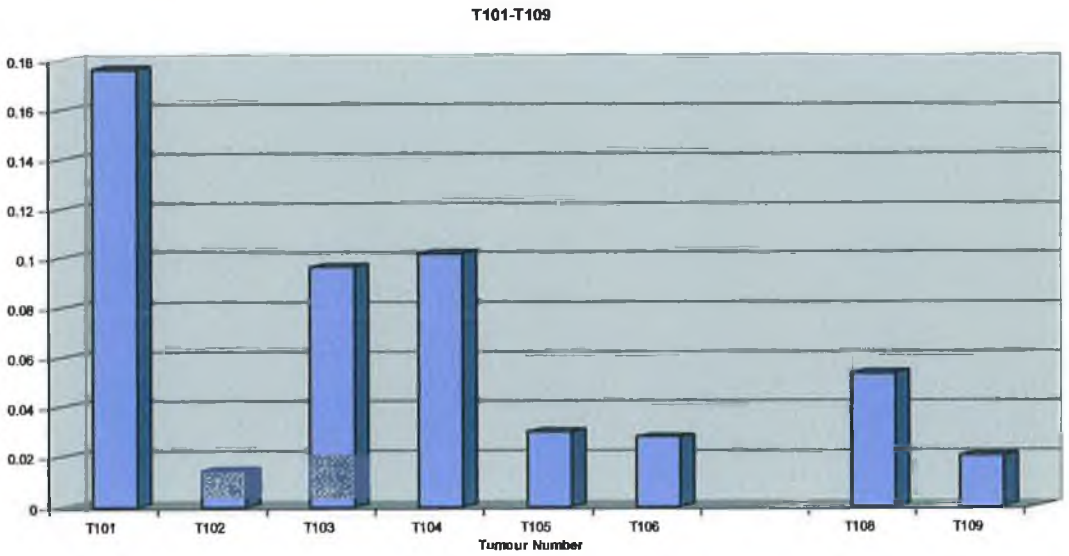
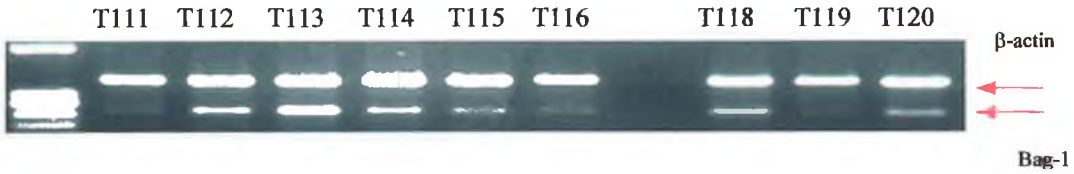


Figure 7.3.4.13a: T111-T120.



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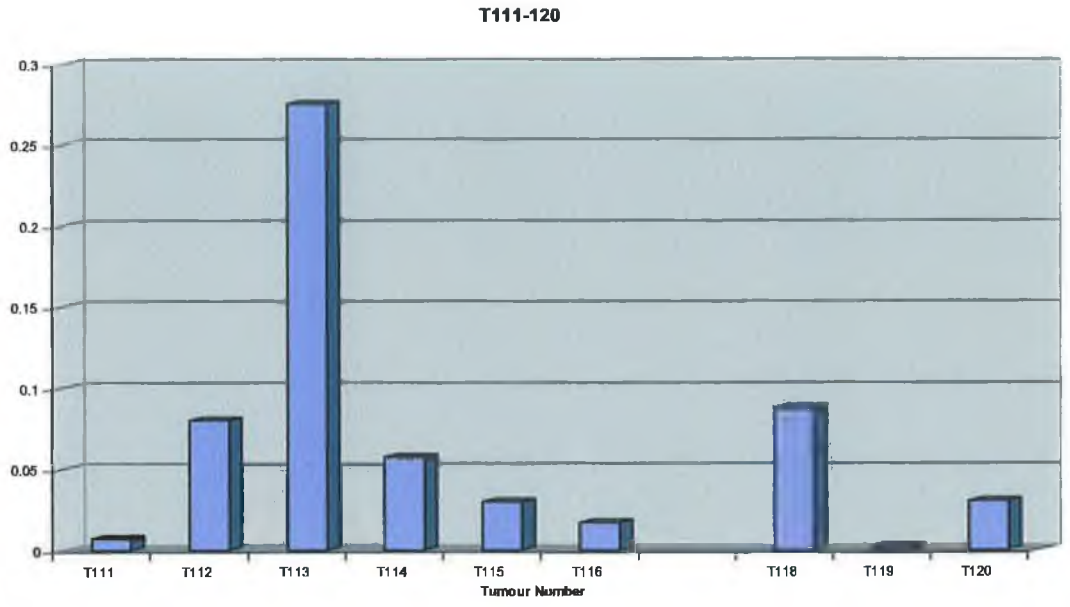

Figure 7.3.4.14a: T121-T130.

Figure 7.3.4.14b:

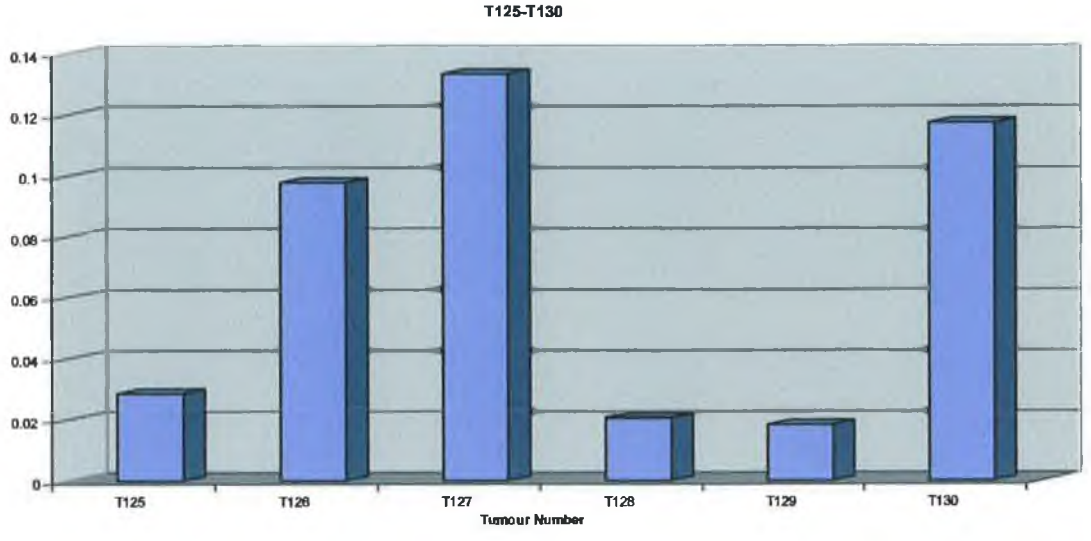

Figure 7.3.4.15a: T131-T134.

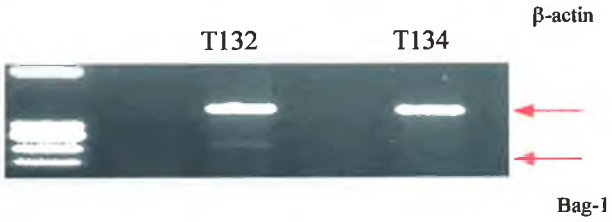

Figure 7.3.4.15b:

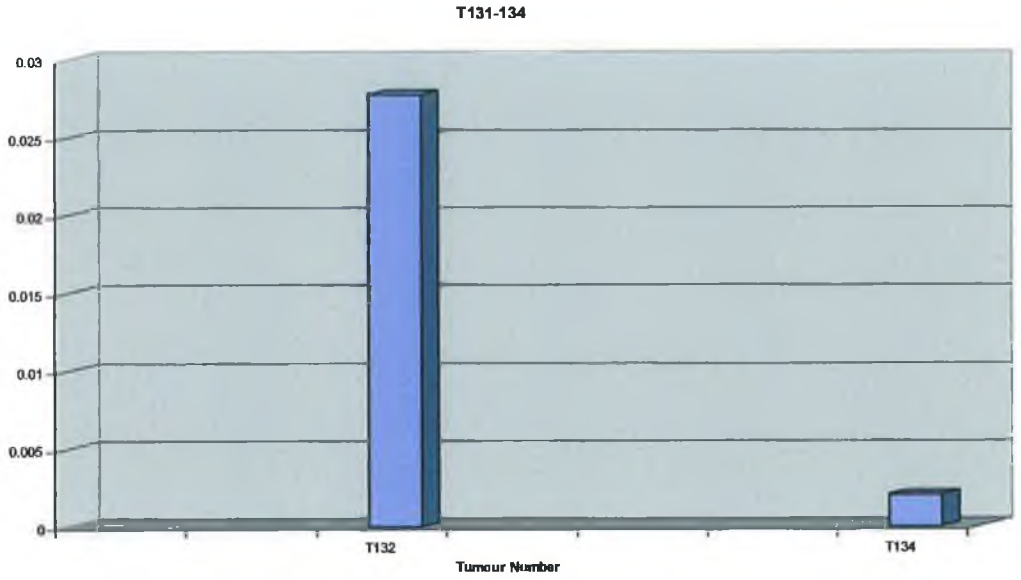

Figure 7.3.4.16a: S1-S10.

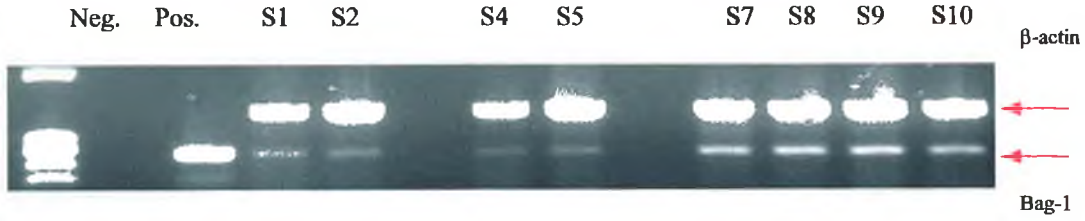

Figure 7.3.4.16b:

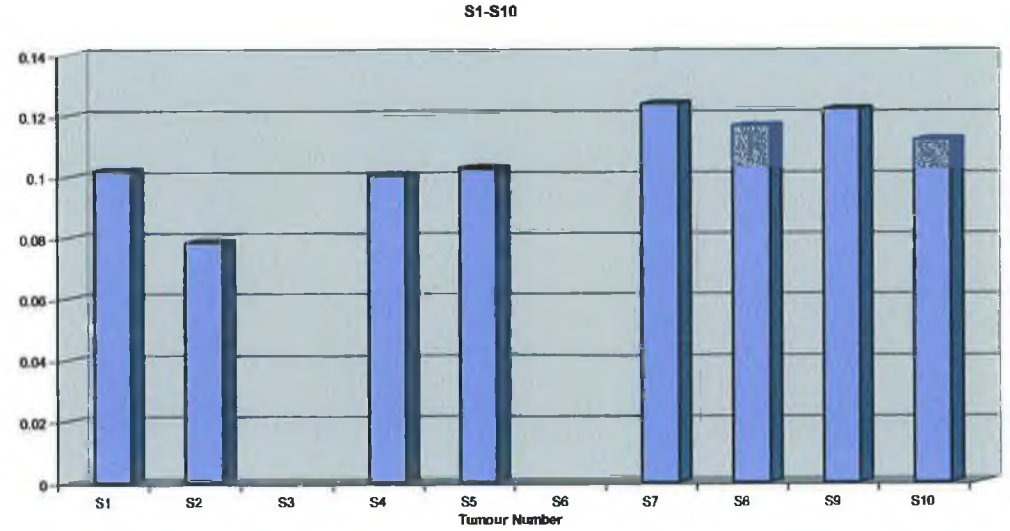

Figure 7.3.4.17a: S11-S20.

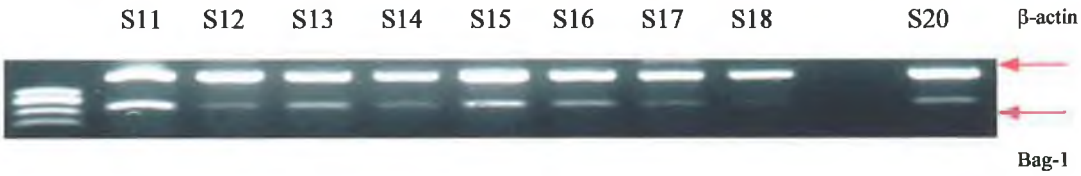

Figure 7.3.4.17b

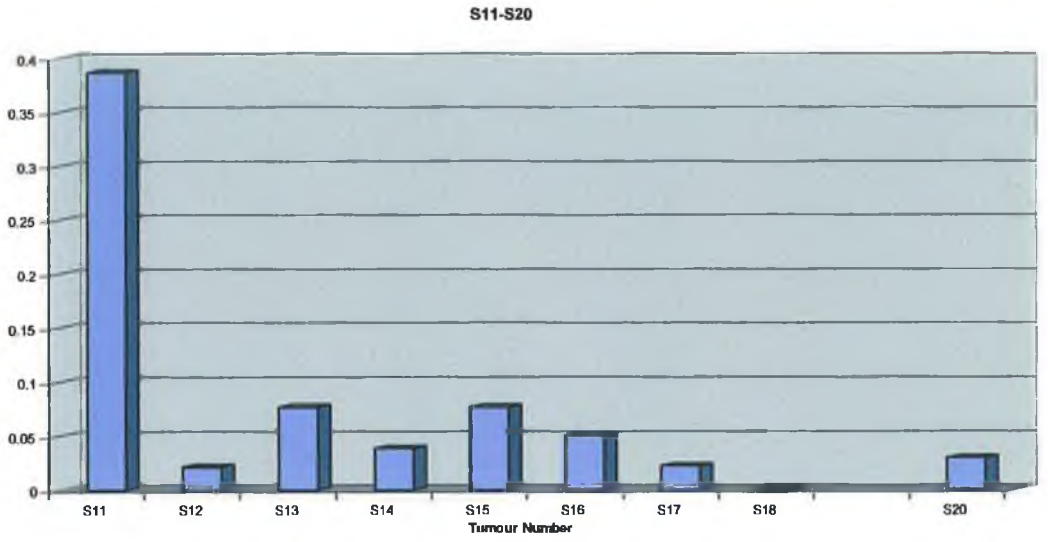

Figure 7.3.4.18a: S21-S30.

Figure 7.3.4.18b:

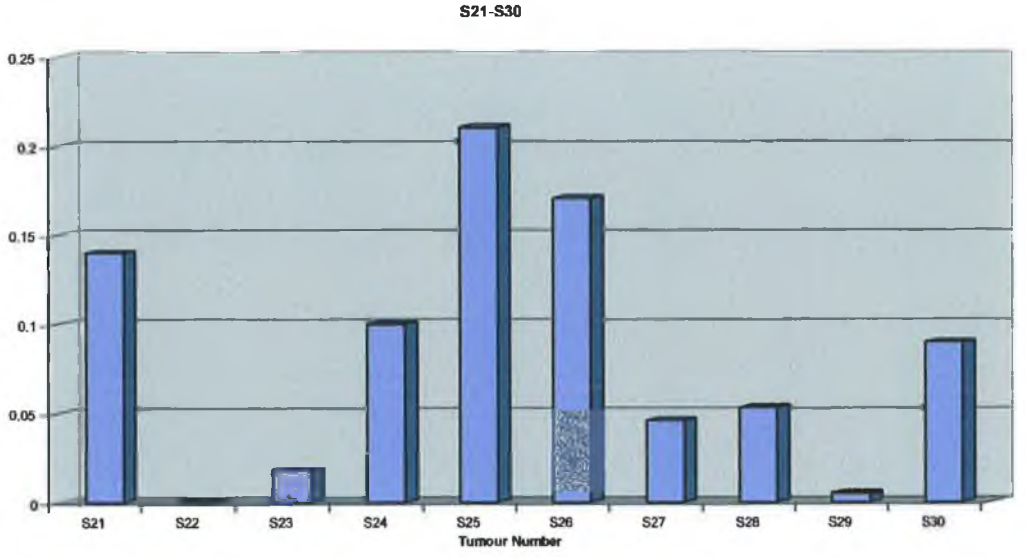

Figure 7.3.4.19a: S31-S38.

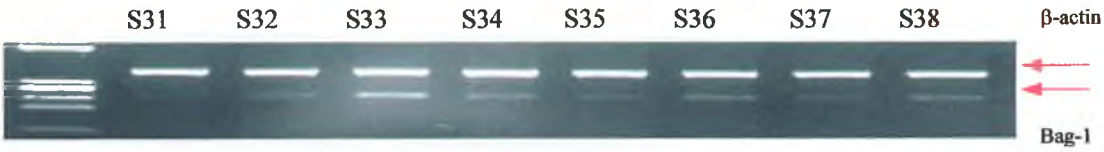

Figure 7.3.4.19b: