The Role of Galectin-3 and Survivin in Invasion and Drug Resistance in Human Cancer

A thesis submitted for the degree of Ph.D.

By

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The experimental work described in this thesis was carried out under the supervision of

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I hereby certify that this material, which I now submit for assessment on the program 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: Rashna Lincham **I.D. No.:** 19970828

Date: 18/9/03

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This Thesis is dedicated to my husband and my parents, and to the memory of my Grandparents, Tetah and Gedo.

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Abbreviations

aa	-	Amino Acid
Ab	-	Antibody
ABC	-	ATP Binding Cassette
ACT-D	-	Actinomycin D
AD	-	Adriamycin
ALL	-	Acute lymphocytic leukaemia
AML	-	Acute myeloid leukemia
ATP	-	Adenosine-triphosphate
ATCC	-	American Tissue Culture Collection
BCRP	-	Breast cancer resistance protein
BSA	-	Bovine Serum Albumin
BIR	-	Baculovirus IAP repeat
BIRPs	-	BIR-domain containing proteins
bp	-	Base pair
CARD	-	Caspase-recruiting doamin
CAM	-	Cell Adhesion Molecule
CARM	-	Carmustine
CCNU	-	cyclohexylchloroethylnitrosourea
CDDP	-	*Cis*-diamminedichloroplatinum(II)
cDNA	-	Complementary DNA
CHLB	-	Chlorambucil
c-IAP	-	Cellular IAP
CISPL	-	Cisplatin
Da	-	Daltons
DEPC	-	Diethyl Pyrocarbonate
DISC	-	Death-inducing signal complex
DMEM	-	Dulbecco's Minimum Essential Medium
DMSO	-	Dimethyl sulfoxide
DNase	-	Deoxyribonuclease
DNA	-	Deoxyribonucleic Acid

DNR	-	Daunorubicin
dNTP	-	Deoxynucleotide triphosphate (N= A, C, T, G or U)
DTT	-	Dithiothreitol
ECM	-	Extracellular matrix
EDTA	-	Ethylene diamine tetracetic acid
EPIR	-	Epirubicin
EPR-1	-	Effector protein receptor-1
FADD	-	Fas-associated death domain
FCS	-	Fetal Calf Serum
FLICE	-	FADD-like IL-1 converting enzyme
5-FU	-	5-Fluorouracil
Gal-3	-	Galectin-3
GAPDH	-	Glyceraldehyde-6-phosphate dehydrogenase
HEPES	-	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HUVEC	-	Human umbilical vein endothelial cells
H-SFM	-	Ham's F12 Serum Free Medium
IAP	-	Inhibitor of apoptosis protein
IC_{50}	-	Inhibitory Concentration 50%
ICC	-	Immunocytochemistry
IHC	-	Immunohistochemistry
Ig	-	Immunoglobulin
IL-1	-	Interleukin-1
IMS	-	Industrial Methylated Spirits
JNK	-	c-jun N-terminal kinase
kDa	-	Kilo Daltons
MAb	-	Monoclonal Antibody
MDR	-	Multiple Drug Resistance
Melph	-	Melphalan
MEM	-	Minimum Essential Medium
min	-	Minute(s)
MITOX	-	Mitoxantrone
MMLV-RT	-	Moloney Murine Leukemia Virus-Reverse Transcriptase
MMPIs	-	MMP inhibitors

MMPs	-	Matrix metalloproteinases
mRNA	-	Messenger RNA
MRP	-	Multidrug Resistance-associated Protein
MTX	-	Methotrexate
MW	-	Molecular Weight Marker
N/A	-	Not applicable
NB	-	Northern blot
NIAP		Neuronal apoptosis inhibitor
NICB	-	National Institute for Cellular Biotechnology
NSAIDS	-	Non-Steroid Anti-Inflammatory Drugs
NSCLC	-	Non-Small Cell Lung Carcinoma
OD	-	Optical Density
Oligos	-	Oligonucleotides
ORF	-	Opening reading frame
P	-	Passage
PAd-T34A	-	Replication-deficient adenovirus encoding T34 dominant-negative mutant
PBS A	-	Phosphate Buffered Saline A
PCR	-	Polymerase Chain Reaction
Pgp	-	P-glycoprotein
PM	-	Plasma membrane
QRT-PCR	-	Quantitative real-time PCR
RCC	-	Renal cell carcinoma
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
RNasin	-	Ribonuclease Inhibitor
RPA	-	Rnase protection assay
rpm	-	Revolution(s) Per Minute
RT-PCR	-	Reverse Transcriptase-PCR
Rz	-	Ribozyme
SB	-	Southern blot
SCLC	-	Small Cell Lung Carcinoma
SDS	-	Sodium Doedecyl Sulphate

sec(s)	-	Second(s)
SF	-	Serum-Free
SFM	-	Serum-Free Medium
SiRNA	-	Small interfering RNA
Surv	-	Survivin
TAX	-	Taxol
TBE	-	Tris-boric acid-EDTA buffer
TBS	-	Tris Buffered Saline
TE	-	Tris-EDTA
TEMED	-	N, N, N', N'-Tetramethyl-Ethylenediamine
TIMPs	-	tissue inhibitors of metalloproteinas
TNF	-	Tumour necrosis factor
TOPOT	-	Topotecan
Tris	-	Tris(hydroxymethyl)aminomethane
TUNEL	-	Tdt-mediated dUTP-X Nick End Labeling
Tx	-	Taxol
TXT	-	Taxotere
UHP	-	Ultra high pure water
UTR	-	Untranslated region
v/v	-	volume/volume
VBL	-	Vinblastine
VCR	-	Vincristine
VEGF	-	Vascular endothelial growth factor
WB	-	Western blot
w/v	-	weight per volume
X-IAP	-	X chromosome-linked IAP

Abstract

Galectin-3 is an apoptosis-related gene previously found to be over-expressed in invasive tumours and to cause *in vitro* invasiveness and metastasis in colon, breast and thyroid follicular cancer cells. Galectin-3 over-expressing clones were obtained following stable transfection of galectin-3 cDNA into the non-invasive human lung carcinoma cell line DLKP. These clones exhibited increased *in vitro* invasiveness and motility and altered cell adhesion properties, but did not exhibit a drug resistant phenotype. Survivin is an anti-apoptotic gene highly expressed in all cancer types and during fetal development, but not in most normal adult tissue. To determine whether survivin over-expression plays a role in drug-resistance, transient transfections of survivin cDNA into SKOV-3 'Tet off', MCF-7 'Tet off' and DLKP cells, were carried out. All three transfections resulted in an over-expression of survivin mRNA, but not survivin protein.

To further investigate the role of galectin-3 and survivin in drug resistance and invasiveness, RT-PCR and western blot analysis were carried out on DLKP and RPMI-2650 cells which had been exposed to sequential pulsing with three (vincristine, taxotere and 5-fluorouracil) and five (vincristine, 5-fluorouracil, CCNU, carboplatin and epirubicin) chemotherapy drugs, respectively. Drug selection of DLKP resulted in two MDR variants, DLKP-taxotere and DLKP-vincristine. The taxotere-resistant variant displayed a highly invasive phenotype. Drug selection of RPMI-2650 cells resulted in unstable MDR variants, with no induction of invasiveness. Galectin-3 expression was examined in the drug-selected variants, along with the expression of the MDR-related genes, by RT-PCR analysis. Galectin-3 mRNA expression was unaltered in all the drug-selected variants of DLKP and RPMI-2650. Survivin expression was examined in the drug-selected variants by RT-PCR and western blot analysis. Survivin protein expression was dramatically down-regulated in DLKP-vincristine and DLKP-taxotere MDR variants. This decrease was not observed at the mRNA level, indicating that this down-regulation of survivin protein may be at the post-transcriptional level in this cell system. In addition, DNA microarray technology was used to investigate differences in gene expression between the DLKP MDR variants and to identify new targets involved in MDR and invasion/metastasis. Results indicated a dramatic increase of Mdr-1 expression and a slight increase of MRP2 expression in DLKP-taxotere and DLKP-vincristine. The expression of other genes studied were unaltered in the MDR variants compared to the parental cells.

Finally, to establish a role for apoptosis-related and drug resistance-related genes as potential clinical markers in breast cancer, the expression of survivin, galectin-3 and MRP1 genes were examined in breast tumour specimens, by RT-PCR analysis. The expression of these genes was correlated with clinicopathological parameters to investigate an association between them and gene expression. As galectin-3 was expressed in almost all the tumour specimens, it was not possible to correlate its expression with any of the parameters. No significant association was detected between disease outcome and the mRNA expression of either survivin or MRP1.

1.0 Introduction

1.1 Treatment of Cancer

Chemotherapy has a major role in controlling metastatic cancers which cannot be cured by surgery. Radiotherapy, also an effective treatment for cancer, has been used alone and in combination with chemotherapy and surgery. Surgery and radiotherapy can often eradicate primary or localised disease but may ultimately fail because the cancer has metastasised to other areas of the body. In such instances, chemotherapy may control or eliminate metastatic disease and reduce mortality. Chemotherapy combined with surgery or radiotherapy (or both), known as adjuvant therapy, has increased survival rates for a number of solid tumours that were previously treated by only one therapeutic modality.

Many of the drugs used in chemotherapy are directed at disrupting the cell cycle, with RNA, DNA and protein molecules as the targets. Most synthetic chemotherapeutic agents currently in use can be categorised according to whether they alkylate DNA (alkylating agents) or antagonise metabolites required for DNA synthesis (antimetabolites). A third group (including vinca alkaloids and epipodophyllotoxins) are natural products from plants and fungi. Different groups of agents will be discussed in section 1.1.1

The ultimate clinical effectiveness of any anticancer drug requires that it kill malignant tumour cells *in vivo* at doses that allow enough cells in the patient's critical tissues to survive so that recovery can occur. In general, anticancer drugs are most useful against malignant tumours with a high proportion of dividing cells. Thus, in practical terms, drugs alone are primarily effective against the leukemias and lymphomas. The most common malignant tumours, however, are "solid" tumours, including those of the colon, rectum, lung and breast. These tumours usually have a low proportion of dividing cells and therefore are less susceptible to treatment by drugs alone (Pratt *et al.*, 1994).

1.1.1 Chemotherapy Drugs involved in Cancer therapy

The first cancer treatment to be administered to a cancer patient was in 1931, when Adair and Bagg applied sulfur mustard, a precursor of nitrogen mustard (a drug developed for use as a war gas), to squamous carcinomas and also injected it directly into tumours in humans. This was however, too toxic for systematic use. In 1942, the first clinical trials began, using nitrogen mustard to treat patients with lymphosarcoma. The second successful anti-cancer drug to be used clinically belongs to a class of drugs called antimetabolites. There are three types of anti-metabolites: antifolates, antipurines and antipyrimidines. Over a decade later, cytotoxic antibiotics were introduced. These include the anthracyclines, which are discussed below. In 1965, the platinum compounds were discovered to inhibit proliferation of *E.coli* and were used in clinical trials seven years later. This group of compound will also be discussed below along with other classes of anti-cancer drugs.

A total of nine chemotherapeutic drugs were utilised in this study to examine the role of certain genes in drug resistance and to examine the effect of drug treatment on the development of multiple drug resistance on cancer cells. The nine drugs utilised here are Vincristine, Taxol (Paclitaxel), Taxotere (Docetaxol), 5-Fluorouracil (5-FU), Adriamycin (Doxorubicin), Epirubicin, Cisplatin (CDDP), Carboplatin, and CCNU (Lomustine); their mechanisms of action will be discussed in the following section.

Common name	**Generic name**
Taxol	Paclitaxel
Taxotere	Docetaxol
Adriamycin	Doxorubicin
Cisplatin	CDDP
CCNU	Lomustine
Etoposide	VP-16

Table 1.1.1 Chemotherapy drugs and their various names.

1.1.1.1 The Vinca Alkaloids

Vincristine (Figure 1.1.1.1) and Vinblastine are complex plant alkaloids isolated from the periwinkle plant *Catharanthus roseus*. They are members of a general class of drugs that function as mitotic inhibitors which act by interfering with the function of microtubules, a class of long, tube-like cellular organelles approximately 250nm in diameter (Pratt and Ruddon, 1994). Microtubules and microfilaments play an important role in the movement of cells relative to each other and in the movement of organelles within the cytoplasm of a single cell. The cytotoxicity of vincristine and vinblastine is attributed to their ability to interrupt cell division in metaphase (Bruchovsky *et al.*, 1965), but other effects could also contribute to cell death. Their action is specific to the M phase of the cell cycle (Wilson *et al.*, 1975).

The vinca alkaloids specifically exert their effect by binding to tubulin and preventing its polymerisation (King *et al.*, 2000). As polymerised microtubules form the spindles that retract chromosomes into daughter cells at mitosis, their disruption results in blocked mitosis. Exposure of mitotic cells to the drugs is followed by the rapid disappearance of the spindle apparatus and the maintenance of the chromosomes in the condensed state. Although the effects are seen at the time, the actual vincristine-tubulin interaction occurs during interphase.

Vincristine and vinblastine have very similar chemical structures and behave in essentially the same way at the level of drug-tubulin interaction; however, there are differences in the spectrum of the antitumour activity of the two drugs in both experimental animal tumours and clinical cancer. No reason for these differences is currently known. These compounds are effective against a broad spectrum of cancers such as lung, ovarian and testicular cancer (Culine *et al.*, 1994; Einhorn, 1997; Schiller, 2001; Schuette, 2001). They induce bone marrow and neural toxicities, and development of drug resistance to both agents is via overproduction of P-glycoprotein (Bradley *et al.*, 1989).

1.1.1.2 The Taxanes

The taxanes are a group of drugs including paclitaxel (taxol) and docetaxol (taxotere). Taxol was originally isolated from the *Taxus brecifolia* yew tree. Despite the elucidation of its broad activity and its unique structure (Wani *et al.*, 1971), it is still in an early stage of clinical development in so far as its role/potential role in the treatment of many cancer types has yet to be conclusively defined. Taxotere (docetaxel) is a semi-synthetic taxane extracted from the needles of *Taxus baccata*, and slightly more soluble than taxol. The chemical structure of taxotere is shown in figure 1.1.1.2. Similar to the vinca alkaloids, the taxanes exert their anti-tumour effect through the disruption of mitosis. They interfere with chromosome changes during the cell cycle and are toxic to proliferating cells. They induce a shift in the physiological equilibrium between microtubules and tubulin toward polymerization and formation of dysfunctional microtubules.

Taxol and taxotere have been shown to be very active in the treatment of refractory ovarian cancer (Rowinsky *et al.*, 1990). Encouraging results have been obtained in patients with metastatic breast cancer (Holmes *et al.*, 1991 and Marty *et al.*, 1999). Like the majority of chemotherapy drugs, the taxanes also have some side effects. Bone marrow suppression (principally neutropenia), complete alopecia (hair loss), and hypersensitivity reactions are the most common does-limiting toxicities for the taxanes (Markman *et al.*, 2003). Other side effects include, hypersensitivity reaction characterised by dyspnea, urticaria, and hypotension. A variety of cardiac abnormalities have also been associated with taxol treatment (Pratt *et al.*, 1994). Six new taxane derivatives are currently in clinical trial. All sharing the same feature, which is a decreased recognition by P-glycoprotein (Lavelle *et al.*, 2002).

Resistance to taxol and taxotere arises through the same mechanisms involved in resistance to vinca alkaloids, i.e. multidrug resistance, resulting from over-expression of P-glycoprotein (Roy *et al.*, 1985 and Greenberger *et al.*, 1987). A second mechanism of drug resistance is due to the mutation of the gene coding for one of the tubulin subunits (Schibler *et al.*, 1986). By this mechanism, it is thought that, in the absence of drug, the equilibrium between free tubulin and microtubules is shifted toward disaggregation (Cabral *et al.*,

1986); therefore the mutant cells have slightly greater tolerance for taxol-induced stabilization than that of the parent cells. Some mutants were not only resistant to taxol, but they also required it for growth, as a result of an extreme shift toward disaggregation (Pratt *et al.*, 1994).

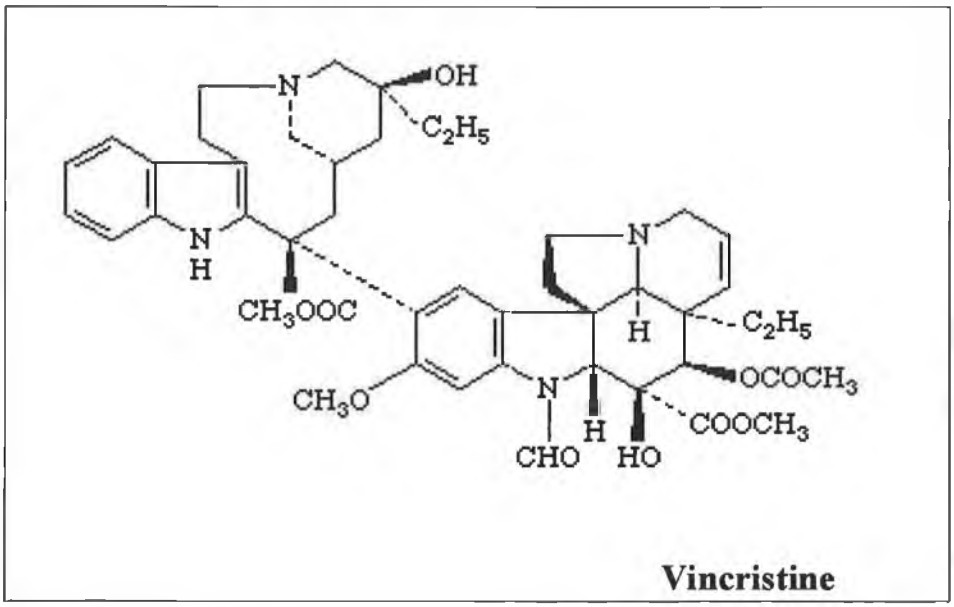


Figure 1.1.1.1 Chemical structure of Vincristine

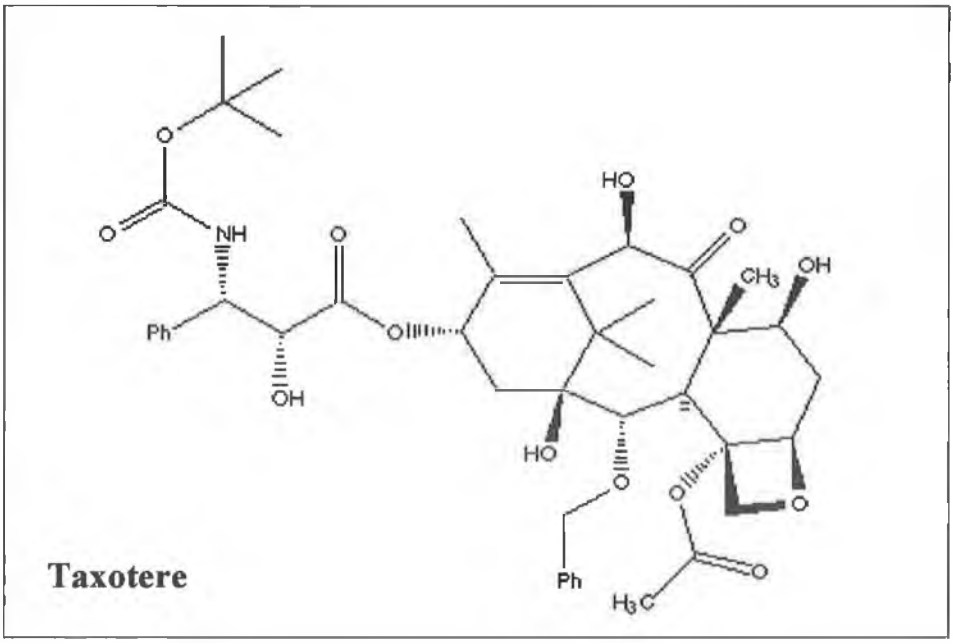


Figure 1.1.1.2 Chemical structure of Taxotere

1.1.1.3 The antimetabolites (5-Fluorouracil)

The antimetabolites are drugs that are chemically related to naturally occurring compounds and interfere with cellular metabolic pathways, especially those involved in the synthesis of DNA. This group of cytotoxic drugs is divided into three groups: 1) folate antagonists, 2) pyrimidine analogues and 3) purine analogues (Skovsgaard *et al.*, 1994).

5-Fluorouracil (5-FU) is an anti-pyrimidine and is one of the most widely used agents in the treatment of human cancer. It is effective in the treatment of the most commonly occurring solid tumours, including colorectal, breast, head and neck, gastric and pancreatic cancers (Pratt *et al.*, 1994). It is more toxic to proliferating than non-proliferating cells, leading to toxic side effects in tissues of the gastrointestinal tract and bone marrow. It is a simple derivative of uracil where the hydrogen at position 5 is replaced by a fluorine atom. 5-FU was first synthesised in 1957 and is a member of the antimetabolite group of drugs. Its major mechanism of action is through inhibition of nucleotide synthesis and through incorporation into RNA (Wilkinson *et al.*, 1973 and Parker *et al.*, 1990). 5-FU is the most important pyrimidine antagonist and can be metabolised to 5-fluorodeoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthesis and consequently DNA synthesis and transcription, translation and intracellular distribution of mRNA.

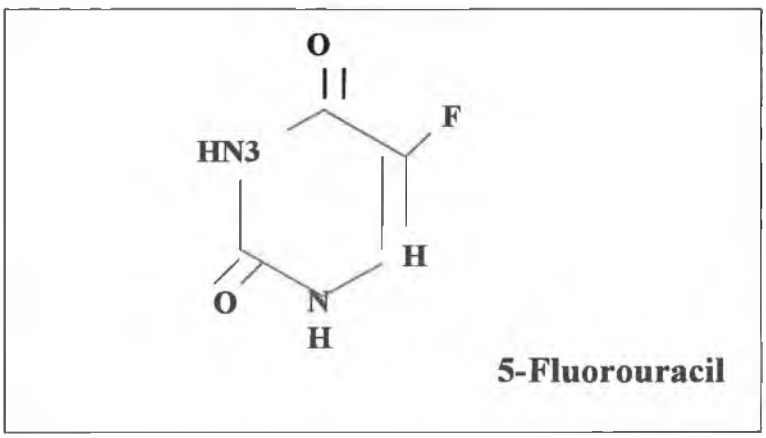


Figure 1.1.1.3 The Chemical structure of 5-Fluoruracil

Another member of the antimetabolite family of drugs is methotrexate. An analogue of folic acid, it is the most important anti-folate as it is successfully used to treat osteogenic sacrcoma and head and neck cancer (Ackland *et al.*, 1987). It is also effective in combination with other drugs for the adjuvant therapy of breast cancer (Stoller *et al.*, 1977) and since its introduction in the clinic in 1984, it is the best understood antineoplastic compound to date. Methotrexate exerts its cytotoxic effect by competitive inhibition of the cytosolic enzyme dihydrofolate reductase (DHFR) (Waltham *et al.*, 1988), which is the key enzyme in the biosynthesis of pyrimdines and purines.

1.1.1.4 The Anthracyclines

The anthracyclines are a class of anti-tumour antibiotics derived from the *Streptomyces* species (Tannock and Hill, 1992). They have a characteristic four-ring structure, linked to the sugar residue by a glycosidic bond (Figure 1.1.1.4). Included in this family are adriamycin (doxorubicin), daunorubicin and epirubicin. Adriamycin is one of the most important agents in the treatment of human cancer (Tritton and Yee, 1982). It is effective against acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), bladder carcinoma, breast, ovarian, lung, bronchi and thyroid cancer as well as non-Hodgkin's lymphoma and neuroblastoma (Carter *et al.*, 1987). Serious side effects are seen with the use of this drug, however. Adriamycin is carcinogenic and mutagenic and causes immunosuppression in patients receiving this treatment. Damage to heart muscle is the worst side effect documented (Tannock and Hill, 1992).

The primary mechanism of action of the anthracyclines is due to the drugs' ability to bind DNA, involving intercalation between the two strands of the double helix, resulting in inhibition of replication, transcription and translation. It is this DNA binding that results in their cytotoxicity. Adriamycin has been shown to induce double strand DNA breaks in cultured mammalian cells (Nelson *et al.*, 1984). The DNA breaks are caused by the enzyme topoisomerase II. This enzyme controls the degree of DNA supercoiling by cleaving and reannealing DNA such that the coil is relaxed by one turn. The observation that a direct correlation between topoisomerase II activity and adriamycin resistance exists in several

cell lines strongly supports the involvement of topoisomerase II in the cytotoxicity pathway (Glisson *et al.*, 1986; Pommier *et al.*, 1986; Deffie *et al.*, 1989).

The most common resistance mechanism in cells treated with adriamycin *in vitro* is increased drug efflux. This is due to the over-expression of a number of proteins including P-glycoprotein, multiple drug resistance protein (MRP) family members and lung resistance protein (LRP) (see section 1.2.1).

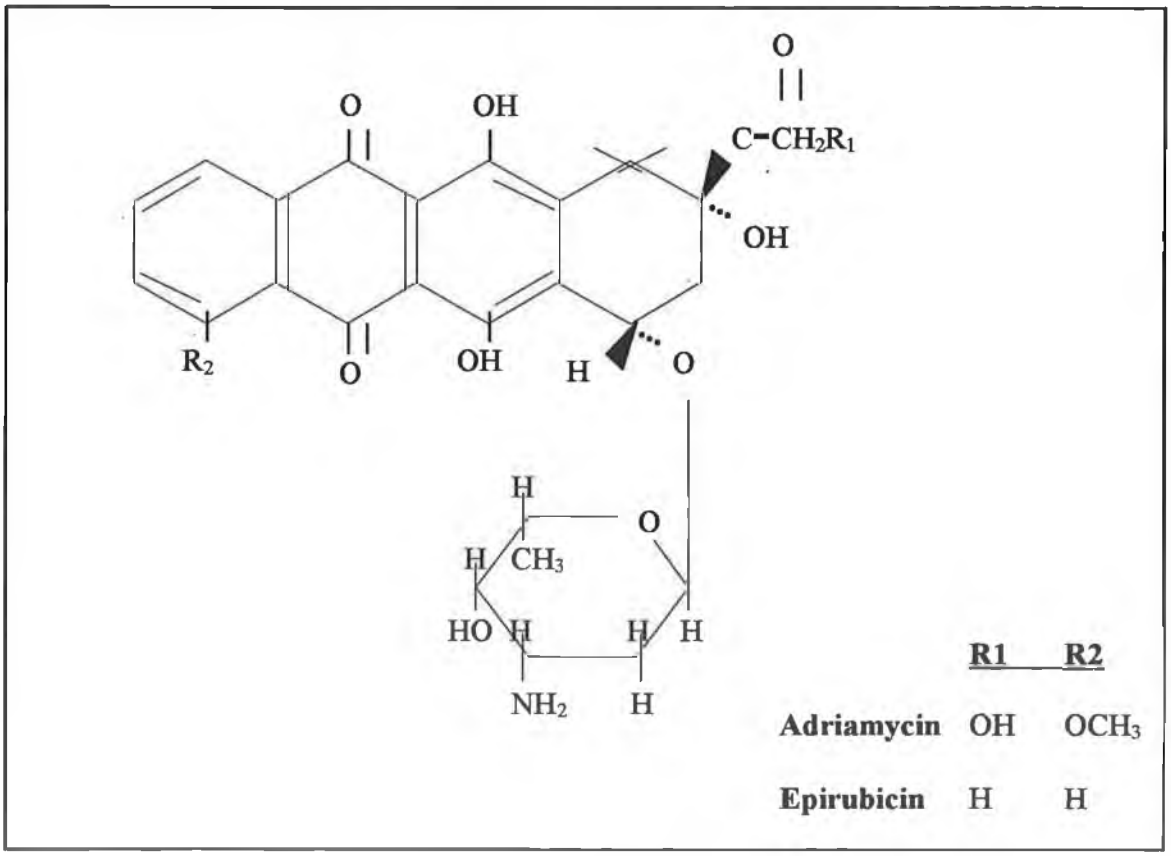


Figure 1.1.1.4 Chemical structure of adriamycin and epirubicin

1.1.1.5 The Platinum Compounds (Cisplatin and Carboplatin)

Platinum compounds were discovered by Rosenberg and co-workers (1969) while studying the effect of electric current on bacterial cell division. They were found to be effective at preventing cell division in *E. coli*. It became clear that cell division was being inhibited by an electrolysis product of the platinum electrode. Rosenberg then tested the anti-tumour activity of several platinum compounds and demonstrated that cisplatin (*Cis*-diamminedichloroplatinum(II) (CDDP)) was effective against leukemia in mice (Rosenberg *et al.*, 1969). This led to clinical trials in 1972.

The platinum compounds exert their toxicity by binding to base pairs of DNA, resulting in the formation of intra-strand cross-links and adducts, and ultimately leads to the disruption and unwinding of the DNA double helix. Cisplatin is currently one of the most effective anti-cancer drugs used in the treatment of ovarian, testicular, head and neck, non-small cell lung and brain tumours (Einhorn, 1997; Gregory et al., 2000; Nardi *et al.*, 2001; Schiller, 2001; Novello & Chevalier, 2001). The side effects of cisplatin include nephrotoxicity, which may ultimately lead to renal failure, gastrointestinal toxicities, myelosuppression, tinnitus and loss of hearing. To avoid these side effects, other platinum compounds, including carboplatin, have been designed. No dominant mechanism of resistance to cisplatin has been identified. Often resistant cells have an increased capacity to repair intrastrand adducts (Fox, 1984), but in many studies the increase in repair capacity has not been sufficient to explain the extent of resistance (Eastman *et al.*, 1987).

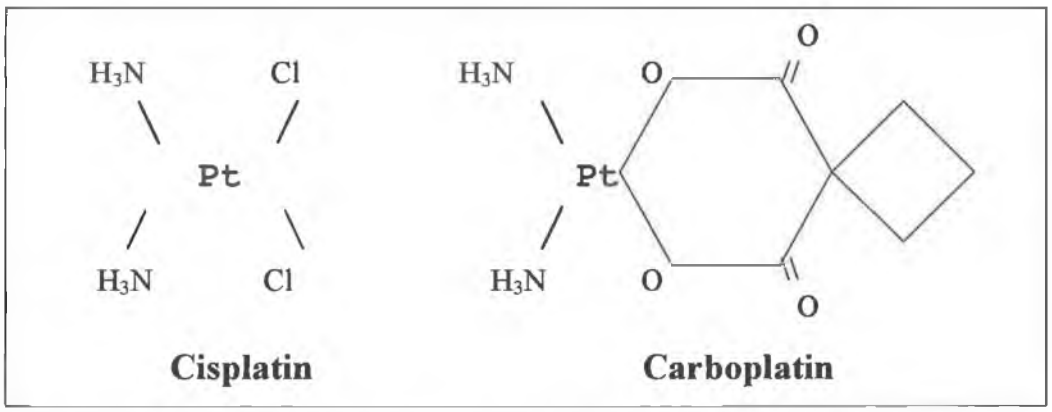


Figure 1.1.1.5 Chemical structure of Cisplatin and Carboplatin

1.1.1.6 Nitrosoureas (CCNU)

This family of alkylating agents, synthesised and tested for anti-tumour activity, consists of four compounds that are available for clinical use. These are: carmustin (BCNU), lomustine (CCNU), Semustine (Methyl-CCNU) and streptozotocin. The chemical structure of CCNU is shown in figure 1.1.1.6. The mechanism of action of CCNU is not very well understood, however, evidence suggests that it can produce interstrand cross-links in DNA (Kohn *et al.*, 1977).

CCNU is rapidly absorbed from the gastrointestinal tract and is routinely administered orally. This group of drugs is very lipophilic and therefore, distributes widely in the tissues. Their lipophilic nature allows them to readily pass across the blood-brain barrier into the cerebrospinal fluid and into brain tumour tissue where drug concentrations can exceed those of plasma (Walker et al., 1976). This has led to clinical application of these drugs in the treatment of brain tumours and meningeal leukemia. Side effects include nausea and vomiting, but major side effects such as, renal damage, CNS toxicity, thrombocytopenia and leukopenia, can also occur.

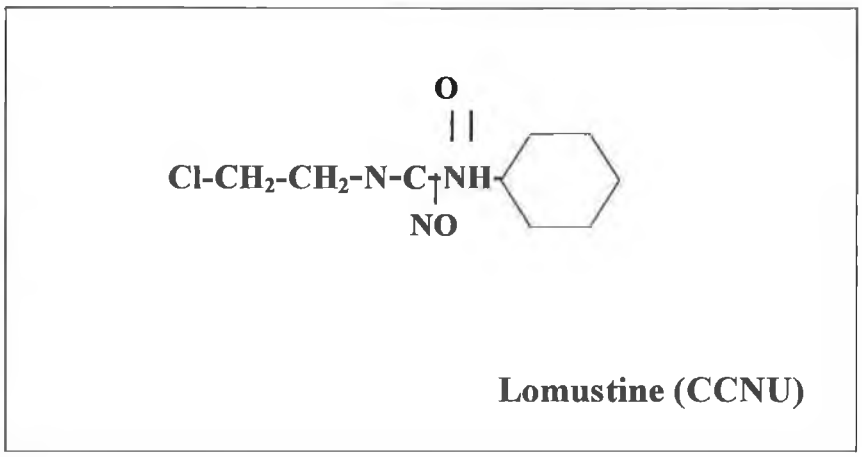


Figure 1.1.1.6 Chemical structure of CCNU

1.2 Multidrug resistance (MDR) in cancer

Cancer treatment using chemotherapy has a limited potential to cure the disease due to acquired or intrinsic resistance of cancer cells to anticancer agents. Resistance to one drug is often associated with resistance to a series of different drugs. This is termed multidrug resistance, (MDR), and it often results in few therapeutic options and so worse prognosis for the cancer that is being treated. Combination chemotherapy using chemically unrelated drugs was introduced to overcome this problem. However, cancers can develop multi-drug resistant variants, which display resistance to a range of chemically unrelated drugs, thus diminishing the effectiveness of combination therapy (Clynes *et al.*, 1990).

Gastrointestinal, hepatobiliary and renal cancers are usually unresponsive to chemotherapy and therefore have a high degree of intrinsic MDR, whereas leukemias, lymphomas, ovarian and breast cancers often respond to treatment initially, but acquire resistance during the course of the disease (Lehne *et al.*, 2000). Multidrug resistance compromises the efficacy of cancer chemotherapy treatment in the clinic. It may occur when tumours that may have been sensitive, become resistant to a variety of anticancer drugs that are structurally unrelated and have diverse cellular targets. MDR may be observed in primary therapy (inherent resistance) or be acquired during or after treatment (acquired resistance) (Yu *et al.*, 1999). Although the majority of tumours show an initial response to chemotherapy, a relapse that neither responds to the drugs initially used nor to other anticancer drugs often follows. *In vitro* studies have shown that MDR is frequently accompanied by reduced intracellular drug accumulation due to increased drug efflux by energy-dependant trans-membrane drug transport proteins (Endicott and Ling, 1989).

One major mechanism of resistance to such drugs is linked to decreased cellular accumulation of anticancer drugs through enhanced cellular efflux of the anti-tumour compounds, as mentioned above. Such multidrug resistance can be conferred *in vivo* and *in vitro*, by a number of proteins. The most important genes mediating drug resistance *in vivo* are the 170kDa P-glycoprotein (encoded by the *MDR*1 gene) (Higgins *et al.*, 1992; Gottesman and Pastan, 1993), the 190kDa MRP1 (Ling *et al.*, 1984; Cole *et al.*, 1992) and its homologues (Kool *et al.*, 1997). All of these proteins are members of the ABC (ATP-binding cassette) transporter family and function as ATP-dependent active transporters.

1.2.1 Mechanisms of MDR

1.2.1.1 P-gp and its role in MDR

One of the most important and commonly reported mechanisms of MDR involves the multidrug transporter, P-glycoprotein (Pgp), a plasma membrane efflux pump with ATP-ase activity. Expression of Pgp enables the cells to survive lethal doses of certain cytotoxic drugs by pumping the drugs out of the cells and, thus, reducing their cytotoxic effect (Juliano *et al.*, 1976; Endicott and Ling, 1989). Several clinically important anticancer drugs may be excluded from neoplastic cells by Pgp-mediated transport, despite the diversity in chemical structures and mechanisms of action of these drugs.

Pgp belongs to the ATP binding cassette (ABC) family of transporter molecules (Germann *et al.*, 1993; Breuninger *et al.*, 1995), which also include the MDR-associated proteins (MRPs) (Cole *et al.*, 1992) and the breast cancer resistant protein (BCRP) (Ross *et al.*, 1997). The gene encoding Pgp belongs to the MDR multigene family, which consists of two highly homologous genes MDR1 and MDR2, situated on chromosome 7q21.1 in humans (Lincke *et al.*, 1991). Pgp transverses the plasma membrane and consists of two homologous halves, each of which contains six hydrophobic domains and a hydrophilic nucleotide binding fold (Figure 1.2.1.1) (Germann *et al.*, 1996). The hydrophobic regions represent putative transmembrane domains forming a pore-like structure (Rosenberg *et al.*, 1997). The two adenosine triphosphate (ATP)-binding folds are located intracellularly and exhibit significant ATPase activity (Ambudkar et al., 1992). The drug binding sites are localized in the transmembrane domains close to the cytosolic surface (Bruggemann *et al.*, 1992). Pgp transports drugs out of the cell, which is a process that requires the presence of two ATP-binding domains. These domains are a defining characteristic of this family of ATP-binding cassette (ABC) transporters. The exact mechanism of drug efflux is not well understood, but might involve either direct transport out of the cytoplasm or redistribution of the drug as it transverses the plasma membrane, resulting in the reduction of intracellular drug concentration. Some of the cytotoxic drugs that are known substrates for Pgp include etoposide, taxol, vincristine, actinomycin D, mitoxantone and adriamycin (Clynes *et al.*, 1993) (see Figure 1.2.1.3).

P-glycoprotein expression is not, however, limited to cancer cells. Its expression has been reported in a variety of normal tissues (van der Valk *et al.*, 1990) and it is thought to play a role in xenobiotics efflux. P-gp is expressed in normal tissue from liver (Fojo *et al.*, 1987; Kamimoto *et al.*, 1989), kidney (Thiebaut *et al.*, 1987), pancreas (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987), adrenal gland (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987; Croop *et al.*, 1989) and intestine (Thiebaut *et al.*, 1987), as well as a subset of normal bone marrow cells. P-gp also plays a role as a chlorine channel and is related to the cystic fibrosis gene product, CFTR, also associated with chloride channel activity (Bremer *et al.*, 1992; Germann *et al.*, 1993).

Overcoming Pgp-mediated MDR has been the focus of many researchers. This has been investigated in many ways. Theoretically, using cytotoxic drugs that are not substrates of Pgp and thus retain activity in cells that express Pgp at high levels would be a useful approach; however, due to the lack of suitable "non-MDR" drugs, in practice this is not always a feasible option. Both anthracyclines and taxanes, which are MDR related drugs, are currently irreplaceable in a range of chemotherapy regimens because of their unique antineoplastic activity. The use of drug sensitisers that interfere with the drug efflux driven by Pgp may restore drug sensitivity in MDR cells. Several classes of modulators have been identified that act as drug sensitisers including calcium channel blockers, calmodulin antagonists, and immunosuppressive agents (Ferry *et al.*, 1996). These are now termed first generation P-gp modulators. The calcium channel blocker, verapamil, was the first agent shown to modify MDR *in vivo* and *in vitro* (Tsuruo *et al.*, 1981), but unfortunately the MDR modulating activity required concentrations that are associated with severe cardiac toxicity in patients (de Faire *et al.*, 1977). Immunosuppressive agents present a similar problem. Although the immunosuppressive agent cyclosporin A has been shown to be a highly potent inhibitor of Pgp, and it inhibits Pgp at clinically tolerable concentrations, the immunosuppression activity restricts its utility in the clinic (Slater *et al.*, 1986). There is a need, therefore, for more potent and less toxic modulators. Second generation P-gp modulators have been developed. These include dexverapamil, dexniguldipine and valspodar. These agents are more potent than the first generation modulators and are also less toxic (Krishna *et al.*, 2000). These compound also have their limitations, however. They significantly inhibit the metabolism and excretion of cytotoxic agents, leading to

toxicity. Third generation P-gp modulators have recently been developed and are currently undergoing clinical trials (Duffy *et al.*, 1998; Touhey *et al.*, 2003; Thomas *et al.*, 2003).

Antisense oligonucleotides to mdr-1 have also been developed and proved a direct causal role for P-gp over-expression in MDR. Antisense oligonucleotides transfections in adriamycin-resistant cells caused partial reversal in adriamycin resistance in DLKP-A resistant cells (Clynes *et al.*, 1992). MDR1 ribozyme studies have also been carried out successfully, inhibiting MDR1 gene expression and preventing the development of drug resistance (Daly *et al.*, 1996). The use of siRNA to inhibit P-gp expression and reverse MDR has recently been demonstrated. SiRNA generated from double-stranded RNA can trigger silencing of homologous gene expression by inducing degradation of the complementary mRNA. This newly identified mechanism is termed RNA interference or post-transcriptional gene silencing (Tuschl *et al.*, 1999) and is emerging as a new tool for down-regulating gene expression, *in vitro*. Through the use of siRNA, expression of endogenous, as well as transfected, mdr-1 gene transcripts were dramatically down-regulated in the breast carcinoma cell line, MCF-7, as seen by RT-PCR and western blot analysis. In addition, the mdr-1-targeted siRNA reversed resistance to P-gp-transportable drugs, such as vinblastine, adriamycin and taxol, but did not affect the sensitivity to hydroxyurea, a non-P-gp substrate. Furthermore, treatment of MDR cells with mdr-1-targeted siRNA increased the intracellular accumulation of taxol and adriamycin (Wu *et al.*, 2003). Similar experiments using siRNA have been carried out recently on pancreatic and gastric cell lines. Mdr-1 expression in these cells was also dramatically reduced due to expression of siRNA constructs, as seen by RT-PCR and western blot analysis. Resistance to daunorubicin was reduced dramatically in both cell lines (Nieth *et al.*, 2003). This method of reversing/preventing MDR is very promising.

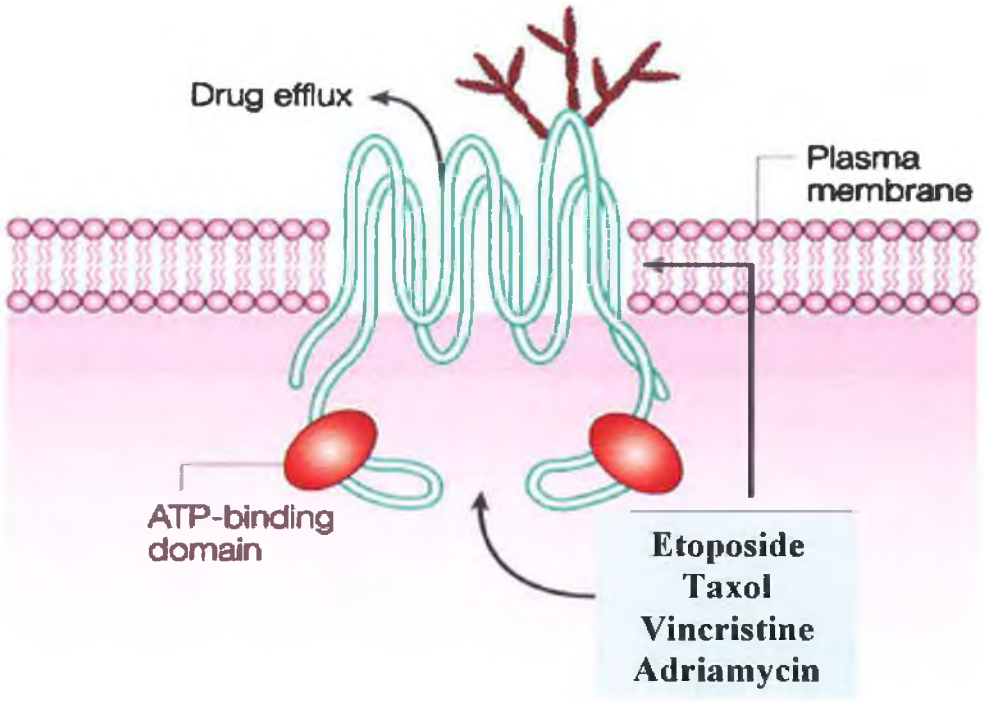


Figure 1.2.1.1 P-glycoprotein (Pgp) as a transmembrane drug efflux pump. Pgp transports drugs out of the cell, which is a process that requires the presence of two ATP-binding domains. These domains are a defining characteristic of this family of ATP-binding cassette (ABC) transporters. Some cytotoxic drugs that are known substrates for Pgp include etoposide, taxol, vincristine and adriamycin. Pgp is modified by sugar moieties (brown) on the external surface of the protein. *(Nature Reviews Cancer* 2; 431-441 (2002)).

1.2.1.2 MRP and its role in MDR

Studies of a Pgp negative multidrug resistant human lung cancer cell line, H69AR, led to the identification of another transmembrane protein, the p-190 multidrug resistance-associated protein (MRP) (Cole *et al.*, 1992). ABC transporters belonging to the MRP family have been identified in a variety of organisms including yeast, nematodes and plants (Jedlitschky *et al.*, 1996 and 1997). The family of human MRPs consists of at least eight members know as MRP1, MRP2 (cMOAT), MRP3, MRP4, MRP5, MRP6, MRP7 and MRP8 (Allikmets *et al.*, 1996; Kool *et al.*, 1997; Hopper *et al.*, 2001). The membrane topology is a common feature to at least four of the six MRP members. In contrast to the 12 transmembrane organisation for P-glycoprotein (section 1.2.1.1 and Figure 1.2.1.1), the MRPs exhibit an additional amino-proximal membrane-spanning domain, represented by an extension of approximately 200 amino acids. The best characterised members of this family are MRP1 and MRP2. MRP1 was cloned from a drug-selected human lung cancer cell line (Cole *et al.*, 1992) and consists of 1531 amino acids. The MRP1 gene is located on chromosome 16p13.12-13 (Grant *et al.*, 1997). MRP1 confers resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids (Cole *et al.*, 1994; Zaman *et al.*, 1994; Breuninger *et al.*, 1995; Lorica *et al.*, 1996), methotrexate (Hooijberg *et al.*, 1999), and actinomycin D (Lorico *et al.*, 1996). Unlike Pgp, MRP1 does not affect resistance to taxol (Cole *et al.*, 1994; Zaman *et al.*, 1994) or mitoxantrone (Cole *et al.*, 1994) (see Figure 1.2.1.3).

MRP2 is an apical isoform of MRP1. A close relationship exists between MRP1 and MRP2, as evident from the comparison of the topology of both transporters. They both consist of an MDR-like core structure of two transmembrane regions and two ATP-binding domains, as well as a third amino proximal transmembrane region (Figure 1.2.1.2) (Buchler *et al.*, 1996). The identification of MRP3, MRP4 and MRP5 was mainly based on expressed sequence tags (EST) database analysis followed by cloning of cDNA fragments (Allikmets *et al.*, 1996; Kool *et al.*, 1997). Recently, a significant overlap in the transport capacity of MRP1 and MRP3 has been found (Bodo *et al.*, 2003). MRP1 over-expression in cells results in multiple drug resistance (Cole *et al.*, 1998). MRP2, which shares a similar substrate spectrum to MRP1, may also confer drug resistance through pumping drug conjugated by glutathione S-transferase out of the cell (Konig *et al.*, 1999). Despite their

similar function in MDR, MRP1 and Pgp show little sequence homology, which is restricted to the ATP binding site. Drug cross-resistance profiles are similar but not identical for MRP1 and Pgp over-expressing cells (Lautier *et al.*, 1996) and agents that reverse Pgp are usually less effective on MRP1.

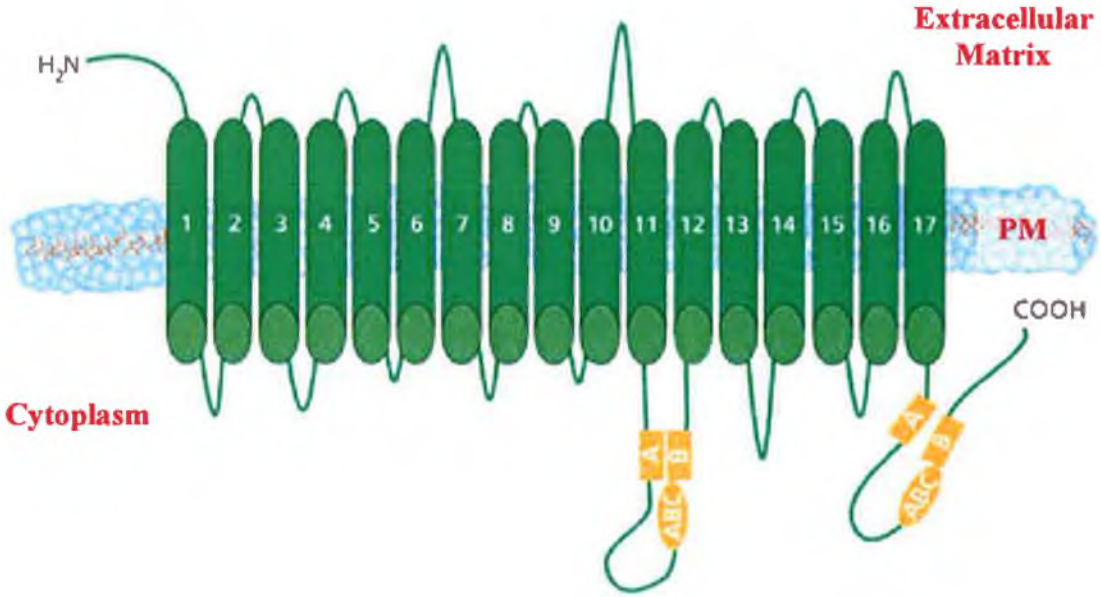


Figure 1.2.1.2 Schematic representation of MRP1. MRP1 is a representative of the second major subfamily, MRP, of multidrug ABC transporters. MRPs have an extra transmembrane domain (adapted from this website: www.sigma-aldrich.com). PM=Plasma membrane; 1-17=Transmembrane domains 1-17; COOH=Carboxy terminus; NH_2=Amino terminus

The predictive significance of MRP in cancer patients is not fully established. Since the discovery of MRP1 in the small cell lung cancer cell line, H69AR (Cole *et al.*, 1992), MRP1 has been identified in non-Pgp multidrug resistant cell lines from a variety of tumour types, including leukemias, fibrosarcoma, non-small cell lung, human small cell lung, breast, cervix, prostate, and bladder carcinomas (Izquierdo *et al.*, 1996). MRP1 has also been detected, either at the protein or mRNA level, in normal human tissues including lung, stomach, colon, peripheral blood macrophages, thyroid, testis, nerve, bladder, adrenal, ovary, pancreas, gall-bladder, duodenum, heart, muscle, placenta, brain, kidney, liver and spleen (Loe *et al.*, 1996a; Zaman *et al.*, 1993; Cole *et al.*, 1992; Kool *et al.*, 1997).

The expression of MRP1 has been detected in almost all tumour types examined, including both solid tumours (lung, gastrointestinal and urothelial carcinomas, neuroblastoma, glioma, retinoblastoma, melanoma, cancers of the breast, endometrium, ovary, prostate and thyroid) (Ito *et al.*, 1998; Canitrot *et al.*, 1998; Chan *et al.*, 1997; Nanashima *et al.*, 1999; Hipfner *et al.*, 1999; Oshika *et al.*, 1998; Loe *et al.*, 1996), and hematological malignancies (Filipits *et al.*, 1997; Abbaszadegan *et al.*, 1994; Loe *et al.*, 1996b). Among the common tumour types, expression of high levels of MRP1 is particularly frequent in the major histologic forms of non-small cell lung cancer (Nooter *et al.*, 1996; Hipfner *et al.*, 1999). There are reports of MRP1 as a marker of poor prognosis in lung cancer (Ota *et al.*, 1995) and neuroblastoma (Bordow et al., 1994), whereas other studies have failed to demonstrate correlation between MRP1 expression and prognosis in colorectal carcinoma (Fillpits *et al.*, 1997), childhood leukemia (den Boer *et al.*, 1998) and breast cancer (Linn *et al.*, 1997; also see section 3.5.3 and 4.5.5 for relevant results arising from this study).

Reports of MRP1 expression in breast cancer have resulted in a lot of conflicting results on the role of MRP1 in disease outcome (for full update see review by Leonessa and Clarke, 2003). MRP1 is expressed in both normal breast tissue and breast cancer as shown by RT-PCR and immunohistochemistry analysis (Linn *et al.*, 1997; Dexter *et al.*, 1998; Filipits *et al.*, 1997 and 1999; Kanzaki *et al.*, 2001). MRP1 expression may correlate with previous chemotherapy treatment in breast cancer patients. Nooter *et al.* (1997) found that in untreated breast cancer patients, the presence of MRP1 protein was associated with a lower response rate, whereas, in patients who were treated with chemotherapy agents, no correlation between MRP1 and response rate was found (Nooter *et al.*, 1997). Furthermore,

patients who had MRP1 negative tumours had longer overall survival and disease free survival compared to patients who had MRP1 positive tumours (Filipits *et al.*, 1999). Further studies of MRP1 expression in breast tumours is required to determine if MRP1 could be a molecular target for the treatment of breast cancer. MRP1 expression pattern and its correlation to prognosis in different human cancers is summarised in Table 1.2.

Table 1.2 Summary of MRP1 Expression in different types of human cancers and its relationship to prognosis.

Cancer Type	Technique	(%)	Prognosis& Correlation	Localisation	Reference
Colorectal	IHC	100	No correlation	Nuclear & cytoplasmic	Filipits *et al.*, 1997
	RT-PCR	82	No correlation	N/A	
Ovarian	IHC	44	No correlation	Cytoplasmic	Arts *et al.*, 1999
	RT-PCR	N/A	Correlated w/ Bad prognosis	N/A	Ohishi *et al.*, 2002
Adult T-cell Leukemia	Slot blot	N/A	Correlated w/ short survival &poor prognosis	N/A	Ohno *et al.*, 2001
Breast	IHC	100	No correlation	Cytoplasmic	Dexter *et al.*, 1998
	IHC	80	Inversely Correlated w/ good prognosis	Cytoplasmic	Stiglbauer *et al.*, 1999
	IHC	100	Correlation w/ Lymph node Metastasis	N/A	Zauchbaur-Muller *et al.*, 2001
	QRT-PCR	N/A	Correlation w/ progression-free survival	N/A	Burger *et al.*, 2003
Urothelial	NB IHC	50	Involved in drug resistance	N/A Cytoplasmic	Kubo *et al.*, 1996

IHC=Immunohistochemistry; QRT-PCR= Quantitative reverse transcrisptase polymerase chain reaction; NB=Northern blot; N/A= Not applicable.

Over-expression of MRP2 (cMOAT) in cancer cells could potentially lead to drug resistance because of its proven ability to transport vinblastine (Borst *et al.*, 1997). However, no correlation has been established, thus far, between MRP2 over-expression and MDR in cultured cells (Lehne *et al.*, 2000), but there was a positive association with cisplatin resistance, raising the possibility that MRP2 might contribute to cisplatin resistance by mediating excretion of cisplatin-glutathione complexes (Borst *et al.*, 1997). MRP2 is found predominantly in the liver, duodenum and, in low levels, in the kidney (Kool *et al.*, 1997; Schaub *et al.*, 1997). Kool *et al.* (1997) and Kiuchi *et al.* (1998) reported that MRP3 mRNA is mainly expressed in the liver, colon, intestine and adrenal gland, and to a lesser extent in several other tissues. Kool *et al.* (1997) reported that MRP4 was found only in a small number of tissues at very low levels. However, Lee *et al.* (1998) demonstrated, using RNA blot analysis, the expression of MRP4 in a wide range of tissues, with particularly high levels in prostate, but almost undetectable levels in the liver. MRP5, like MRP1, is readily detected in several tissues with highest levels in skeletal muscle, intermediate levels in kidney, testis, heart and brain and low levels in most other tissues, including lung, liver, spleen, thymus, prostate, ovary and placenta (Belinsky *et al.*, 1999).

Recent investigations have shown that MRP6 is predominantly expressed in liver and kidney cells and to a lesser extent in other tissues (Kool *et al.*, 1999). MRP6 does not appear to contribute to the cisplatin-resistance of the cell lines analyzed by Kool *et al.* Over-expression of MRP6 was detected in adriamycin-resistant lung cancer cell lines known to have high over-expression of MRP1 (Zaman *et al.*, 1993; Barrand *et al.*, 1994; Eijdems *et al.*, 1995). In each case the over-expression is associated with co-amplification of MRP1 and MRP6 (Kool *et al.*, 1999). MRP7 is a recently described member of the ABC family of ATP binding cassette proteins (Hopper *et al.*, 2001). A splicing variant of human MRP7, MRP7A, expressed in most human tissues, has recently been characterised (Kao *et al.*, 2003). MRP8, which contains high sequence homology with MRP5, has also been recently identified. It is highly expressed in breast cancer and is expressed at moderate levels in normal breast and testis and at very low levels in liver, brain, and placenta (Bera *et al.*, 2001).

1.2.1.3 BCRP and its role in MDR

Breast cancer resistance protein (BCRP) was first identified in the human MCF-7 breast cancer cell line, which displayed an ATP-dependant reduction in intracellular anthracycline accumulation (Doyle *et al.*, 1998). Since then, it has been characterised and identified in a number of human tissues (Maliepaard *et al.*, 2001a). BCRP mRNA encodes a protein of 663 amino acids, which was also identified as a member of the ABC family of transporters that encompass the MRP homologues. A previous study by Ma *et al.* (1998) had identified a novel mechanism of multidrug resistance in the ovarian cancer cell line IGROV1 selected in topotecan. Further research (Maliepaard *et al.*, 1999; Scheffer *et al.*, 2000; Yang *et al.*, 2000) confirmed the expression of the BCRP gene in the IGROV1 and MCF-7 cell lines following selection in topotecan. Scheffer *et al.* (2000) also localised the protein in the plasma membrane of the IGROV1 cell line. BCRP expression has also been observed in cell lines following exposure to mitoxantrone (Maliepaard *et al.*, 1999; Ross *et al.*, 1999), and the BCRP gene is sometimes referred to as the Mitoxantrone resistance gene (MXR). Further studies identified expression of BCRP in a flavopiridol-resistant MCF-7 subline (Robey *et al.*, 2001)

Expression of BCRP has been linked with resistance to mitoxantrone, methotrexate (MTX), adriamycin, daunorubicin bisantrene and topotecan (Doyle *et al.*, 1998; Volk *et al.*, 2000; Litman *et al.*, 2000) (see Figure 1.2.1.3). A clinical study Ross *et al.* (1999) observed a sufficiently high level of BCRP gene expression in patients with AML to warrant additional investigation. Little is currently known of how BCRP mediates cross-resistance to these drugs.

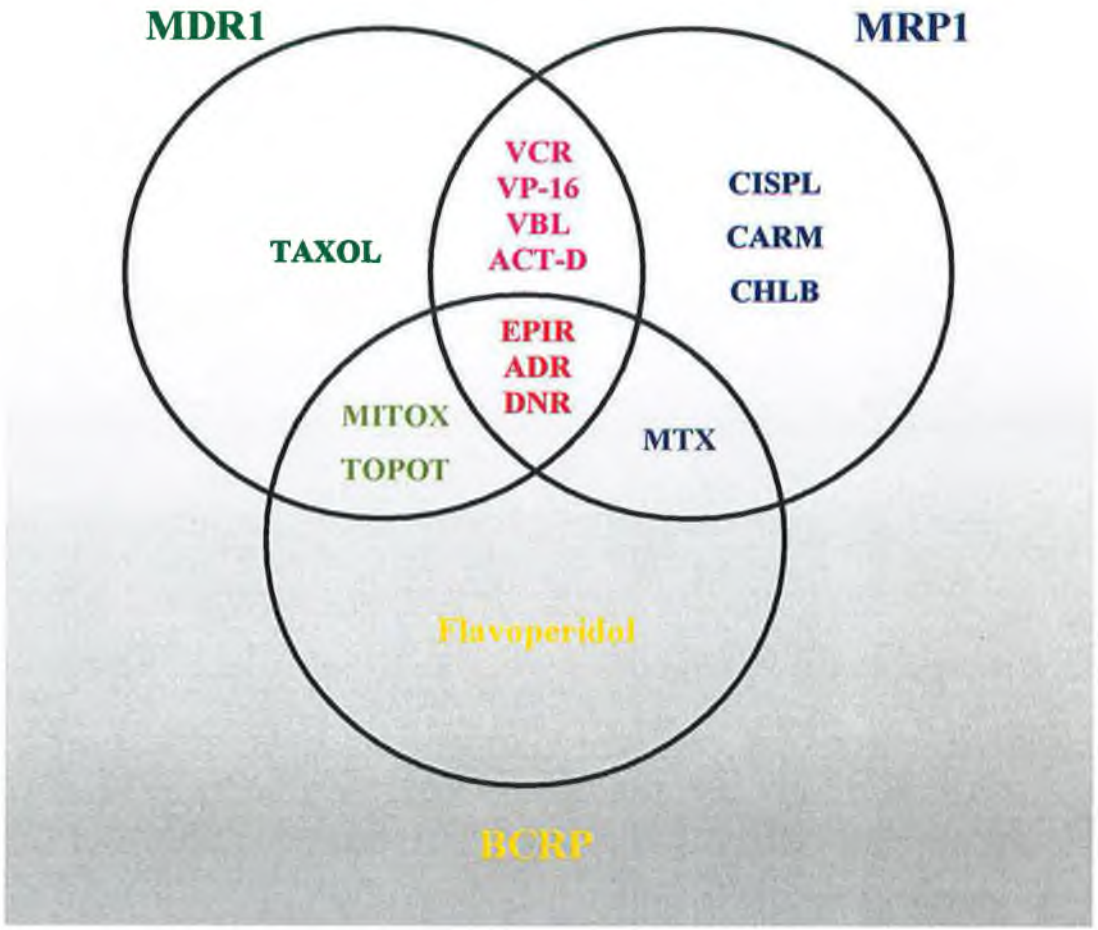


Figure 1.2.1.3 Some anticancer drugs which are substrates for MDR1, MRP1 and BCRP. VCR = vincristine; VBL = Vinblastine; ACT-D = Actinomycin D; EPIR = Epirubicin; ADR = Adriamycin; DNR = Daunorubicine; CISPL = Cisplatin; CARM = Carmustine; CHLB = Chlorambucil; MTX = Methotrexate; MITOX = Mitoxantrone; TOPOT = Topotecan (adapted from www.solvo.hu/scientific-background.php).

1.3 Invasion and metastasis

Metastasis is the spread of cancer from a primary tumour to distant sites of the body. A distinguishing feature of malignant cells is their capacity to invade surrounding normal tissues and metastasise through the blood and lymphatic systems to distant organs. This process of metastasis is the most devastating aspect of cancer. It is estimated that in nearly 50% of cancer patients, surgical excision of a primary tumour is not curative, as a result of metastasis (Fidler *et al.*, 1994). In most patients, metastases develop many years after the resection of the primary neoplasm. This may be caused by a sudden conversion of a tumour to an angiogenic phenotype (Folkman, 1992).

Cancer metastasis has been described as a complex series of sequential processes that involve: (1) the initial transforming event; (2) proliferation of transformed cells; (3) the ability of cancer cells to avoid destruction by immune mechanisms; (4) nutritional supply to the tumour mass requiring the release of tumour angiogenesis factors; (5) local invasion and destruction of extracellular matrix components and parenchymal cells; (6) migration of tumour cells away from the primary tumour mass; (7) penetration of cancer cells through the blood vessel wall; (8) embolisation of cancer cells in 'clumps' to distant organs; (9) arrest of cancer cells in the lumen of small blood vessels or lymphatics; (10) reverse penetration of blood vessels; (11) repetition of the process beginning at step 2, resulting in the formation of a secondary tumour (metastases) (Fidler *et al.*, 1994).

One of the most critical steps in metastasis is invasion. Invasion is the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular barriers (Liang *et al.*, 2002). Liotta *et al.* (1977) has proposed a three-step theory of invasion: (1) tumour cell attachment *via* cell surface receptors which specifically bind to components of the extracellular matrix; (2) the anchored tumour cell secretes hydrolytic enzymes, such as matrix metalloproteinases, to degrade the extracellular matrix; (3) tumour cell locomotion into the region of the matrix modified by proteolysis. Cancer invasion involves a number of changes in cell behavior, in particular the production of enzymes, called proteases, that will break down surrounding tissue as shown in Figure 1.3.1.

Proteases are enzymes that hydrolyse peptide bonds and therefore, lead to the disassembly of proteins. There are four known categories of proteases i.e. serine, cysteine, aspartic proteinases, and metalloproteinases (see section 1.3.1), which have all been implicated in the invasive process.

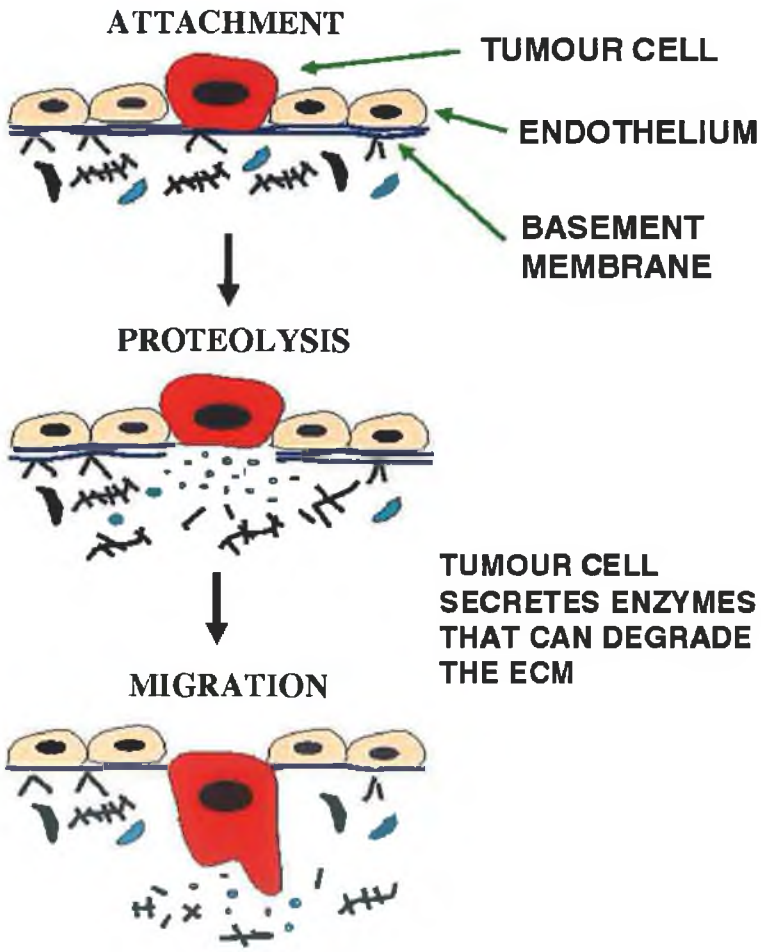


Figure 1.3.1 The process of invasion of a tumour cell through the endothelium and extracellular matrix (ECM) by secretion of degradation enzymes (www.Ibora.ac.uk).

1.3.1 The extracellular matrix

The extracellular matrix (ECM) is a complex structure of carbohydrate- and protein-containing components that make up the basement membrane underlying epithelial tissues and that surround structural tissues such as bone and muscle (Yurcheno *et al.*, 1990). The extracellular matrix not only provides a supportive and nutritive function for the development and organisation of tissues, but it also serves as a physical barrier to limit the migration of most normal cells away from their sites of origin. The extracellular matrix consists of a supramolecular aggregate of connective tissue proteins including collagens, elastin, glycoproteins (laminin, fibronectin, enactin, nidogen) and glycosaminoglycans (ground substances-heparan sulfate proteoglycans), which interact with one another through covalent, and non covalent bonds to form highly insoluble materials (Zern and Reid, 1993). Changes in ECM synthesis, deposition, metabolism and matrix receptors are important components in cancer development and metastasis (Stetler-Stevenson *et al.*, 1993).

The basement membranes of epithelial tissues serve a complex role in cell-cell adhesion and in the regulation of cell proliferation and differentiation. The ECM that makes up the basement membranes is a target for the lytic enzymes secreted by metastatic cancer cells. Cell attachment to specific glycoproteins of the extracellular matrix such as fibronectin, collagen, laminin and entactin, is mediated through tumour cell receptors of the integrin and non-integrin variety. Cell-cell adhesion in normal tissue involves interactions between numerous proteins on the cell surface. Cell adhesion molecules (CAM) of the cadherin family have an inhibitory role in the process of metastasis. Cadherins are calcium ion dependent CAMs that mediate cell-cell binding. Three subtypes have been identified in mammals (E-cadherin, P-cadherin, and N-cadherin). Cadherin proteins may be diminished in cancer cells, leading to a loss of intercellular adhesion.

1.3.2 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases. Their primary function is degradation of proteins in the ECM. Currently at least 19 members of this family are known to exist (Chambers *et al.*, 1997; Duffy *et al.*, 1998). Physiologically, these enzymes play a role in normal tissue remodeling events, such as embryonic development, angiogenesis and wound healing. Abnormal expression of these enzymes, however, appears to contribute to various pathological processes including tumour growth, invasion and metastasis (Chambers *et al.*, 1997).

All MMPs possess specific domains that are conserved between different members. Most MMPs are synthesised and secreted in a zymogen form. Activation is usually accompanied by loss of a 10-kDa amino-terminal domain (Duffy *et al.*, 2000). Most MMPs cleave at least one component of the ECM, as listed in Table 1.3. MMPs are divided into four main subgroups: the interstitial collagenases, gelatinases, stromelysins and membrane MMPs (Chambers *et al.*, 1997; Duffy *et al.*, 1998).

The collagenases comprise interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase 3 (MMP-13) (Table 1.3). These MMPs catalyze degradation of fibrillar forms of collagen (i.e. types I, II and III). MMP-1 shows a preference for the type III form, MMP-8 preferentially degrades type I collagen, and MMP-13 has highest affinity for type II collagen (Balbin *et al.*, 1999). The gelatinases, which are also known as type IV collagenases, degrade and types IV, V, VII, IX and X collagen. This subgroup has two distinct members, known as gelatinase A (MMP-2) and gelatinase B (MMP-9) (see Table 1.3). Generally, these two gelatinases are thought to have similar substrate specificity with respect to ECM substrates, but may have different specificity toward growth factor receptors (Levi *et al.*, 1996). The stromelysins (i.e. stromelysin-1 [MMP-3], stromelysin-2 [MMP-10], stromelysin-3 [MMP-11] and matrilysin [MMP-7]) have relatively broad substrate specificity, catalyzing degradation of many different substrates in the ECM (Chambers *et al.*, 1997). The substrates include proteoglycans, noncollagenous proteins such as laminin, fibronectin and the non-helical regions of collagen IV (see Table 1.3).

The fourth group consists of the membrane-type MMPs, which possess a transmembrane domain (Seki *et al.*, 1999). Five members of this group have been described, the best characterised being the transmembrane-type 1 MMP. This MMP ahs been shown to catalyse activation of progelatinase A (Ohuchi *et al.*, 1997).

Increased MMP activity is detected in a wide range of cancers and seems to correlate to their invasive and metastatic potential. MMPs, therefore, are an attractive target for diagnostic and therapeutic purposes (Denis and Verweij, 1997). Inhibition of these proteinases may lead to prevention of cancer development and inhibit dissemination (Duffy *et al.*, 2000). Two main types of MMP inhibitors (MMPIs) exist: the tissue inhibitors such as tissue inhibitors of metalloproteasas (TIMPs) and low molecular-weight synthetic inhibitors (Hiraoka *et al.*, 1998). TIMPs display a multiplicity of actions (lack specificity) and may not, therefore, be widely used as anticancer molecules. Several synthetic MMPIs have been developed and preclinical studies are promising as they suggest inhibition of several steps in the metastatic process (Denis and Verweij, 1997).

Table 1.3 MMPs, their alternative names and their substrates (adapted from www.rndsystems.com)

MMP	Alternative Names	Substrates
MMP-1	• Collagenase	Collagens (I, II, III, VII, VIII and X); Gelatin
MMP-2	• 72-kDa Gelatinase • Type IV Collagenase	Collagens (I, IV, V, VII, X, XI and XIV); Gelatin; elastin; fibronectin; laminin-1, laminin-5; galectin-3
MMP-3	• Stromelysin-1 • Transin	Collagens (III, IV, V, IX); Gelatin
MMP-7	• Matrilysin • PUMP	Collagen IV and X; Gelatin; fibronectin and laminin
MMP-8	• Neutrophil Collagenase • Collagenase I	Collagens (I, II, III, V, VII, VIII and X); Gelatin
MMP-9	• 92 kDa Gelatinase • Gelatinase B	Collagens (IV, V, VII, X and XIV); Gelatin; elastin; galectin-3
MMP-10	• Stromelysin-2	Collagens (III, IV and V); Gelatin; casein; aggrecan; elastin;
MMP-11	• Stromelysin-3	Laminin; fibronectin; gelatin; collagen IV and carboxymethylated transferrin
MMP-12	• Macrophage Metalloelastase	Collagen IV; Gelatin; elastin and κ-elastin; fibronectin; laminin; plasminogen
MMP-13	• Collagenase-3	Collagens (I, II and III, IV, IX, X and XIV); Gelatin
MMP-14	• MT-MMP-1	Collagen I, II and III, progelatinase A
MMP-15	• MT-MMP-2	Fibronectin; laminin
MMP-16	• MT-MMP-3	Collagen III; Gelatin; casein; fibronectin
MMP-17	• MT-MMP-4	Collagen I, II and III
MMP-19	N/A	Gelatin
MMP-20	• Enamelysin	Amelogenin

1.3.3 MDR and Invasion/Metastasis

An increasing body of evidence indicates that the tumour micro-environment may contribute to cancer progression (Shain and Dalton, 2001). Soluble factors such as cytokines, hormones and growth factors (Klein *et al.*, 1995), as well as interactions between tumour cells and ECM molecules (O'Brien *et al.*, 1996) may play a role in pathogenesis and progression of human cancers. Studies suggest that these same environmental factors may also contribute to the survival of cancer cells after initial therapy, allowing resistant cells to proliferate and acquire multiple drug resistance (see section 1.2).

The relationship between invasion/metastasis and drug resistance has been widely studied since the 1960s. In 1961, enhancement of metastases was observed after mice were treated with nitrogen mustard (Kondo *et al.*, 1961). Drug resistance and cancer invasion and metastasis are two phenotypes that result in chemotherapy failure and disease progression. Evidence is growing that the two phenotypes are linked and this has been demonstrated by two types of observations: firstly, some tumour cells selected for resistance to chemotherapeutic drugs become more invasive relative to sensitive parental cells (Scaddan *et al.*, 1993; Haga *et al.*, 1997; De Larco *et al.*, 2001; Liang *et al.*, 2001and manuscript in prep); secondly, in some cases, secondary metastatic tumours are more resistant to chemotherapeutic drugs than primary, non-metastatic tumours (Donelli *et al.*, 1967; Tanigawa *et al.*, 1984; Staroselsky *et al.*, 1990; Furukawa *et al.*, 2000).

Pgp, as discussed in section 1.2.1.1, is a membrane glycoprotein whose over-expression has been detected in many multi-drug resistant cell lines. Over-expression of Pgp has been detected in clinical cancer specimens of many histological types (Goldstein *et al.*, 1989; Chan *et al.*, 1991; Leonessa *et al.*, 2003). Pgp protein expression was examined in early and advanced breast cancer by immunohistochemistry and was found to be expressed in 10% of early operable breast cancer compared to 60% of locally advanced breast cancer, suggesting a correlation between Pgp expression and breast cancer metastasis (Linn *et al.*, 1997). In addition, the expression of Pgp during stepwise progression to rat liver cancer was examined to investigate the possible role of Pgp in carcinogenesis. A strong elevation

of Pgp mRNA expression was observed as the malignant phenotype progressed (Bradley *et al.*, 1992).

Although there is strong evidence that drug resistance and cancer invasion/metastasis phenotypes are linked to each other in a number of biological models of cancer, and perhaps also in clinical disease, it is important to address the fact that there are also observations which suggest that these phenotypes are un-related. It has been demonstrated that a murine cyclophosphamide-resistant tumour obtained *in vivo* by repeated cyclophosphamide treatment of mouse ovarian sarcoma, displayed similar metastatic rate and slower growth rate when compared with the drug-sensitive parent sarcoma. In addition, the survival time of drug-resistant tumour-bearing mice was longer than that of drug-sensitive tumour-bearing mice (Sheng *et al.*, 1997).

Studies in our laboratories indicate that taxol did not promote *in vitro* invasiveness in the human nasal carcinoma cell line, RPMI-2650, whereas melphalan exposure did (Liang *et al.*, 2001). Furthermore, mitoxantrone, 5-FU, methotrexate, BCNU, cisplatin and chlorambucil induced an invasive phenotype in the human lung carcinoma cell line, DLKP, whereas VP-16, vincristine, taxotere and CCNU did not (Liang *et al.*, manuscript in prep.). Results in this thesis show that taxotere-resistance is associated with *in vitro* invasiveness in DLKP cells, whereas vincristine-resistance is not (see sections 3.3.3, 4.4.2.3 and 4.4.3.3).

A better understanding of the correlation between drug resistance/drug exposure and invasion/metastasis is needed to help identify improved cancer treatment regimens and better drug targets to circumvent these two phenotypes, simultaneously.

1.4 Apoptosis

Apoptosis, or programmed cell death, is a set of ordered events that enables the selective removal of cells from tissue and is essential for homeostasis and proper function of multicellular organisms. Such a control mechanism is required for organ formation during development, cellular homeostasis in adulthood, and proper function of the immune system (Los *et al.*, 2003). Numerous genes are involved in the regulation of apoptosis. There are two main pathways in apoptosis. The mitochondrial pathway involves the release of cytochrome *c* from the mitochondria. The cell surface pathway is stimulated by cell surface death receptors such as Fas receptor (see below). Both pathways share activation of caspases, which are considered as crucial effectors of the cell death machinery (Kania *et al.*, 2003). A number of caspases have been identified to date. However, it is unclear whether they are all involved in the apoptosis mechanisms. Recent evidence suggest that caspases that are most closely related to CED-3 of the C. *elegans*, such as capase-3, are the most involved in the execution and signaling events of apoptosis (Gruter *et al.*, 2000).

Certain cells have unique sensors, termed death receptors, on their surface, including the Fas receptor and the tumour necrosis factor (TNF) receptor (Sartorius *et al.*, 2001). These death receptors detect the presence of extracellular death signals and rapidly activate the cell's intrinsic apoptosis machinery. This biochemical process is regulated by a group of protein-cutting enzymes, termed Caspases, which break up the cell into fragments that can be disposed of, allowing cell division and renewal of cells to take place. Caspases act at two levels in carrying out cell death; the initial caspases (initiators) are activated in response to signals indicating that the cell has been damaged. The initiators in turn activate another family of caspases, the effectors, which go on to make selected cuts in key proteins that then break up the cell. In mammals, there are two major pathways for caspase activation. One is engaged by tumour necrosis factor receptor-family members such as Fas, also known as CD-95 (Scaffidi *et al.*, 1998) and that involves recruitment to the receptor and activation of proximal caspases, particularly caspase-8. Bringing together three or more Fas receptors on the surface of a cell by FasL recruits the adaptor protein FADD (Fas-associated death domain) (see Figure 1.4.1). This occurs through interaction between the death domains of FADD and Fas.

The death-effector domain of FADD in turn recruits procaspase-8, which is cleaved to generate active caspase-8. This complex is called the DISC (death-inducing signal complex). Caspase-8 activates executioner caspases (such as caspases-3 and -7), which cleave substrates within the cell. Nucleases are then activated, chromosomal DNA is degraded and the cell dies by apoptosis. Another protein that contains a death-effector domain is RIP, which can bind to FADD and generates other types of signal, including one that results in caspase-independent cell death. Signaling through Fas can be inhibited by the action of c-FLIP, which resembles procaspase-8 but has no active site for the protease. The full-length form of c-FLIP probably also provides signals of other types to the cell. RIP, receptor interacting protein; FLIP, FLICE (FADD-like IL-1 converting enzyme)-inhibitory protein (see Figure 1.4.1)

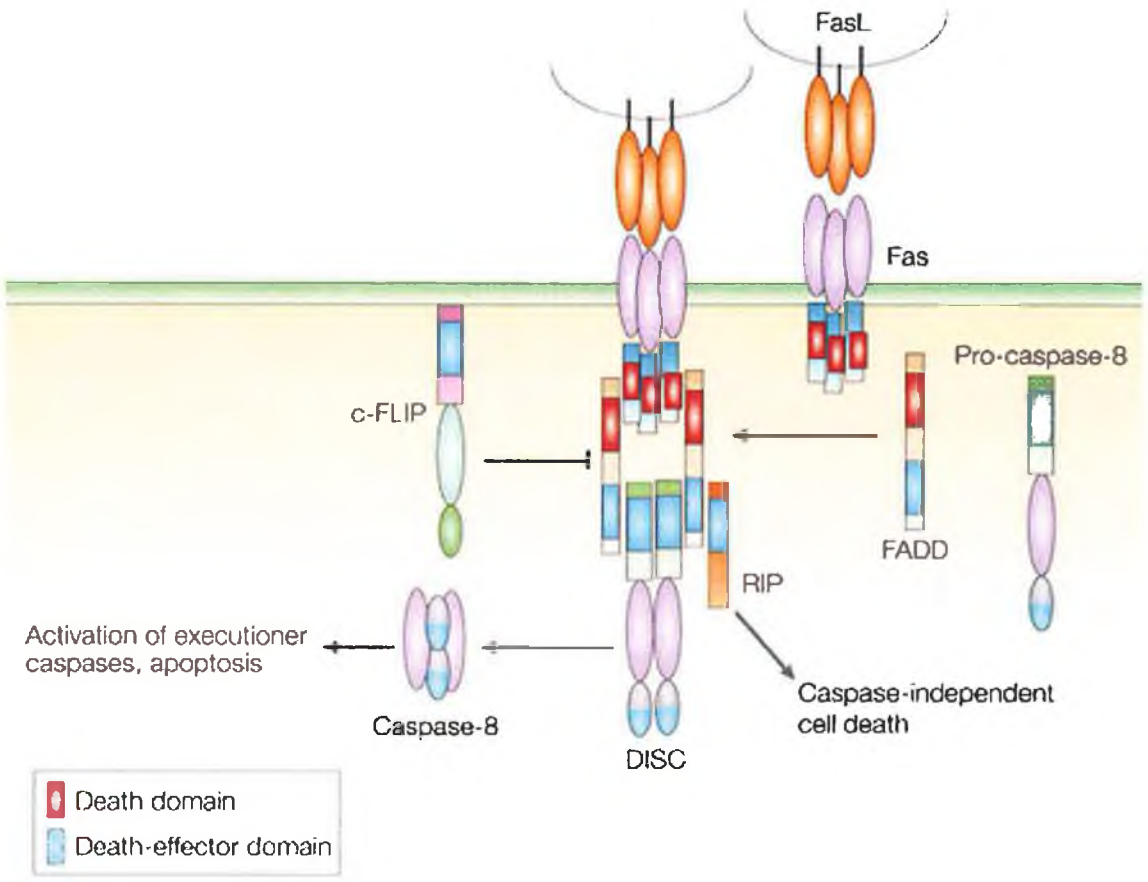


Figure 1.4.1 Proposed pathway of Fas/FasL-mediated death. (*Nature Reviews Molecular Cell Biology* **2**; 917-924 (2001)).

The second pathway involves the release of cytochrome *c* from the mitochondria, resulting in binding of cytochrome *c* to the apoptotic protease factor, Apaf-1, in the cytoplasm, and the activation of pro-caspase-9. Both of these pathways converge on downstream effector caspases (Nicholson *et al.*, 1999). Cytochrome *c* is an essential component of the mitochondrial respiratory chain, and it is normally located in the inner mitochondrial membrane. It has been shown by Kluck *et al.* (1997) that cytochrome *c* is released into the cytosol during the early stages of apoptosis, thus supporting a central role for cytochrome *c* in the induction of apoptosis (Ashkenazi *et al.*, 1998). Apoptosis is not, however, always triggered by receptors receiving specific signals from outside the cell. Cells can commit suicide, for example, if they are deprived of growth factors or if they are damaged by radiation (Barinaga *et al.*, 1998) or chemotherapy agents (Hickmann *et al.*, 1992; Drive *et al.*, 1992).

The nematode *Caenorhabditis elegans* has been a good model organism for studying the core components of the cell death machinery. Three *C. elegans* gene products are essential for apoptosis: CED-3 and CED-4 promote apoptosis, whereas CED-9 inhibits apoptosis (Smith *et al.*, 1975). CED-3, a caspase, is a cysteine protease that cleaves certain proteins after specific aspartic acid residues; it exists as a zymogen, which is activated through self-cleavage. Pro-CED-4 binds to Pro-CED-3 and promotes CED-3 activation, whereas anti-CED-9 binds to CED-4 and prevents it from activating CED-3. Normally, CED-9 is complexed with CED-4 and CED-3, keeping CED-3 inactive. Apoptosis stimuli cause CED-9 dissociation, allowing CED-3 activation and thereby committing the cell to die by apoptosis. Vertebrates have gene families that resemble *C. elegans* cell death genes. Mammalian caspases are similar to CED-3 (Keller *et al.*, 1985). The products of the mammalian Bcl-2 gene family are related to CED-9 but include two subgroups of proteins that either inhibit or promote apoptosis (Ingo *et al.*, 1998).

1.4.1 Chemotherapy and Cell Death

As discussed in section 1.2, MDR in cancer is associated with an over-expression of ATP-binding cassette proteins (P-gp and MRP) and it may be intrinsic or acquired (induced by certain chemotherapy drugs). Many chemotherapeutic agents also induce apoptosis or programmed cell death in cancer cells (Hickmann *et al.*, 1992 and Drive *et al.*, 1992). MDR is associated with resistance to apoptosis induced by chemotherapy agents (Reed *et al.*, 1995). There is a strong correlation between the expression of genes regulating apoptosis and those conferring MDR in cancer cells (Kim *et al.*, 1997). Kim *et al.* (1997) reported that MDR HL60 cells over-expressing MRP or P-gp, expressed reduced levels of apoptosis promoters including Fas, Bcl-xs and Bax, and an increased level of apoptosis repressor, Bcl-xl, compared to the parental, drug-sensitive cell line.

Cisplatin and VP-16 were used to study the induction of apoptosis in Panc-1 cells and the effect of these drugs on the expression level of Bax (a pro-apoptotic gene) and Bcl-2 (an anti-apoptotic gene) was determined (Lee *et al.*, 1997). The group found up-regulation of Bax expression, but no change in the expression of Bcl-2 in the cells. Cisplatin exposure also caused prolonged cell cycle arrest, accompanied by induction of the p21 gene. Cisplatin was observed to induce expression of the ERCC-1 gene in human ovarian A2780 cells. ERCC-1 is an important gene in nucleotide excision repair. The capability of vincristine and adriamycin to induce apoptosis was studied in murine T cell leukemia cell lines. Resistance to these two drug resulted in a decrease in apoptosis, while cyclosporin-A and its analogue PSC 833, which are P-gp modulators (and, as discussed in section 1.2.1.1, are immunosuppressive agents) that inhibit P-gp, increased sensitivity of MDR cells to anticancer agents, as well as triggering apoptosis in these cell lines. Failure to induce apoptosis is a recognized pathway for multiple drug resistance that may explain some aspects of cancer treatment failure (Lopes *et al.*, 2003).

Further knowledge of apoptosis-regulating genes and their role in multiple drug resistance may greatly improve chemotherapy treatment, if the correct approach to targeting these genes can be achieved. (For a more comprehensive discussion on drug resistance and apoptosis see review paper in Clynes *et al.*, 1998).

1.5 Galectin-3

Animal lectins are proteins involved in cell-cell interactions and cell-extracellular matrix interactions. These events are very important during invasion and metastasis as discussed in section 1.3. Galectins are a family of galactose-specific lectins (Barondes *et al.*, 1994). To date, 14 mammalian galectins have been identified (Liu *et al.*, 2002). They are galactoside-binding proteins that play an important role in tumour progression by promoting cancer cell invasion and metastasis (Akahani *et al.*, 1997; Berberat *et al.*, 2001; Takenaka *et al.*, 2003). Galectin-1 and Galectin-3 are the most widely studied members of the galectin family, with molecular weights of 14.5 kDa and approx. 30 kDa, respectively (Xu *et al.*, 1995).

Galectin-3 has been reported to bind laminin, a glycoprotein involved in development and tumour invasion and metastasis (Hughes *et al.*, 1994) and its expression has been noted in many cell types and tissues (Barondes *et al.*, 1994b) as discussed in section 1.5.3. Galectin-3 has a number of intracellular activities, including: regulation of the cell cycle through G1 or G2/M arrest; regulation of cell growth through an anti-apoptotic effect, i.e. protects cells from a variety of death signals, including Fas receptor cross-linking and loss of cell anchorage; nuclear splicing of pre-mRNA (Liu *et al.*, 2002) and cell adhesion and migration. There is also evidence of galectin-3 being involved in neoplastic progression and cancer metastasis (Bresalier *et al.*, 1997; Nangia-Makker *et al.*, 1998; Song *et al.*, 2002). The role of galectin-3 in apoptosis and invasion/metastasis will be discussed in more detail in sections 1.5.2 and 1.5.3, respectively.

1.5.1 Intracellular localisation of Galectin-3

A number of studies have documented galectin-3 localisation in both the nucleus and the cytoplasm of various cell types (Wang *et al.*, 1995; Hubert *et al.*, 1995; Craig *et al.*, 1995). The localisation of galectin-3 in fibroblasts was found to be in both the nucleus and the cytoplasm. The distribution of galectin-3 protein was dependent on the proliferation state of the cells under analysis. Galectin-3 was expressed in the cytoplasm of fibroblasts in their quiescent state. However, when the cells were proliferating, galectin-3 was highly expressed in the nucleus of the cells (Moutsatsos *et al.*, 1987). In colonic epithelia, the progression from normal mucosa, to adenoma, to carcinoma is characterised by down-regulation of galectin-3 and a shift of expression from the nuclei to the cytoplasm. Sanjuan *et al.* (1997) found strong nuclear expression of galectin-3 in normal colorectal mucosa, which was down-regulated in neoplastic progression. In later phases of tumour progression, galectin-3 shows increased cytoplasmic expression in colorectal cancer (Sanjuan *et al.*, 1997). Similar findings were previously documented by Lotz *et al.* (Lotz *et al.*, 1993), while galectin-3 was highly expressed in the nucleus of cancerous thyroid cells (Paron *et al.*, 2003).

1.5.2 Role of Galectin-3 in Apoptosis

Several researchers have demonstrated the involvement of galectin-3 in cell growth and proliferation, suggesting a role for galectin-3 in inhibiting apoptosis and promoting cell growth. Transfection of the human T lymphoma Jurkat cells with galectin-3 caused an increase in growth rate of these cells compared to the control cells, which do not express galectin-3. The cells were also more resistance to apoptosis induced by anti-Fas receptor antibody (which cross-links Fas receptor causing apoptosis) and by staurosporine (a protein kinase inhibitor) (Yang *et al.*, 1996). Similarly, transfection of a human breast carcinoma cell line, Evsa-T, with galectin-3 cDNA, caused the cells to be more resistant when exposed to apoptotic stimuli such as cycloheximide/TNF-α and UVB irradiation (Mataresse *et al.*, 2000). Galectin-3 expression in another breast carcinoma cell lines, BT549, also resulted in resistance to cisplatin-induced apoptosis (Akahani *et al.*, 1997).

Studies of galectin-3 over-expression in the human breast carcinoma cell line, BT549, have been carried out by Kim *et al.* (1999). They found that galectin-3 protects the cells against apoptosis induced by anoikis (the loss of cell anchorage). In this study, wild-type BT549 cells readily underwent anoikis, whereas transfectants over-expressing galectin-3 responded to the loss of cell adhesion by cell cycle arrest without detectable apoptosis (Kim *et al.*, 1999). Similarly, the transfectants underwent cell cycle arrest without cell death when treated with genistein, an isoflavonoid compound found to inhibit protein tyrosine kinase and other critical enzymes involved in signal transduction and induce apoptosis. Parental cells underwent apoptosis in response to genistein without detectable cell cycle arrest (Lin *et al.*, 2000). Further studies on this breast carcinoma cell line, demonstrate that galectin-3 over-expression protects these cells from nitric oxide-induced cytotoxicity during liver ischemia (Moon *et al.*, 2001; Lee *et al.*, 2002). Galectin-3 inhibited apoptosis by protecting mitochondrial integrity, inhibition of cytochrome *c* release and caspase activation (Moon *et al.*, 2001).

Further evidence for a role of galectin-3 in cell growth and apoptosis is demonstrated through the use of antisense oligonucleotides to suppress galecin-3 expression. Galectin-3 antisense was transfected into the breast carcinoma cell lines, MDA-MB435, and resulted in decreased cell proliferation compared to parental cells (van den Brule *et al.*, 1997; Honjo *et al.*, 2001), proving that when the anti-apoptotic activity of galectin-3 is blocked, cell growth is greatly reduced, due to spontaneous apoptosis. RNA interference experiments have recently been carried out pituitary tumour cells, causing an inhibition of galectin-3 expression, thereby resulting in a decrease of cell proliferation and an increase of apoptosis, suggesting that galectin-3 plays a role in cell proliferation and tumour progression of pituitary tumours (Riss *et al.*, 2003).

1.5.2.1 Mechanism of galectin-3's role in apoptosis

The mechanism of action of galectin-3 in inhibiting apoptosis may be linked to the anti-apoptotic gene, Bcl-2 (Akahani *et al.*, 1997). The reason for this may lie in the fact that galectin-3 shares significant sequence similarity with Bcl-2. Both proteins are rich in proline, glycine and alanine in the N-terminal region and contain an NWGR quartet in the

C-terminal region. The NWGR quartet is found in the BH1 domain of Bcl-2 and has been shown to critical for anti-apoptotic function of Bcl-2 (Reed *et al.*, 1998). This sequence is highly conserved among galectin-3 from different animal species and is essential for the carbohydrate-binding activity of this protein. Substitution of glycine to alanine in this motif abrogates its anti-apoptotic activity (Akahani *et al.*, 1997). In addition, similar to other Bcl-2 family members, which form heterodimers with Bcl-2, galectin-3 binds Bcl-2 *in vitro* (Yang *et al.*, 1996).

Another possible mechanism of action for anti-apoptotic activity of galectin-3 may be linked to the JNK (c-jun N-terminal kinase) pathway. Studies of cells from galectin-3 deficient mice have provided further evidence of the anti-apoptotic function of galectin-3 (Hsu *et al.*, 2000). Bone marrow-derived mast cells from galectin-3-deficient mice were found to be more susceptible to apoptosis than those of wild-type mice when grown in the absence of growth factors. These cells were also defective in the expression of JNK, which is one of the mitogen-activated kinases (MAPK). Since JNK is a regulator of apoptosis, it is possible that galectin-3 exerts its anti-apoptotic activity by regulating the level of JNK. However, this mechanism is not applicable to all cell types, as other cell types in galectin-3-deficient mice are not defective in JNK expression (Liu *et al.*, 2002).

The proposed mechanisms of action for the anti-apoptotic role of galectin-3 would suggest that galectin-3 regulates cell survival through intracellular processes. However, extracellular process may also be involved. It is possible that galectin-3 exerts its anti-apoptotic activity through its adhesion mechanisms, since increased cell adhesion can protect cells from apoptosis (as mentioned in section 1.5.2). The role of galectin-3 in adhesion, invasion and metastasis will be discussed in section 1.5.3. Further investigation into the molecular mechanisms by which galectin-3 regulates intrinsic and extrinsic apoptosis is needed for a better understanding of the role of this protein in apoptosis inhibition.

1.5.3 Role of Galectin-3 in Invasion and Metastasis

Adhesion and migration of cells are mediated by the interaction between cells and extracellular matrix (ECM) glycoproteins and they are involved in inflammation and metastasis of cancer cells. Galectins are found on the cell surface and within the extracellular matrix (Perillo *et al.*, 1998) as well as in the cytoplasm and the nucleus of cells (as discussed earlier). Galectins bind specifically to oligosaccharide ligands, which can be found on a number of glycoproteins. However, despite the specific binding of galectins to these glycoproteins, controversies still remain as to whether galectin-3 expression facilitates or inhibits cell adhesion. Laminin, an ECM protein, has been shown to be a receptor for galectin-3. Galectin-3 has been shown to mediate cell-to-cell and cell-to-matrix adhesion; however, it doesn't behave consistently as an adhesion molecule. Galectin-3 mediates the adhesion of neutrophils (Kuwabara *et al.*, 1996), but not melanoma cells (van den Brule *et al.*, 1995), to laminin (Perillo *et al.*, 1998).

Recently, there is increasing evidence that galectin-3 is in fact involved in invasion and metastasis of cancer cells *in vitro*. This has been demonstrated in non-small-cell lung cancer cell line, PC9 (Yoshimura *et al.*, 2003), squamous cell lung cancer cell line, DLKP (O'Driscoll *et al.*, 2002), breast carcinoma cell line, MDA-MB-435 (Khaldoyanidi *et al.*, 2003) and BT549 (Song *et al.*, 2002), colon cancer cell lines, HM7 and LS174T (Bresalier *et al.*, 1998), thyroid follicular cell line, TAD-2 (Takenaka *et al.*, 2003) and metastatic liver adenomcarcinoma cell lines, XK4-A3 and RPMI4788 (Inufusa *et al.*, 2001).

Down-regulation of galectin-3 using antisense methods resulted in marked decrease in the metastatic potential of a highly metastatic colon cell line, HM7, while the up-regulation of galectin-3 enhanced the metastatic potential of a low metastatic colon cell line, LS174T (Bresalier *et al.*, 1998). In a separate study, human breast cancer cell line, BT549, was transfected with galectin-3 cDNA and injected into the spleen of nude mice. The mice developed tumours in both the spleen and the liver, indicating that galectin-3 can enhance the metastatic potential of BT549 cells (Song *et al.*, 2002). Furthermore, the over-expression of galectin-3 also confers a malignant phenotype on TAD-2 thyroid follicular cells (Takenaka *et al.*, 2003). TAD-2 cells transfected with galectin-3 showed anchorage-

independent growth and loss of contact inhibition, suggesting that galectin-3 plays an important role in malignant transformation in thyroid follicular cells (Takenaka *et al.*, 2003).

Stable transfection of galectin-3 cDNA into the lung cancer cell line, DLKP, resulting in galectin-3 over-expression caused an increase in invasion, motility and adhesion to ECM, fibronectin and laminin, compared to the DLKP parent cell line (O'Driscoll *et al.*, 2002) (as discussed in section 4.1.4). A similar study was carried out on the breast carcinoma cell line, BT549, where it was found that galectin-3 over-expressing clones were more adherent to laminin, collagen IV and fibronectin. In addition, they were also able to invade through matrigel-coated filters at approx. three times the rate of parental cells. Galectin-3 was found to be critical for adhesion to laminin and collagen IV, but not to fibronectin by breast carcinoma cells (Warfield *et al.*, 1997). Galectin-3 was also found to play a role in the interactions between breast carcinoma cells and elastin (Ochieng *et al.*, 1999).

1.5.4 Galectin-3 Expression

There have been many conflicting reports regarding the expression of galectin-3 in tumour and normal cells and tissues. Some groups have reported galectin-3 down-regulation in cancer (van den Brule *et al.*, 1994; Castronovo *et al.*, 1996; Idikio *et al.*, 1998; Mollenhauer *et al.*, 2003), compared to normal tissue, which correlates with disease progression and metastasis, while others have reported galectin-3 up-regulation in cancer compared to normal tissue (Xu *et al.*, 1995; Schaffert *et al.*, 1998; Berberat *et al.*, 2001; Riss *et al.*, 2003), especially in metastatic and/or advanced cancer, which, in some cases, correlated with poor prognosis (Lotan *et al.*, 1994; Fernandez *et al.*, 1997; Strik *et al.*, 2001; Miyazaki *et al.*, 2002). (There is increasing evidence for the latter scenario).

Galectin-3 expression has been shown to be down-regulated in colon (Castronovo *et al.*, 1992; Lotz *et al.*, 1993), ovary (van den Brule *et al.*, 1994), breast and endometrium carcinoma (van den Brule *et al.*, 1995). This down-regulation seems to be associated with tumour progression. Castronovo *et al.* (1996) found high levels of galectin-3 mRNA and protein expression in normal breast tissue and in benign lesions. The expression of galectin-

3 was dramatically decreased in *in situ* carcinoma and further down-regulation was observed in invasive ductal carcinoma. It is speculated that this down-regulation could enable cancer cells to interact with laminin, thereby facilitating invasion and metastasis (Castronova *et al.*, 1996). Down-regulation of galectin-3 was found to be a prognostic marker for poor outcome in laryngeal squamous-cell carcinoma patients (Piantelli *et al.*, 2002).

In contrast, galectin-3 expression has been found in thyroid tumours, but not normal thyroid tissue or benign thyroid adenomas (Xu *et al.*, 1995), suggesting a role for galectin-3 in thyroid cancer. In thyroid follicular cells, TAD-2, galectin-3 cDNA transfection induced a malignant phenotype (Takenaka *et al.*, 2003). Galectin-3 expression has been found in normal human monocytes, which increases dramatically as the monocytes differentiate into macrophages *in vitro* (Liu *et al.*, 1995). Nakamura *et al.* (1999) reported galectin-3 expression in normal and tumour colorectal mucosa. Expression of galectin-3 was at higher levels in primary lesions of cancer than in normal mucosa. Furthermore, galectin-3 expression increased dramatically in liver metastatic lesions and correlated with progression of disease, liver metastasis and poor prognosis (Nakamura *et al.*, 1999). In gastric cancer, galectin-3 expression correlated significantly with tumour progression and was dramatically stronger in metastatic lymph nodes than in primary gastric cancers (Miyazaki *et al.*, 2002). In pancreatic cancer, galectin-3 mRNA and protein was predominately expressed in cancer cells, with very weak expression in normal tissue. Galectin-3 expression was strongest in metastatic pancreatic cancer cells (Berberat *et al.*, 2001) (see Table 1.5 for summary of galectin-3 expression in human cancer and its correlation with prognosis).

Galectin-3 expression has also been demonstrated in a large number of cancer cell lines, including breast (Ochieng *et al.*, 1999; Honjo *et al.*, 2001; Lahm *et al.*, 2001; Khaldoyanidi *et al.*, 2003), colorectal (Huflejt *et al.*, 1997; Lahm *et al.*, 2001), lung (Lahm *et al.*, 2001; O'Driscoll *et al.*, 2002; Yoshimura *et al.*, 2003), brain (Lahm *et al.*, 2001), prostate (Lahm *et al.*, 2001), pancreatic (Schaffert *et al.*, 1998), nasal (O'Driscoll *et al.*, 2002) and ovarian carcinomas (Lahm *et al.*, 2001), as well as melanoma (Lahm *et al.*, 2001) and leukemia cell lines (Lahm *et al.*, 2001). Further analysis of galectin-3 expression in invasive carcinomas is needed for a better understanding of this gene.

Table 1.5 Summary of Galectin-3 Expression in different types of human cancers and its relationship to prognosis.

Cancer Type	Technique	(%)	Prognosis& Correlation	Localisation	Reference
Larynx	IHC	57.5	Correlated w/ Good prognosis	N/A	Piantelli *et al.*, 2002
Breast	IHC	69	Inversely Correlated w/ progression	N/A	Castronovo *et al.*, 1996
Colorectal	IHC	68	Correlation w/progression & poor prognosis	Cytoplasmic	Nakamura *et al.*, 1999
	IHC	59C 49N	Inversely Correlated w/ progression	Cytoplasmic &Nuclear	Sanjuan *et al.*, 1997
Pancreas	IHC	95	N/A	Ductal cells	Scharffert *et al.*, 1998
	IHC	N/A	No correlation	Ductal cells	Berberat *et al.*, 2001
	NB	77	N/A	N/A	
Gastric	IHC	84	Correlation w/ Tumour progression	Nuclear	Miyazaki *et al.*, 2002
Thyroid	IHC	100	Correlation w/ malignant transformation	Cytoplasmic	Xu *et al.*, 1995
	WB	100			

IHC=Immunohistochemistry; WB=Western blot; N/A= Not applicable; C=Cytoplasmic; N=Nuclear.

1.6 The Inhibitors of Apoptosis (IAP) family

Inhibitors of apoptosis proteins (IAPs) are an evolutionary conserved family of proteins that interfere with the process of cell death by inhibiting caspases. IAPs were discovered in baculovirus by Miller *et al.* in 1993. These researchers found that viruses deploy such proteins to keep host cells alive while the viruses replicate and spread. In 1994, Alex Mac Kenzie *et al.* reported the first cellular IAP, a protein that inhibits apoptosis in nerve cells, namely NIAP. Since then, researchers have reported a number of IAPs in mammalian cells alone. The identifying signature of these molecules is the presence of at least one approximately 70 amino acid zinc finger module, designated baculovirus IAP repeat (BIR). Several groups have shown that IAPs, including XIAP, NIAP, $cIAP_1$, $cIAP_2$, Livin and Survivin (see figure 1), bind to caspases and block their activity. It is the BIR region that, in several mammalian IAPs, binds initiator and effector caspases, thus suppressing the enzyme's activity and /or interfering with its activation (Deveraux *et al.*, 1999). However, like Bcl proteins, IAPs may function in more than one way. In studies investigating inhibitors of apoptosis, Miller *et al.* (1999) found that IAPs apparently can arrest apoptosis before the caspases are involved.

1.6.1 The BIR motif

BIR (baculovirus IAP repeat) was identified originally as a sequence of approximately 70 amino acids that was tandemly repeated in a class of proteins blocking apoptosis-induced during baculovirus infection (Birrnbaum *et al.*, 1994). At least one BIR motif is essential for anti-apoptotic activity of members of the IAP family. Some IAPs also contain a C-terminal RING (C_3HC_4) finger, but the requirement of this RING finger for anti-apoptotic activity differs depending on the IAP and /or the nature of the apoptotic stimulus (Cleu *et al.*, 1994, Takahash *et al.*, 1998, Vucic *et al.*, 1997; Hay *et al.*, 1995). Structure analysis shows that a BIR folds into a structure with a highly hydrophobic center that includes a C_2HC motif coordinating a zinc ion. This core structure includes a number of highly conserved residues, which are required for anti-apoptotic activity (Li *et al.*, 1998). As a number of IAPs interact with caspases through regions encompassing BIRs, it is often

assumed that BIRs are caspase-interacting regions. Why there are multiple BIR domains in some IAPs and BIRPs (BIR proteins) is unknown, although both BIRs are required for full anti-apoptotic activity of Op-IAP (*Orgyia pseudotsugata*). However, minimal anti-apoptotic activity is associated with the C-terminal BIR motif of Op-IAP and the middle BIR (BIR2) of XIAP, and survivin has only a single BIR (Figure 1.6.1).

1.6.2 Function of IAPs

Recombinant purified mammalian IAPs, XIAP, cIAP1 and cIAP2 directly inhibit the terminal effector proteases, caspase-3 and –7, providing evidence for the specific mechanism of action for these mammalian cell-death suppressors (Deveraux *et al.*, 1997 and Roy *et al.*, 1997). Not all BIR-containing proteins (BIRPs) are anti-apoptotic. Recent genetic analysis of *C. elegans* BIR-1 demonstrated an essential role in cytokinesis instead of apoptosis (Fraser *et al.*, 1999). In yeasts containing no caspases, BIRPs are found whose gene knockouts result in the defects in meiosis and mitosis (Uren *et al.*, 1999). These results suggest that BIRs have evolved to acquire divergent biological roles (Shin *et al.*, 2001).

1.6.2.1 Mammalian IAPs as Caspase Inhibitors

Eight IAP-family members have been identified to date including NIAP (neuronal apoptosis inhibitor), cIAP1 (cellular IAP 1), cIAP2, XIAP (X Chromosome-linked inhibitor of apoptosis), Livin, Bruce, apollon and Survivin (see Figure 1.6.1). The spectrum of apoptosis stimuli that are blocked by these IAPs is broad and includes anti-fas antibody (Duckett *et al.*, 1996), TNFα (tumour necrosis factor), viral infection (Duckett *et al.* 1996), chemotherapy agents, exposure to UV radiation (Duckett *et al.*, 1998), serum withdrawal (Liston *et al.*, 1996), and over-expression of caspase family proteins (Duckett *et al.*, 1996; Uren *et al.*, 1996), with XIAP having the broadest and strongest anti-apoptotic activity. XIAP, cIAP1 and cIAP2 have been shown to bind to specific cell death proteases, i.e. caspases-3 and 7 and procaspase-9 and to inhibit their proteolytic activity *in vitro* (Deveraux *et al.*, 1997; Roy *et al.*, 1997). XIAP contains three BIR domains. BIR2 and the

amino acids immediately N-terminal to BIR2 interact with caspase-3, while the BIR3 domain binds capase-9 thereby preventing activation of the intrinsic apoptotic cascade (Takahashi *et al.*, 1998; Silke *et al.*, 2001). As will be discussed later (see section 1.6.8), survivin has been proposed to interact physically with caspase-3 and -7 (Conway *et al.*, 2000; Shin *et al.*, 2001; Suzuki *et al.*, 2001).

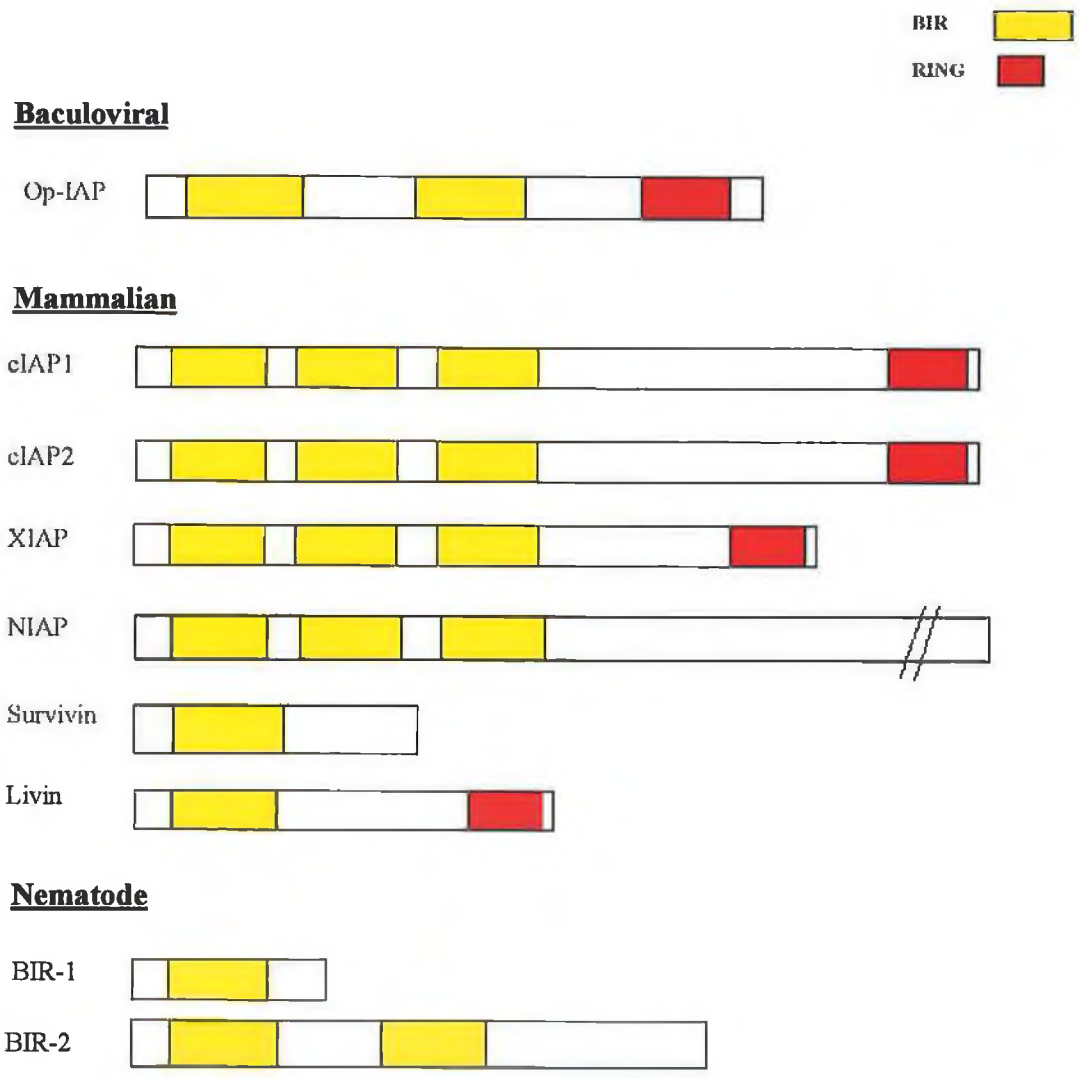


Figure 1.6.1 Schematic representation of selected baculovirus IAP repeat (BIR) containing proteins. BIR motifs (yellow boxes) occupy the N-terminal portion of the proteins. RING finger domain (red boxes) are present at some C-termini.

1.6.3 Survivin

Survivin is the smallest IAP cloned to date, consisting of only 142 amino acids. It is a 16.5 kDa protein, containing only a single N-terminal BIR domain and it lacks a C-terminal RING finger (Ambrosini *et al.*, 1997). Survivin was originally identified by hybridisation screening of human genome libraries with the cDNA of a factor Xa receptor, Effector Protease Receptor 1 (EPR1) (Ambrosini *et al.*, 1997), a protein involved in blood coagulation (Zaman and Conway, 2000). Although survivin has generally been described as a cytoplasmic protein (Ambrosini *et al.*, 1997; Kawasaki *et al.*, 1998; Lu *et al.*, 1998; Adida *et al.*, 1998; Tanaka *et al.*, 2000; Adida *et al.*, 2000; Muzio *et al.*, 2001; Satoh *et al.*, 2001; Sasaki *et al.*, 2002; Adida *et al.* 2002; Trieb *et al.*, 2003), nuclear localisation has also been reported (Ito *et al.*, 2000; Okada *et al.*, 2001; Trieb *et al.*, 2003; Kennedy *et al.*, 2003).

Survivin is expressed during fetal development and at low levels in normal, adult differentiated, tissue (Ambrosini *et al.* 1997; Adida *et al.*, 1998a). Adida *et al.* (1998) reported strong expression of survivin in several apoptosis-regulated fetal tissues, including the stem cell layer of stratified epithelia, endocrine pancreas, and thymic medulla; exhibiting a pattern that did not overlap with that of Bcl-2. Survivin was also detected in human fetal lung, liver heart, kidney and gastrointestinal tract (Adida *et al.*, 1998). This suggests that survivin expression in embryonic and fetal development may contribute to tissue homeostasis and differentiation, independently of Bcl-2.

Interestingly, survivin becomes prominently expressed in all of the most common human cancers, including breast (Tanaka *et al.*, 2000; Nasu *et al.*, 2002; Kennedy *et al.*, 2003), bladder (Swana *et al.*, 1999), blood (Moriai *et al.*, 2001; Adida *et al.*, 2000), colon (Sarela *et al.*, 2000; Kawasaki *et al.*, 1998; 2001; Lin *et al.*, 2003), esophageal (Kato *et al.*, 2001; Ikeguchi *et al.*, 2003), liver (Ito *et al.*, 2000), brain (Nakagawara *et al.*, 1998; Islam *et al.*, 2000), lung (Monzo *et al.*, 1999; Choi *et al.*, 2001), pancreas (Satoh *et al.*, 2001), Gastric (Lu *et al.*, 1998; Okada *et al.*, 2001; Ikeguchi; Kaibara 2001; Krieg *et al.*, 2002; Kania *et al.*, 2003), ovarian (Hattori *et al.*, 2001; Chen *et al.*, 2003) prostate (Xing *et al.*, 2001),

kidney (Takamizawa *et al.*, 2001) and high-grade lymphomas but not low-grade lymphomas (Ambrosini *et al.*, 1997; Adida *et al.*, 1998a; Lu *et al.*, 1998).

Survivin is predominantly expressed in cancer, and has been associated with cancer prognosis and diagnosis (see Table 1.6), making it a very interesting drug target as will be discussed in section 1.6.8. Recently, however, survivin was detected in several normal tissues, such as bone marrow (Fukuda *et al.*, 2001), endometrium (Takai *et al.*, 2002), thymus (Kobayashi *et al.*, 2002) and colonic mucosa (Gianani *et al.*, 2001). In a study by Shiozaki *et al.* (2003), survivin mRNA and protein was expressed in cytotrophoblasts of normal human placenta (Shiozaki *et al.*, 2003). Survivin mRNA is expressed at low levels in human liver, with up-regulation in liver tissue of hepatitis or cirrhosis under the stress of inflammatory cytokines or regeneration (Shiraki *et al.*, 2003). Survivin is also expressed at a high basal level in normal human (and rat) gastric mucosa (Chiou *et al.*, 2003). The gastric mucosa undergoes continuous surface epithelial cell renewal with a turnover rate of 3-5 days. The results by Chiou *et al.* suggest that survivin expression may be important for regulating this process and may help to promote mucosal surface epithelial cell viability and regeneration after ulceration or injury by damaging agents, such as alcohol and NSAIDS (Chiou *et al.*, 2003). In addition, survivin is reported to be a crucial regulator of normal smooth muscle cells apoptosis after acute vascular injury (Blanc-Brude *et al.*, 2002). Taken together, these findings suggest that survivin plays an important role in controlling cell proliferation, differentiation, and death in human non-transformed cells, as well as malignant cells.

1.6.4 Discovery of Survivin

First discovered when screening a human genomic library with EPR-1 (see section 1.6.3), the survivin gene has a coding strand that is highly homologous to the sequence of EPR-1, but oriented in the opposite direction (Ambrosini et al., 1997). This suggests the existence of two separate genes duplicated in a head-to-head configuration with potential functional interaction between these two transcripts (Ambrosini *et al.*, 1997).

Ambrosini *et al.* (1998) reported successful down-regulation of survivin by transiently transfecting an EPR-1 cDNA into tumour cells, resulting in an increase in apoptosis and inhibited growth of transformed cells. Further studies included a stable transfection of Hela cells with an EPR-1 cDNA under the control of a metallothionein-inducible promoter. It was found that $ZnSO_4$ induction of EPR-1 mRNA suppressed the expression of endogenous survivin. Furthermore, antisense down-regulation of survivin resulted in massive apoptosis (detected by TUNEL assay) in growth factor-deprived Hela cells (Ambrosini *et al.*, 1998). Suppression of Survivin by an endogenous EPR-1 transcript potentially acting as a natural antisense may overcome the drawbacks of limited specificity and insufficient delivery commonly observed with antisense oligonucleotides (Henry *et al.*, 1997). Elucidation of the mechanisms regulating survivin and EPR-1 gene expression should further facilitate the selective disruption of this novel anti-apoptosis pathway in cancer without affecting viability of normal tissue.

Shinozawa *et al.* (2000) reported an investigation of the mutual expression of survivin/EPR-1 genes in a variety of hematological malignancies. Results showed that expression of survivin/EPR-1 genes occur in many types of hematologically malignant cells and that coordination of the EPR-1 and survivin gene expression may be independent (Shinozawa *et al.*, 2000), leaving a doubt as to whether EPR-1 mRNA can down-regulate survivin expression by acting as a natural antisense transcript.

1.6.5 Survivin Structure

As the interest in survivin's function and expression in human tissue develops, increasing efforts have been invested in elucidating its crystal structure. Both human and mouse survivin crystal structures have been described by three independent research groups (Muchmore *et al.*, 2000; Chantalat *et al.*, 2000; Verdecia *et al.*, 2000). The N-terminal Zn^{2+}- binding BIR domain of survivin consists of a three-stranded anti-parallel β-sheets surrounded by four small α-helices (Muchmore *et al.*, 2000, Chantalat *et al.*, 2000, Verdecia *et al.*, 2000). There have been some discrepancies between the identity of the dimeric interface. Chantalat and Verdecia's groups report a bow-tie model with a predominantly hydrophobic interface on the BIR domain of each survivin monomer. On the other hand, Muchmore's group reports a zinc chelation model whereby a zinc atom was observed to mediate the dimeric interface. Despite the discrepancies, however, the three structures reveal important insights on survivin and provide a molecular framework to help design future experiments to clarify survivin's biological functions (Shi *et al.*, 2000). Contrary to Ambrosini's coiled-coil model of the C-terminal (Ambrosini *et al.*, 1997), it was found that the c-terminal domain of survivin forms a long, extended helix (α6) and is not involved in the dimeric formation; elimination of this interface by deleting the bulk of α6, did not affect the formation of a survivin homodimer in solution (Verdecia *et al.*, 2000; Chantalat *et al.*, 2000).

The survivin structures reveal three chemically distinct surfaces, the first comprising many acidic residues, the second containing basic residues, and the third rich in hydrophobic residues on helix α6. Expression of a survivin mutated form of the negatively charged residues (Asp 71A) caused spontaneous apoptosis in Hela cells (Muchmore *et al.*, 2000), consistent with the functional significance of this region. Survivin associates with and is phosphorylated on Thr^{34} by, $p34^{cdc2}$-cyclin. This modification is required for apoptosis inhibition (Fortugno *et al.*, 2002; O'Connor *et al.*, 2000). A Thr 34A mutation, which results in the expression of a phosphorylation-defective survivin mutant, caused spontaneous apoptosis, are seen by flow cytometry, preventing T^{34} phosphorylation. Interestingly, two mutations, H 80A and E 76A, both affecting the reported zinc-chelating

residues, also led to spontaneous apoptosis in the cells. In contrast, other mutations exhibited less (E51A) or no (L64A) effect on cell survival (Muchmore *et al.*, 2000). These data suggest that the negatively charged surface may be important for interactions with other proteins (Shi *et al.*, 2000). The determination of the three-dimensional structure of survivin and the elucidation of structural requirements of the apoptosis/cell division control pathway maintained by survivin in cancer cells should facilitate the identification of selective antagonists of this cytoprotective pathway in cancer (Ambrosini *et al.*, 1997; Shi *et al.*, 2000).

1.6.5.1 Survivin Splice Variants

While carrying out a study on survivin expression in renal cell carcinoma (RCC), Mahotka *et al.* (2000) discovered two novel alternatively processed survivin transcripts. These were designated Survivin-ΔEx3 (lacking exon 3) and survivin-2B (retaining a part of intron 2 as a cryptic exon) (Figure 1.6.3). Mahotka's group found that the expression of both survivin and survivin-ΔEx3 was observed in all RCC cell lines tested, with most RCCs additionally expressing survivin-2B. Survivin splice variants have also been reported by Fogt *et al.* (2001) in normal and tumour colon cells.

Transient transfection of HepG2 cells, revealed marked differences in the anti-apoptotic activity of the novel survivin splice variants. Transfection of HepG2 cells with survivin resulted in a large increase of cell survival after exposure to methotrexate. This was also the case when transfected with survivin-ΔEx3, although the exon 3 deletion results in a frameshift with truncation of the BIR domain in addition to a new COOH-terminal protein segment, which in turn might affect functional properties such as subcellular localisation. Retention of anti-apoptotic activity by the structurally modified survivin-ΔEx3 cannot be explained (Mahotka *et al.*, 1999). In contrast, HepG2 cells transfected with survivin-2B showed a marked reduction of cell survival after exposure to methotrexate (Mahotka *et al.*, 1999). This suggests differential anti-apoptotic properties of the novel survivin splice variants, showing a nearly complete preservation (survivin-ΔEx3) or a marked reduction (survivin-2B) of the anti-apoptotic effects known for the regulatory spliced survivin

isoforms. The identification of the two splice variants will have important implications for the understanding of survivin actions.

(a) Survivin **(142aa; 16.5 kDa)**

(b) Survivin-2B **(165aa; 18.6 kDa)**

(c) Survivin-ΔEx3 **(137aa; 15.7 kDa)**

Figure 1.6.3 The three transcripts of the Survivin gene. (a) the survivin transcript with four exons, (b) survivin-2B with an additional exon (exon 2B) inserted between exon 2 and 3, and (c) survivin-ΔEx3, showing a loss of exon 3 as well as a frame-shift, with extension of the reading frame into the open reading frame (ORF) of the 3' un-translated region (UTR). aa=amino acids; Kda =Kilo Daltons (Mahotka *et al.*, 1999).

1.6.5.2 Livin: A close relative of Survivin

A novel member of the IAP family has been identified by several groups. It has been designated ML-IAP by Vucic *et al.* (2000), livin by Kasof and Gomes (2001), and KIAP by Lin et al. (2000). (This protein will be referred to as livin in this thesis). Livin encodes a protein with a single BIR domain and a COOH-terminal RING domain and it is reported to be approximately 26% homologous to survivin. Expression of livin inhibits apoptosis, whereas a livin antisense construct has been shown to induce apoptosis. The livin antisense, however, did not reduce the levels of survivin, suggesting that the construct has a specific effect on livin and not on related IAP family members (Kasof and Gomes, 2001).

Similar to survivin, livin was found to be capable of binding to caspase-3 and –7 and it has been shown to inhibit the proteolytic processing of caspase-9 *in vitro* (Kasof and Gomes, 2001). Livin's expression, like survivin's, was undetected in many normal adult tissues, but was present in developmental tissue and several cancer cell line, with highest levels in melanoma cell lines. Livin, however, seems to have more restricted expression than survivin with a narrower distribution (i.e. fewer cancer types) than survivin in cancer (Kasof and Gomes, 2001; Vucic *et al.*, 2000).

In the study reported by Kasof and Gomes in 2001, Hela cells were transfected with apoptotic genes in the Ptracer vector containing a GFP marker for accessing viability. Transfection with pro-apoptotic genes, e.g. Bax, led to ~ 90% reduction in viability. Co-transfection of Bax with either livin or survivin caused a 4-6-fold increase in viability. It was found that livin showed more anti-apoptotic activity than survivin. Survivin has been shown previously to be less effective at inhibiting apoptosis that other IAP family members (Tamm *et al.*, 1998). Kasof *et al.* (2000) reported that livin was observed predominantly in the nucleus and in a filamentous pattern throughout the cytoplasm and that survivin was observed in the same pattern, indicating that livin and survivin have similar subcellular localisation (Kasof *et al.*, 2001). In a separate study by Ashhab *et al.* in 2001, two splice variants of livin were identified i.e. livin α and β, representing the longer and shorter variants, respectively. The group found the two isoforms to have different anti-apoptotic properties (similar to the findings of Mahotka *et al.* on the survivin variants).

Differences in expression patterns of livin α and were also reported. Livin β, but not livin α was found in fetal and adult kidney and in several other fetal tissues, indicating that it may play a physiological role during fetal development. Expression of both variants is most prominent in the placenta (Ashhab *et al.*, 2001). Furthermore, it was found that livin α, but not β protects cells from apoptosis induced by staurosporine, but in contrast, apoptosis initiated by etoposide was blocked only by the β isoform. The findings from these two studies, suggest that there is no correlation between the expression of livin and survivin.

1.6.6 Survivin expression, localisation and mechanism of action

The subcellular localisation and function of survivin has resulted in much controversy and has attracted a lot of interest in recent years. Studies reveal that survivin is localised at the mitotic spindle, binds caspases, and can thus protect cells from apoptosis. Some studies have demonstrated that survivin is expressed at the G2/M phase of the cell cycle (Li *et al.*, 1998 and Kobayashi *et al.*, 1999) and inhibition of human survivin has been associated with cell cycle defects (Li *et al.*, 1998). In addition, survivin expression has been found in embryonic tissue and in proliferating hematopoietic stem cells, as well as reproductive tissue (Konno *et al.*, 2000; Endoh *et al.*, 2001), confirming its cell cycle-specific function. Survivin may also protect cells from apoptosis by binding to caspases. The types of caspases involved, however, are still disputed (Banks *et al.*, 2000; Conway *et al.*, 2000; O'Connor *et al.*, 2000; Shin *et al.*, 2001).

1.6.6.1 Survivin as a caspase inhibitor

There is much discrepancy regarding the role of survivin as an inhibitor of caspase activity. Survivin has been proposed to interact physically with caspase-3 and -7 (Conway *et al.*, 2000; Shin *et al.*, 2001; Suzuki *et al.*, 2001). However, recombinant survivin expressed as an N-terminal histidine-tagged protein was unable to bind caspase-3 or to inhibit caspase-3 activity *in vitro* (Verdecia *et al.*, 2000). Other reports also suggest that survivin does not inhibit caspase-3 activity (Banks *et al.*, 2000; Reed, 2001).

Whether the interaction between survivin and caspase-3 is direct or not is still unclear. However, there is evidence of an inverse correlation between the two proteins. Over-expression of survivin in gastric tumours and adjacent mucosa is accompanied by a down-regulation of caspase-3, indicating that these changes may play a role in the transformation from normal gastric mucosa to gastric cancer (Kania *et al.*, 2003). In ovarian cancer cells, exogenous expression of procaspase-3 leads to up-regulation of wild-type survivin expression, while over-expression of procaspase-3 alone does not induce apoptosis. When survivin expression is blocked by a dominant negative mutant (T34A), apoptosis is induced. Furthermore, this induction of apoptosis was caused by a caspase-9/ caspase-3-mediated pathway as shown by western blot analysis (McKay *et al.*, 2003).

1.6.6.2 Survivin as a regulator of cell cycle

Survivin has been identified as an interface between cell-cycle progression and control of apoptosis. It may be required to counteract a constitutive pathway that induces apoptosis during mitosis. It is expressed primarily during the G2/M phase and associates with microtubules of the mitotic spindle during metaphase and with mid-bodies during late telophase. Association with polymerised microtubules is mediated by a C-terminal coiled-coil domain, not the BIR domain (Li *et al.*, 1997).

Survivin appears to be closely linked to the mitotic process. In normal tissue it is only expressed at detectable levels at the G2/M phase of the cell cycle, survivin protein then migrates to the microtubules in a process similar to chromosomal passenger proteins (Skoufias *et al.*, 2000; Speliotes *et al.*, 2000; Suzuki *et al.*, 2000, Adams *et al.*, 2001). Survivin has also been classified as a chromosomal passenger protein (Temme *et al.*, 2003) that interacts with Aurora-B and INCENP (Skoufias *et al.*, 2000; Uren *et al.*, 2000; Wheatley *et al.*, 2001; Bolton et al., 2002) to form a chromosomal passenger complex that is proposed to play a crucial role in the execution of cytokenesis. Survivin localises Aurora-B to its substrates and enhances it kinase activity *in vitro* and *in vivo* (Chen *et al.*, 2003). Survivin is also essential for chromosome alignment and checkpoint responses elicited by drugs that interfere with tension. Cells lacking survivin entered mitosis with normal kinetics and formed bipolar spindles, but were delayed in a prometaphase state. They also were unable to align their chromosomes, failed to recruit Aurora B to kinetochores, and polyploid at a very high frequency (Lens *et al.*, 2003).

One of the critical requirements for survivin function is the phosphorylation on Thr34 by the mitotic kinase p34cdc2-cyclin B1 (O'Connor *et al.*, 2000), as shown when a phosphorylation-mimetic survivin mutant strongly inhibited p53-induced apoptosis (Hoffman *et al.*, 2002). This method of preventing survivin phosphorylation results in caspase-9-dependent apoptosis and anti-cancer activity (Mesri *et al.*, 2001). Furthermore, inhibition of survivin phosphorylation on Thr34, by the cyclin-dependent kinase inhibitor flavopiridol, resulted in loss of survivin expression and enhanced tumour cell apoptosis induced by anti-cancer agents and it also suppressed tumour growth, without toxicity, in a breast cancer xenograft model *in vivo* (Wall *et al.*, 2003).

1.6.7 Survivin's Role in Cancer

Lung and breast cancers are leading causes of cancer death, and their incidence continues to rise. The main reason for the unfavorable prognosis of these tumours is their tendency to metastasize early and develop resistance to a wide range of functionally unrelated anticancer agents. Interestingly, lung and breast cancer cells express the highest levels of Survivin found in human tumours (Tamm *et al.*, 1998, Ambrosini *et al.*, 1997). Survivin's expression, its correlation to prognosis, and its association with other genes in different types of cancer is summerised in Table 1.6.

Survivin may be a molecular marker for osteosarcoma, the most common malignant tumour of bone (Trieb *et al.*, 2003). Its localisation within the tumour cell may be a prognostic factor of this type of cancer. 70% of osteosarcoma patients with no survivin nuclear staining died of their disease, while 80% of patients with survivin-positive nuclear staining were alive after an average follow-up of eight years. Thus, survivin localisation could be an important prognostic marker for the prediction of the survival of patients with high grade osteosarcoma (Trieb *et al.*, 2003). This finding is in agreement with other findings in gastric cancer (Okada *et al.*, 2001), and in breast cancer (Kennedy *et al.*, 2003), where survivin nuclear staining is associated with a more favourable prognosis.

As well as being a potential molecular marker for predicting disease outcome, survivin may also play an important role in tumour response to chemotherapy. Chemotherapy and radiation induce apoptosis in neoplastic cells. In patients with rectal cancer, who were treated by neoadjuvant radiochemotherapy, survivin expression was correlated inversely with spontaneous apoptosis rate (Rodel *et al.*, 2003). High survivin expression was associated with a significantly higher risk of tumour recurrence after preoperative radiochemotherapy, indicating that over-expression of survivin may confer a certain degree of radio-resistance to rectal cancer cell *in vivo* (Rodel *et al.*, 2002). Furthermore, this was confirmed *in vitro* in colorectal cancer cell lines. The cell lines expressing the highest level of survivin mRNA and protein had the lowest rate of spontaneous and radiation-induced apoptosis and were the most resistant to irradiation, whereas the cell lines with the lowest level of survivin mRNA and protein expression, showed the highest level of apoptosis and

were the most sensitive to irradiation, suggesting that survivin may act as a constitutive radio-resistant factor in colorectal cancer (Rodel *et al.*, 2003). A similar relationship between survivin expression and radio-resistance was reported in pancreatic cells and in melanoma cells, *in vitro*, where an inverse relationship between survivin expression levels and radio-sensitivity was found (Asanuma *et al.*, 2000; Pennati *et al.*, 2003, respectively). Such an association was also reported between survivin and chemoresistance in advance esophageal, lung and skin cancer (Kato *et al.*, 2001; Olie *et al.*, 2000; Mesri *et al.*, 2001, respectively).

Furthermore, survivin up-regulation in ovarian cancer cells, by survivin cDNA transfection, caused a large increase in cell resistance to taxol and taxotere (Zaffaroni *et al.*, 2002). In addition, this drug resistance was reflected *in vivo* where high levels of survivin protein expression, detected by immunohistochemistry, were highly associated with clinical resistance to taxol/platinum-based regimen, but unrelated to non-taxol based treatment, suggesting a link between survivin expression and tumour cell susceptibility to taxol (Zaffaroni *et al.*, 2002). Studies of drug resistant leukemia cell line, HL60R, revealed an increase in survivin mRNA expression compared to parental, drug sensitive, cells (Notarbartolo *et al.*, 2002). In two separate studies by Ikeguchi *et al.* (2001 and 2002), exposure of gastric cancer cell line, MKN-45, to cisplatin resulted in a large increase in survivin mRNA (Ikeguchi *et al.*, 2001 and 2002) and protein (Ikeguchi *et al.*, 2002). Taken together, these findings suggest that survivin plays an important role in chemo- and radio-resistance of malignant cells.

Table 1.6 Summary of Survivin Expression in different types of human Cancers and its relationship prognosis.

Cancer type	Technique	(%)Survivin Expression	Prognosis & Correlation	Localisation	Reference
Lung	RT-PCR	85	Poor survival.	N/A	Monzo *et al.*, 1999
Breast	IHC	70.7	Poor survival; Strong correlation With Bcl-2.	Cytoplasm	Tanaka *et al.*, 2000
	RT-PCR	90.2	N/A	N/A	Nasu *et al.*, 2002
	IHC	60	Favourable prognosis	Nuclear mainly	Kennedy *et al.*,2003
Colorectal	IHC	53.2	Poor survival; Strong correlation With Bcl-2.	Cytoplasm	Kawasaki *et al.*, 1998
	RT-PCR	62	N/A	N/A	Sarela *et al.*, 2000
	IHC & RT-PCR	100 in all normal & Neoplastic Colonic mucosa.	N/A	N/A	Gianani *et al.*, 2001
	IHC	0% in normal 63.2% high-grade dysplasia	Correlation with stage	Cytoplasm	Lin *et al.*, 2003
Skin	IHC	93	More aggressive tumour.	N/A	Grossman *et al.*, 1999(a)
	IHC	81 in BCC 92 in SCC	N/A	N/A	Grossman *et al.*, 1999(b)
	IHC + WB	64 in skin SCC 56 in oral SCC	N/A	Cytoplasm	Muzio *et al.*, 2001
Gastric	IHC	35.4	Correlation with Bcl-2 & p53 expression.	Cytoplasm	Lu *et al.*, 1998.
	IHC	82-88	Nuclear staining associated with favourable prognosis.	Nuclear + Cytoplasm	Okada *et al.*, 2001
	RT-PCR	13.3	N/A	N/A	Ikeguchi and Kaibara, 2001
	RT-PCR	100	Survivin2B inversely Correlated with stage	N/A	Krieg *et al.*, 2002
	RT-PCR & WB	N/A	Inversely correlated with caspase-3	N/A	Kania *et al.*, 2003
Kidney	RPA	50 recurred 18 didn't recur	Correlation with recurrence	N/A	Takamizawa *et al.*, 2001
	RT-PCR	100	Survivin 2B inversely Correlated with stage	N/A	Mahotka *et al.*, 2002
Ovarian	RT-PCR	86	N/A	N/A	Hattori *et al.*, 2001
	IHC	73	Correlation with resistance Taxol/platinum	N/A	Zaffaroni *et al.*, 2002
	IHC	73.5	Correlation with grade & histological type	Nuclear	Cohen *et al.*, 2003

Table 1.6 continued

Cancer type	Technique	Survivin Exp (%)	Prognosis & Correlation	Localisation	Reference
Bladder	IHC	78	Correlation with recurrence & tumour grade.	N/A	Swana *et al.*, 1999
	RT-PCR	100	Correlation with tumour grade	N/A	Smith *et al.*, 2001
Diffuse large B-cell Lymphoma	IHC	55	N/A	Cytoplasm	Ambrosini *et al.*, 1997
	IHC	60	Poor survival	N/A	Adida *et al.*, 2000
Pancreas	IHC	76.9	Higher levels of Surv. in malignant than in benign.	Cytoplasm	Satoh *et al.*, 2001
	IHC	88	Correlation with high proliferation index, low apoptotic index&Bcl-2	Cytoplasm	Sarela *et al.*, 2002
Liver	IHC	70	N/A	Primarily Nuclear, weak cytoplasmic.	Ito *et al.*, 2000
	RT-PCR	87.5	N/A	N/A	
Esophageal	RT-PCR	70.6 (47 in paired normal tissue)	Poor survival	N/A	Kato *et al.*, 2001
	RT-PCR	N/A	Correlation with p53 & proliferative activity	N/A	Ikeguchi *et al.*, 2003
Acute Myeloid Leukemia (AML)	IHC	60	Poor survival, Surv. Expression strongly associated with lower WBC	Cytoplasm	Adida *et al.*, 2000
	WB	88.8	N/A	N/A	Carter *et al.*, 2001
Neuro-blastoma	IHC	47	Poor survival	Cytoplasm	Adida *et al.*, 1998
	Quant. PCR	N/A	Poor prognosis	N/A	Tajira *et al.*, 2001
	RPA	90 recurred 27.7 didn't recur	Correlation with recurrence	N/A	Sandler *et al.*, 2002
	RPA	100 recurred 0 didn't recur	Correlation with recurrence	N/A	Azuhata *et al.*, 2001
Osteosarcoma	IHC	57.5 50	no correlation Correlation with prolonged survival	Cytoplasm Nuclear	Trieb *et al.*, 2003

IHC=Immunohistochemistry; RPA=Rnase protection assay; WB=western blot; N/A=not applicable.

1.6.8 Survivin as a Cancer Therapeutic Target

Components of the apoptosis signaling pathway, along with several other triggers and regulators, are among the most promising targets for pharmacological modulation of cell death and inflammation (Evan *et al.*, 2001; Schattner *et al.*, 2002). Caspases are among the apoptosis regulators that are being targeting for cancer therapy, as well as the caspase inhibitors, IAPs (Los *et al.*, 2003).

The notion that survivin is over-expressed in most common tumours, but absent in the majority of normal adult tissue, with the few exceptions discussed in section 1.6.3, has led to the proposal of survivin as a promising therapeutic target for novel anticancer therapies. Survivin down-regulation may affect the growth of transformed cells, preventing them from continuous growth and metastasis. Experiments targeting survivin with specific ribozymes (Pennati *et al.*, 2002 and 2003; Choi *et al.*, 2003), antisense oligonucleotides (Ambrosini *et al.*, 1998; Grossman *et al.*, 1999a and 1999b; Olie *et al.*, 2000; Chen *et al.*, 2000; Mesri *et al.*, 2001; Shankar *et al.*, 2001; Guan et al., 2002; Xia *et al.*, 2002), or with survivin dominant negative mutants (Grossman *et al.*, 1999a and 1999b; Mesri *et al.*, 2001; Tran *et al.*, 2002; Asanuma *et al.*, 2000; Wall *et al.*, 2003; Zhu *et al.*, 2003; McKay *et al.*, 2003) induced spontaneous and/or chemotherapy-induced apoptosis in various cancer cell lines, and/or eliminated drug resistance. Based on these promising results, Isis Pharmaceuticals and Abbott Laboratories have launched the development of antisense-based strategies that target the expression of survivin (Los *et al.*, 2003).

Strategies to inhibit survivin expression by anti-sense oligonucleotides or ribozyme may emerge as a new gene therapeutic treatment option in a variety of tumour types. This may result in the enhancement of the sensitivity profile of radiation-refractory human malignancies (Pennati *et al.*, 2003).

Understanding the mechanisms of survivin function could, potentially, lead to the development of therapeutic strategies for cancer and other diseases.

1.7 Aims of Thesis

Cell death *via* apoptosis may be induced in tumours following exposure to toxic agents such as chemotherapeutic drugs. Cells which develop resistance to chemotherapeutic drugs do not undergo apoptosis at the same rate as non-resistant cells, indicating that apoptosis may be inhibited in these cells. The first aim of this thesis was to use these MDR variants to identify the roles (if any) played by the anti-apoptotic genes galectin-3 and survivin in multiple drug resistance and *in vitro* invasiveness.

Previous studies have shown that galectin-3 induces *in vitro* invasiveness and inhibits apoptosis induced by cisplatin in the breast cancer cell line, BT549, and blocks staurosporin-induced apoptosis in human leukemia T-cells. Studies have also demonstrated that survivin up-regulation protects cancer cells from apoptosis induced by cisplatin and taxol in endothelial cells (section 1.5.2). To further examine the role of these two genes in drug resistance and invasion/metastasis, cDNA transfections of galectin-3 into the drug sensitive lung cancer, DLKP, and survivin into the drug sensitive lung, ovarian and breast cancer cell lines, DLKP, SKOV-3 and MCF-7, respectively, were carried out. SKOV-3 and MCF-7 cell lines have previously been transfected with an inducible vector (Tet Off), which allows regulation of gene expression. It was hoped that over-expression of these genes (galectin-3 and survivin) followed by analysis of the transfected cell lines using RT-PCR, western blotting, *in-vitro* toxicity testing and *in vitro* invasion experiments, would elucidate the roles played by these genes in drug sensitivity and invasiveness.

A third aim of this thesis was to establish drug resistant variants of DLKP and RPMI-2650 cell lines. The purpose of carrying out this procedure was two-fold. Firstly, it would allow us to analyse the expression of galectin-3 and survivin, as well as the established multi-drug resistance-related genes (Mdr-1, MRP1 and BCRP), in these sub-lines, allowing further insight into the roles they play in drug resistance. Secondly, these studies would contribute to an understanding as to whether the development of drug resistance consistently induces an invasive phenotype in cancer cell lines or if this correlation is cell-type or drug-specific. In addition, this research aimed to use the recently established

technique of DNA microarray analysis to analyse gene expression in the DLKP MDR variants and to possibly identify novel MDR-related and invasion-related genes for future studies.

The final aim of this thesis was to use RT-PCR technique to analyse the gene expression of galectin-3, survivin and MRP-1 in a panel of invasive breast tumour biopsies and to correlate the results with clinicopathological parameters, in an attempt to investigate whether any of these genes may serve as prognostic or predictive indicators in breast cancer disease.

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 initial activated carbon, pre-filtration and anti-scaling. 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, utilising 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 proteineous 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 0.8/0.2μm filters were used for non-serum/protein solutions.

2.1.4 Media Preparation

The basal media used during routine cell culture were prepared, by Mr. Joe Carey, according to the formulations shown in Table 2.1.1. 10x media were 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 were 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 2.2.8.

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 media were supplemented with 2mM L-glutamine (Gibco, 25030-024) and 5 or 10% foetal calf serum 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)**	**MEM (Modified Eagle Medium) (mls) (Gibco, 21430-020)**
10X Medium	Powder	Powder	500
Ultrapure H_2O (UHP)	4300	4700	4300
1M HEPES*	100	100	100
7.5% $NaHCO_3$	45	45	45

* 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.

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 which involved 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. Cell 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 this work

Cell Line	Source of Cell Line	Media	Cell Type
DLKP	NCTCC	ATCC* (5% FCS, 1% L-glut)	Poorly differentiated human lung squamous carcinoma
RPMI-2650	ATCC$^{\Psi}$	MEM (10% FCS, 1% L-glut, 1% NEAA, 1%Sodium pyruvate)	Human nasal squamous carcinoma cell line
SKOV3 'Tet off'	ATCC$^{\Psi}$	ATCC* (10% FCS, 1%L-glut)	Human ovarian carcinoma
MCF-7 'Tet off'	ATCC$^{\Psi}$	ATCC* (10% FCS, 1% L-glut)	Human breast carinoma

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

ATCC$^{\Psi}$ = American Tissue Culture Collection.

NCTCC = National Cell and Tissue Culture Centre.

L-glut = L-glutamine

NEAA = Non-essential amino acids

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 which 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. 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 a 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 which 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) which 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 warm 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 efficiency of the freezing/thawing procedures.
6. Thawed cells were then placed into $25cm^2$ tissue culture flasks with 7mls of the appropriate 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 were sterility checked at least 2 days prior to use by incubating samples at 37°C and assessing as before.

2.2.8 *Mycoplasma* Analysis

Mycoplasma examinations were carried out routinely (at least every 3 months), by Mr. Michael Henry and Ms. Aine Adams, 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 33258 stain (BDH) 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 the dark 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 min. 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. Sample 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 were 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.

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 (or $2x10^4$ cells/ml for RPMI-2650) 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, on 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 (for concentrations see Appendix B) in cell culture medium and 100 µl volumes of the drug dilutions were added to each well using a multichannel pipette. Plates were mixed gently as above.
4. Cells were incubated for 5-7 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.

Table 2.3.1 Chemotherapeutic drugs used in study

Cytotoxic drug	Storage	Disposal
Adriamycin	Store at 4^0C in darkness	Incineration
Vincristine	Store at 4^0C in darkness	Incineration
5-Fluorouracil	Store at RT in darkness	Incineration
Carboplatin	Store at RT in darkness	Incineration
Cisplatin	Store at RT in darkness	Incineration
Taxol	Store at RT in darkness	Incineration
Taxotere	Store at RT in darkness	Incineration
CCNU	Store at RT in darkness	Incineration
Epirubicin	Store at 4^0C in darkness	Incineration

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 twice 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 aluminium foil 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.

2.3.2 Detection of Apoptosis

2.3.2.1 TUNEL analysis

DNA strand breaks were identified using *In Situ* Cell Death Detection Kit with a fluorescein tag (Roche, 1684817). Drug-treated cells or untreated (control) cells were trypsinised at the desired times points into single cell suspension, and washed twice with sterile PBS. Cytospins were prepared on a glass slide using a 100µl aliquot of cell suspension of $5x10^4$cells/ml. After fixing in 4% formaldehyde, the slides were washed and immersed in 0.1% sodium acetate (Sigma, S8625), 0.1% Triton X-100 (BDH, 30632) solution to permeabilise the cells. The slides were washed again and TUNEL mixture was added to each sample, following manufacturer's instructions. The glass slides were incubated at 37°C in the dark for 1 hour, after which time, they were then mounted using fluorescence mounting medium (Dako, S3023) and covered with coverslips (Chance Propper, 22 x 22 mm). Fluorescent staining was visualised using a fluorescence microscope (Nikon).

2.3.3 Adaptation of Drug-selected Variants

In this study, two cell lines, DLKP and RPMI-2650, were selected with various chemotherapeutic drugs and a number of MDR variants were developed. This was achieved by pulse selection.

2.3.3.1 Pulse selection

Cells were grown to 50-60% confluency in $75cm^2$ flasks. In the case of RPMI-2650 selection, flasks were set up in duplicate for each selection agent. Cells were exposed to an initial drug concentration which was equivalent to approx. 90% kill for cells in 96-well plate miniature toxicity assay (see section 2.3.1). The cells were exposed to the drug for 4 hours, after which time the drug was removed and the cells were rinsed with fresh media. The cells were then grown in drug-free media at 37°C for 6 days. Cells were re-fed once during those 6 days. Drug concentration was gradually increased when the cells appeared

healthy. Cells were trpysinised as normal when they reached 70% confluent as outlined in section 2.2.3. Drug exposure was repeated for approx. 10 pulses (in the case of RPMI-2650, some cells received 12 or 14 pulses). After this time, sensitivity to the selecting drug was monitored using miniaturized toxicity assay (section 2.3.1).

2.4 Analytical Techniques

2.4.1 Western Blot analysis

2.4.1.1 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. Further treatment of the cell pellet, to isolate protein, was carried out either on the same day or the pellet was stored at –80°C until required.

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 200-500 µls of this diluted solution was added to the pellet, depending on the size of 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 ensure 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 8 µls with UHP for protein quantification. Sonicated cell extracts were either used immediately in Western blots or were stored at –80°C.

2.4.1.2 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 prepare a standard curve. 10 µl samples were diluted into Eppendorfs, in a stepwise fashion, from 0 – 2 mg/ml BSA. The Biorad 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 µls samples were diluted 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 10-50 μg protein per lane was loaded.

2.4.1.3 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 2.4.1 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, including 5μls of molecular weight broad weight protein markers (New England Biolabs, 7708S), were boiled for 5 minutes before being loaded. The gels were run at 250V, 45mA for approximately 1 hour. When the bromophenol red dye front was seen to have reached the end of the gels, electrophoresis was stopped.

Table 2.4.1 Preparation of electrophoresis gels

Components	Resolving gel (7.5%)	Resolving gel (15%)	Stacking gel
Acrylamide stock*	3.8 mls	7.6 mls	0.8 mls
Ultrapure water	7.3 mls	5.3 mls	3.9 mls
1.875M-Tris/HCl, pH 8.8	3.75 mls	1.88 mls	-
1.25M-Tris/HCl, pH 6.8	-	-	0.31 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	5 µls

* 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).

2.4.1.4 Western blotting

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris (Sigma T-8524), 192mM glycine (Sigma, G-7126) pH 8.3-8.5 without adjusting) for 10 min. Proteins were transferred from gels 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 semi-dry blotting apparatus (Biorad). Excess air was removed from between the filters by rolling a universal tube 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 pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling the universal tube over the filter paper. The proteins were transferred from the gel to the nitrocellulose 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 (Bellydancer, Stovall) 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) with 0.5% Tween (Sigma, P-1379), pH 7.5. After blocking, the membranes were rinsed once in 1X TBS and incubated with 10 mls diluted primary antibody (see Table 2.4.2). Bound antibody was detected using enhanced chemiluminescence (ECL).

Table 2.4.2 Antibodies used for different proteins

1°Antibody	Supplier	Dilution	2° Antibody	Dilution
Galectin-3	Gift from Dr. Fu-Tong Liu	1:1000	Anti-Rabbit HRP	1:1000
Survivin	Alpha Diagnostics SURV11A	1:500	Anti-Rabbit HRP	1:2000
P-gp (C219)	Alexis 801-002-C100	1:1000	Anti-mouse HRP	1:1000
MRP-1 (MRPr1)	Alexis 801-007-C125	1:50	Anti-mouse HRP	1:1000
MRP-2 (M_2III-6)	Alexis 801-016-C250	1:50	Anti-mouse HRP	1:1000
E-cadherin	R&D BTA1	1:200	Anti-mouse HRP	1:1000
α-Tubulin	Sigma T5168	1:2000	Anti-mouse HRP	1:1000

2.4.1.5 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions. The membrane 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 was 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 intensity (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 was then fixed (Kodak, FX-40, diluted 1:5 in water), for 5min. The film was transferred to water for 5 min and then air-dried. For details of densitometric analysis see section 2.4.3.7.

2.4.2 Immunocytochemistry

The avidin-biotin complex (ABC) immunoperoxidase technique combined with the diaminobenzidine (DAB) visualisation procedure was used in all immunocytochemistry experiments. The ABC method involves application of a relevant biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-peroxidase complex, which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains peroxidase molecules. The peroxidase enzyme then reacts with a DAB solution to give an insoluble, brown-coloured precipitate. The formation of this brown-coloured precipitate is indicative of primary antibody reactivity.

The procedure used is as follows:

Cell preparations on 6-well tissue culture plates (which had been previously fixed in methanol and frozen at -20^{0}C) were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle cells in the tissue culture plates, to contain the various solutions involved. The cells were incubated for 5 minutes with a 3% H_2O_2 solution, to quench any endogenous peroxidase activity that may be present in the cells and that could lead to false positive results. The cells were then rinsed with UHP and placed in TBS for 5 minutes. The plates were then incubated for 20 minutes at room temperature (RT) with an appropriate serum diluted 1:5 in TBS to block non-specific binding. This was removed and 30-50µl of optimally diluted primary antibody added. The slides and tissue-culture plates were placed on a tray containing moistened tissue paper and incubated at 37^{0}C for 2 hours. The primary antibodies used in the study are listed in Table 2.4.2. The wells were then rinsed in TBS/ 0.1% Tween, x3 for 5 min each, and then incubated for 30 min with a biotinylated secondary antibody diluted in TBS. The wells were rinsed as before and incubated with strepABComplex/ Horse Radish Peroxidase (HRP) (DAKO, K377) for 30 min at RT, after which they were rinsed x3 in TBS/ 0.1% Tween. The cells were then incubated with a DAB solution (DAKO, S3000) for 7-10 min. Excess DAB solution was then rinsed off with UHP water. The wells were then mounted using a commercial mounting solution (DAKO, S3023).

2.4.2.1 Immunofluorescence

Frozen 6 well sample plates were removed from the –20°C freezer and allowed to come to room temperature (~10 minutes). Grease circles (DAKO pen, DAKO Cat S2002) were then drawn within the wells (to contain the solutions used in the subsequent analysis). The cells were re-hydrated using 1X TBS for 5 minutes. This was tapped off and the relevant serum (diluted 1/5) was added as a 'blocker' for 20 minutes at room temperature. The serum was removed at this point and the primary antibody was applied (antibodies and dilutions are listed in Table 2.4.2). This was incubated overnight at 4°C in a moist environment. The following day the primary antibody was removed and the wells were washed three times in TBS-0.1% Tween at 5 minutes per wash. The fluorescent secondary antibodies were prepared in the dark room under dim conditions and were coated in foil upon dilution with TBS (they are light sensitive). The secondary antibodies (Table 2.4.2) were incubated for 60 minutes and the plates were wrapped in foil to maintain dim conditions. All work from this point onwards was carried out in the dark to prevent 'quenching' fluorescent signal. After 60 minutes incubation the antibodies were removed and the plates were washed three times in TBS-0.1% tween. The wells were then mounted using fluorescence mounting medium (Dako, S3023) and covered with coverslips (Chance Propper, 22 x 22 mm). Fluorescent staining was visualised using a fluorescence microscope (Nikon) and the appropriate filters, i.e. for FITC labelled mouse IgG (Dako, F0261) the B2 filter was used.

2.4.3 RNA Analysis

2.4.3.1 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.

2.4.3.2 RNA Isolation

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

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 were allowed to attach and grow 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 pellet 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.

2.4.3.3 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 were 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 *et al.*, 1989). In these studies approximately 200 µg RNA per 175cm^2 flask was retrieved. RNA samples were diluted to 500 ng/µl and stored at –80°C.

2.4.3.4 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.

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

2.4.3.5.1 Reverse Transcription of isolated RNA

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

To form the cDNA, the following reagents were mixed in a 0.5ml Eppendorf tube (Eppendorf, 0030 121 023), heated to 72°C for 5 min and then chilled on ice.

1µl oligo (dT) primers (1µg/µl)*
2µl total RNA (500ng/µl)
2µl DEPC water

After chilling on ice, the following reagents were added:

*Exception: For survivin RT reaction, specific reverse transcript primers were used (see appendix A).

4µl of a 5X buffer (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100) (Sigma, B-0175)
2µl DTT (Sigma, D-6059)
1µl dNTP (10mM of each dNTP) (Sigma, DNTP-100)
1µl Rnasin (40U/µl) (Sigma, R-2520)
1µl MMLV-RT (40,000U/µl) (Sigma, M-1427)
6µl DEPC UHP

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 then inactivated by heating to 95°C for 2mins. The cDNA was stored at -20°C until required for use in PCR reactions as outlined in Section 2.4.3.5.2.

2.4.3.5.2 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 0.5ml Eppendorf tubes were used, as for the RT reactions. All reagents had been aliquoted and were stored at -20°C. A complete list of all PCR primers and reaction conditions are included in Appendix A.

A typical PCR reaction contained the following:

5µl	10x buffer (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100) (Sigma, P-2317)
3µl	25mM-$MgCl_2$ (Sigma,M-8787)
1µl	each of first and second strand target primers[1] (250ng/ml)
1µl	each of first and second strand endogenous control primer (250ng/ml) (β-actin)
1µl	dNTPs (10mM each of dATP, dCTP, dGTP and dTTP)
0.5µl	5U/µl *Taq* DNA polymerase enzyme (Sigma, D-4545)
31.5µl	UHP
5µl	cDNA

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

	95°C for 3 min	-	denaturation
30 cycles:	95°C for 30 sec.	-	denaturation
	X[2]°C for 30 sec.	-	annealing
	72°C for 30 sec.	-	extension
And finally,			
	72°C for 7 min.	-	extension

[1] 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.
[2] Temperature dependent on primer type. See Appendix A for specific details.

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

2.4.3.6 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 (BioRad). 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 product 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.

2.4.3.7 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 selected and scanned and a value was calculated by the software 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 $1cm^2$) was corrected for background of an identical set area. The corrected reading, for a given amplified band of interest was then normlaised by dividing with a normalised reading obtained from an endogenous control band (usually β-actin). As a result, these O.D. readings having arbitrary units.

2.4.3.8 Isolation of RNA from Tumour/Normal Specimens

1. Breast tumour samples (both Tumour (T) and Normal (N)) were obtained from the pathology department under the supervision of Dr. Susan Kennedy, St. Vincent's University hospital, Dublin.
2. These were archival tumour biopsies that were stored at –80°C until RNA isolation.
3. All manipulations of the human material was carried out inside a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) to prevent contamination and to protect the operator. The floor of the cabinet was lined with two sheets of aluminium foil and then covered with two large plastic autoclavable sheets.
4. All implements (e.g. scissors, forceps, aluminium foil, test tubes, etc.) used in the RNA isolation were baked @ 200^0C overnight prior to use.
5. The Tumour/Normal specimens was removed from their wrapping.
6. The specimens were bisected using a sterile blade and forceps and approx. half the tissue was placed in a test tube with 2mls of Tri Reagent. This was then exposed to homogenisation using a Braun potter S886 homogeniser, for 5 minutes on ice, 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 cellular debris.
8. The specimens then underwent the TRI REAGENT™ protocol for RNA isolation (Section 2.4.3.2).

2.4.4 Plasmid DNA manipulation

2.4.4.1 Plasmids and oligonucleotides used

The galectin-3 cDNA was cloned into the pREB9 plasmid and was kindly donated by Dr. Fu-Tong Liu (The Scripps Research Institute, CA, USA). The survivin cDNA was cloned into the pTarget plasmid and kindly donated by Dr. Kevin Scanlon (Berlix Inc, CA). The survivin cDNA was also sub-cloned (by Cytomyx) into the pTRE plasmid for transfection into the inducible cell lines.

2.4.4.2 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.

2.4.4.3 DNA miniprep of plasmid DNA

This was carried out to generate material for subsequent restriction enzyme digestion, in order to determine the orientation of the inserted DNA sequence in the transformed plasmid.

1. Single colonies were selected off the plates and incubated in universals containing 5 mls LB/Amp shaking at 180rpm at 37^0C 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.

2.4.4.4 Restriction enzyme digestion of plasmid DNA

5 µls of each isolated plasmid sample was run out on a 1% agarose gel to check for degradation. Restriction digestion was then carried out to confirm orientation of the insert. All digestions were carried out using the protocol as outlined in Table 2.4.3. Digestion enzymes used and the size of the insert that was cut out are listed in Table 2.4.4. For plasmid map see Figures 2.4.1, 2.4.2 and 2.4.3. For gene cDNA sequence see Appendix A.

Table 2.4.3 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

Table 2.4.4 Digestion Enzmyes used and size of insert

Plasmid	**Enzyme**	**Size**
Survivin cDNA in pTarget	EcoR1	550bp
Survivin cDNA in pTRE	EcoR1	642bp
Galectin-3 cDNA in pREB9	EcoR1	427bp

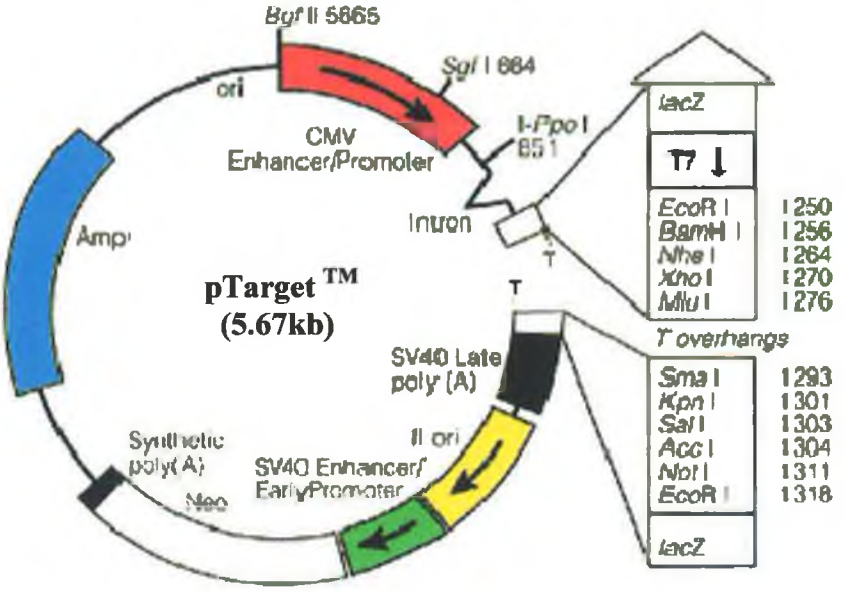


Figure 2.4.1 Schematic map of pTarget™ Vector, showing location of enhancer/promoter (CMV), polyadenylation site (SV40 poly A), multiple cloning sites (T overhang), and ampicillin and neomycin-resistant genes (Amp^r and Neo, respectively) (www.promega.com).

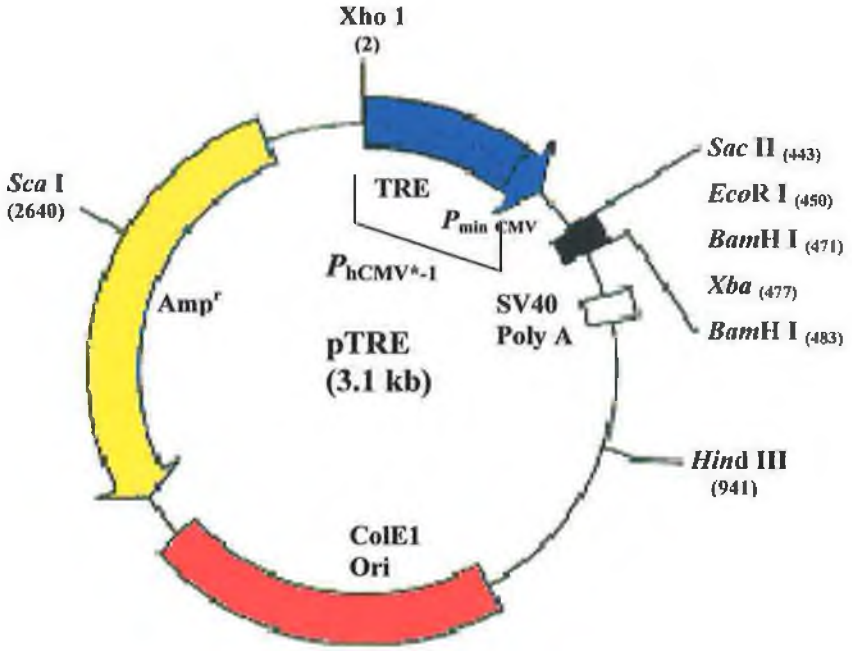


Figure 2.4.2 Schematic map of pTRE Vector, showing the Tet-responsive promoter ($P_{hCMV*-1}$), the minimal CMV promoter (P_{mimCMV}), the ampicillin-resistant gene (Amp^r), the polyadenylation site (SV40 poly A), and multiple cloning sites (adapted from www.clontech.com).

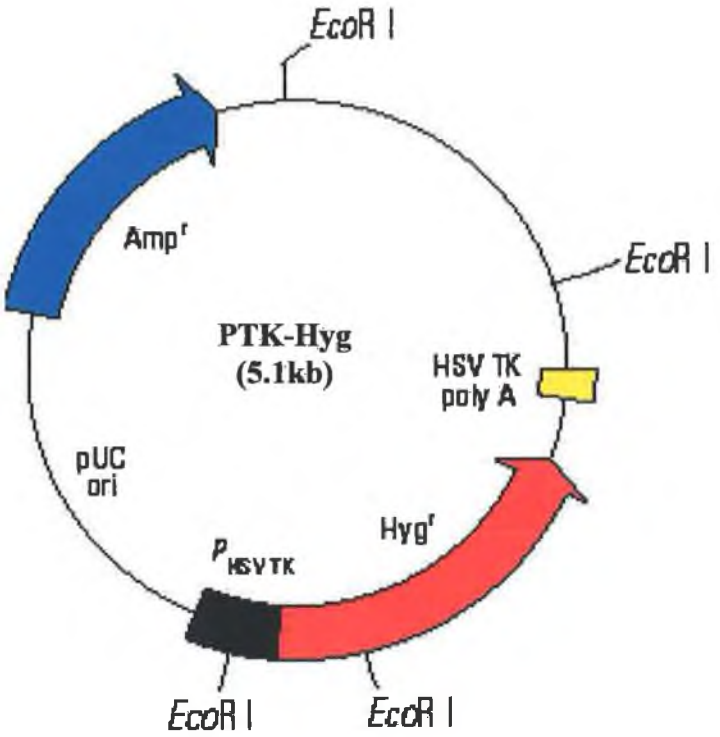


Figure 2.4.3 Schematic map of pTK-Hyg Vector, showing the ampicillin-resistant gene (Amp^r), and the hygromycin resistant gene (Hyg^r) (adapted from www.clontech.com).

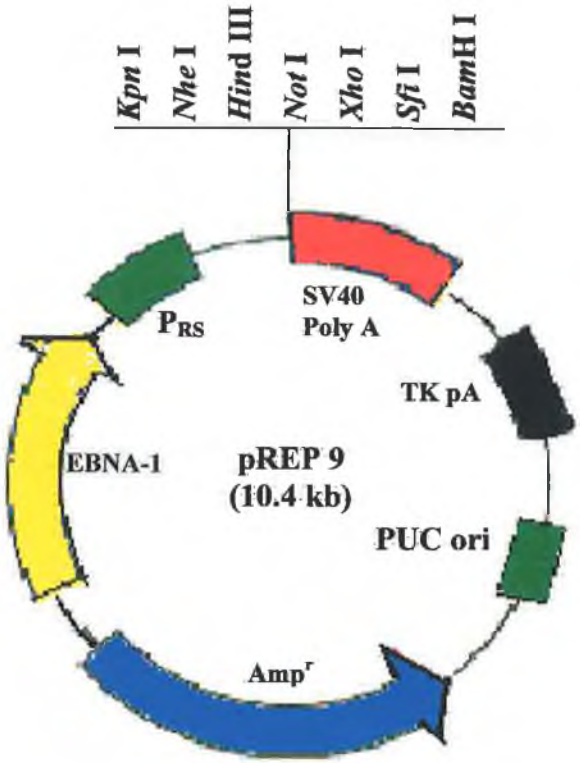


Figure 2.4.4 Schematic map of pREP9 Vector, showing multiple cloning site (bases 632-691), SV40 polyadenylation signal (SV 40 poly A), TK polyadenylation signal (TkpA), ampicillin resistance gene (Amp^r), pUC origin: (PUC ori) and the EBNA-1 gene (adapted from www.invitrogen.com).

2.4.4.5 Large scale plasmid preparation

A single colony (Section 2.4.4.2) was inoculated into 10ml of LB containing Ampicillin at 50μg/ml and was 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) with Ampicillin at 50μg/ml and left to grow overnight at 37°C for large scale isolation of plasmid from JM109 cells. The following day the cells were pelleted and 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. This was 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 1xTris-EDTA, pH 8.0 (TE). The DNA concentration was determined by measuring the OD_{260nm}.

2.4.5 Transfection of mammalian cells with exogenous DNA

2.4.5.1 Stable transfection of DNA using lipofectin reagent

On the day prior to transfection, the cells to be transfected were prepared as a single cell suspension and were seeded into 25cm^2 flasks at 3x10^5 cells per flask. On the day of the transfection, the plasmid(s) to be transfected were prepared along with the lipid transfection reagents, according to the manufacturers protocols (Lipofectin – GibcoBRL, 18292-011). The cells were transfected for four hours in the absence of serum, after which the medium was replaced with serum-containing medium. Cells were incubated at 37°C.

2.4.5.2 Cloning Procedure

Examining the effect of transfection of cDNAs of interest (i.e. galectin-3 and survivin) involved the selection and establishment of stably-transfected clonal cell lines, where RNA, protein and drug profiles were only assayed when the new cell line(s) were obtained.

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) (or hygromycin B (Clontech 8057-1) in the case of survivin-pTRE/pTK-Hyg transfection). The plasmids used had a geneticin-resistant (or hygromycin-resistant) gene, therefore, only those cells containing the plasmid should survive treatment with geneticin. Two 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 cells is 65μg/ml; cells were fed with media containing 200μg/ml geneticin). When the cells grew readily in this concentration of geneticin, the concentration was increased step-wise to a final concentration of 1mg/ml. The final concentration used of hygromycin B used was 140μg/ml. At this stage the cells were plated out in 96-well plates (Costar, 3596) at a clonal density of one cell/3 wells. Clonal populations were propagated from these wells, and transfected cells were periodically challenged with geneticin to maintain stability of transfectants and prevent cross-contamination with non-transfected cells.

2.4.5.3 Optimisation of plasmid transfection protocol

Before transient transfections were carried out, the transient transfection protocol was 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 a 6-well plate, at two different cell concentrations (i.e. $1x10^5$ cells and $2x10^5$ cells in 1ml medium). Following incubation overnight at 37°C, 5% CO_2, the cells were transfected according to the transfection protocol for the transfectant used, i.e. Fugene 6 (Boehringer Mannheim, 1 814 443). Only the volumes of transfectant and concentration 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), 2.5ml 0.1 EDTA (pH 8.0) (Sigma, E-0396), 0.1ml 1.0M $MgCl_2$ (Sigma, M-8266), 37ml UHP) for 10 mins. The cells were then washed for 10 mins. in wash solution (40ml 0.5M Phosphate buffer (pH 7.3), 10ml 1.0M $MgCl_2$ (Sigma, M-8266), 20mg Sodium deoxycholate (Sigma, D-4297), 40µl Nonidet P-40 (Sigma I-3021), 160ml UHP). Staining was carried out on the cells using 2.5ml of stain solution (10ml rinse solution, 0.4ml 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 highest number of blue colonies, was thereafter used for that cell line.

2.4.5.4 Transient transfection of DNA using Fugene 6 reagent

The day before the transfection experiment, cell were seeded into a 25cm^2 flask at a cell density of 1.5 x 10^5 cells/ml (in 4ml medium). Fugene 6 reagent:DNA comlex was used at a 3:2 ratio which was found to be an optimal ratio (other ratios included 3:1 and 6:1). The Fugne:DNA complex was made up according to manufacturer's recommendations and cells were transfected will 100µl of the complex mixture in a dropwise fashion. Cells were returned to a 37°C incubator until the following day. Cells were harvested for RNA and protein at 24, 48, 72 hour and 5day time intervals, or seeded in 96-well plates for *in vitro* toxicity testing (Section 2.3.1.1), or used for preparing cytospins on glass slides for TUNEL assay (Section 2.3.2.1) for detection of apoptosis.

2.5 Invasion Techniques

2.5.1 Invasion Assay

Invasion assays were performed using the method of Albini (1998). ECM (Sigma E-1270) (11mg/lml) was diluted to 1mg/ml in serum-free DMEM. 100µl of 1mg/ml ECM was placed into each invasion chamber/insert (Falcon 3097) (8.0µm pore size, 24 well format) which was placed in a 24-well plate (Costar). This was carried out on ice to prevent the ECM from solidifying during the process. The inserts were incubated overnight at 4°C. The following day, the cells were harvested and re-suspended in media-containing fetal calf serum, at a concentration of $1x10^6$ cell/ml. The inserts were washed with serum-free DMEM, then 100µl of the cell suspension were added to each insert and 300µl of media containing serum was added to the well underneath the insert. Cells were incubated at 37°C for 48 hours. After this time, the inner side of the insert was wiped with a wet cotton swab while the outer side of the insert membrane was stained with 0.25% crystal violet for 10 minutes and then rinsed with PBS and allowed to dry. The inserts were then viewed under the microscope

The procedure for the ready-coated inserts was very similar to the procedure described above, with the exception that the inserts were not coated with ECM (as they were pre-coated with matrigel), but were re-hydrated for 2 hours prior to use, by adding serum-free media and incubating at 37°C, following the manufacture's instructions (Beckton Dickinson).

For a quantitative method of invasion analysis, assessment of cell migration to the outer side of the insert was determined by acid phosphatase assay (Section 2.3.1.2). Cells were incubated for the time indicated above, after which, the inner side of the insert was wiped with a wet cotton swab and the media in the well was replaced with 200ul 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). The plates were wrapped in aluminium foil and incubated in the dark at 37°C, 5% CO_2, for 2 hours.

The enzymatic reaction was stopped by the addition of 50 µl of 1M NaOH to each well. 100µl aliquots were removed from the wells, placed in a 96-well plate, and read in a dual beam plate reader at 405 nm with a reference wavelength of 620 nm.

Another quantitative method was also used in come cases whereby the crystal violet dye was eluted with 200 µl of 33 % glacial acetic acid/well. 100 µl aliquots were transferred from each well to corresponding wells of a 96 well plate and the absorbance was read at 570 nm.

2.5.1.1 Invasion Inhibition Assays

The procedure for carrying out MMP inhibition assays was identical to that of invasion assays (section 2.5.1), with the exception that 200µg/ml MMP inhibitor I, 10µg/ml MMP inhibitor III or 10 µg/ml MMP-2 inhibitor I was added both to the cell suspension in the insert and to the medium in the well underneath the insert before the cells were incubated at 37°C, 5%CO_2, for 48 hours. All three MMP inhibitors were purchased from CalBiochem (Nottingham, UK). Table 2.5.1 illustrates details provided by manufactures about the function of each inhibitor

Table 2.5.1 MMP inhibitors used in invasion inhibition assays and the MMP whose activity they inhibit.

Inhibitor	**MMP inhibited**	**Catalogue number**
MMP inhibitor I	MMP-1, -3, -8 and –9	444250
MMP inhibitor III	MMP-1, -2, -3, -7 and –13	444264
MMP-2 inhibitor I	MMP-2	444244

2.5.2 Motility Assay

The procedure for carrying out motility assays was identical to the procedure used for invasion assays (section 2.5.1) with the exception that the inserts were not coated with ECM. Incubation times were 12, 24 and 48 hours. The procedure for quantitative analysis was identical to that of the invasion assays.

2.5.3 Adhesion Assay

Adhesion assay was preformed using the method of Torimura *et al.* (1999). Collagen Type IV (Sigma C-5533), fibronectin (Sigma F-2006) and laminin (Sigma L-2020) were reconstituted in PBS to a stock concentration of 500μg/ml. Stocks were aliquoted into sterile Eppendorfs. Fibronectin and collagen stocks were stored at –20°C and laminin stocks were stored at –80°C. ECM (Sigma E-1270) was diluted to 1mg/ml in serum-free DMEM, aliquoted and stored at –20°C. ECM undergoes thermally activated polymerization when brought to 20-40°C to form a reconstituted basement membrane.

When the solutions were ready for use, each of the ECM proteins, collagen, fibronectin and laminin, was diluted to 25μg/ml with PBS, while ECM was diluted to 1mg/ml with serum-free DMEM. 250μl aliquots were placed into wells of a 24-well plate. The plates were tapped gently to ensure that the base of each well was completely covered with solution. The plates were then incubated at 4°C overnight. The ECM solutions were then removed from the wells and the wells were rinsed twice with sterile PBS. 0.5ml of a sterile 0.1% BSA/PBS solution was dispensed into each well to reduce non-specific binding. The plates were incubated at 37°C for 20 minutes and then rinsed twice with PBS.

Cells were harvested and resuspended in serum-free DMEM medium. The cells were then plated at a concentration of 2.5×10^4 cells per well and incubated at 37°C for 60 minutes. After that time, the media was removed from the wells and the wells were rinsed gently with PBS. The cells were then stained with 0.5ml of 0.25% crystal violet dye for 10 minutes. The plates were then rinsed and allowed to dry. The dye was eluted with 200μl of

33% glacial acetic acid and 100µl aliquots were transferred to a 96-well plate and the absorbance was read at 570 nm (Torimura *et al.*, 1999).

2.5.4 Zymography

Zymography was used to assess the level of proteolytic activity of different proteinases. The choice of substrate incorporated into the resolving gel depends on substrate specificity of the species of enzyme to be detected (Johansson *et al.*, 1986). Gelatin is a substrate for matrix metalloprotienases (MMPs), serine and cysteine proteinases.

The gel was prepared by incorporating gelatin within the polymerized acrylamide matrix. 10% acrylamide gels were used. The quantities for one gel is given in Table 2.5.4a.

Table 2.5.4a Preparation of resolving and stacking gel for zymography

Components	**Resolving gel (10%)**	**Stacking gel**
Acrylamide stock*	3.3 mls	0.5 mls
3mg/ml Gelatin	2.5 mls	-
1.875M-Tris/HCl, pH 8.8	2.5 mls	-
1.25M-Tris/HCl, pH 6.8	-	0.8 mls
Ultrapure water	1.7 mls	2 mls
10% Ammonium persulphate (Sigma, A-1433)	33 µls	33 µls
TEMED (Sigma, T-8133)	5 µls	5 µls

* 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), TEMED, N,N,N,N'-tetramethylethylenediamine (T-8133) and Gelatin (G-8150).

Cells were grown in cell culture Petri dishes (Greiner). When the cells were 80% confluent, they were rinsed twice with sterile PBS, followed by 4-hour incubation with serum-free medium. The cells were then grown in fresh serum-free medium for another 24-72 hours (depending on their growth rate). After the relevant time period, supernatants were collected as samples. Samples were mixed 3:1 with 4X sample buffer (10% glycerol; 0.25M Tris-HCl, pH 6.8; 0.1% (w/v) bromophenol blue) and were loaded onto the gel. 5µl of boiled Broad size range protein marker (New England Biolabs, 7708S) was also loaded onto the gel. The gels were run at 30 mA per gel in running buffer (as in section 2.4.1.3) until the dye front reached the bottom of the gel. Following electophoresis, the gels were soaked in 2.5% Triton-X-100 with gentle shaking for 30 minutes at room temperature. The gels were then rinsed in substrate buffer (50mM Tris-HCl, pH8.0; 5mM $CaCl_2$) and then incubated for 24 hours in substrate buffer at 37°C. The gels were then stained with Coomassie blue (2.5mg/ml) for 2 hours by shaking and destained in destain solution (50ml acetic acid; 150ml isopropanol; 300 ml distilled water) until clear bands were visible. The gels were then scanned.

To identify the different classes of proteinases that are being secreted, inhibitors of proteinases were added to 2.5% Triton-X-100 in substrate buffer. The inhibitors used in this study are listed in Table 2.6.4b.

Table 2.5.4b Inhibitors of different classes of proteinases used in gelatin zymography

Inhibitor	Enzyme inhibted	Concentration used
EDTA	MMPs	30 mM
PMSF	Serine proteinases	1 mM

2.6 DNA Microarray analysis technique

2.6.1 RNA Extraction

High quality RNA was isolated from the DLKP parental cells and the DLKP MDR variants using the Rneasy mini kit (Qiagen 74104). The Rneasy extraction is based on guanidine thiocyanate method of extraction. The procedure was carried out according to the manufacturer's recommendations. Affymetrix recommends a minimum of 5μg of total RNA at a concentration 0.5μg /μl. 10μg of RNA was used for all cell lines included in this study.

2.6.2 Sample, Array and Data processing

After RNA isolation, quantification, and purification, cDNA was synthesised using the GeneChip T7-Oligo(dT) Promoter Primer kit (Affymetrix; 900375). First strand cDNA synthesis was then carried out using the Superscript choice kit (Biosciences 11917-010). First strand cDNA synthesis involved "primer hybridisation" where the T7-oligo(dT) primer was incubated with the RNA and DEPC-treated H_2O at 70°C for 10mins, followed by a short incubation on ice; "temperature adjustment" where 5X first strand buffer, DTT and dNTP mix were added to the RNA mix and incubated at 42°C for 2mins; and "first stand synthesis" where SuperScript II RT was added to the mix and incubated at 42°C for 1hr. Second strand cDNA synthesis was carried out and purified using GeneChip Sample Cleanup module (Affymetrix 900371) as recommended by the manufacturers.

cRNA was synthesised and biotin-labelled using the Enzo BioArray HighYield RNA Transcript Labelling Kit (Affymetrix 900182). Biotin-labelled cRNA was purified using the GeneChip Cleanup module kit (Affymetrix 900371) and quantified. The value obtained was adjusted to reflect carry over of unlabeled total RNA. A sample of biotin-labelled cRNA was taken for gel electrophoresis analysis. The labelled cRNA was then fragmented before hybridisation onto GeneChip probe arrays. An aliquot of fragmented and un-fragmented cRNA was checked by gel electrophoresis. Fragmented sample RNA was stored at –20°C (for a maximun of 48hrs, if possible) until ready to perform the

hybridisation. Hybridisation of RNA onto GeneChip probe human arrays (Hu133A; Affymetrix) was carried out in the Conway Institute, University College Dublin, where the Affymetrix Hybridisation Oven and Fluidics Station is set up along with the Affymetrix Scanner, which exported the data directly to the Affymetrix analysis software, MicroArray Suite 5.1 (MAS 5.1).

The Affymetrix Genechips have short oligonucleotides synthesised *in situ* on glass slides, with 22 spots (11 Perfect Match and 11 Mismatch) for each gene. The mismatch oligos have a single base difference to the perfect match oligos in the middles of the strand. The values for each of the 22 spots are considered by the software to produce a Flag Call (e.g. Present or Absent) and a Raw Value (in Affymetrix units) for each probe set.

The data from the MAS 5.1 is transferred into the Genespring, a Gene Expression Analysis program, where it was first normalised so that the results from different chips can be compared with each other. There are three stages in the normalization: The first stage is a 'data transformation' where negative numbers are converted to a small positive value, such as 0.0001. Negative numbers may be a problem because the analysis programs may try to log transform a column of data which contained some negative numbers and would fail. The second stage is a 'Per Chip' normalisation. This centres all of the chips in the experiment around the same median and enables us to compare one chip with another. It is done to eliminate minor differences in hybridisation conditions etc. In this case, each gene on the chip is normalised to the 50^{th} percentile of all the measurements on that chip. The final step is a 'Per Gene' normalisation, where the measurement for a particular gene is divided by its measurement in a designated control sample. This allows us to discuss the level of a particular transcript relative to a particular control condition.

In this analysis, the data was loaded into Genespring and the levels of a particular list of genes that had already been investigated by PCR were looked at. Only changes of 2- or greater –fold were considered to be 'real changes'.

3.0 Results

3.1 Analysis of Galectin-3 expression in RPMI Drug-resistant cell lines

Two MDR variants of RPMI-2650 (a nasal carcinoma cell line) were established in the NICB (Y. Liang, PhD, 1999) and were termed RPMI-taxol and RPMI-melphalan. Both cell lines exhibited increased multiple drug resistance. In addition, RPMI-melphalan resistant cell line exhibited increased invasiveness and metastasis compared to the non-invasive parental cell line.

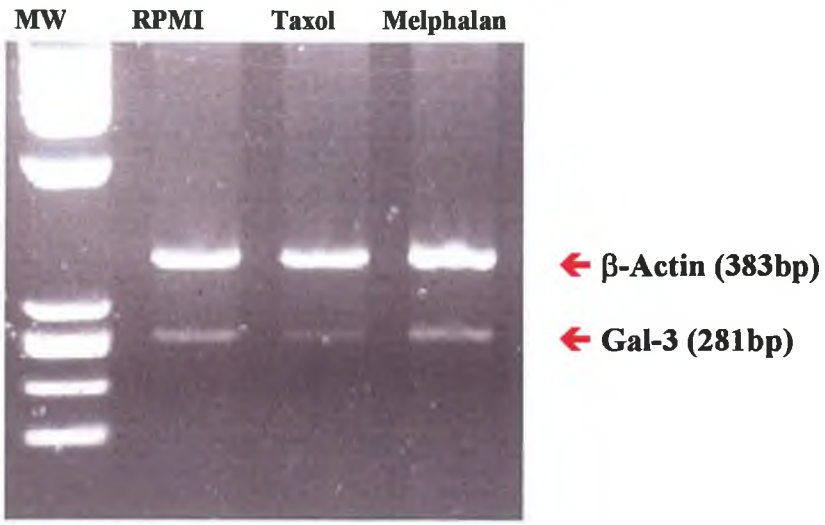
In order to determine the effect of induced MDR in cancer cell lines on gene expression, an investigation was carried out to establish the change in the expression level of two anti-apoptotic genes, galectin-3 (section 3.1.1) and survivin (section 3.2.1).

3.1.1 Galectin-3 expression in RPMI-Taxol and RPMI-Melphalan

3.1.1.1 Investigation of Galectin-3 expression in RPMI-Taxol and RPMI-Melphalan resistant cell lines using RT-PCR analysis

RNA was extracted from the RPMI-2650 cell lines using the TRI Reagent method (section 2.4.3.2). RT-PCR was carried out using galectin-3 primers, which amplified a band of 281 bp and β-actin primers, acting as in endogenous control, which amplified a band of 383 bp (see section 2.4.3.5 for RT-PCR technique). Galectin-3 mRNA expression was shown to increase slightly in the RPMI-melphalan variant compared to RPMI parent, while galectin-3 mRNA expression was down-regulated in the RPMI-taxol variant (Fig 3.1.1.1A). Densitometry was carried out on the results and the galectin-3 bands were normalised to the β-actin bands (Fig. 3.1.1.1B). RT-PCRs were carried out in triplicate and the results were reproducible. The molecular weight marker "ϕ-X174" Hae III digest was used as a size reference (Figure 3.1.1.1).

(A)



(B)

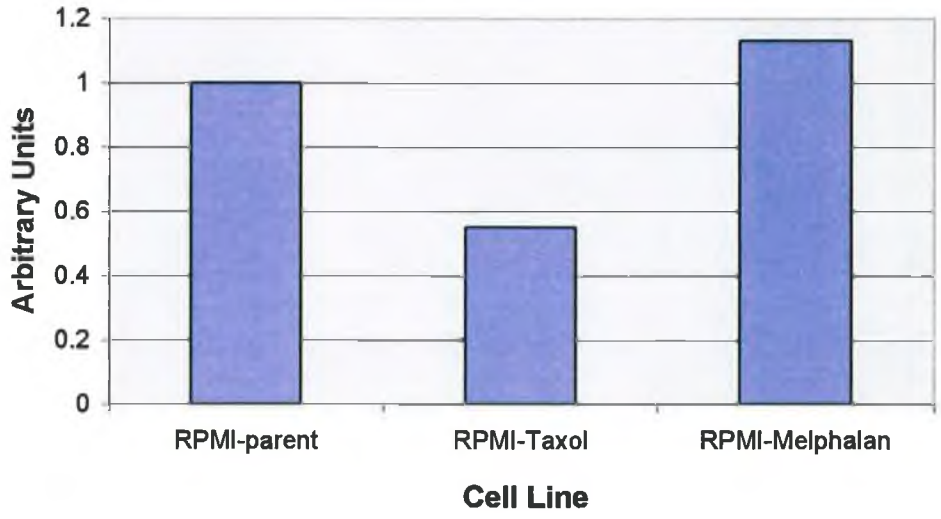
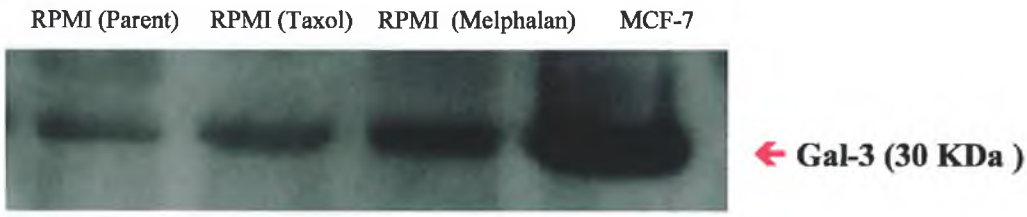


Figure 3.1.1.1 **(A)** Gel electrophoresis of galectin-3 RT-PCR results on RPMI, RPMI-taxol and RPMI-melphalan resistant cell lines. MW=molecular weight marker; **(B)** Densitometric analysis of RT-PCR results. (n=3).

3.1.1.2 Investigation of Galectin-3 expression in RPMI-Taxol and RPMI-Melphalan resistant cell lines using Western blot analysis

Protein was isolated from the RPMI-2650 cell lines and Western blot was carried out on the RPMI-2650 parental cell line and the RPMI-2650-taxol and RPMI-2650-melphalan variants using a galectin-3 primary antibody, which detects a band of 30 kDa, corresponding to galectin-3 protein (see section 2.4.1 for western blotting technique). Galectin-3 protein expression was shown to increase in the RPMI-melphalan variant compared to RPMI parent and RPMI-taxol. MCF-7 cell line was used a positive control for galectin-3 protein expression (Figure 3.1.1.2). Experiments were carried out in triplicate and results were reproducible.

(A)



(B)

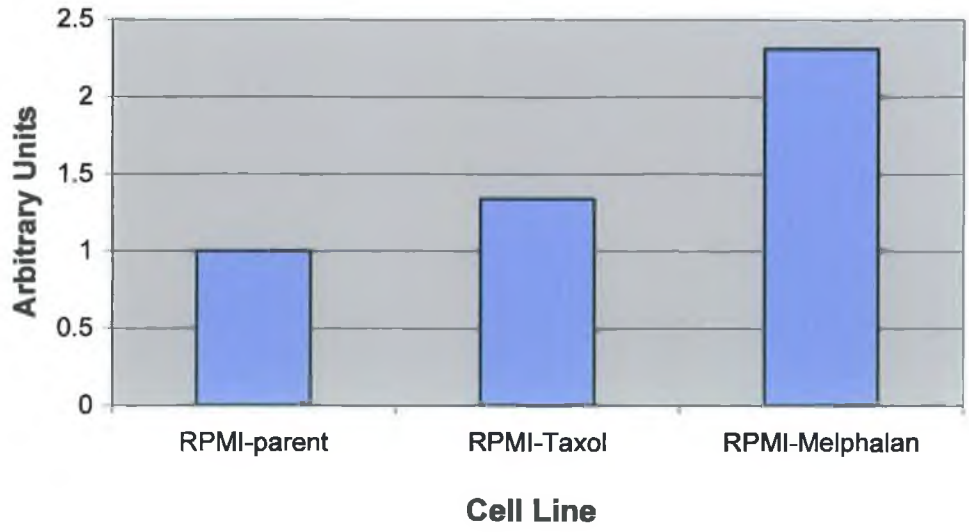


Fig. 3.1.1.2 **(A)** Galectin-3 Western blot analysis on RPMI, RPMI-taxol and RPMI-melphalan resistant cell lines. MCF-7 cell line was used as a positive control; **(B)** Densitometric analysis of western blot results. (n=3)

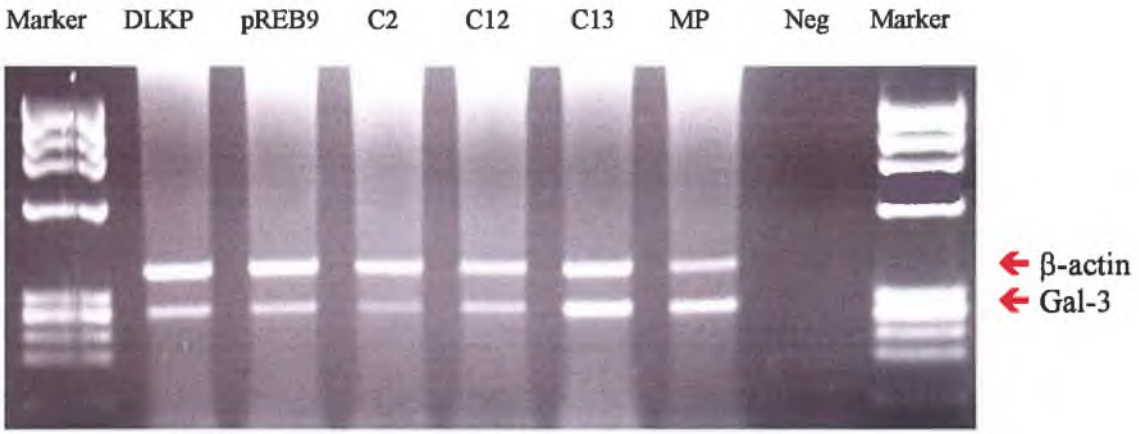
3.1.2 Galectin-3 cDNA Transfection into DLKP cells.

To further elucidate the role of galectin-3 in cancer cells, galectin-3 was transfected into the lung carcinoma cell line, DLKP, a cell line developed in the NICB.
Galectin-3 cDNA in pREB9 plasmid was transfected into DLKP cells using Lipofectin. Selection using geneticin was carried out until a mixed population (MP) and 3 clones (C2, C12 and C13) were obtained. The concentration of geneticin at which this was achieved was 1 mg/ml. The mixed population along with the three clones were characterised by RT-PCR and Western blot analysis. The sensitivity of the galectin-3 clones to a range of chemotherapeutic drugs was determined and compared to DLKP parent cell line drug sensitivity by carrying out toxicity assays using the acid phosphatase method (see section 2.3.1).

3.1.2.1 Analysis of DLKP- Galectin-3 clones using RT-PCR for Galectin-3

RNA was extracted from the DLKP-galectin-3 clones and RT-PCR analysis was carried out. Results indicate that DLKP-Gal-3 clone 12, clone 13 and the mixed population over-express galectin-3 mRNA, whereas clone 2 had similar levels as the parent cell line and the empty pREB9 vector in the mock transfection (Fig. 3.1.2.1A). Densitometry was carried out on the results by normalising the galectin-3 bands to the β-actin bands (Fig. 3.1.2.1B). RT-PCR analysis were carried out at least three times in order to maximise the accuracy of the results. As mentioned in section 3.1.1.1, the molecular weight marker "ϕ-X174" Hae III digest was used as a size reference. Sterile water was used as a negative control in the majority of RT-PCR results presented in this thesis.

(A)



(B)

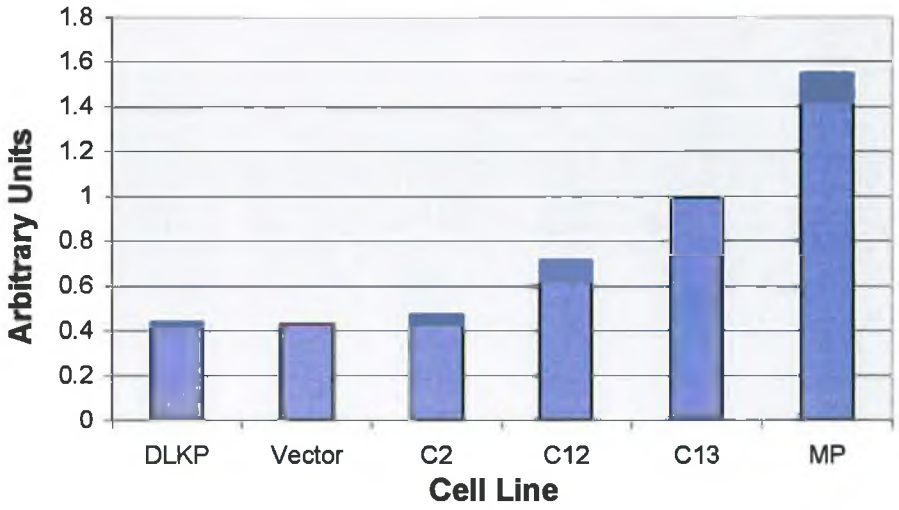
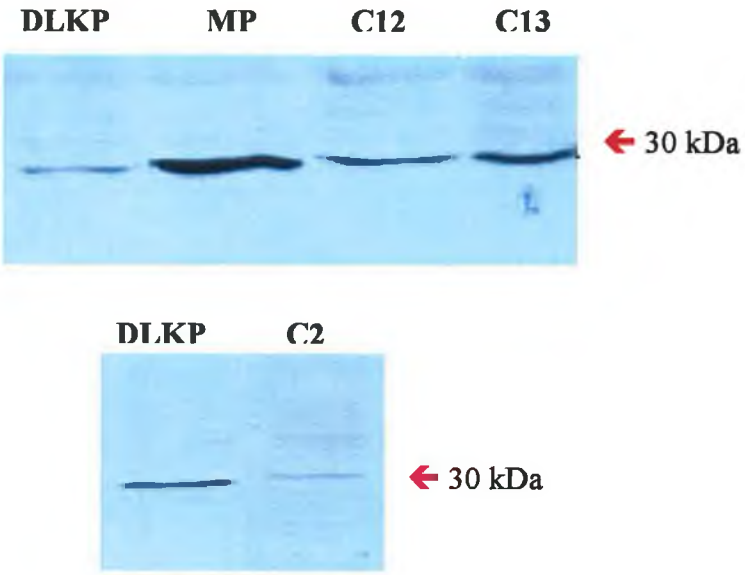


Fig. 3.1.2.1 **(A)** Gel electrophoresis of Gal-3 RT-PCR results on DLKP-Gal-3 cDNA transfectants. Neg = sterile water; **(B)** Densitometric analysis of RT-PCR results. (n>3)

3.1.2.2 Analysis of DLKP- Galectin-3 clones using Western blotting for Galectin-3

Protein was isolated from the clones, the mixed population and the parent cell line, DLKP, and Western blot was carried out using a galectin-3 primary antibody. The results were consistent, to a certain extent, with those of the RT-PCR analysis and showed that DLKP-Gal-3 clone 12, clone 13 and the mixed population over-expressed galectin-3 protein, whereas clone 2 shows a down-regulation of galectin-3 protein compared to the parent cell line (Fig. 3.1.2.2A). Densitometry was carried out on the results comparing the clones and MP to the DLKP parent cell line (Fig. 3.1.2.2B). Western blot analysis was carried out over three times and results were reproducible.

(A)



(B)

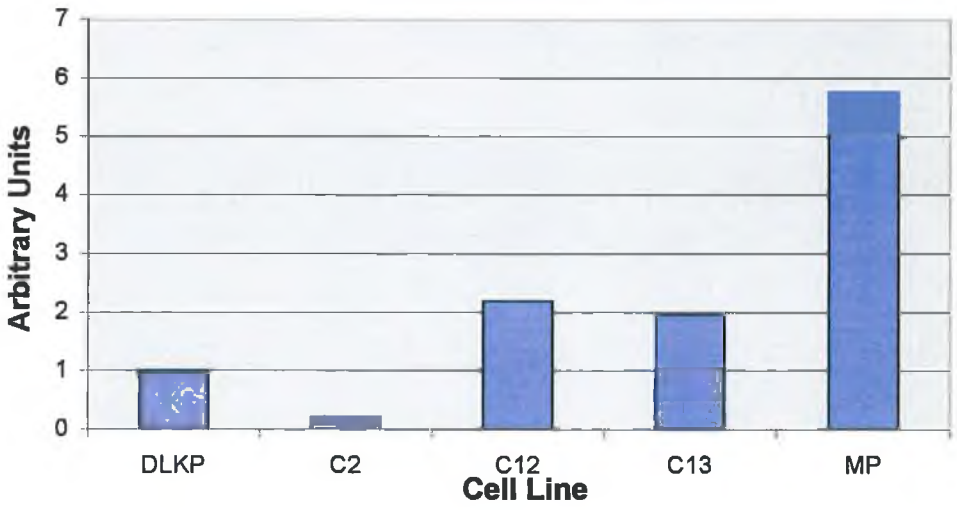


Fig. 3.1.2.2 **(A)** Galectin-3 Western blot analysis of DLKP-Gal-3 cDNA transfectants; **(B)** Densitometric analysis of Western blot results. (n>3)

3.1.2.3 Analysis of DLKP-Gal-3 cDNA transfectants using *in vitro* toxicity testing

The sensitivity of the galectin-3 transfectants to a range of chemotherapeutic drugs was determined and compared to DLKP parent cell line drug sensitivity. Cells were exposed to a selection of chemotherapeutic drugs. The chosen drugs were carboplatin, adriamycin and taxol. The cells were exposed to the drugs over a range of concentrations, which included the IC_{50} values for each drug for the DLKP cell line. At the end of the 5-7 day toxicity assay, the cells were analysed using the acid-phosphatase assay (section 2.3.1). The results represent a mean of at least three repeats of the experiment and the standard deviation (see Table 3.1.2.3A).

Whereas galectin-3 over-expression did not affect cell sensitivity to adriamycin and taxol, cells over-expressing galectin-3 tended to be more resistant to carboplatin (Table 3.1.2.3B).

(A)

Cell Line	Adriamycin (ng/ml)	Carboplatin (μg/ml)	Taxol (ng/ml)
DLKP	14.2±0.78	1.86±0.12	2.11±0
Vector	14.91±0.94	1.73±0.08	1.18±0.08
C2	20.45±0.61	1.12±0.06	0.97±0.05
C12	10.51±0.86	2.36±0.12	1.06±0.04
C13	9.23±0.74	2.83±0.11	1.08±0.06
MP	13.92±0.92	4.41±0.28	2.51±0.2

Table 3.1.2.3A Table of IC_{50} values for DLKP parent cells and DLKP Galectin-3 transfectants to a range of chemotherapeutic drugs.

(B)

Cell Line	Adriamycin	Carboplatin	Taxol
DLKP	1	1	1
Vector	1.05	0.93	0.56
C2	1.44	0.6	0.46
C12	0.74	1.27	0.5
C13	0.65	1.52	0.51
MP	0.98	2.37	1.19

Table 3.1.2.3B Table of fold resistance of DLKP Galectin-3 transfectants to a range of chemotherapeutic drugs, compare to the parental cell line.

3.1.2.4 Analysis of DLKP-galectin-3 transfectants using *in vitro* Invasion assays.

In order to determine the effect of galectin-3 over-expression on the invasive properties of DLKP cell line, invasion assays were carried out using ECM-coated inserts. Cells were grown in the inserts for 48 hours before being stained with crystal violet, as described in section 2.5.1.

It was found that galectin-3 over-expression in DLKP correlated with increased invasiveness. DLKP parental cells are considered to be non-invasive. This was also the case with DLKP cells transfected with the empty vector. DLKP-C2, which does not over-express galectin-3, was also non-invasive. However, C12, C13 and the MP, which all over-express galectin-3, were found to have developed an invasive phenotype due to galectin-3 transfection. RPMI-2650, a nasal carcinoma cell line, was used as a negative control in this experiment as they have been found to lack invasive properties. HT-1080, a highly invasive fibrosarcoma was used as a positive control.

For a quantitative analysis of the assay, the crystal violet dye was eluted from the inserts using 33 % glacial acetic acid and the absorbance was read at 570 nm. Results from this elution, as shown in figure 3.1.2.4b correlated with the photographic representation in figure 3.1.2.4a.

Invasion assays were carried out three times and the results were found to be reproducible.

(a)

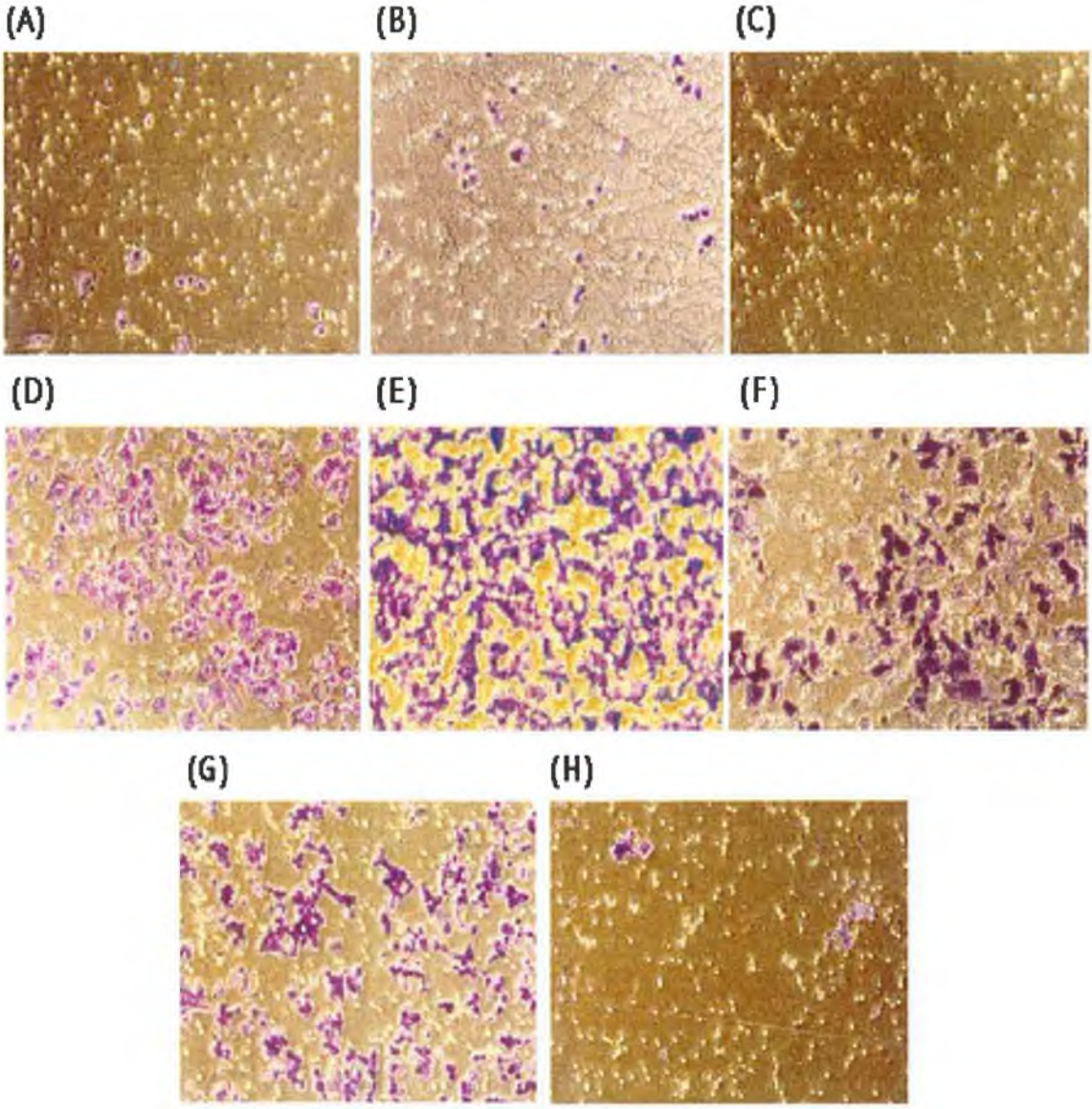


Figure 3.1.2.4a Invasion assays of **(A)** DLKP parent; **(B)** DLKP-vec; **(C)** DLKP-C2; **(D)** DLKP-C12; **(E)** DLKP-C13; **(F)** DLKP-MP, **(G)** HT-1080; **(H)** RPMI-2650 (4X). (n=3).

(b)

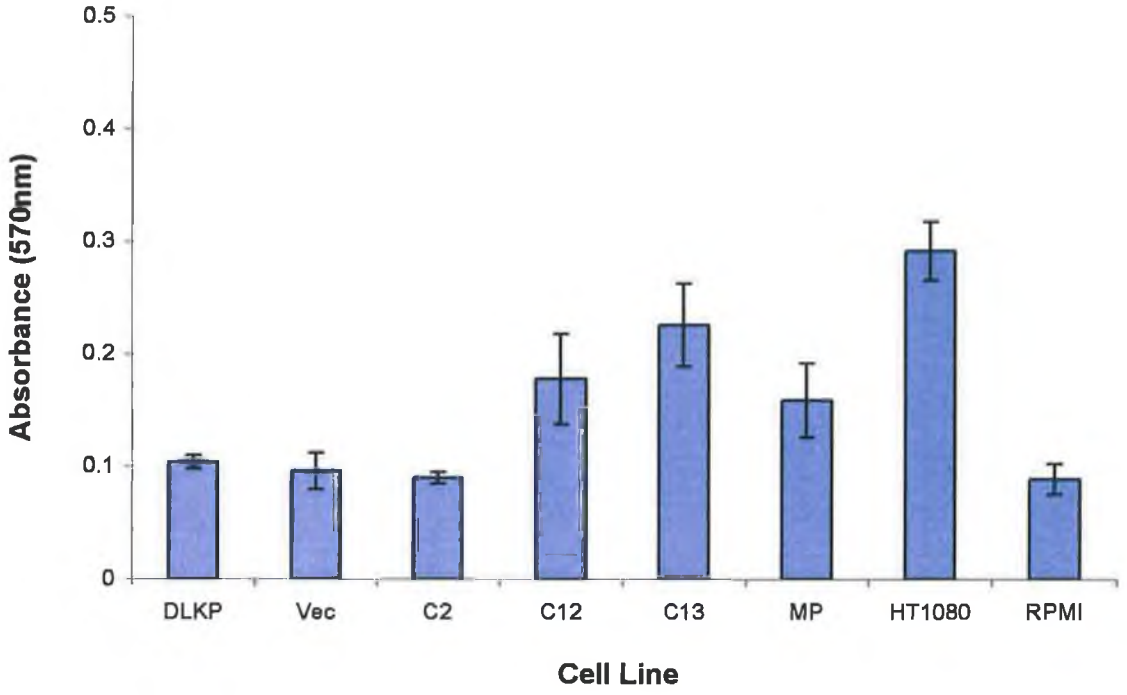


Figure 3.1.2.4b Absorbance values of each eluted insert from invasion assays carried out on the DLKP transfectants. Read at 570 nm. Results represent the mean of three separate experiments +/- standard deviations.

3.1.2.5 Analysis of DLKP-galectin-3 transfectants using *in vitro* Motility assays.

In order to determine the effect of galectin-3 over-expression on the motility of DLKP cell line, motility assays were carried out using the same method as that used for invasion assays, except that the inserts were not coated with ECM (section 2.5.2). Cells were grown in the inserts for 48 hours before being stained with crystal violet.

It was found that galectin-3 over-expression in DLKP correlated with increased motility. This also correlated with the invasion assay results i.e. the more invasive the cells, the more motile they were found to be. DLKP parental cells had low motility, as were DLKP cells transfected with the empty vector. DLKP-C2, which does not over-express galectin-3 was also non-motile. However, C12, C13 and the MP, which all over-express galectin-3 were found to have increased motility due to galectin-3 transfection. RPMI-2650 was used as a negative control in this experiment as they have been found to be non-motile. HT-1080 was used as a positive control.

For a quantitative analysis of the assay, the crystal violet dye was eluted from the inserts using 33 % glacial acetic acid and the absorbance was read at 570 nm. Results from this elution, as shown in figure 3.1.2.5b, correlated with the photographic representation in figure 3.1.2.5a.

Motility assays were carried out three times and the results were found to be reproducible.

(a)

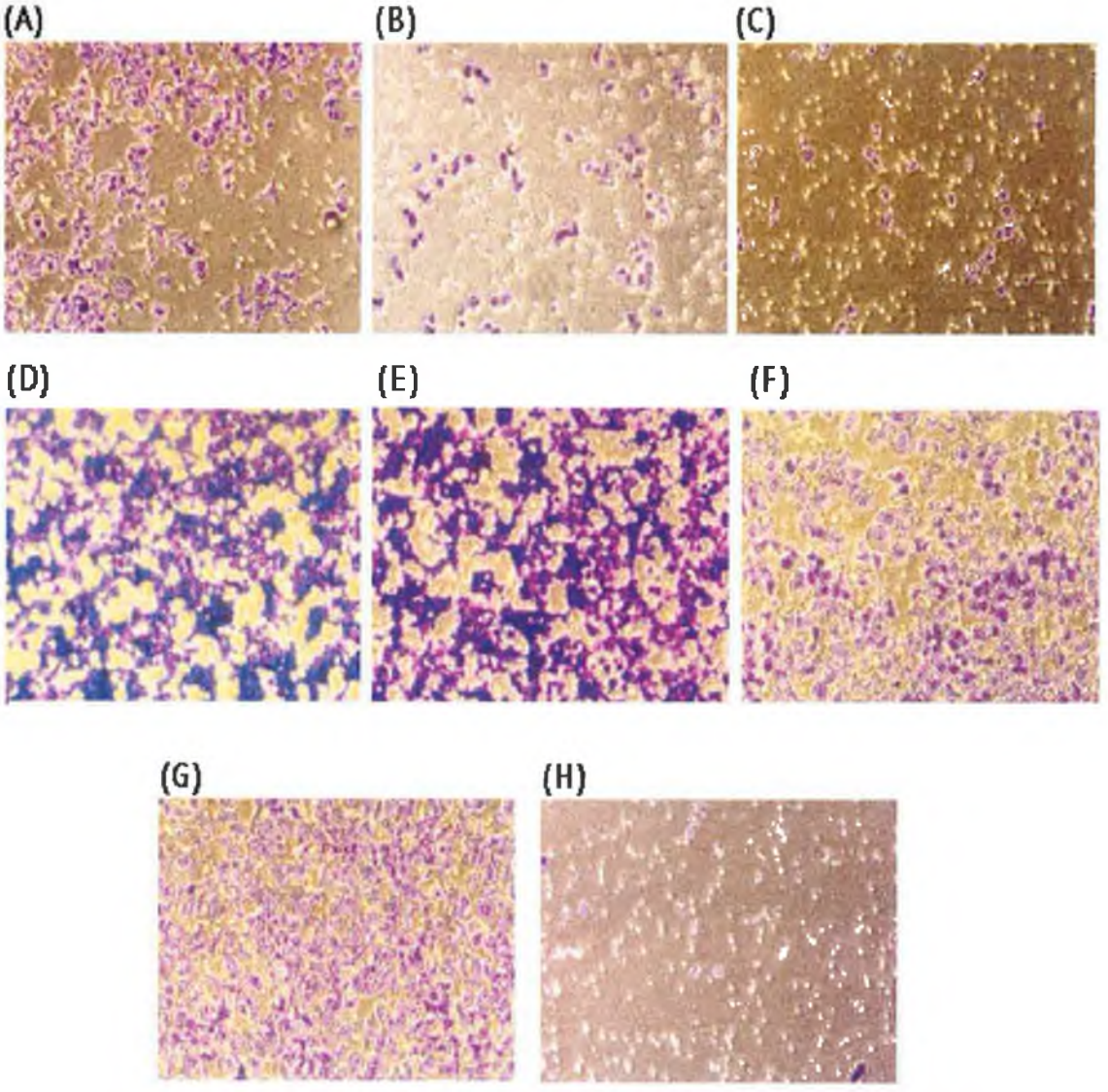


Figure 3.1.2.5a Motility assays of **(A)** DLKP parent; **(B)** DLKP-vec; **(C)** DLKP-C2; **(D)** DLKP-C12; **(E)** DLKP-C13; **(F)** DLKP-MP; **(G)** HT-1080; **(H)** RPMI-2650 (4X). (n=3)

(b)

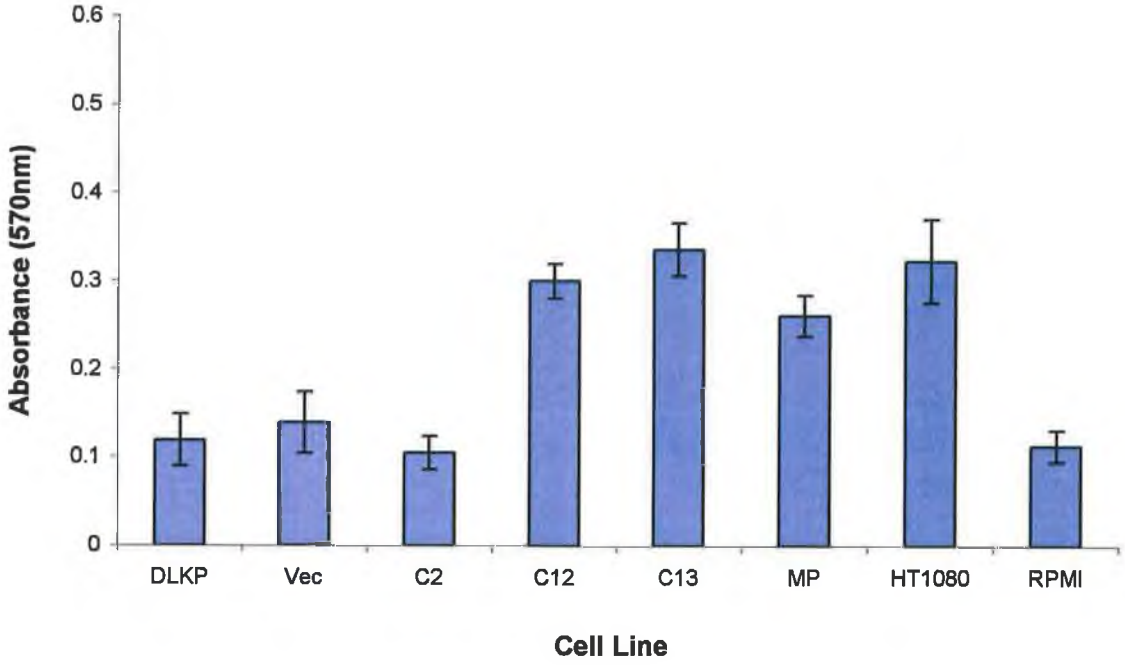


Figure 3.1.2.5b Absorbance values of each eluted insert from motility assays carried out on the DLKP transfectants. Read at 570 nm. Results represent the mean of three separate experiments +/- standard deviations.

3.1.2.6 Analysis of DLKP-galectin-3 transfectants using *in vitro* Adhesion assays.

To detect the adhesiveness of the DLKP parental cell line compared to the DLKP-galectin-3 transfectants to extracellular matrix and its components, collagen type IV, laminin and fibronectin, adhesion assays were carried out as described in section 2.5.3. As with invasion and motility assays, RPMI-2650, was used as a negative control in this experiment. HT-1080 was used as a positive control.

The adhesion assays with ECM, as shown in Figure 3.1.2.6, demonstrates that DLKP-gal-3 C12, C13 and MP were the most adhesive to ECM, therefore suggesting that galectin-3 over-expression supports DLKP adhesion to ECM. DLKP parent, DLKP-vec and DLKP-gal-3 C2 were all less adhesive. RPMI-2650 was less adhesive to ECM while, HT-1080 was as adhesive as MP. As shown in figure 3.1.2.6, all cell lines were equally adhesive to collagen IV, suggesting that galectin-3 over-expression does not correlate with DLKP adhesion to collagen IV. As with ECM, galectin-3 over-expression correlates with DLKP adhesion to fibronectin. DLKP-gal-3 C12, C13 and MP were the most adhesive to fibronectin. DLKP-gal-3 C12, C13 and MP were slightly more adhesive to laminin than the parental cell line, DLKP-vec and DLKP C2 (Figure 3.1.2.6). Adhesion assays were carried out in triplicate and results were reproducible.

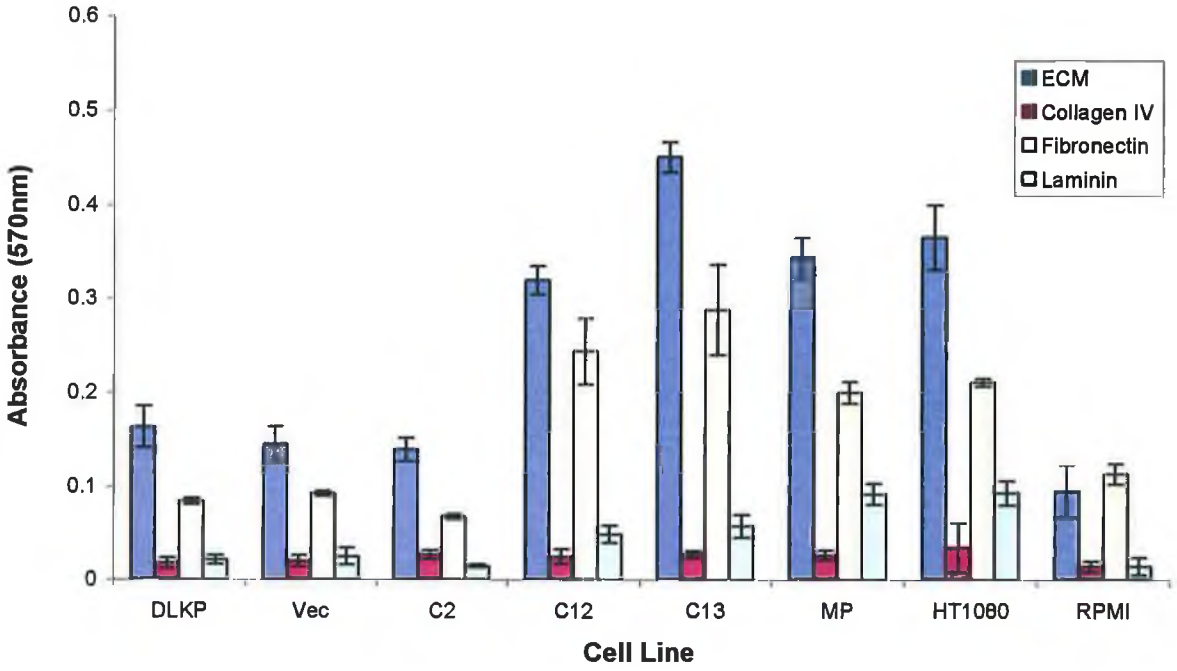


Figure 3.1.2.6 Absorbance values of each eluted insert from adhesion assays to ECM, collagen IV, fibronectin and laminin, carried out on the DLKP transfectants. Read at 570 nm. Results represent the mean of three separate experiments +/- standard deviations.

3.1.2.6 Proliferation rate of DLKP-galectin-3 transfectants

To determine the proliferation rate of cells, their growth is monitored over consecutive 24 hour time periods, as described in section 2.5.

Doubling times were determined for all DLKP transfectants including the parent cells. As shown in Table 3.1.2.7, doubling times did not differ greatly between the cell lines and did not correlate with galectin-3 over-expression. Although the doubling time for MP was similar to the other transfectants, the lag time before exponential growth was longer for MP than the other transfectants.

Cell Line	Doubling Time (Hour)
DLKP	28.9 ± 1.6
Vector	31.8 ± 2.2
C2	30.3 ± 1
C12	30.7 ± 1.8
C13	24.2 ± 2.1
MP	32.3 ± 3

Table 3.1.2.7 Doubling times of DLKP galectin-3 transfectants. Results represent the mean of three separate experiments +/- standard deviations.

3.2 Analysis of Survivin expression in RPMI-2650 drug-resistant cell lines

In order to determine the effect of induced MDR on survivin expression in RPMI-2650 cells, an investigation was carried out to establish the change in the expression level of this anti-apoptotic gene.

The two MDR variants of RPMI-2650, RPMI-taxol and RPMI-melphalan resistant cell lines, as previously mentioned, exhibit increased multiple drug resistance compared to the parent cell line. In addition, RPMI-melphalan resistant cell line exhibited an increase in invasiveness compared to the non-invasive parental cell line.

3.2.1 Survivin expression in RPMI-Taxol and RPMI-Melphalan

3.2.1.1 Investigation of Survivin expression in RPMI-Taxol and RPMI-Melphalan resistant cell lines using RT-PCR analysis

RNA was extracted from the RPMI-2650 cell lines using the TRI Reagent method (section 2.4.3.2). RT-PCR was carried out using survivin primers, which amplifies the three splice variants of survivin (survivin-2B (500bp), survivin (431bp), and survivin-ΔEx3 (329bp)). Survivin RT-PCR was carried out on RPMI-2650 parent cell line, RPMI-Taxol and RPMI-melphalan resistant cell lines. The results showed a decrease in survivin mRNA expression in RPMI-taxol compared to RPMI parent and RPMI-melphalan. As in section 3.1.2.1, β-actin was used as an endogenous control and sterile water was used at a negative control. The molecular weight marker "ϕ-X174" Hae III digest was used as a size reference (Fig. 3.2.1.1). RT-PCR analysis was carried out in triplicate and was reproducible.

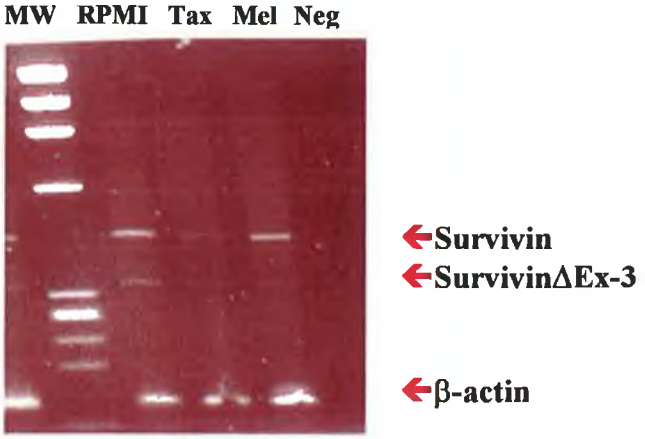


Figure 3.2.1.1 Gel electrophoresis of survivin RT-PCR results on RPMI, RPMI-taxol (Tax) and RPMI-melphalan (Mel) resistant cell lines. MW=molecular weight marker and Neg = negative control (n=3)

3.2.1.2 Investigation of Survivin expression in RPMI-Taxol and RPMI-Melphalan resistant cell lines using Western blot analysis

Survivin Western blots were also carried out on the RPMI variants using a survivin anti-rabbit antibody (see Table 2.4.2) which detects a band of 16.5KDa, and in agreement with the RT-PCR analysis of mRNA levels, survivin protein was down-regulated in RPMI-taxol compared to RPMI parent and RPMI-melphalan. α-Tubulin was used as an endogenous control to ensure equal protein loading (Fig. 3.2.1.2). Western blot analysis was carried out in duplicate and was reproducible.

(A)

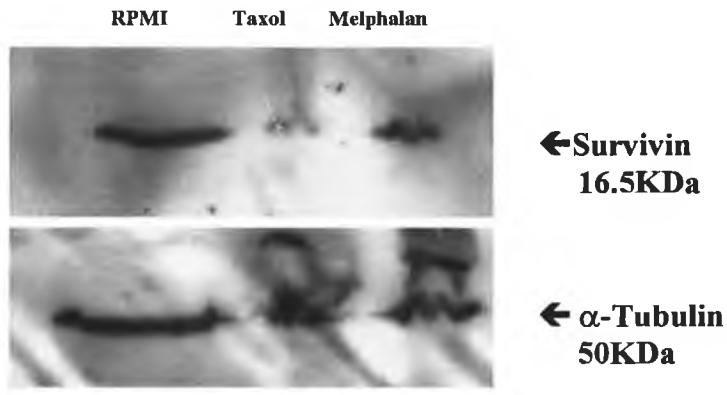


Fig. 3.2.1.2 Survivin and α-tubulin Western blot analysis on RPMI, RPMI-taxol and RPMI-melphalan resistant cell lines. (n=2)

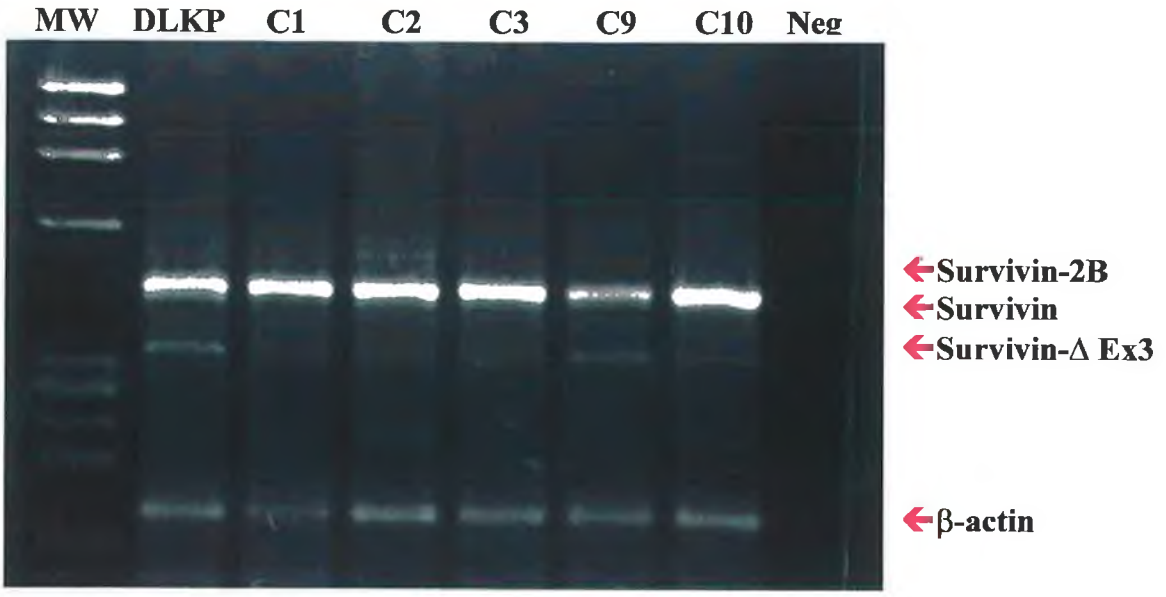
3.2.2 Survivin cDNA Stable Transfection into DLKP cell.

To investigate the role of survivin in lung cancer cells, survivin cDNA in pTarget plasmid was transfected into DLKP cells using Lipofectin. Selection using geneticin was carried out until 5 clones, C1, C2, C3, C9 and C10, were obtained. The concentration of geneticin at which this was achieved was 1 mg/ml. The five clones were characterised by RT-PCR and Western blot analysis. The sensitivity of the survivin clones to a range of chemotherapeutic drugs was determined and compared to DLKP parent cell line drug sensitivity by carrying out toxicity assays using the acid phosphatase method (section 2.3.1).

3.2.2.1 Analysis of DLKP-survivin clones using RT-PCR

RNA was extracted from the clones, the mixed population and the parent cell line, DLKP, and RT-PCR was carried out using survivin primers, which amplified survivin and its two splice variants, survivin 2B and survivin-delta Exon 3. Bands of 431 bp, 500bp and 329bp were amplified for each of the splice variants, respectively. β-actin primers, acting as endogenous control, were included amplifying a band of 142 bp. Results showed that none of the DLKP transfectants over-expressed survivin mRNA (Fig. 3.2.2.1A). Densitometry was carried out on the results by normalising the survivin bands to the β-actin bands (Fig. 3.2.2.1B). As in section 3.1, RT-PCRs were carried out at least three times and sterile water was used as a negative control. The molecular weight marker "ϕ-X174" Hae III digest was used as a size reference

(A)



(B)

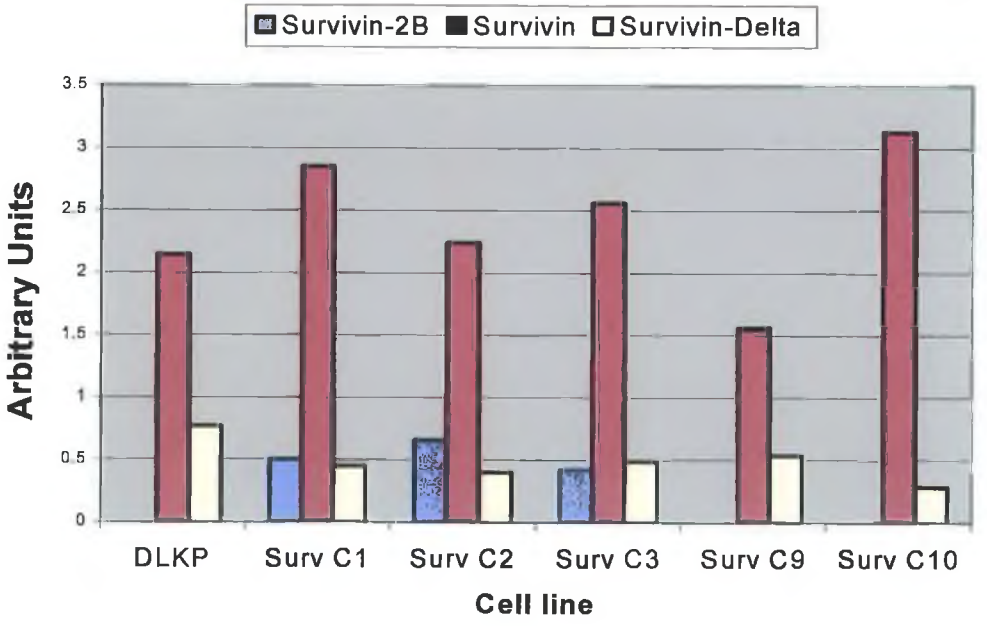


Fig. 3.2.2.1 **(A)** Gel electrophoresis photograph of survivin RT-PCR results on DLKP-survivin cDNA transfectants. Neg=negative control and MW=molecular weight marker; **(B)** Densitometric analysis of RT-PCR results (n=3).

3.2.2.2 Analysis of DLKP-survivin clones using Western Blot

Protein was isolated from the clones and the parent cell line, DLKP (see section 2.4.1.1), and Western blot (section 2.4.1) was carried out using a survivin primary antibody, supplied by Alpha Diagnostics (see Table 2.4.2), which detects a band of 16.5 kDa, corresponding to survivin protein. The results, as seen with the RT-PCR analysis, showed no up-regulation of survivin protein (Fig. 3.2.2.2). As in section 3.2.1.2, α-tubulin was used to ensure equal loading of protein. Western blot analysis was carried out in triplicate and results were reproducible.

(A)

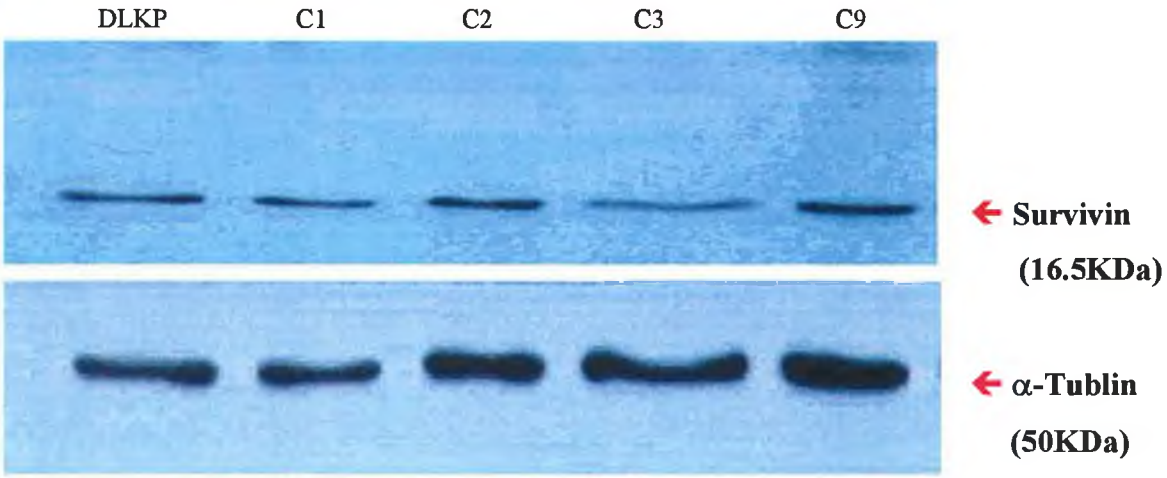


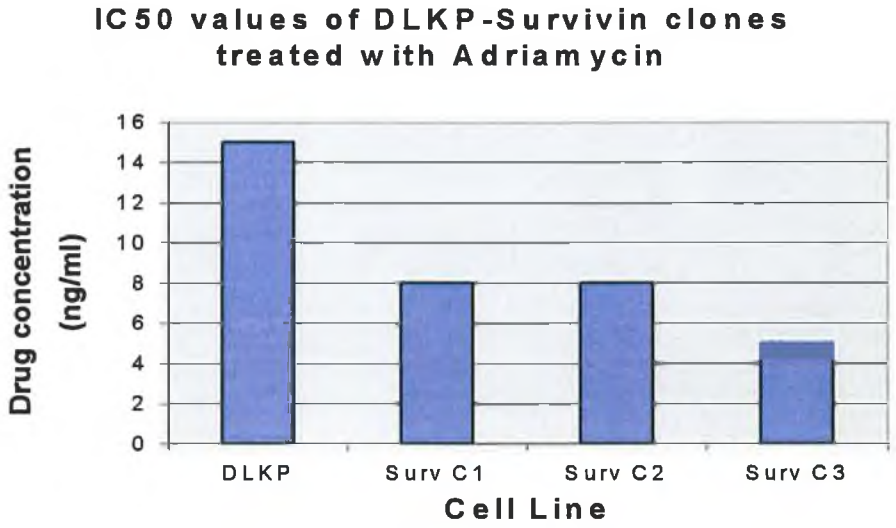
Fig. 3.2.2.2 Survivin and α-tubulin Western blot analysis of DLKP-survivin cDNA transfectants (n=3).

3.2.2.3 Analysis of DLKP-survivin cDNA transfectants using *in vitro* toxicity testing

The sensitivity of the survivin transfectants to a range of chemotherapeutic drugs was determined and compared to DLKP parent cell line drug sensitivity. As in section 3.1.2.3, cells were exposed to chemotherapeutic drugs. The chosen here drugs were adriamycin and carboplatin. The cells were exposed to the drugs over a range of concentrations, which included the IC_{50} values for each drug for the DLKP cell line. At the end of the 5-7 day toxicity assay, the cells were analysed using the acid-phosphatase assay as in section 2.3.1.

No resistance to any of the drugs was observed when compared to the parent cell line. (Fig. 3.2.2.3A and B). Toxicity assays were carried out once with adriamycin and twice with carboplatin.

(A)



(B)

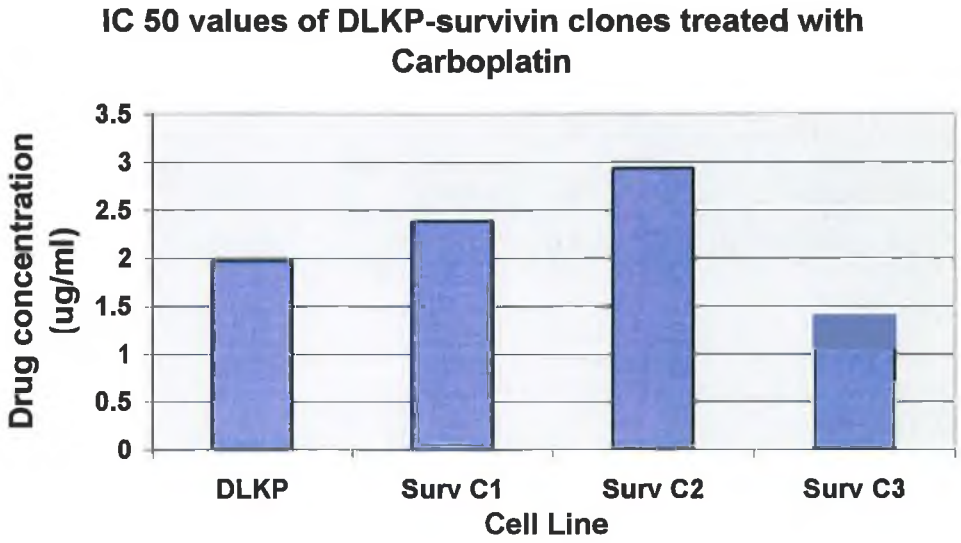


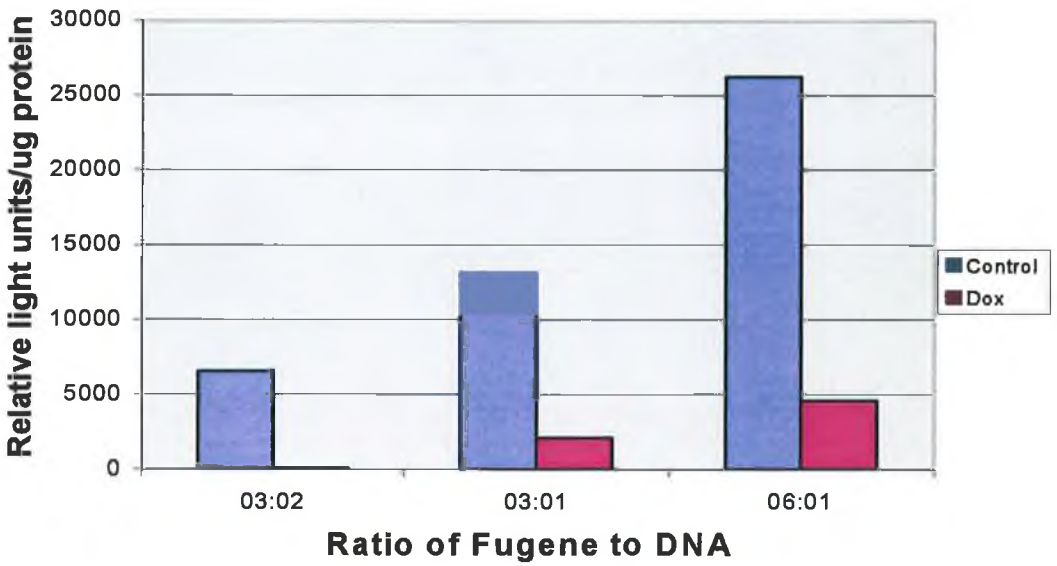
Fig. 3.2.2.3 **(A)** Effect of survivin on Adriamycin resistance in DLKP-survivin cDNA clones (n=1); **(B)** Effect of survivin on carboplatin resistance in DLKP-survivin cDNA clones (n=2).

3.2.3. SKOV-3- Tet off: (The inducible system)

A second attempt was made at creating another stably transfected cell line over-expressing survivin. The cell line chosen was the ovarian carcinoma cell line, SKOV3. This was chosen for two reasons, firstly, this cell line was found to express intermediate levels of survivin compared to DLKP and MCF-7; and secondly this cell line was stably transfected with the Tet-Off (T.O) by Helena Joyce, M.Sc., in the NICB. The advantage of this gene expression system is that it allows regulation of mammalian gene expression with tetracycline or tetracycline derivatives. For the purpose of these studies, doxocycline was used. In the Tet-Off system, gene expression is turned on when doxocycline (Dox) is removed; in contrast to the Tet-On system, where expression is turned on by the addition of Dox.

Initial work on SKOV-3-Tet Off cell line involved checking its inducibility with the addition of doxocycline. A transient transfection of the Luciferase gene, using Fugene 6, was carried out for that purpose and the cell line was found to be inducible with all concentrations of Fugene to DNA (Fig. 3.2.3.1A). The ratio of fugene to DNA that gave the highest fold difference between 'on' and 'off' of gene expression was the 3:2 ratio (see Figure 3.2.3.1B) This ratio was the one used when survivin cDNA was being transfected (see section 2.4.5.4). These results indicate that the SKOV-3-Tet Off cell line was most inducible at the 3:2 ratio of Fugene to DNA. This optimization procedure was carried out once.

(A)



(B)

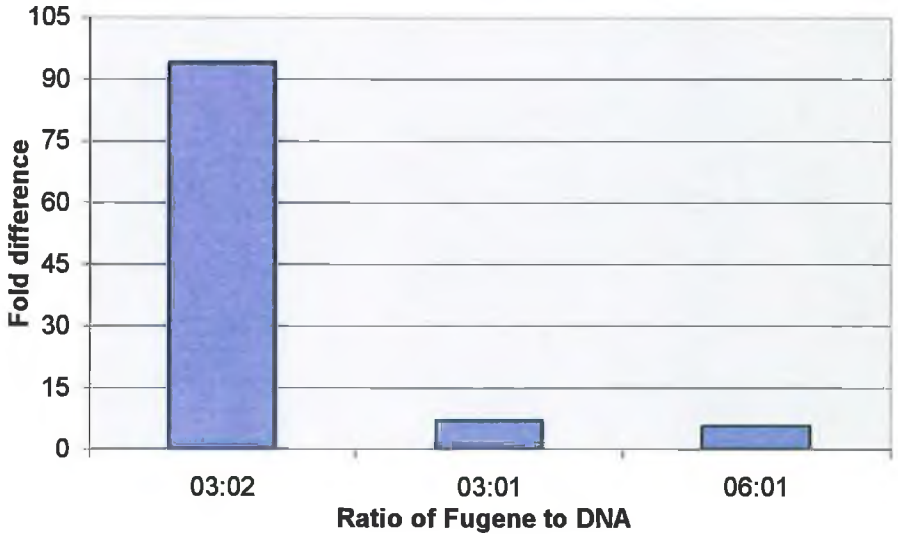


Fig. 3.2.3.1 **(A)** Results of Luciferase assay in the presence and absence of doxocycline as read on a luminometer; **(B)** Representation of the fold difference in inducicilty between the different Fugene:DNA ratios. (n=1).

3.2.3.1 Survivin cDNA Stable Transfection into SKOV3 'Tet off' cell line

Survivin cDNA was sub-cloned into the pTRE plasmid by Cytomyx and it was co-transfected with the selection plasmid, PTK-Hyg into SKOV3 'Tet off' cells, using Fugene 6 as the transfection agent. Selection using hygromycin was carried out, starting at 75ug/ml and increased until a mixed population and 12 clones were obtained. The concentration of hygromycin at which this was achieved was 140 ug/ml. The twelve clones were characterised by RT-PCR and Western blot analysis in the presence and absence of doxocycline. The sensitivity of the survivin clones to two chemotherapeutic drugs was determined and compared to SKOV3-T.O. parent cell line drug sensitivity by carrying out toxicity assays using the acid phosphatase method.

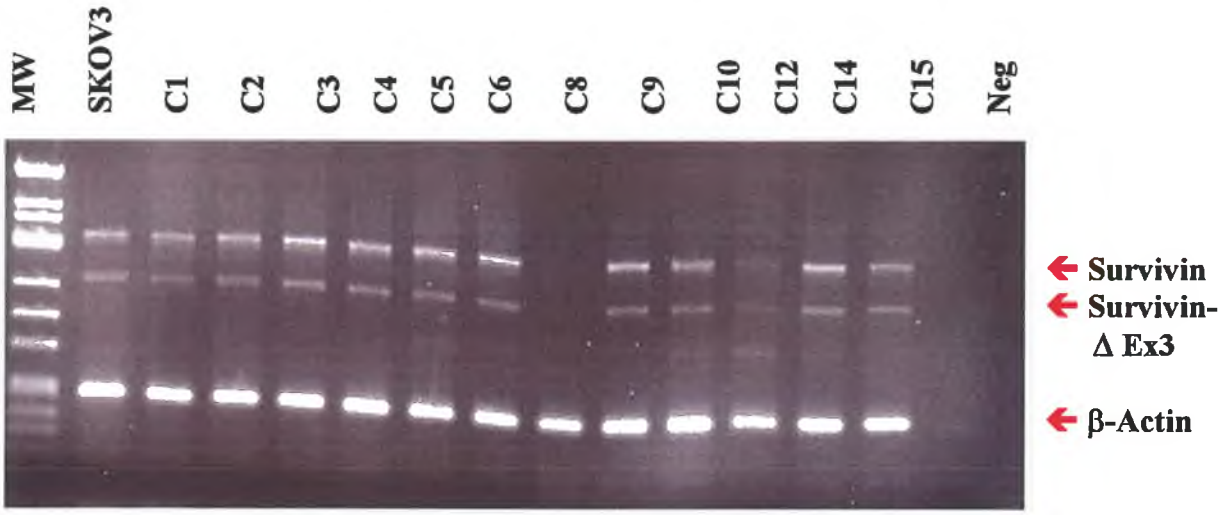
3.2.3.1.1 Analysis of SKOV3-T.O.- survivin clones using RT-PCR

RNA was extracted from the SKOV3-'T.O.' survivin clones and the mixed population, and RT-PCR was carried out using the same survivin primers as mentioned in section 3.2.2.1. The same β-actin primers as used in section 3.2.2.1 were also used. Results from the gel electrophoresis and the densitometric analysis show that the SKOV3-survivin clones fail to over-express survivin mRNA (Fig 3.2.3.1.1A and B).

In addition, clones 2 and 5 and the mixed population were grown in the presence and absence of doxocycline and RT-PCR was carried out on them in their "off" and "on" states, respectively. This, however, seemed to have little or no effect on their expression of survivin mRNA (Fig. 3.2.3.1.1C and D).

As previously mentioned all RT-PCRs were carried out in triplicate. The gel shown in Figure 3.2.3.1.1 is not, however, a good representation of the repeat experiments for clone 8 (C8). Survivin mRNA expression was not always found to be down-regulated in this clone. Results from the repeat experiments taken together suggest no change in survivin mRNA expression in any of the clones. Sterile water used as a negative control. The molecular weight marker Hinf I digest of ϕ-X174 DNA was used as a size reference.

(A)



(B)

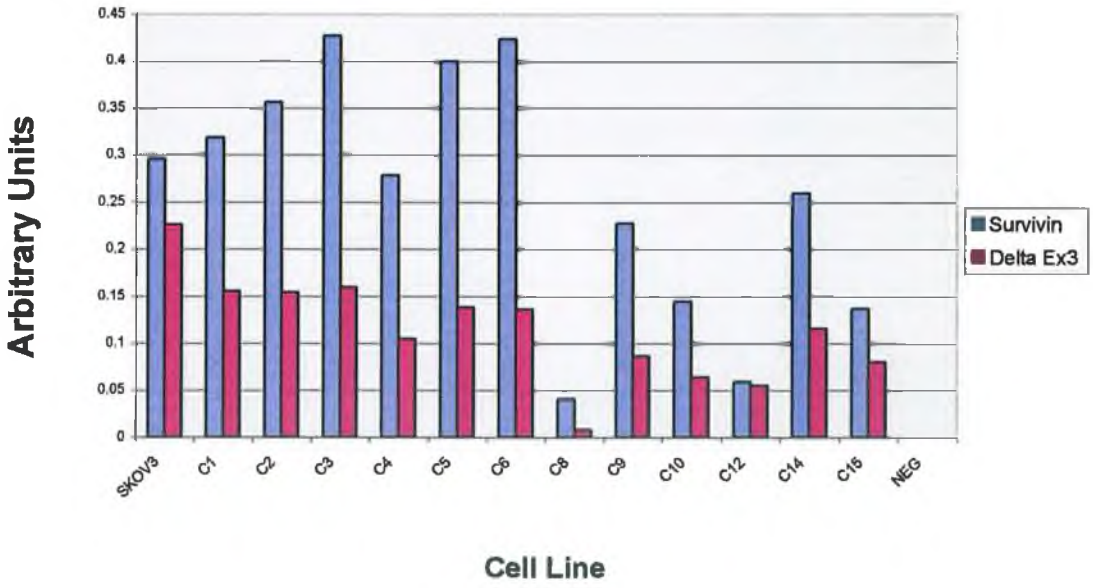
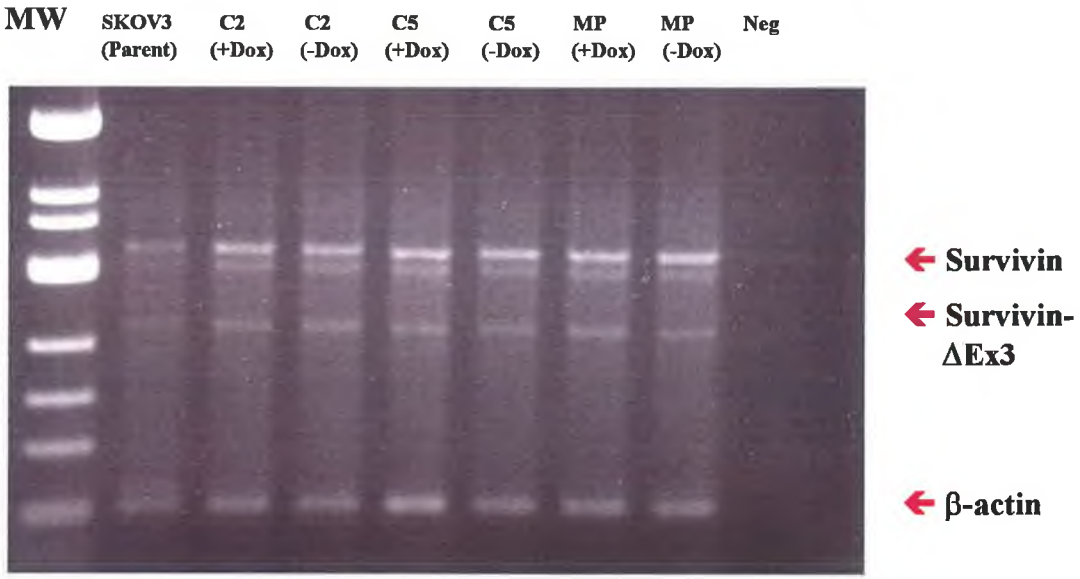


Fig. 3.2.3.1.1 **(A)** Gel electrophoresis photograph of survivin RT-PCR results on SKOV3-T.O. survivin cDNA clones. Neg=negative control and MW=molecular weight marker; **(B)** Densitometric analysis of RT-PCR results (n=3)

(C)



(D)

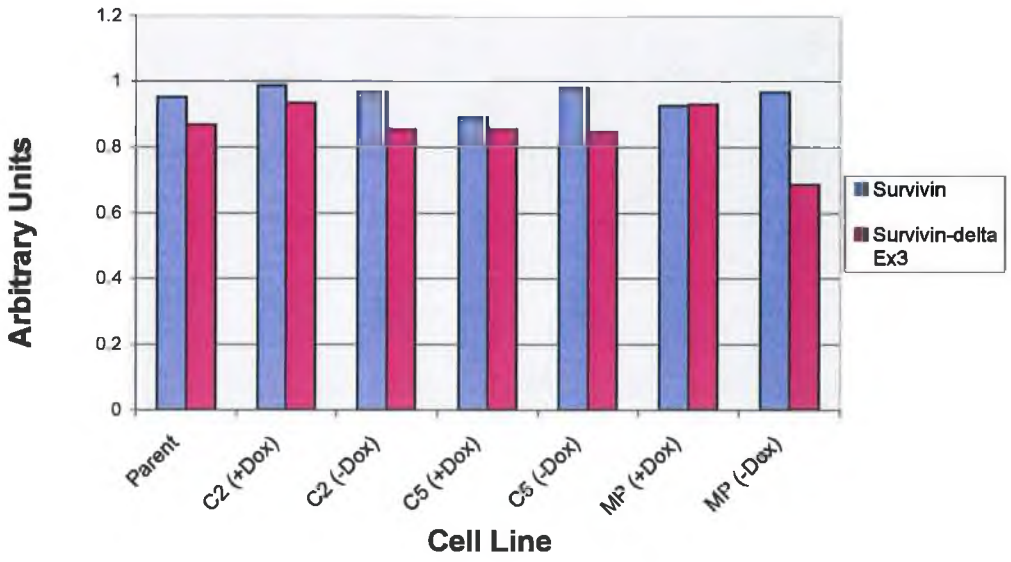


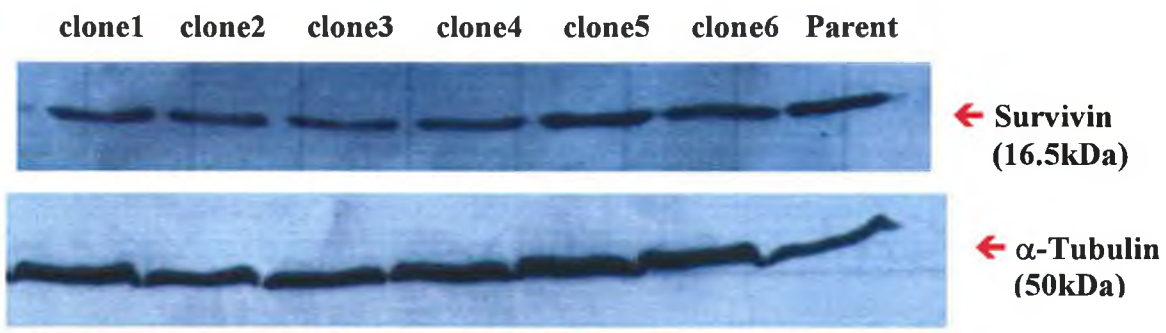
Fig. 3.2.3.1.1 **(C)** Gel electrophoresis of survivin RT-PCR results on SKOV3-T.O. survivin clone 2, clones 5 and the mixed population in the presence and absence of doxocycline. Neg=negative control and MW=molecular weight marker; **(D)** Densitometric analysis of RT-PCR results (n=3).

3.2.3.1.2 Analysis of SKOV3-T.O.- survivin clones using Western Blot

Protein was isolated from the clones and the parent cell line, SKOV-3 'T.O' (see section 2.4.1.1), and Western blot was carried out using the same survivin primary antibody as in sections 3.2.1.2 and 3.2.2.2. The clones failed to increase in survivin protein levels and the results were not very reproducible. Figures 3.2.3.1.2A and B show the results of the survivin western blot, in addition to the α-tubulin western blot used to ensure equal protein loading.

As in section 3.2.3.1.1, clones 2 and 5 and the mixed population were grown in the presence and absence of doxocycline and western blotting was carried out on them in their "off" and "on" states, respectively. Similar to the RT-PCR results, this didn't seem to effect their expression of survivin protein. α-tubulin was used as an endogenous control to ensure equal protein loading and densitometry was carried out by normalising the survivin bands to the α-tubulin bands (Fig. 3.2.3.1.2C). Western blots for all SKOV-3 'T.O' clones were carried out in duplicate and both experiments showed no survivin protein over-expression.

(A)



(B)

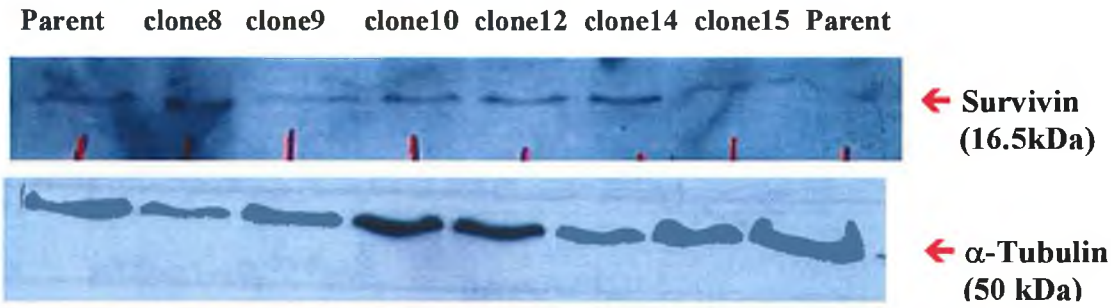
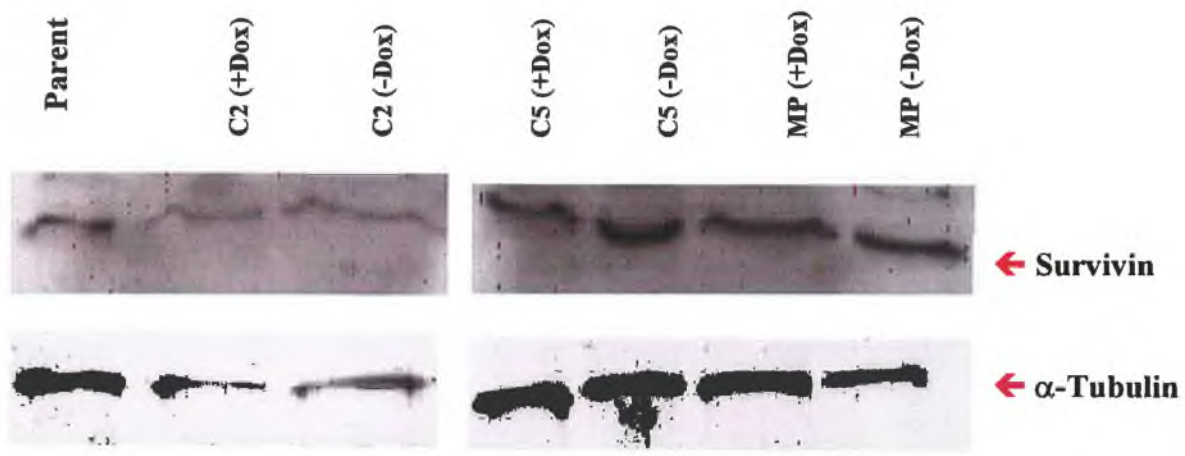


Fig. 3.2.3.1.2 **(A)** Survivin and α-tubulin Western blot analysis of SKOV3-T.O. survivin clones 1-6; **(B)** Survivin Western blot of SKOV3-T.O. survivin clones 8-15 (n=2).

(C)



(D)

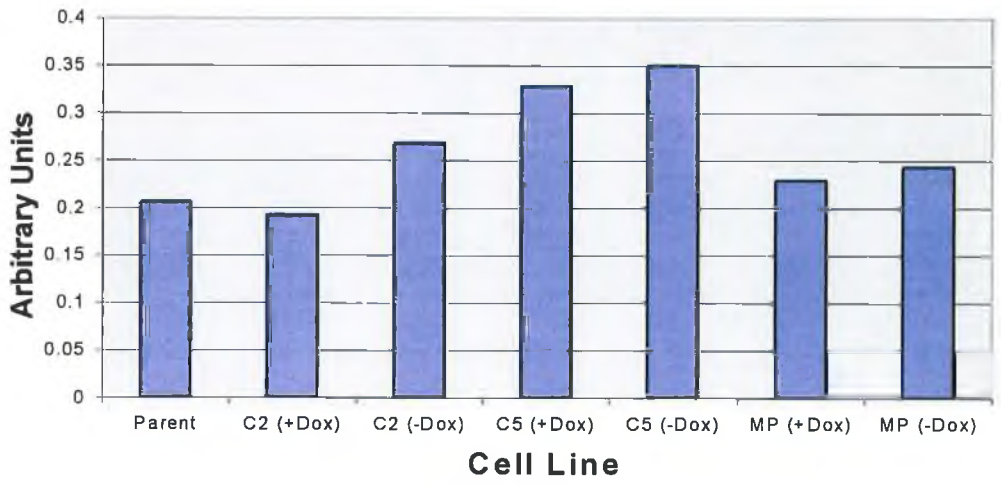


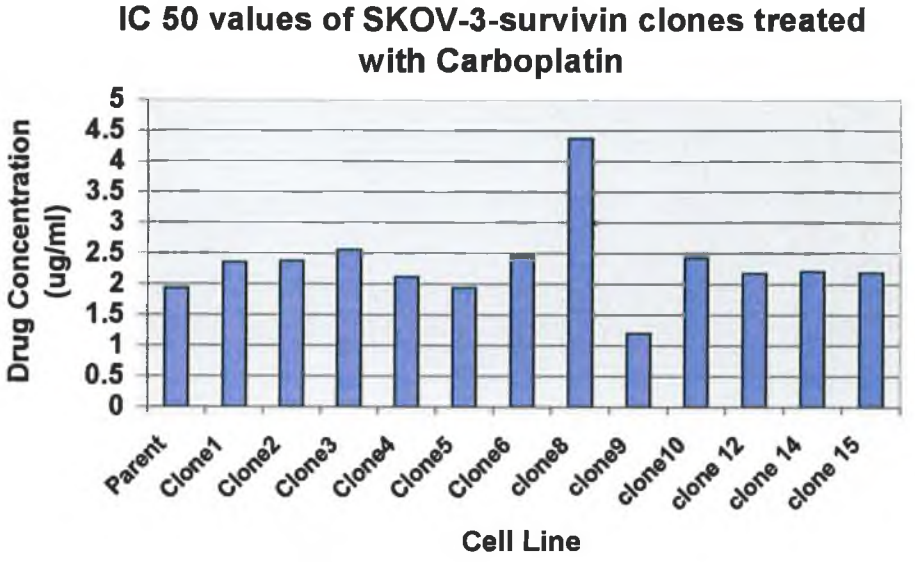
Fig. 3.2.3.1.2 **(C)** Survivin and α-tubulin Western blot analysis of SKOV3-T.O. survivin clone 2, clones 5 and the mixed population in the presence and absence of doxocycline; **(D)** Densitometric analysis of Western blot (n=2).

3.2.3.1.3 Analysis of SKOV3-survivin cDNA transfectants using *in vitro* toxicity testing

The sensitivity of the survivin transfectants to carboplatin and taxol was determined and compared to SKOV3-'T.O.' parent cell line drug sensitivity. As in section 3.2.2.3, the cells were exposed to the drugs over a range of concentrations, which included the IC_{50} values for each drug for the SKOV3-'T.O.' cell line. At the end of the 5-7 day toxicity assay, the cells were analysed using the acid-phosphatase assay. This was carried out on all clones in the absence of doxocylcine (i.e. in their "on" state), as shown in Figures 3.2.3.1.3 A and B, and on two clones and the mixed population in both the presence and absence of doxocyline (i.e. in their "off" and "on" states, respectively) (Figures 3.2.3.1.3 C and D).

With the exception of the resistance of clone 8 to carboplatin (Fig 3.2.3.1.3A), the remaining survivin clones failed to show any increase in resistance to either carboplatin or taxol when compared to the parent cell line (Fig. 3.2.3.1.3A and B). However, there seemed to be a slight increase in resistance to carboplatin between the "on" and "off" states of clone 5 (C5) (Fig. 3.2.3.1.3C) and to taxol in clone 2 (C2) and clone 5 (C5) (Fig 3.2.3.1.3D). The results are a mean of IC_{50} values taken from two experiments.

(A)



(B)

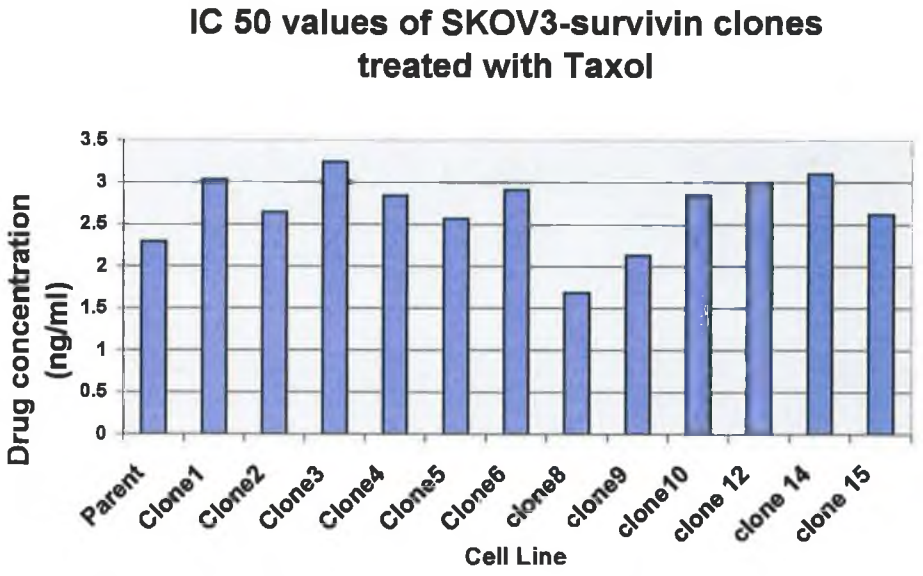
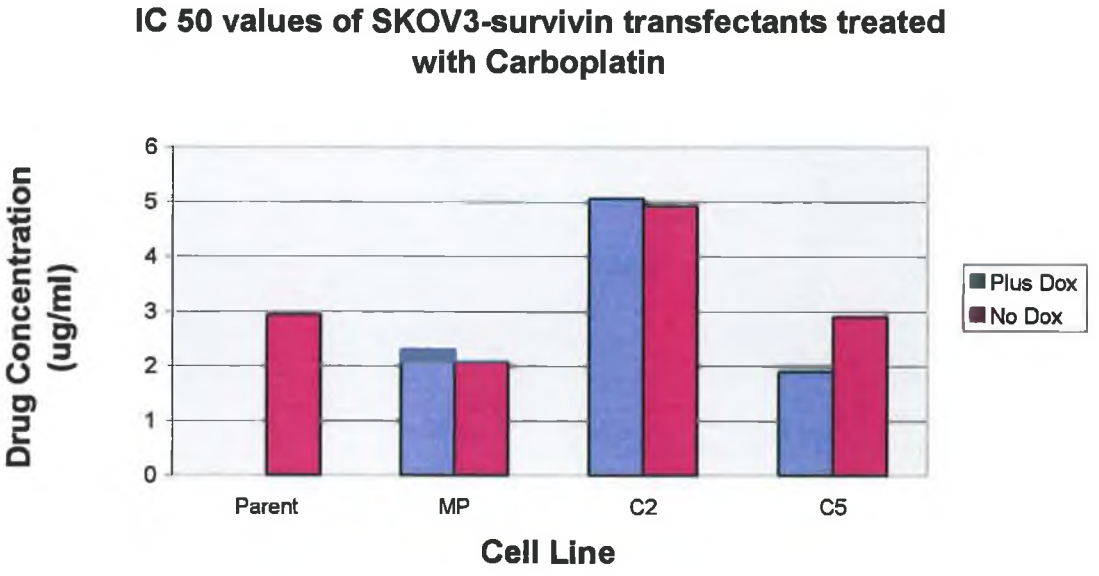


Fig. 3.2.3.1.3 **(A)** Effect of survivin on Carboplatin resistance in SKOV3-survivin cDNA clones; **(B)** Effect of survivin on Taxol resistance in SKOV3-survivin cDNA clones (n=1).

(C)



(D)

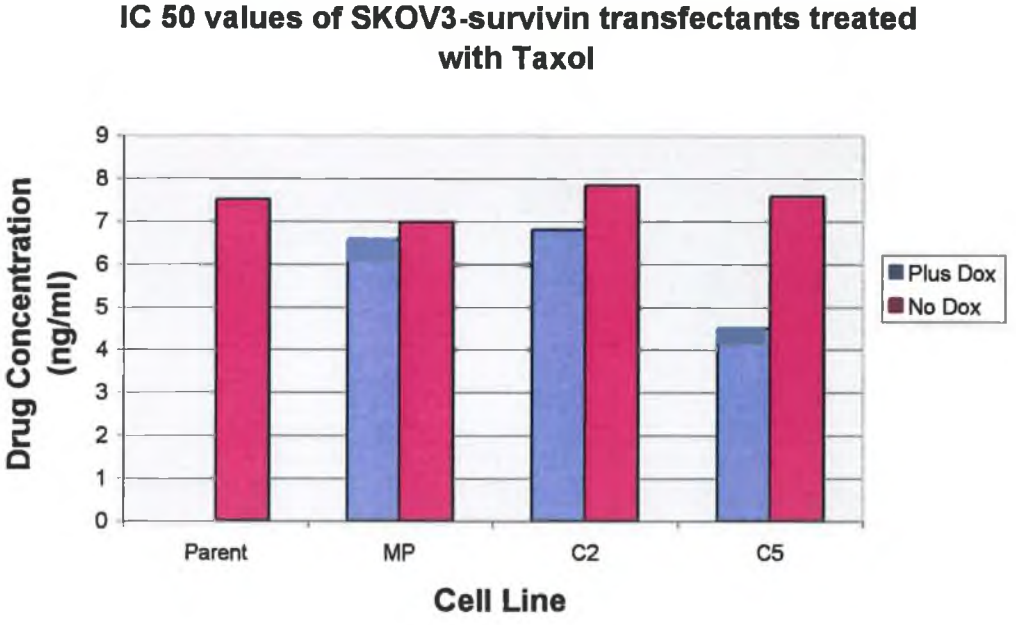


Fig. 3.2.3.1.3 **(C)** Effect of survivin on Carboplatin resistance in SKOV3-survivin cDNA clones 2 and 5 and the mixed population in the presence and absence of doxocycline; **(D)** Effect of survivin on Taxol resistance in SKOV3-survivin cDNA clones 2 and 5 and the mixed population in the presence and absence of doxocycline. (n=2).

3.2.4 SKOV-3 Transient Transfection

As described in sections 3.2.2 and 3.2.3, two separate stable transfections of survivin cDNA into two cell lines, DLKP and SKOV3-'T.O.', were carried out and both failed to show over-expression of survivin mRNA or protein. Also neither transfection rendered the cells more resistant to chemotherapeutic drugs. This led to the belief that perhaps there is a mechanism within cells whereby the level of survivin expression is kept constant at all times, making it impossible to stably over-express, or down-regulate, survivin expression.

Transient transfections were then carried out to try over-expressing survivin in SKOV3-T.O. cells over a short period of time.

3.2.4.1. Transfection efficiency

A transfection efficiency experiment was set up first to check the percentage transfection efficiency of the SKOV-3 cell line. As described in section 2.4.5.3, the PCH110 plasmid which codes for β-galactosidase activity was transfected into SKOV3-'T.O.' cells. The cells were stained at 6 and 24 hours post-transfection. Successfully transfected cells were stained blue/green as shown in figure 3.2.4.1A.

SKOV3-T.O. was found to have a 30% transfection efficiency. Cells were set up at 2 different concentrations: $1x10^5$ and $1x10^2$ in 2ml of media. Example results are shown in Figure 3.2.4.1 for concentration of $1x10^5$. This experiment was carried out in duplicate.

(A)

(i) (ii)

(B)

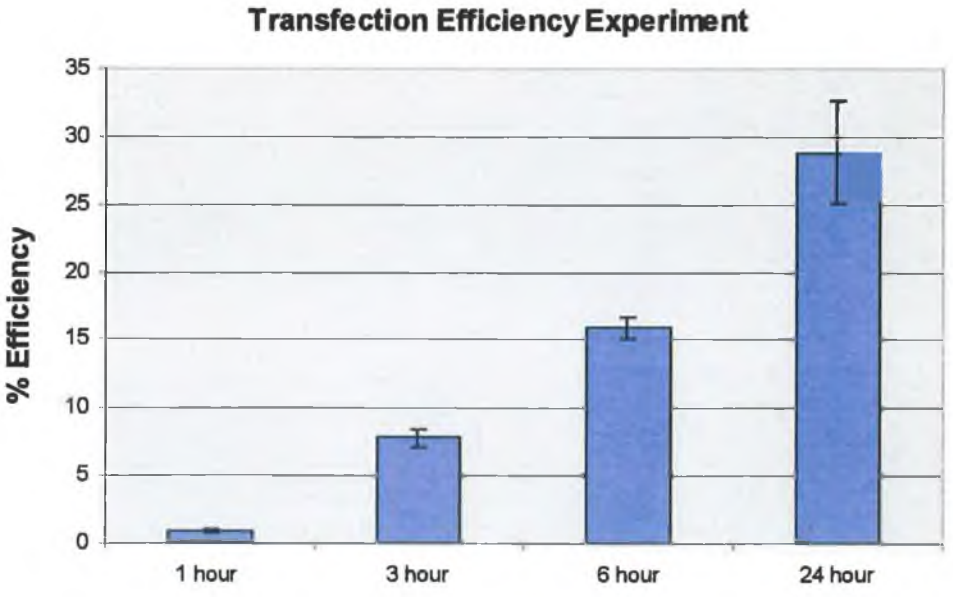


Fig. 3.2.4.1 **(A)** Cells stained with X-gal β-galactosidase (i) 6 hours and (ii) 24 hours after transfection; **(B)** β-galactosidase staining increasing with incubation time. (n=2).

3.2.4.2 Transient Transfection of Survivin cDNA into SKOV-3 'Tet off' cells

Once the transfection efficiency had been determined and was considered to be of a sufficiently high percentage, transient transfections were carried out using Fugene 6 as a transfection agent at the ratio of 3:2 of fugene to DNA (see section 2.4.5.4), as mentioned in section 3.2.3.

Cells were set up in $25cm^2$ flasks at a concentration of $1.5x10^5$ cells/ml, transfected with cDNA, or the empty pTRE vector, and taken down at several time points for analysis by RT-PCR and Western blot analysis. These time points were: 24 hours, 48 hours, 72 hours and 5 days after the start of transfection. Furthermore, toxicity assays were carried out using taxol and carboplatin. Immunofluorescence and invasion assays were also carried out on the transient transfections of survivin cDNA.

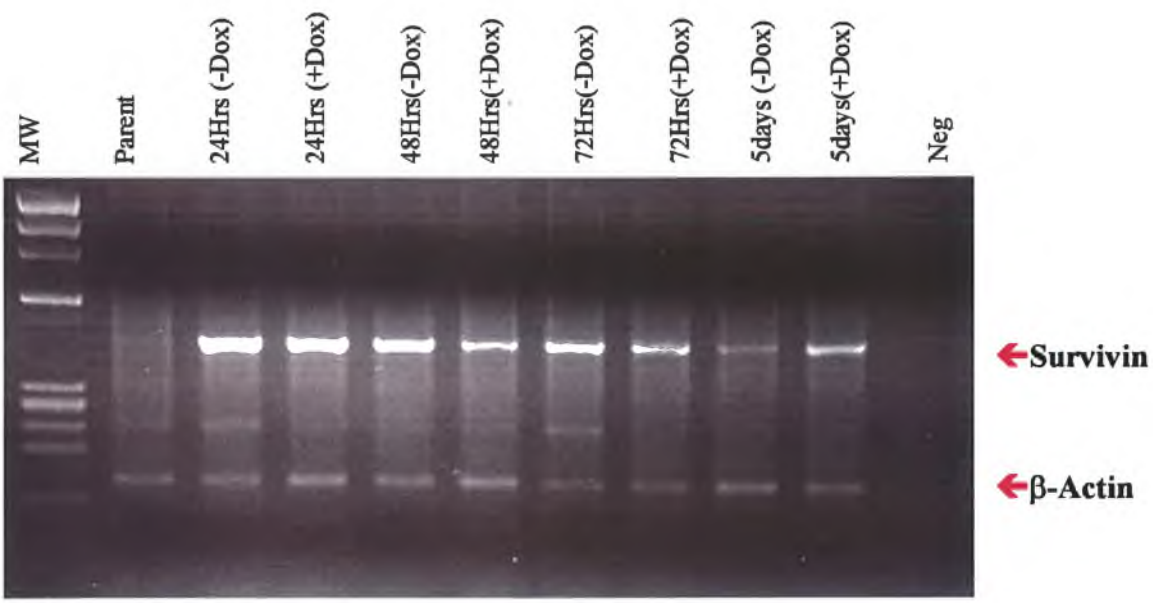
3.2.4.2.1 Analysis of SKOV3-T.O. Survivin cDNA transfectants using RT-PCR

Cells were harvested and RT-PCR was carried out on the SKOV3-'T.O.' survivin cDNA transfections at the time points mentioned above. Cells were treated with dox. and compared to untreated cells. Results showed that at 24 hours after transfection, in the absence of dox., survivin mRNA expression had increased by approximately 18 fold (see Figure 3.2.4.2.1A and B). In the presence of dox., the increase detected was approximately 9-fold, suggesting that there was a 50 % inhibition of survivin mRNA expression when the expression was turned off. At 48 hours after transfection, the increase in survivin mRNA expression in the absence of dox., was approximately 8-fold. Similarly the inhibition of expression was 50 % in the presence of dox. The difference in expression between the 'on' and 'off' states is not as obvious at 72 hours and at 5 days the effect of the transfection disappears as survivin mRNA returns to almost basal level. The effect of turning expression 'off' at the 5 day time-point seems to be reversed in all experiments carried out. Survivin mRNA expression seems higher in the 'off' state than in the 'on' state at the 5 day time-point (Fig. 3.2.4.2.1A and B).

To ensure that this finding was due to survivin cDNA transfection, a control transient transfection was carried out using the pTRE empty plasmid. Results showed a constant level of expression of survivin mRNA, with no over-expression (Fig. 3.2.4.2.1C and D), proving that the effect seen with the cDNA transfection is a real effect. β-actin was used as an endogenous control and densitometry was carried out on the results by normalising the survivin bands to the β-actin bands (Figure 3.2.4.1B and D). As with most RT-PCRs presented, sterile water was used as a negative control and RT-PCRs were carried out in triplicate and found to be reproducible. The molecular weight marker Hinf I digest of ϕ-X174 DNA was used as a size reference.

The PCR presented in Figure 3.2.4.2.1A was carried out at 18 cycles to avoid saturation of the product bands due to the dramatic over-expression of survivin mRNA at 24 hours. This, therefore, resulted in a weak survivin band for the parental cell line. The PCR in Figure 3.2.4.2.1C, however, was carried out at 30 cycles because there was no risk of saturation of the product bands as survivin mRNA was not being over-expressed in the control transfection. This resulted in a strong survivin band for the parental cell line as well as the three time-points. RT-PCR analysis should be repeated for the survivin cDNA transfections and the pTRE mock transfections using the same cycle number. At present it is not known if the expression of survivin-Ex3 in the mock transfection (Figure 3.2.4.2.1C), but not in the survivin cDNA transfection (Figure 3.2.4.2.1A), was due to differences in cycle number or due to different transfected plasmid.

(A)



(B)

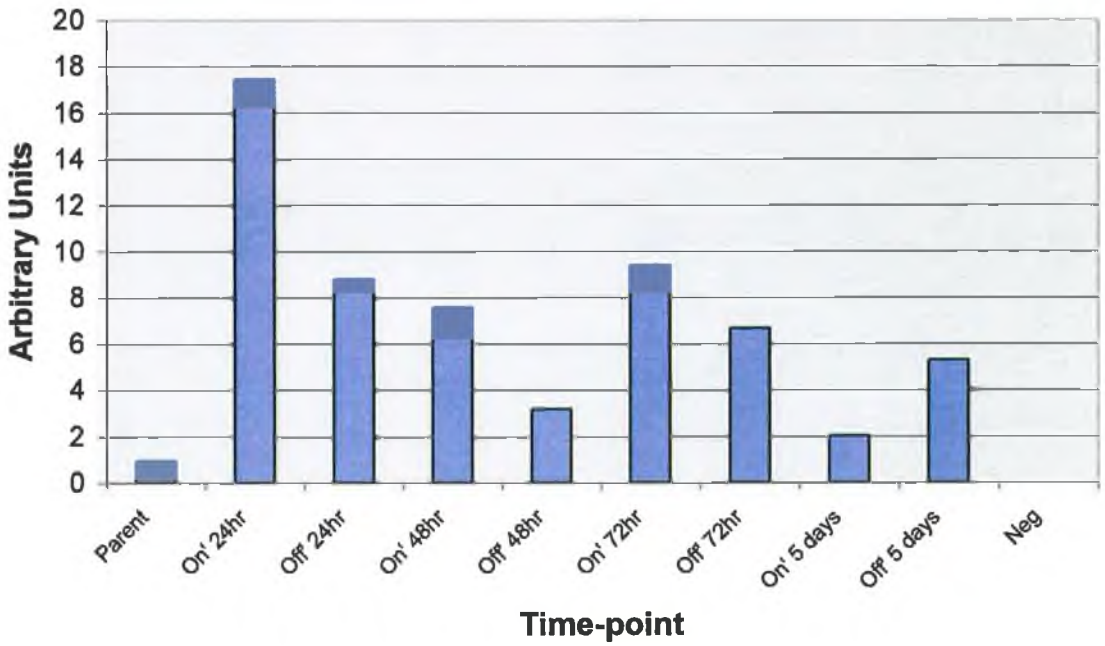
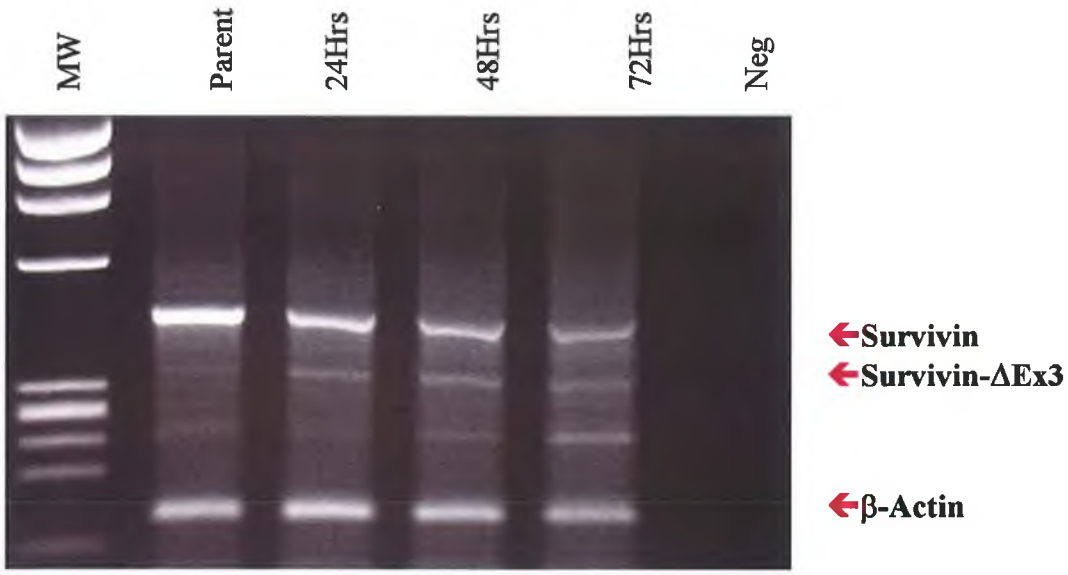


Fig. 3.2.4.2.1 **(A)** Gel electrophoresis photograph of survivin RT-PCR results on SKOV3-T.O. survivin cDNA transient transfection at 24,48,72hrs and 5day time points in the presence and absence of doxocycline. Neg=negative control and MW=molecular weight marker; **(B)** Densitometric analysis of the RT-PCR results (n=3).

(C)



(D)

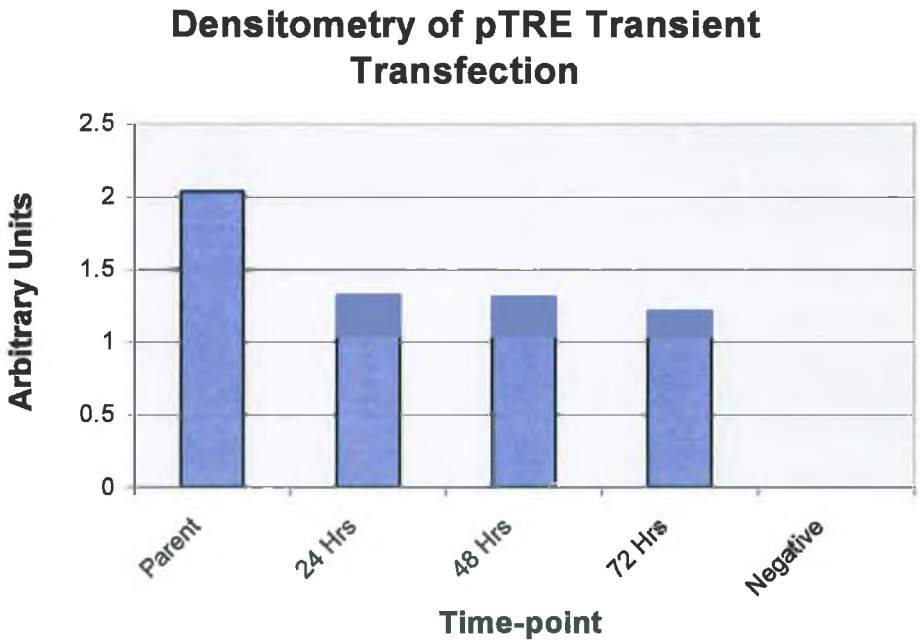


Fig. 3.2.4.2.1 **(C)** Gel electrophoresis photograph of survivin RT-PCR results on SKOV3-T.O. pTRE mock transfection at 24, 48 and 72 hour time points. Neg=negative control and MW=molecular weight marker; **(D)** Densitometric analysis of the RT-PCR results (n=3).

3.2.4.2.2 Analysis of SKOV3-T.O. Survivin cDNA transient transfectants using Western blotting

Cells were harvested, protein was extracted and western blotting was carried out (see section 2.4.1) on the SKOV3-'T.O.' survivin cDNA transfections 24hr, 48hr, 72hr and 5 days after transfection. No change in survivin protein expression as observed at any of the time-points. Transfections experiments were repeated more than three times and Western blots were carried using a range of protein concentrations. These included 10μg, 20μg and 30 μg of protein, but there was still no change in survivin protein expression (Figure 3.2.4.2.2 A, B and C). At a concentration of 30 μg protein, there seems to be an increase in survivin protein at 48 hours post-transfection (Figure 3.2.4.2.2C). This was not reproducible and was not, therefore, considered to be a 'real' result. α-Tubulin was used as an endogenous control to ensure equal protein loading. Each western blot was carried out in duplicate for each protein concentration.

Western blot analysis was also included on cells treated with dox. Results, however, showed no increase in survivin protein expression at any of the four time points. There was also no difference between cells plus or minus dox. (Figure 3.2.4.2.2D). α-Tubulin western blot analysis was carried out to ensure equal protein loading and densitometry was carried out on this western blot by normalising the survivin bands to the α-Tubulin bands (Figure 3.2.4.2.2E). Western blot analysis was carried out in duplicate.

In addition, western blot analysis was carried out on a control transfection with the empty pTRE plasmid and there was no change in survivin protein expression (Figure 3.2.4.2.2F). α-Tubulin was used as an endogenous control to ensure equal protein loading. Western blot for the control transfection was carried out once.

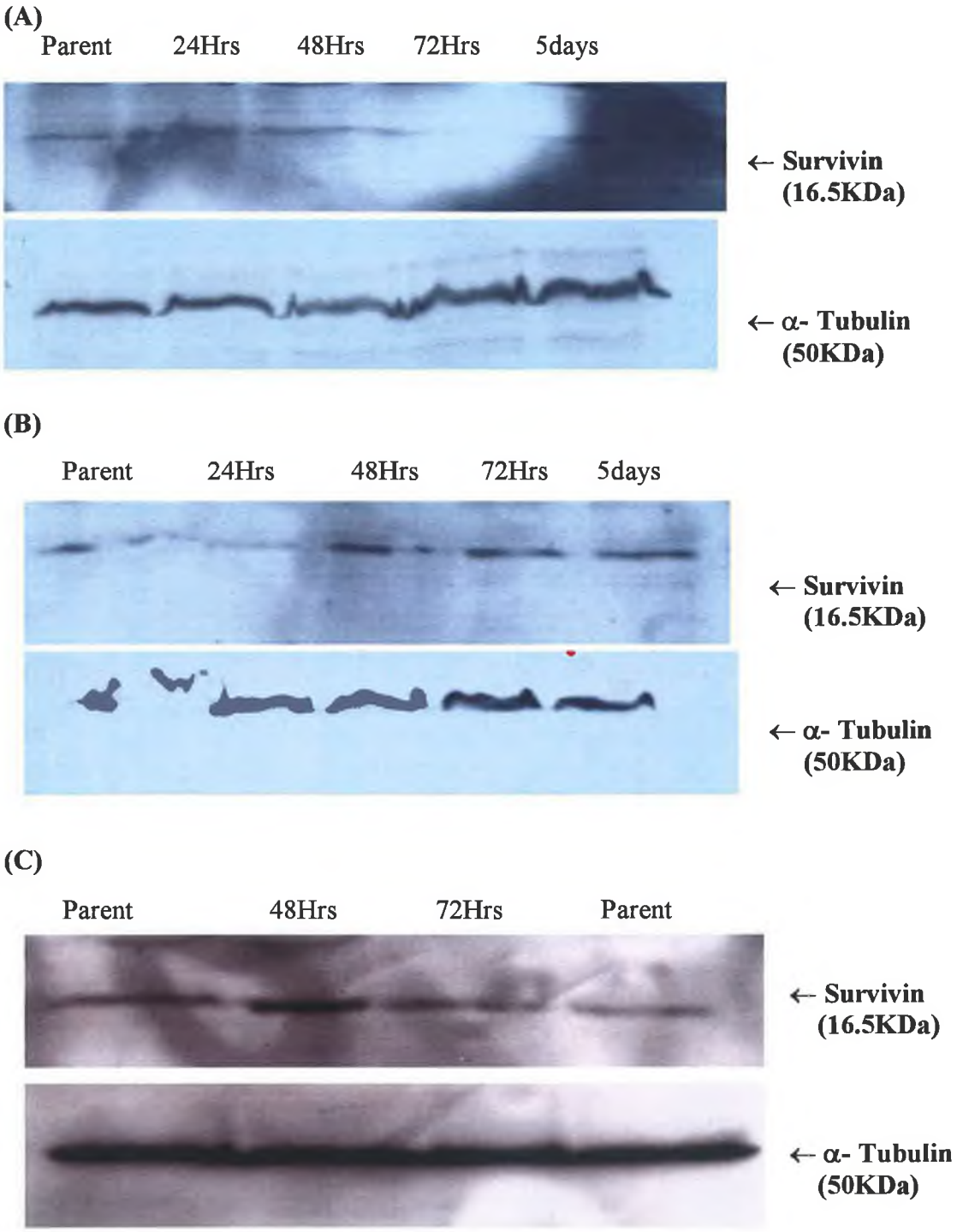
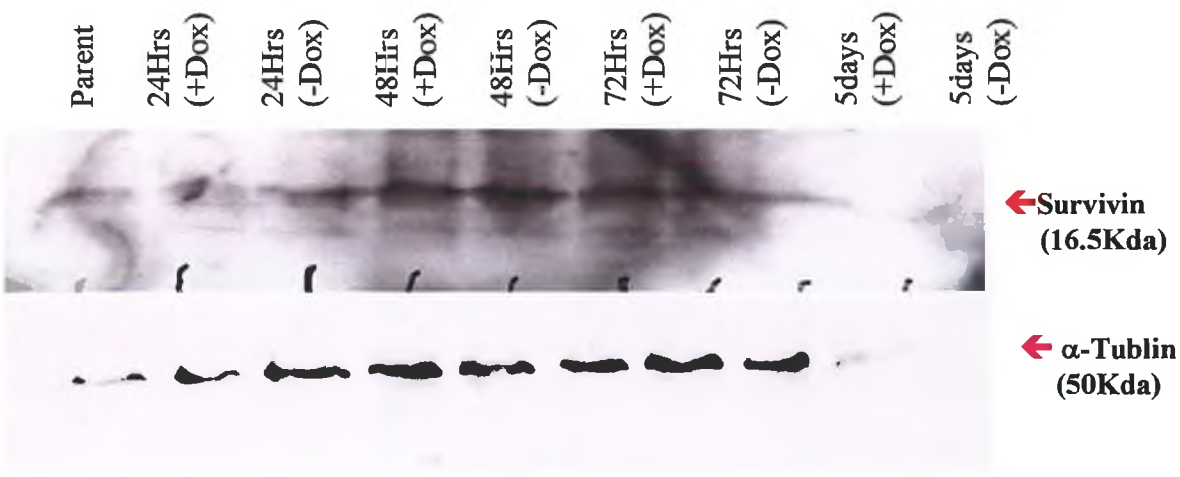


Fig. 3.2.4.2.2 **(A)** Survivin and α-Tubulin western blot on SKOV3-T.O. survivin cDNA transient transfection at 24,48,72hrs and 5days at a conc. of 10μg protein; **(B)** Survivin Western blot on SKOV3-T.O. survivin cDNA transfection at 24,48,72hrs and 5days at a conc. of 20μg protein; **(C)** Survivin Western blot on SKOV3-T.O. survivin cDNA transfection at 48,72hrs and 5days at a conc. of 30μg protein (n=2).

(D)



(E)

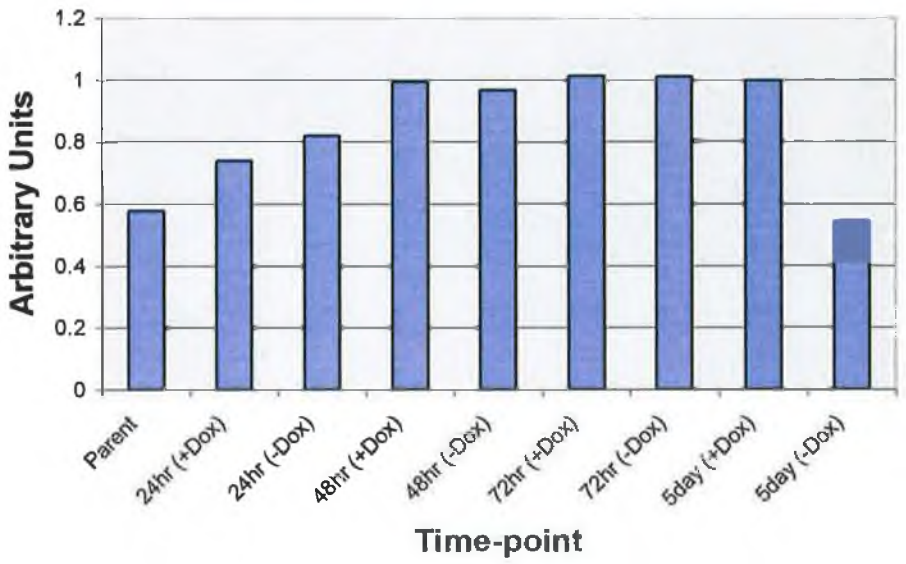


Fig. 3.2.4.2.2 **(D)** Survivin and α-Tubulin western blot on SKOV3-T.O. survivin cDNA transfection at 24,48,72hrs and 5day time points in the presence and absence of doxocycline; **(E)** Densitometric analysis of the Western blot results (n=2).

(F)

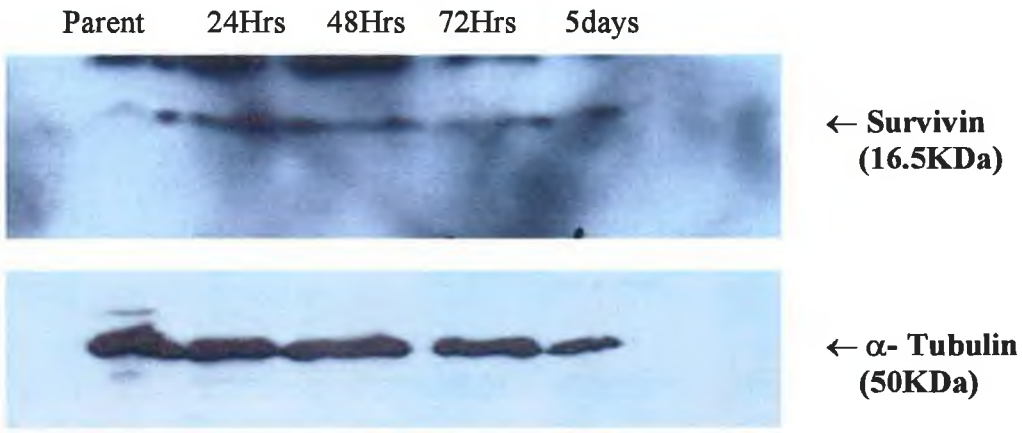
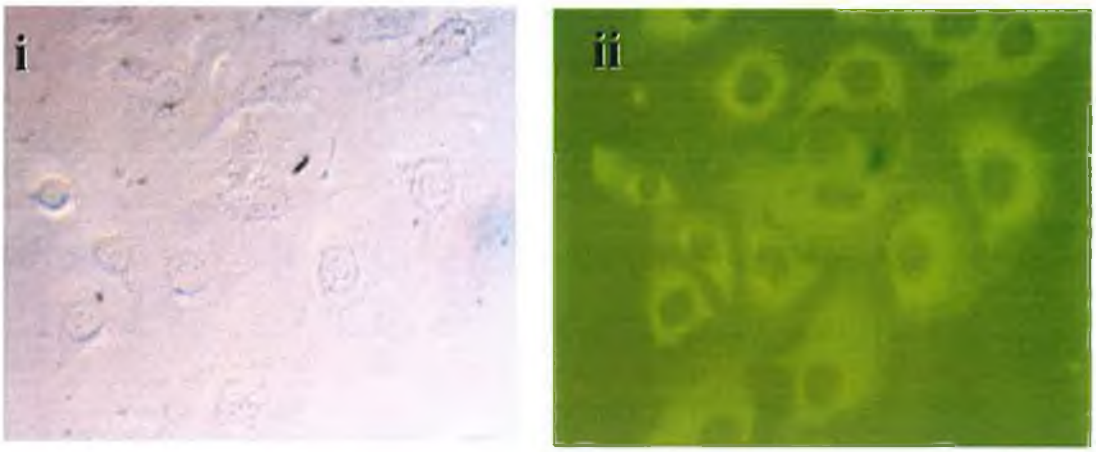


Fig. 3.2.4.2.2(F) Survivin and α-Tubulin western blot on SKOV3-'T.O.' pTRE transfection at 24,48,72hrs and 5day time-points. (n=1).

3.2.4.2.3 Analysis of SKOV3-T.O. Survivin cDNA transient transfectants using Immunofluorescence

Further analysis of the SKOV3-'T.O.' survivin cDNA transient transfections for protein expression included immunofluorescence (see section 2.4.2.2). Cells were seeded in 6-well plates, transfected, and after 48 hours were fixed with methanol and analysed by immunofluorescence. A control transfection was also carried out. Results showed survivin protein expression in the cells' cytoplasm following the survivin cDNA transfection and the pTRE transfection (Fig. 3.2.4.2.3). In addition, the staining was of similar intensity in both transfections. Therefore there was apparently no survivin protein over-expression, in correlation with the results obtained by western blot analysis. This experiment was carried out once.

(A)



(B)

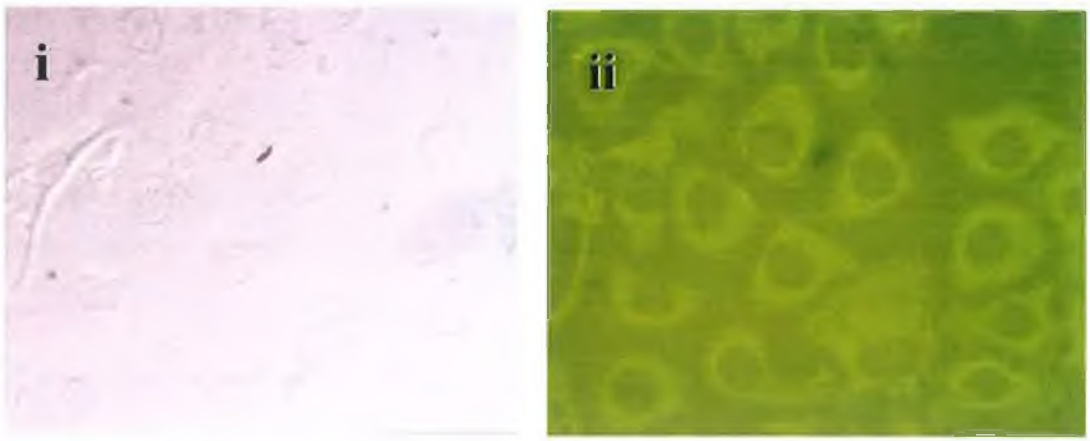


Fig. 3.2.4.2.3 **(A)** Immunofluorescence on SKOV3-'T.O.' survivin cDNA transient transfection under (i) white light and (ii) fluorescent light; **(B)** Immunofluorescence on SKOV3-T.O. pTRE control transfection under (i) white light and (ii) fluorescent light (n=1).

3.2.4.2.4 Analysis of SKOV3-T.O. survivin cDNA transient transfections using in vitro toxicity testing

The sensitivity of the survivin transient transfectants to taxol and cisplatin was determined and compared to SKOV3-T.O. parent cell line drug sensitivity, using *in vitro* toxicity assays (section 2.3.1).
Cells were transfected in 96-well plates, and drug was added to the plates at 0, 24, 48 and 72 hours after transfection. Cells were exposed to the drugs for 5-7 days, and were then analysed using the acid-phosphatase assay. This was carried out on transfected cells plus and minus dox. and on pTRE-transfected cells. Control plates are plates without any plasmid or transfection agent.

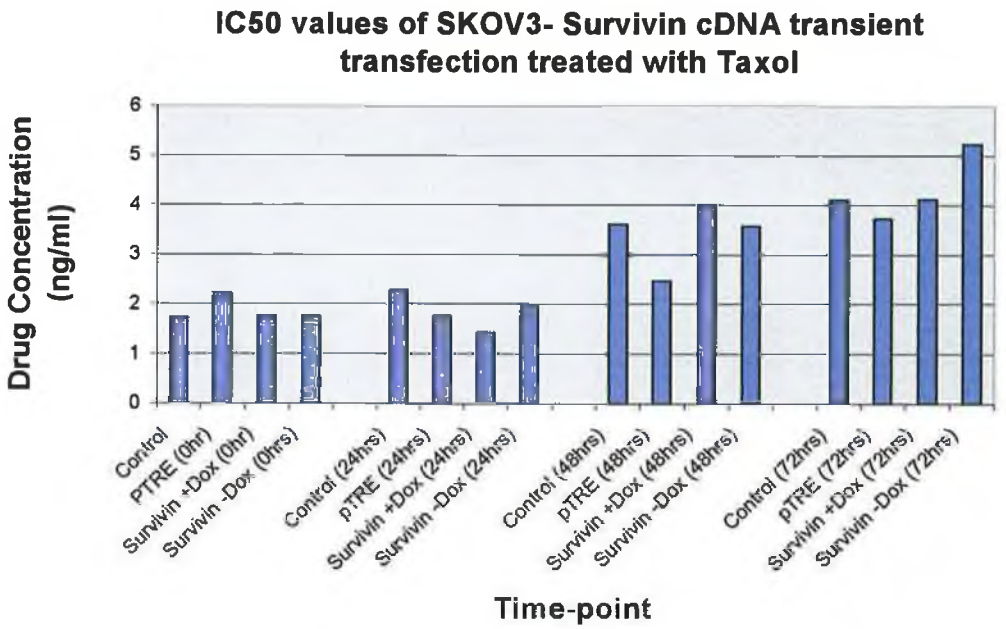
The results showed no increase in the IC_{50} values of the survivin cDNA transfected cells compared to pTRE transfected cells or non-transfected cells to taxol and cisplatin. There was also no difference between the IC_{50} values of cells transfected in the presence and absence of dox. as shown in Figure 3.2.4.2.4. Toxicity assays were carried out in duplicate.

Therefore, survivin cDNA transient transfectants are not more resistant to chemotherapy drugs, taxol and cisplatin, than the parental population.

3.2.4.2.5 Analysis of SKOV3-T.O. survivin cDNA transient transfections using TUNEL assay

TUNEL assay was carried out on the SKOV3-T.O. parental cells and SKOV3-'T.O.'-survivin cDNA (see section 2.3.2.1) to determine whether or not the transfected cells had developed resistance to apoptosis. Taxol was used to induce apoptosis in the cells for 48 hours, after which the cells were harvested and TUNEL technique was carried out as described in section 2.3.21. Slides were analysed using a fluorescence microscope. The results were difficult to analyse, due to non-specific labeling of the dividing cells. However, there was no apparent difference in viability between the parental cells and the transfected cells (Figure 3.2.4.2.5). The technique was difficult to optimise and was carried out successfully once.

(A)



(B)

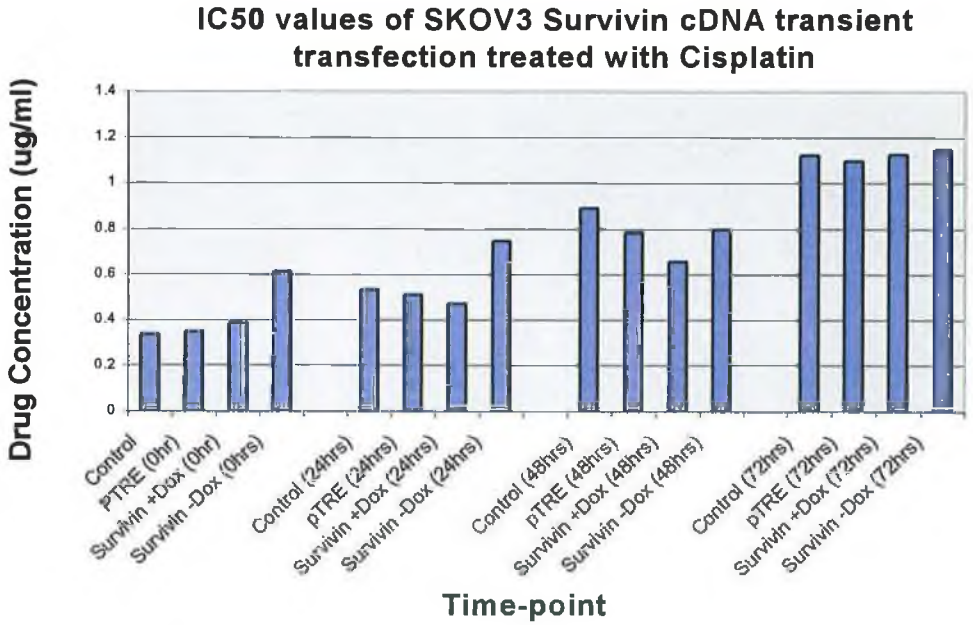


Fig. 3.2.4.2.4 **(A)** Effect of survivin on taxol resistance in SKOV3-survivin cDNA transfectants; **(B)** Effect of survivin on cisplatin resistance in SKOV3-survivin cDNA transfectants (n=2).

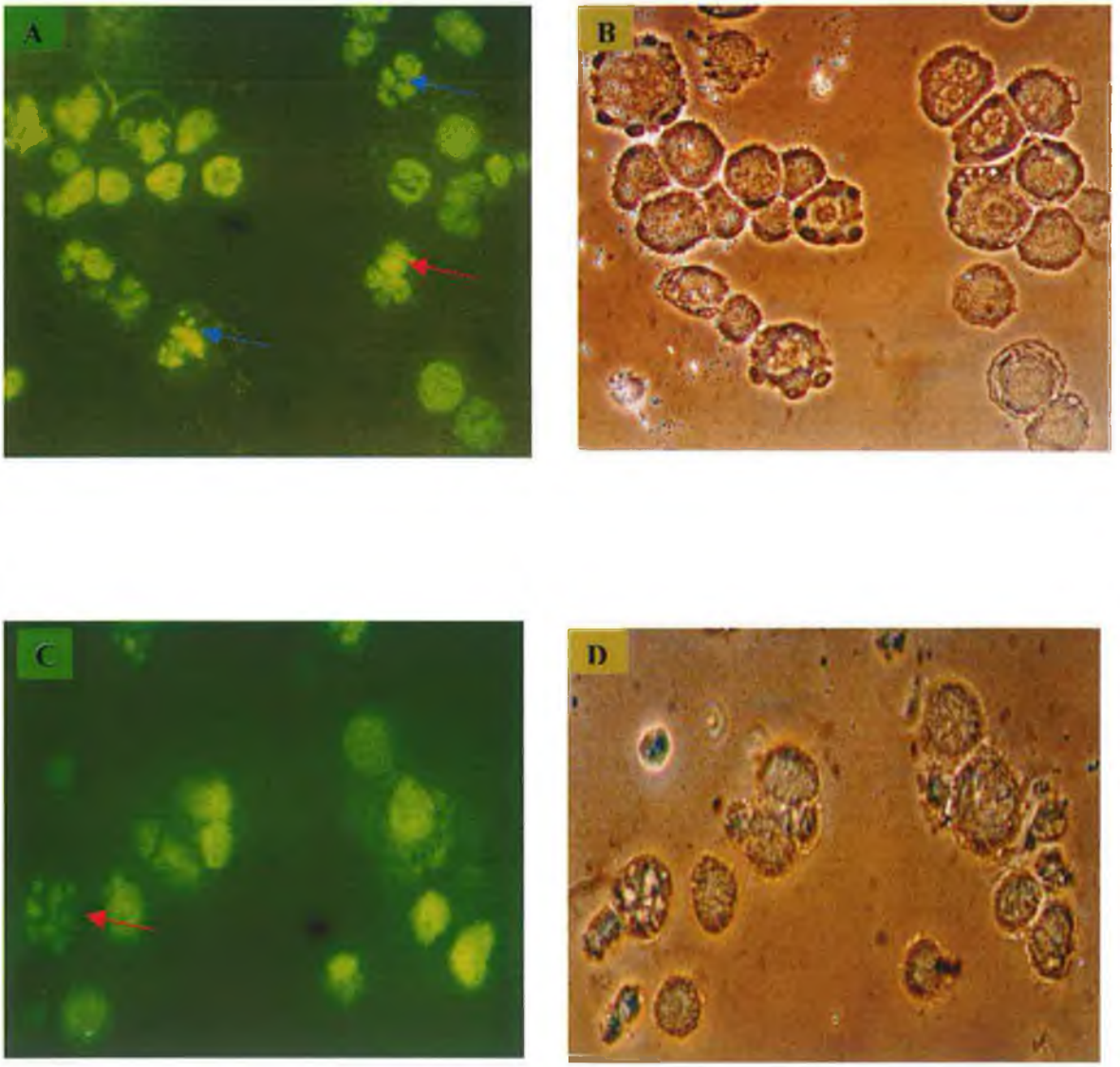


Figure 3.2.4.2.5 **(A)** SKOV3 'T.O.' parent cells under fluorescent light (blue arrows indicate dividing cells, red arrow indicates apoptotic cell); **(B)** SKOV3 'T.O.' parent cells under white light; **(C)** SKOV3 'T.O'-survivin cDNA under fluorescent light; **(D)** SKOV3 'T.O'-survivin cDNA under white light. (n=1)

3.2.4.2.6 Analysis of SKOV3-T.O. survivin cDNA transient transfections using *in vitro* Invasion Assays

To investigate survivin's role in invasion, cells were set up and transfected with survivin cDNA, in the presence and absence of dox., and with pTRE empty vector. Invasion assays were set up a day after transfection and cells were incubated for 48 hours before they were stained using crystal violet (see section 2.5.1).

At first there seemed to be a decease in invasiveness of cells transfected with survivin cDNA compared to cells transfected with pTRE or non-transfected cells (Fig. 3.2.4.2.6). These results, however, were not reproducible and it now seems that there is in fact no change in invasiveness.

RPMI-2650 parent cells were used a negative control, while RPMI-2650 melphalan-resistant cells were used as a positive control.

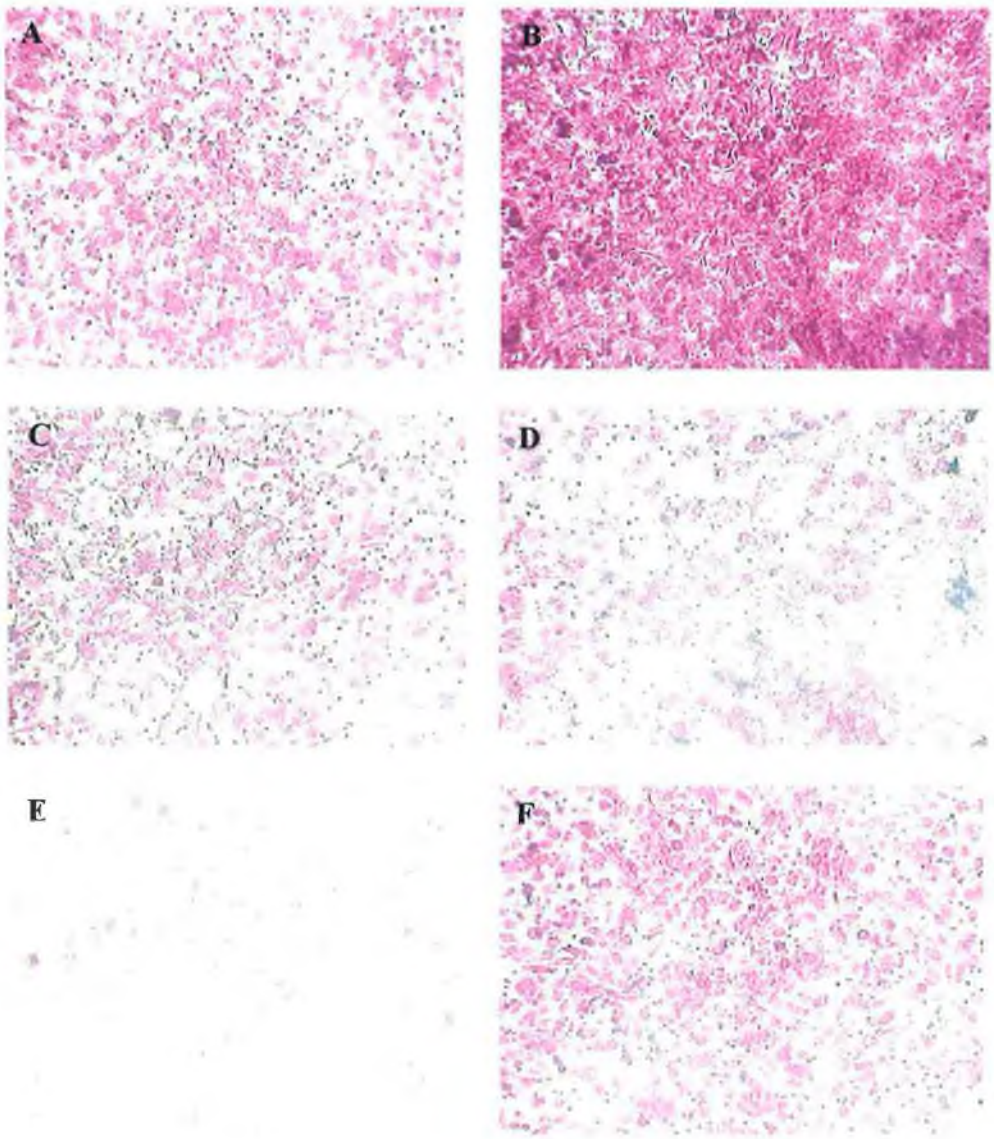


Fig. 3.2.4.2.6 Invasion assays of (A) SKOV3-T.O. parent cells; **(B)** SKOV3-T.O.- pTRE; **(C)** SKOV3-T.O.-survivin cDNA (+Dox); **(D)** SKOV3-T.O.-survivin cDNA (-Dox); **(E)** RPMI (parent); **(E)** RPMI (Melphalan) (n=3).

3.2.5 Transient Transfection of Survivin cDNA into DLKP cells

To try and determine the reason for survivin mRNA over-expression, but lack of survivin protein over-expression in SKOV-3 'T.O.' transient transfections, a second cell line was chosen to study this effect. DLKP was also transiently transfected with survivin cDNA. Cells were set up in 25cm^2 flasks as described in section 2.4.5.4, transfected with cDNA in pTarget vector, and taken down at several time points for analysis by RT-PCR and Western blot analysis. These time points were 24 hours, 48 hours, 72 hours and 5 days after the start of transfection.

3.2.5.1 Analysis of DLKP Survivin cDNA transfectants using RT-PCR

Cells were harvested and RT-PCR was carried out on the DLKP survivin cDNA transfections at the time points mentioned above. Results showed that at 24 hours after transfections, survivin mRNA expression had increased dramatically and at 48 hours after transfection, there was a slight drop in survivin mRNA expression, which continued until the 5 day time point (see Figure 3.2.5.1). β-actin was used as an endogenous control. Sterile water was used as a negative control and the molecular weight marker "ϕ-X174" Hae III digest was used as a size reference. RT-CPR analysis was carried out once.

3.2.5.2 Analysis of DLKP Survivin cDNA transient transfectants using Western blotting

Cells were harvested, protein was extracted and Western blotting was carried out on the DLKP survivin cDNA transfections at 24hr, 48hr and 72hr post-transfection. Results, however, showed no increase in survivin protein expression at any of the four time points. Similar to the results obtained from the SKOV3-'T.O.' transient transfections, the DLKP survivin cDNA transient transfection showed a significant increase in survivin mRNA expression, but no increase in survivin protein expression. α-Tubulin was used as an endogenous control to ensure equal protein loading (see Figure 3.2.5.2). This suggests that the same mechanism being used by the SKOV3-'T.O.' cells to block survivin translation is being used by DLKP cell. This experiment was carried out once.

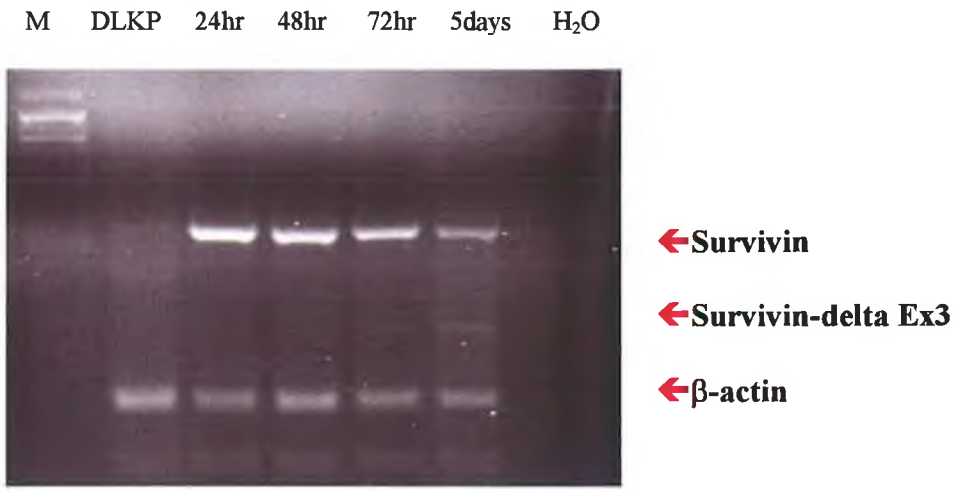


Fig. 3.2.5.1. Gel electrophoresis photograph of survivin RT-PCR results on DLKP survivin cDNA transient transfection at 24,48,72hrs and 5day time points. M=molecular weight marker. (n=1)

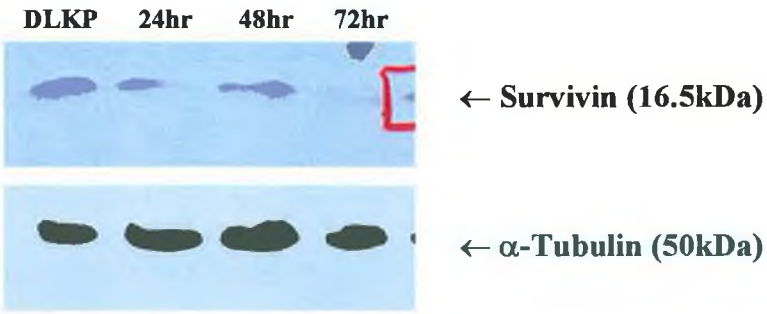


Fig. 3.2.5.2 Survivin and α-Tubulin western blot on DLKP. Survivin cDNA transfection at 24,48, and 72hrs post-transfection. (n=1).

3.2.6 Transient Transfection of Survivin cDNA into MCF-7 'Tet off' cells

A third attempt was made at transiently over-expressing the survivin protein and the cell line chosen for this was MCF-7 'Tet off'. Like SKOV-3 'Tet off', this cell line was stably transfected with the Tet-Off plasmid by Helena Joyce, M.Sc., in the NICB. As discussed in section 3.2.3, the advantage of this gene expression system is that it allows regulated mammalian gene expression with tetracycline or tetracycline derivatives, in this case, doxocycline. In the Tet-Off system, gene expression is turned on when doxocycline (Dox) is removed; in contrast to the Tet-On system, where expression is turned on by the addition of Dox.

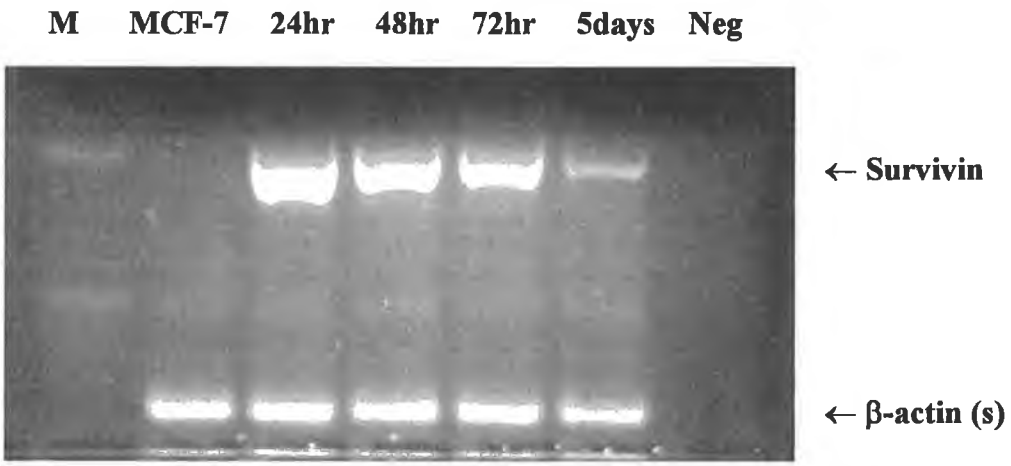
As in the previous transient transfection experiments, cells were set up in $25cm^2$ flasks, transfected with cDNA or the empty pTRE vector, and taken down at several time points for analysis by RT-PCR and Western blot analysis. These time points were: 24 hours, 48 hours, 72 hours and 5 days after the start of transfection.

3.2.6.1 Analysis of MCF-7 T.O. survivin cDNA transient transfections using RT-PCR

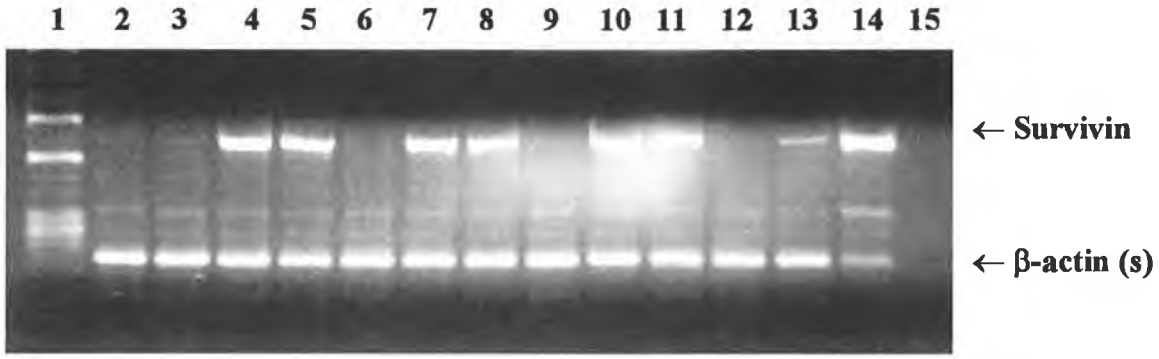
Cells were harvested and RT-PCR was carried out on the MCF-7 T.O. survivin cDNA transfections at the time points mentioned above. Results showed that at 24 hours after transfections, survivin mRNA expression had increased dramatically (Figure 3.2.6.1A). To ensure that this finding was due to survivin cDNA transfection, a mock transient transfection was carried out using the pTRE empty plasmid.

Results showed a constant level of expression of survivin mRNA, proving that the effect seen with the cDNA transfection is a real effect. The transfection was also carried out in the presence and absence of Dox, to determine whether the inducible system working in the MCF-7 cells, as it was in the SKOV3 cells. This was however, found not be the case, as RT-PCR results showed no difference in the two states of expression (i.e. in the 'on' and 'off' states) (Figure 3.2.6.1B). The molecular weight marker "ϕ-X174" Hae III digest was used as a size reference. β-actin was used as an internal control in all RT-PCR analysis. As with most RT-PCRs presented here, sterile water was used as a negative control and RT-PCRs were carried out in triplicate.

(A)



(B)



Lane 1: MW marker, Lane 2: MCF-7 parent, Lane 3: pTRE 24hr,
Lane 4: Surv –dox 24hr, Lane 5: Surv +dox 24hr, Lane 6: pTRE 48hr
Lane 7: Surv –dox 48hr, Lane 8: Surv +dox 48hr, Lane 9: pTRE 72hr
Lane 10: Surv –dox 72hr, Lane 11: Surv +dox 72hr, Lane 12: pTRE 5days
Lane 13: Surv –dox 5days, Lane 14: Surv +dox 5days, Lane 15: Neg

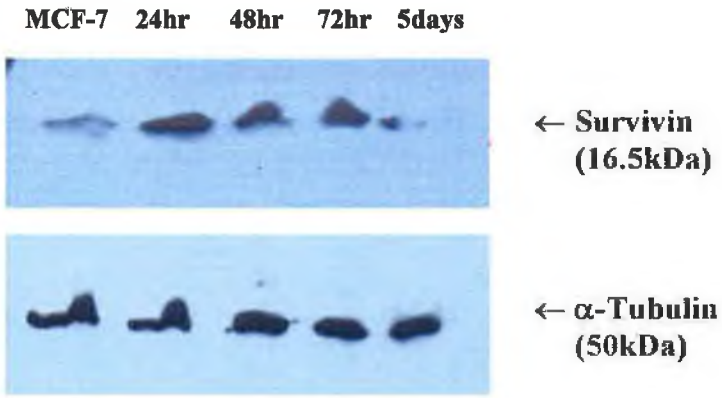
Fig. 3.2.6.1 **(A)** Gel electrophoresis photograph of survivin RT-PCR results on MCF-7 'T.O.' survivin cDNA transient transfection at 24,48,72hrs and 5day time points. **(B)** Gel electrophoresis photograph of survivin RT-PCR results on MCF-7-'T.O.' survivin cDNA transient transfection in the presence and absence of Dox. MW=molecular weight marker and Neg=negative control. (n=3).

3.2.6.2 Analysis of MCF-7 T.O. Survivin cDNA transient transfectants using Western blotting

MCF-7 'T.O.' transient transfection was carried out more than three times. The first time, as can be clearly seen in Figure 3.2.6.2A, there was a large increase in survivin protein at the 24 hour time-point as seen by western blot analysis. However, when this was repeated, the survivin protein over-expression was lost (Figure 3.2.6.2B), leaving only an over-expression in survivin mRNA. α-Tubulin was used as an endogenous control to ensure equal protein loading. Transfections of MCF-7-'T.O'and western blot analysis were carried out five times. Western blot analysis was carried out on cells treated with dox. Results showed no change in survivin protein expression at any of the four time points. There was also no difference between cells plus or minus dox. (Figure 3.2.6.2B). This was carried out three time, using α-Tubulin as an internal control to ensure equal protein loading.

Control transfections using the empty pTRE vector were carried out and the level of survivin protein remained constant (Figure 3.2.6.2C). As with all survivin western blot analysis, α-Tubulin was used as an endogenous control to ensure equal protein loading. Control transfections were carried out in triplicate.

(A)



(B)

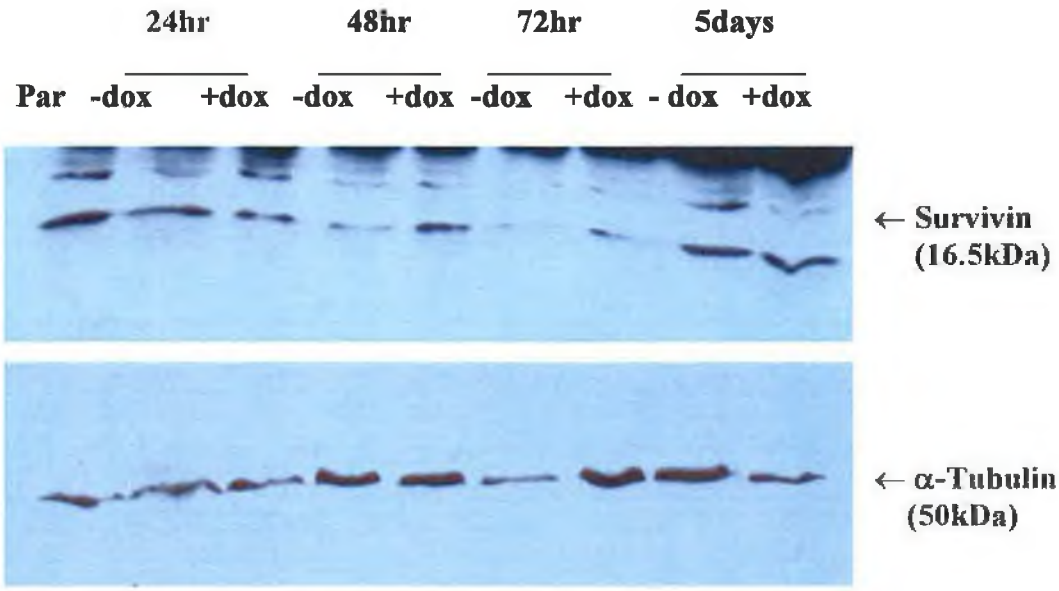


Fig. 3.2.6.2 **(A)** Survivin and α-Tubulin western blots on MCF-7-'T.O.' survivin cDNA transfection at 24, 48, 72hrs and 5day time points. (n=5); **(B)** Survivin and α-Tubulin western blot on MCF-7-'T.O.'. survivin cDNA transfection at 24, 48, 72hrs and 5 day time points in the absence and presence of Dox, when experimented were repeated. (n=3).

(C)

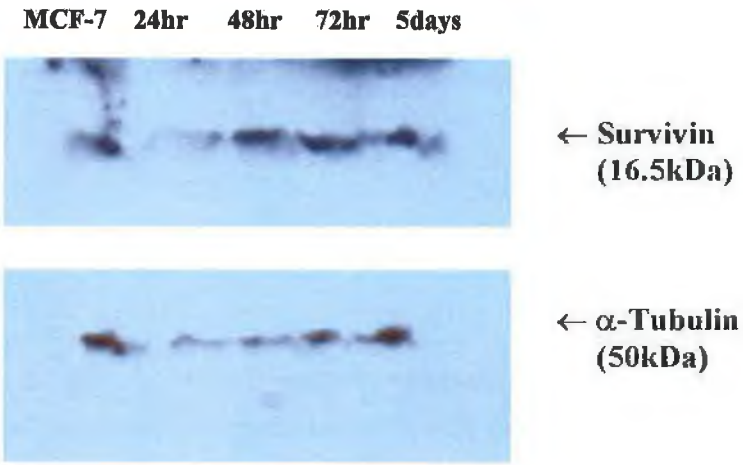


Fig. 3.2.6.2 **(C)** Survivin and α-Tubulin western blots on MCF-7-'T.O' pTRE control transfection at 24, 48, 72hrs and 5 days post-transfection. (n=3).

3.3 Analysis of DLKP Drug-resistant Variants

DLKP is a well characterised squamous lung carcinoma cell line developed in the NICB. In this section of the thesis, drug resistant variants of this cell line were developed by pulse exposure of cells, to increasing concentrations of chemotherapeutic drug, following weekly 4-hour exposure to the drug (see section 2.3.3). Starting concentration and highest concentration of drug used are presented in Table 3.3.1.

Selection Drug	Starting Conc.	Highest Conc.
Vincristine (μg/ml)	0.04	40
5-Fluorouracil (μg/ml)	10	250
Taxotere (ng/ml)	2	8000

Table 3.3.1 Concentrations of chemotherapeutic drug used for the selection of DLKP cells.

3.3.1 Analysis of DLKP resistant variants using *in vitro* toxicity testing

To investigate the resistance profiles of the established variants of DLKP cell line, toxicity assays were carried out as described in section 2.3.1. The chemotherapeutic agents included the selective drug, taxotere, 5-FU or vincristine, as well as other chemotherapy drugs such as, adriamycin, carboplatin, taxol and CCNU.

3.3.1.1 Toxicity Profile of each variant to its selection drug

Table 3.3.1.1.a represents the IC_{50} values of each of the chemotherapeutic agents that the DLKP parent cell line were selected with, resulting in 3 variants, DLKP-5-Fluorouracil (DLKP-5-FU), DLKP-Taxotere (DLKP-TXT) and DLKP-Vincristine (DLKP-VCR). The results demonstrate that DLKP-VCR and DLKP-TXT variants exhibited resistance to vincristine and taxotere, respectively, while DLKP-5FU didn't show resistance to 5-Fluorouracil as shown in Table 3.3.1.1.

Cell line	VCR (ng/ml)	5FU (μg/ml)	Taxotere (ng/ml)
DLKP Parent	0.266±0.02	0.92±0.1	0.3±0.1
Drug resistant variant	1417.6±296	0.93±0.1	134±41

Table 3.3.1.1 (a) IC_{50} values for DLKP, DLKP-vincristine, DLKP 5-FU and DLKP-taxotere variants to selection drug. (n=3).

Cell line	VCR	5FU	Taxotere
DLKP Parent	1	1	1
Resistant Variant	5329.3	1.01	421

Table 3.3.1.1 (b) Fold resistance of the DLKP variants to their selecting drug relative to the parental DLKP cell line. (n=3).

3.3.1.2 Cross resistance profile of the DLKP variants to range of drugs

The resistance of the DLKP variants to a range of chemotherapy drugs, as well as their selection agents, was carried out to determine whether the variants have become multiple drug resistant. The drugs selected for determining this were: Taxotere, Taxol Vincristine, 5-FU, Adriamycin, CCNU, and Cisplatin. Table 3.3.1.2(a) represents the IC_{50} values of each of the chemotherapeutic agents for the DLKP variants and DLKP parental cell line. The results demonstrate that the DLKP-vincristine resistant and DLKP-taxotere resistant variants exhibited cross resistance to vincristine, taxotere, taxol and adriamycin, all of which are substrates to P-glycoprotein.

Table 3.3.1.2(b) represents the fold differences of DLKP variants compared to the parental cell line to each chemotherapeutic drug.

Cell line	Taxotere (ng/ml)	Taxol (ng/ml)	Vincristine (ng/ml)	Adriamycin (μg/ml)	Cisplatin (μg/ml)	CCNU (μg/ml)	5FU (μg/ml)
DLKP	0.3±0.1 (n=3)	2.2±0.5 (n=3)	0.266±0.02 (n=3)	13.9±0.7 (n=3)	0.24±0.04 (n=4)	5.7±0.9 (n=3)	0.92±0.1 (n=3)
DLKP-VCR	74±48 (n=3)	272±13.1 (n=3)	1417.6±296 (n=3)	364.6±26 (n=3)	0.06±0.002 (n=4)	4.1±0.4 (n=3)	1.02±0.2 (n=3)
DLKP-5FU	0.3±0.1 (n=3)	1.35±0.4 (n=3)	0.24±0.002 (n=3)	6.4±1.1 (n=3)	0.138±0.006 (n=4)	5.6±0.6 (n=3)	0.93±0.1 (n=3)
DLKP-TXT	134±41 (n=3)	463±96 (n=3)	1771±235 (n=3)	725±116 (n=3)	0.3±0.08 (n=4)	7.6±1.8 (n=3)	0.8±0.1 (n=3)

Table 3.3.1.2 (a) IC_{50} values for DLKP, DLKP-vincristine, DLKP-5-FU and DLKP-taxotere variants to a range of drugs.

Cell line	Taxotere	Taxol	Vincristine	Adriamycin	Cisplatin	CCNU	5FU
DLKP	1	1	1	1	1	1	1
DLKP-VCR	233	124	5329	26	0.25	0.72	1.17
DLKP-5FU	1	0.61	0.9	0.5	0.6	0.98	1.02
DLKP-TXT	421	210	6657	52	1.2	1.3	0.9

Table 3.3.1.2 (b) Fold resistance of DLKP variants to a range of chemotherapy drugs relative to the parental DLKP cell line.

3.3.2 Morphological Analysis of DLKP and its MDR variants

Following exposure to the chemotherapeutic drugs, taxotere, vincristine and 5-fluorouracil, the morphology of the DLKP variants was examined and was found to change in some cases. During the selection procedure, the DLKP 5-FU variant became very elongated compared to the parental cell line. The DLKP-VCR variant also became elongated, but to a much lesser extent than the DLKP-5-FU variant. The DLKP-TXT variant changed very slightly in morphology, also becoming slightly elongated compared to the parental cells. However, unlike DLKP-5-FU and DLKP-VCR, this change was barely noticeable (see figure 3.3.2.1).

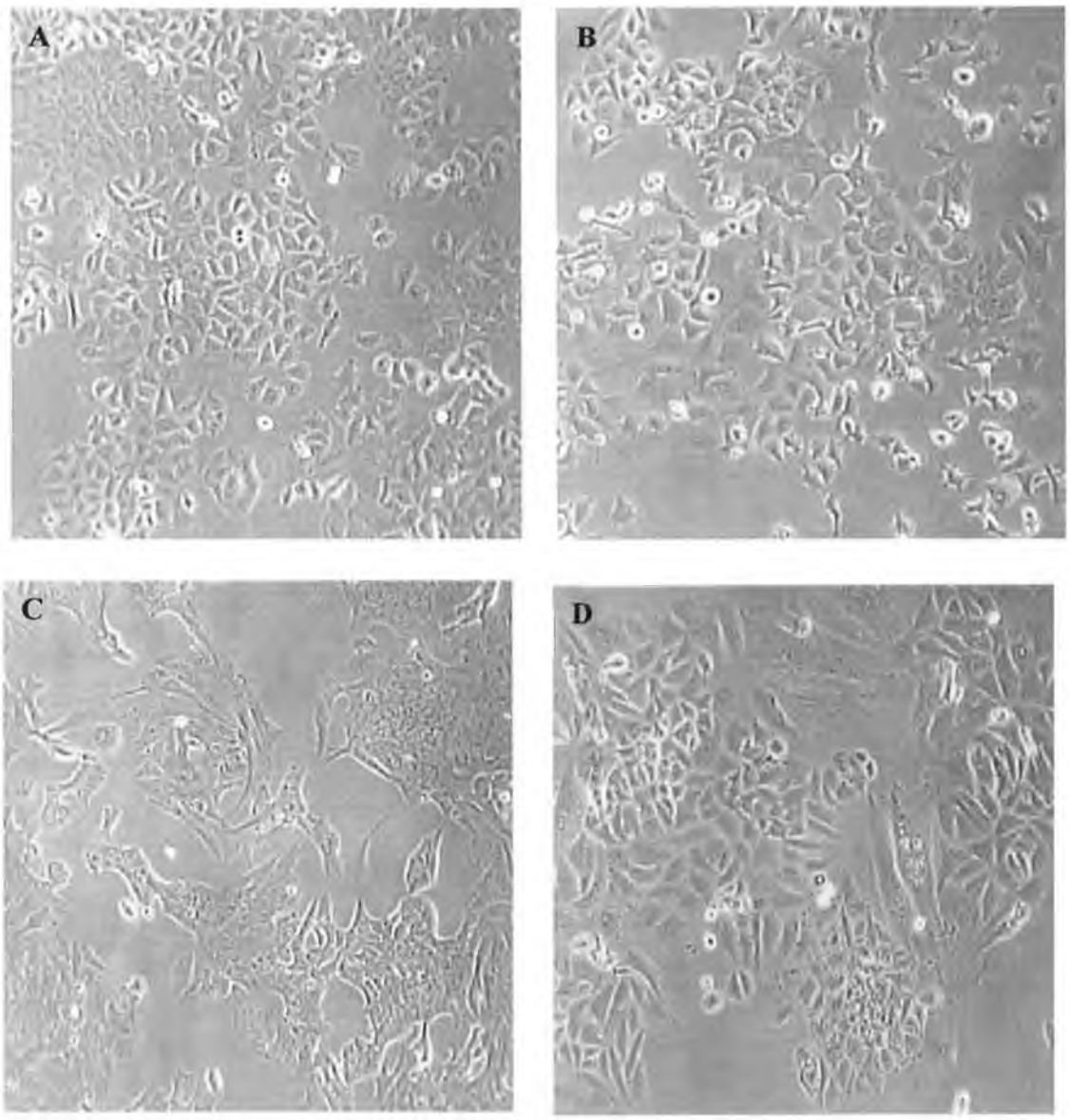


Fig. 3.3.2.1 The morphology of DLKP and its MDR variants. **(A)** DLKP parental cell line, **(B)** DLKP-TXT, **(C)** DLKP-5-FU, **(D)** DLKP-VCR *(10X)*.

3.3.3 Analysis of DLKP resistant variants using *in vitro* Invasion assays

In order to investigate the invasive ability of the DLKP MDR variants compared to the parental cell line, invasion assays were carried out as described in section 2.5.1. Cell culture inserts were coated with ECM before cells were added.

Invasion assays have been carried out in triplicate on ready coated inserts available from Becton-Dickinson (see Figure 3.3.3.1) results indicate that DLKP-vincristine and DLKP-5FU are non-invasive, while DLKP-taxotere (termed DLKP-TXT-1 in sections 3.3.3, 3.3.4 and 3.3.5) is highly invasive (Fig. 3.3.1.C). This has been confirmed by invasion assays carried out, more than three times, on standard inserts coated with two different batches of ECM as shown in Figure 3.3.3.3. Findings from DLKP-5FU and DLKP-TXT-1 are contradictory to Dr. Liang's finding from her previous selections of DLKP-5FU and DLKP-taxotere (termed DLKP-TXT-2 in sections 3.3.3, 3.3.4 and 3.3.5) as shown in Figure 3.3.3.1, while the findings from DLKP-vincristine are in agreement with those reported by Dr. Liang. The invasiveness of two batches of DLKP parent was also tested and it was found that one batch (termed DLKP-1 in section 3.3.3, 3.3.4 and 3.3.5) exhibited lower invasiveness than the second (termed DLKP-2 in sections 3.3.3, 3.3.4 and 3.3.5). The nasal carcinoma cell line, RPMI-2650, was used as a negative control as it is considered to be a non-invasive. DLKP–2 and DLKP-TXT–2 are used only in invasion, inhibition and motility assays for comparison purposes and were not included in any other part of the analysis.

A quantitative method for detecting invasion in these assays was developed, whereby cell on the bottom of the invasion chamber are exposed to acid phosphatase enzyme, which detects viable cells and a yellow colour is observed. This is then read on plate reader at 405nm. The results were plotted on a histograph as shown in figure 3.3.3.2. The graph shows that DLKP-TXT-1 exhibits the highest level of invasiveness, followed by DLKP-2. This is in agreement with the photographic representation in Figure 3.3.3.1 and 3.3.3.3. DLKP-5FU and DLKP-VCR exhibit that lowest level of invasiveness and RPMI is almost non-invasive. Quantitative analysis was carried out once on the ready-coated insert (i.e. on the photographs shown in Figure 3.3.3.1, but not photographs in Figure 3.3.3.3).

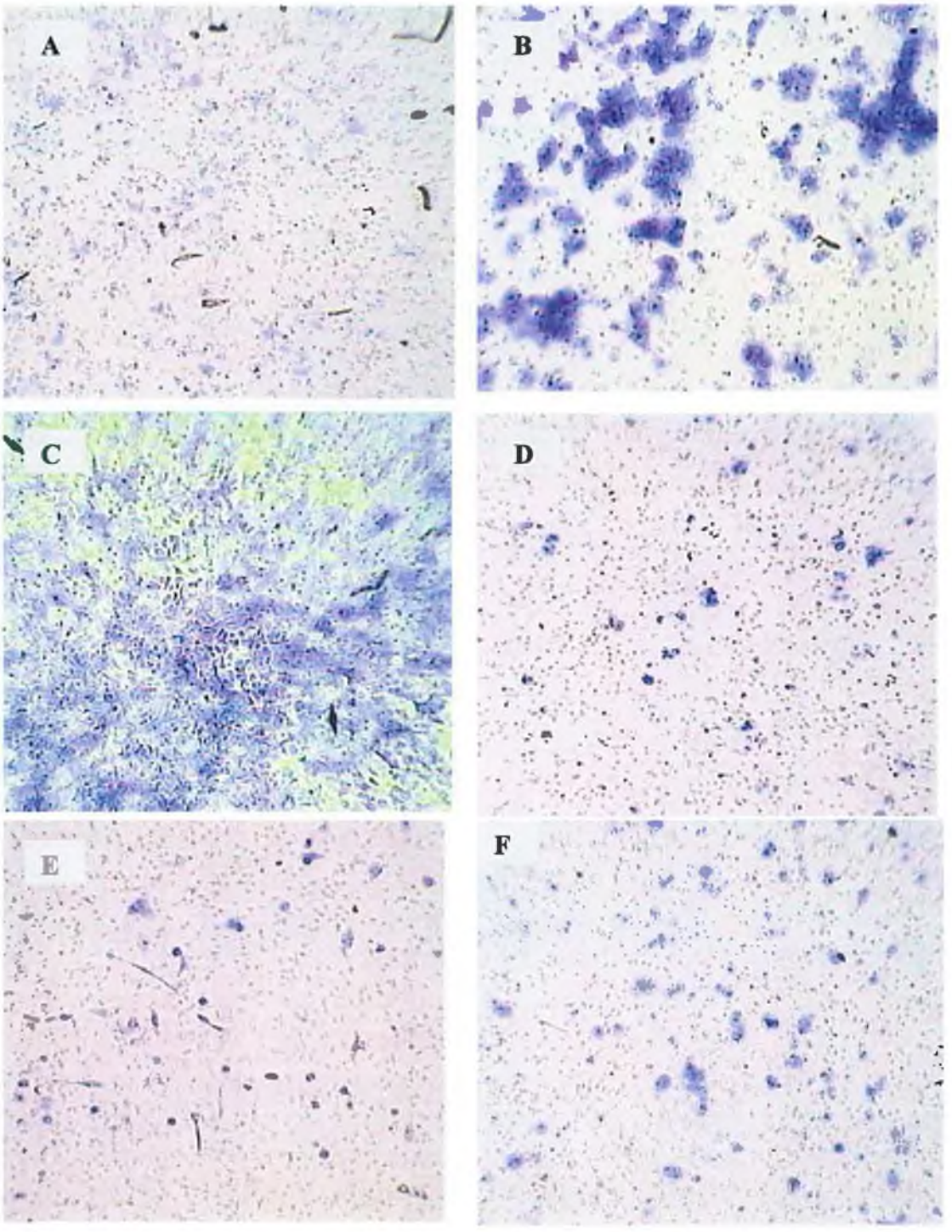


Figure 3.3.3.1 Invasion assays of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-TXT-2, **(E)** DLKP-5-FU, **(F)** DLKP-VCR using the ready-coated invasion chambers *(4X)* (n=3).

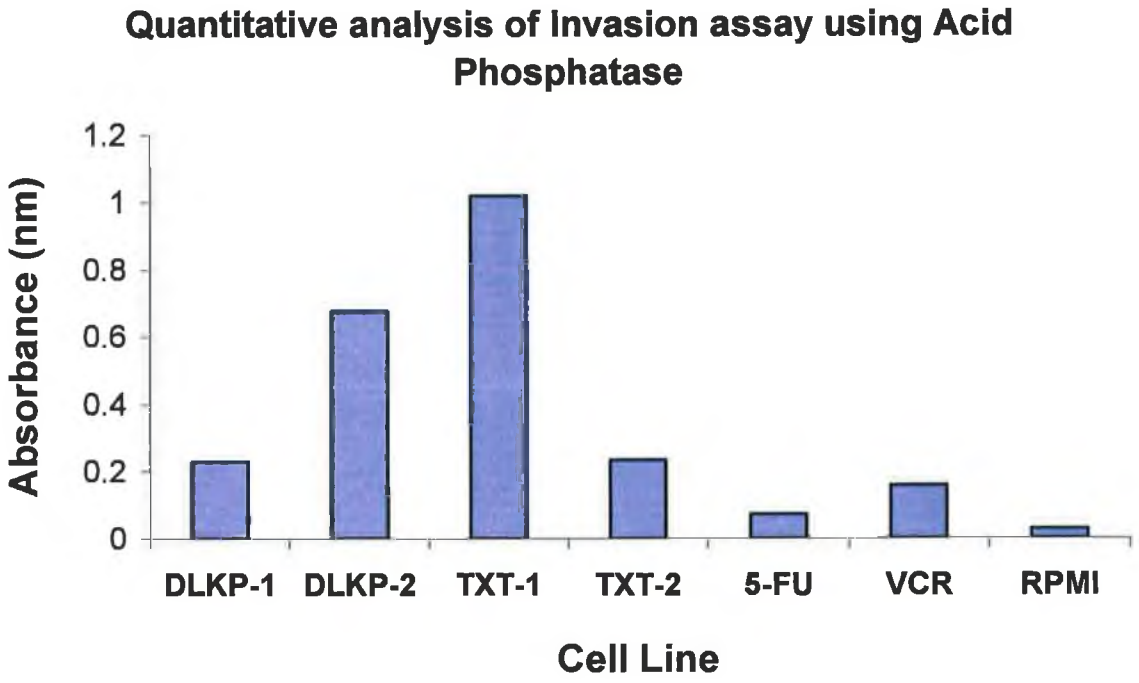


Figure 3.3.3.2 Absorbance values of invasion assay results using Acid Phosphatase and reading at 405nm (n=1). A photograph of RPMI-2650 invasion assay is shown in figure 3.3.4.2.

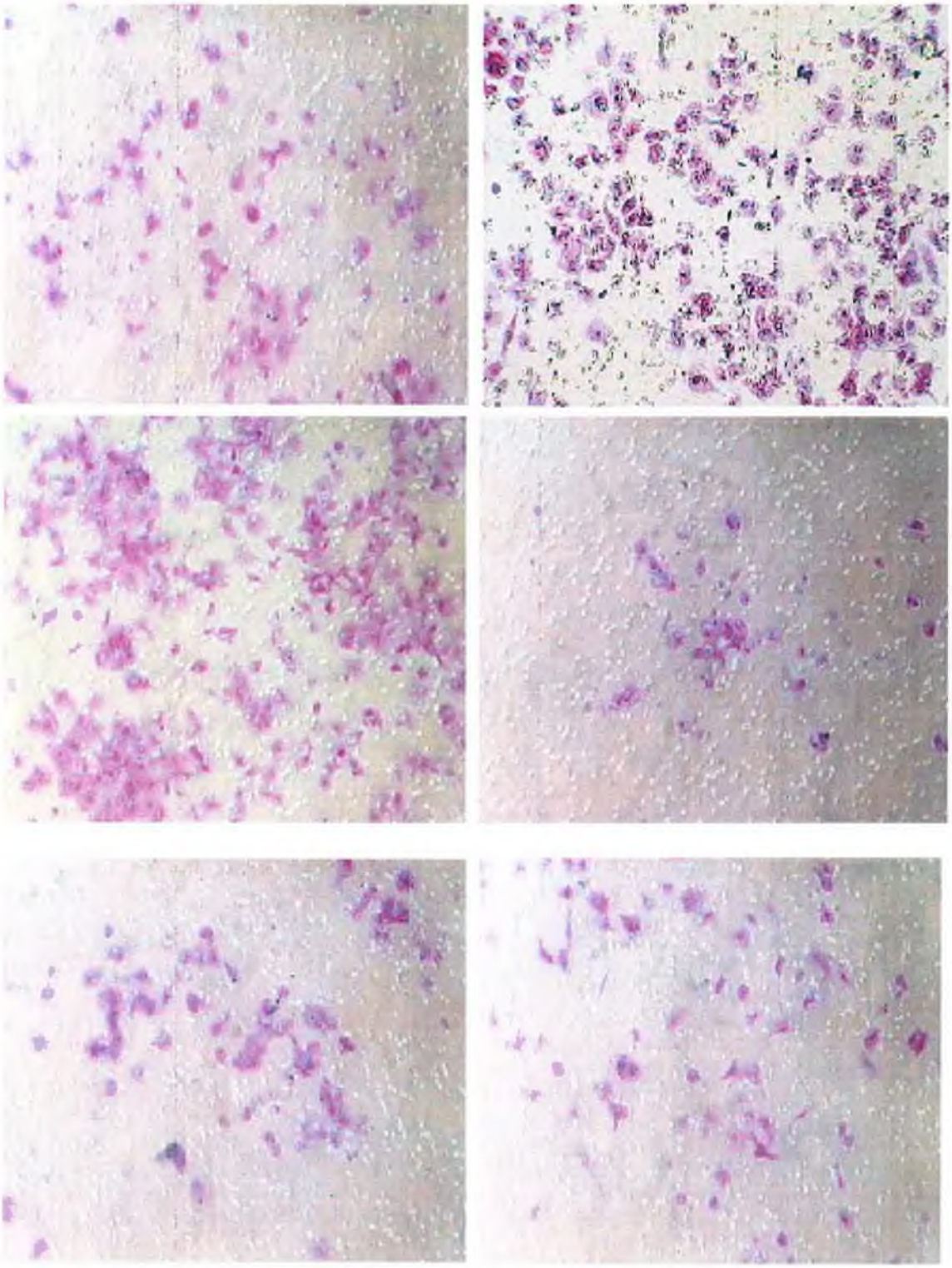


Figure 3.3.3.3 Invasion assays of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-TXT-2, **(E)** DLKP-5-FU, **(F)** DLKP-VCR using standard invasion chambers *(10X)* (n>3).

3.3.4 MMP Inhibition Assays on DLKP and its MDR variants

Invasion assays were carried on the MDR variant, DLKP-TXT-1, in the presence and absence of matrix metalloproteases inhibitors (see section 2.5.1.1). When the cells were incubated with MMP inhibitor I, which inhibits MMP-1, -3, -8 and MMP-9, or MMP-2 inhibitor I, which inhibits MMP-2, cell invasion was decreased compared to cells that were not incubated with any inhibitor. When cells were incubated with MMP inhibitor III, which inhibits MMP-1, -2, -3, -7 and MMP-13, the inhibition of cell invasion was more pronounced than the decease caused by MMP inhibitor I or MMP-2 inhibitor I (Figure 3.3.4.1). This assay was carried out once.

Further inhibition assays were carried out using MMP inhibitor III on the DLKP-1 and DLKP-2, as well as DLKP-TXT-1, DLKP-5-FU and DLKP-VCR variants (Figure 3.3.4.2). Quantitative analysis was carried out on this assay using acid phosphatase, as described in section 3.3.3 and in section 2.5.1 of the material and methods section. A graph representing the findings is shown in Figure 3.3.4..3. This shows that MMP inhibitor III caused a decease in cell invasion of DLKP-TXT-1 and DLKP-2 and to a lesser extent, DLKP-1. The inhibitor had no great effect on DLKP-5FU and DLKP-VCR, which are non-invasive. This indicates that MMP inhibitor III does not inhibit cell invasion, but slows it down. This assay was carried out once.

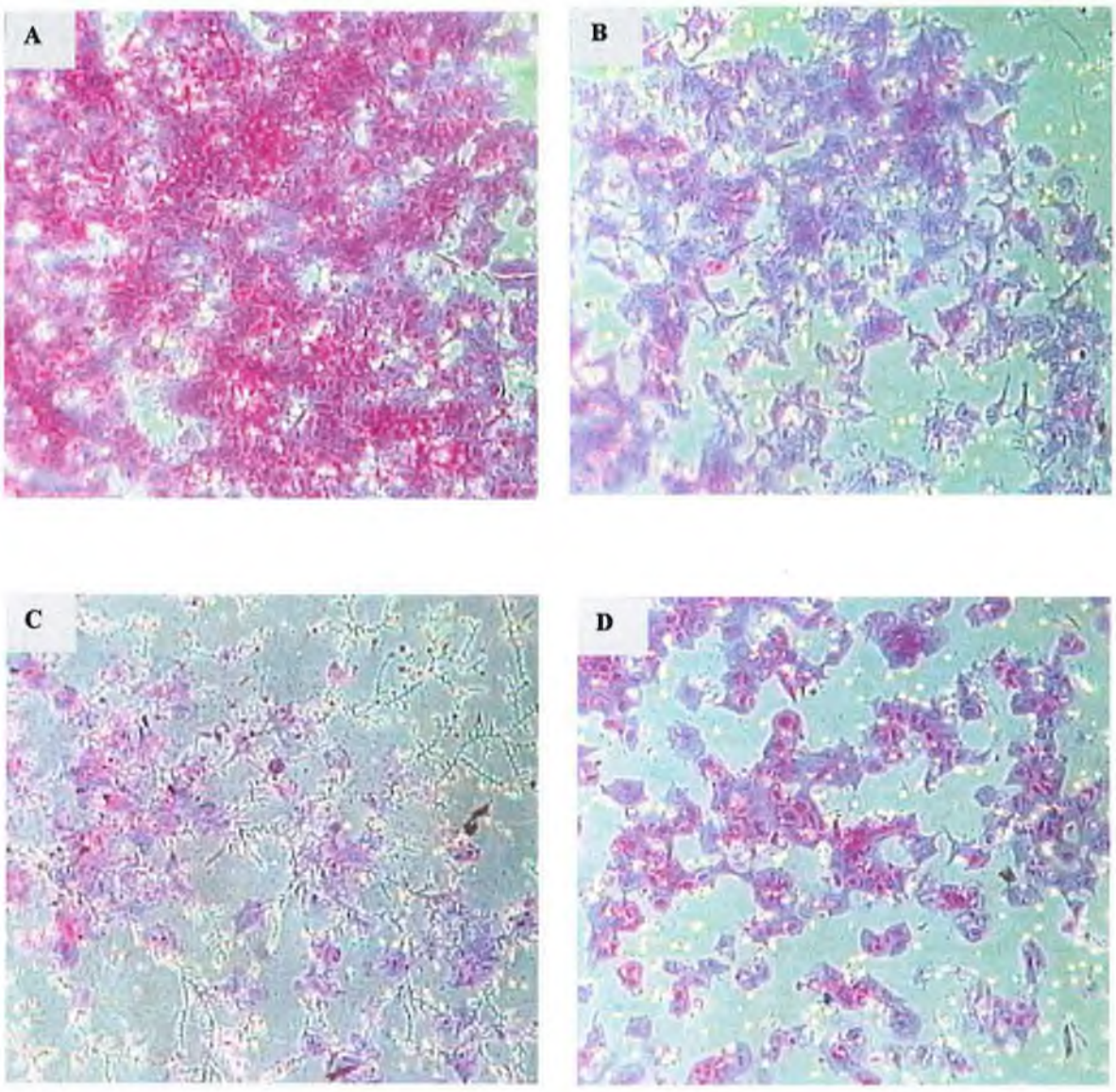


Figure 3.3.4.1 MMP Inhibition assays of **(A)** DLKP-TXT-1 with no inhibitors, **(B)** DLKP-TXT-1 with MMP inhibitor I, **(C)** DLKP-TXT-1 with MMP inhibitor III, **(D)** DLKP-TXT-1 with MMP-2 inhibitor I *(10X)* (n=1).

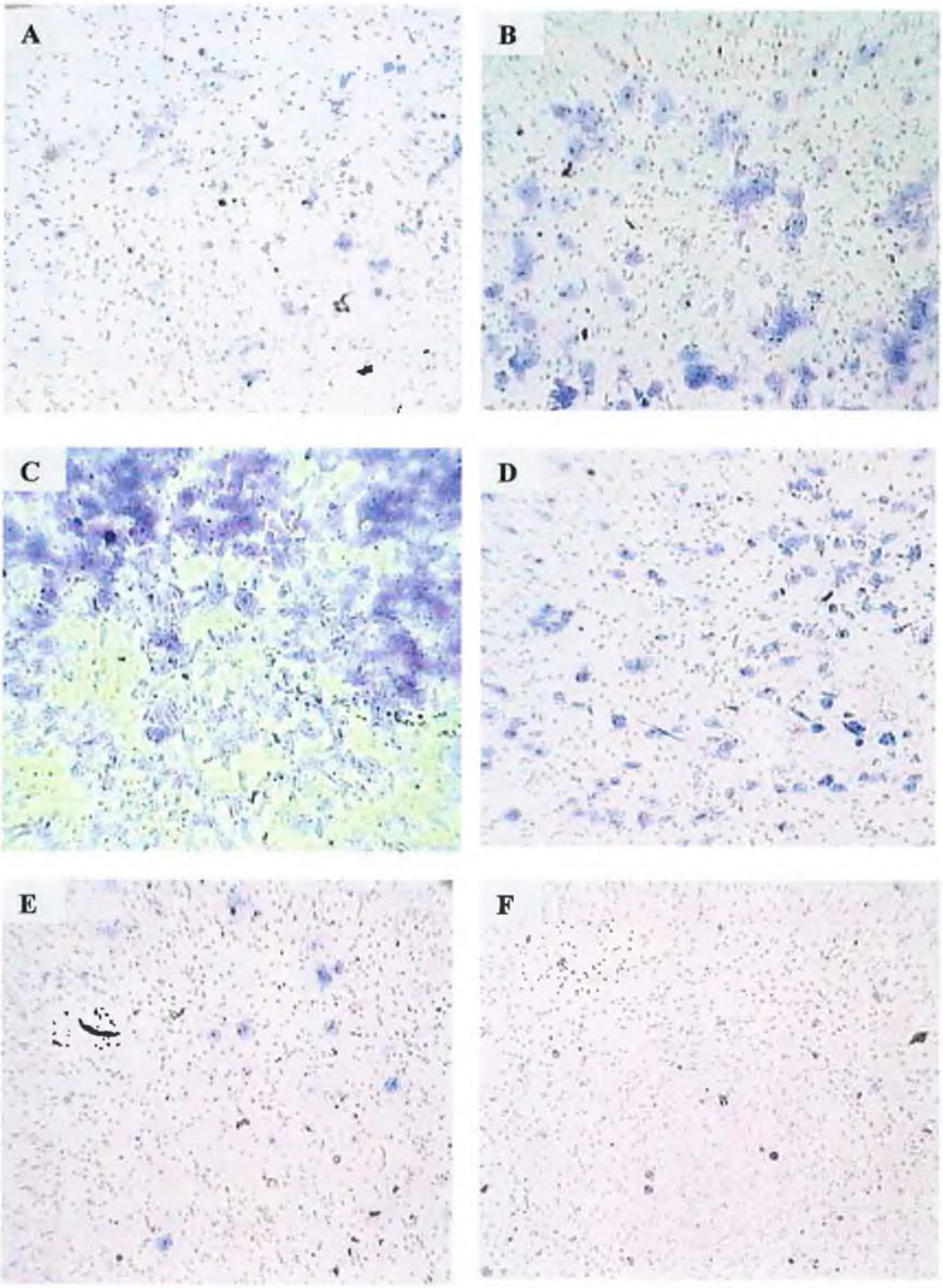


Figure 3.3.4.2 MMP Inhibition assay, with MMP inhibitor III, of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-5-FU, **(E)** DLKP-VCR, **(F)** RPMI-2650 negative control for invasion and inhibition assays *(4X)* (n=1)

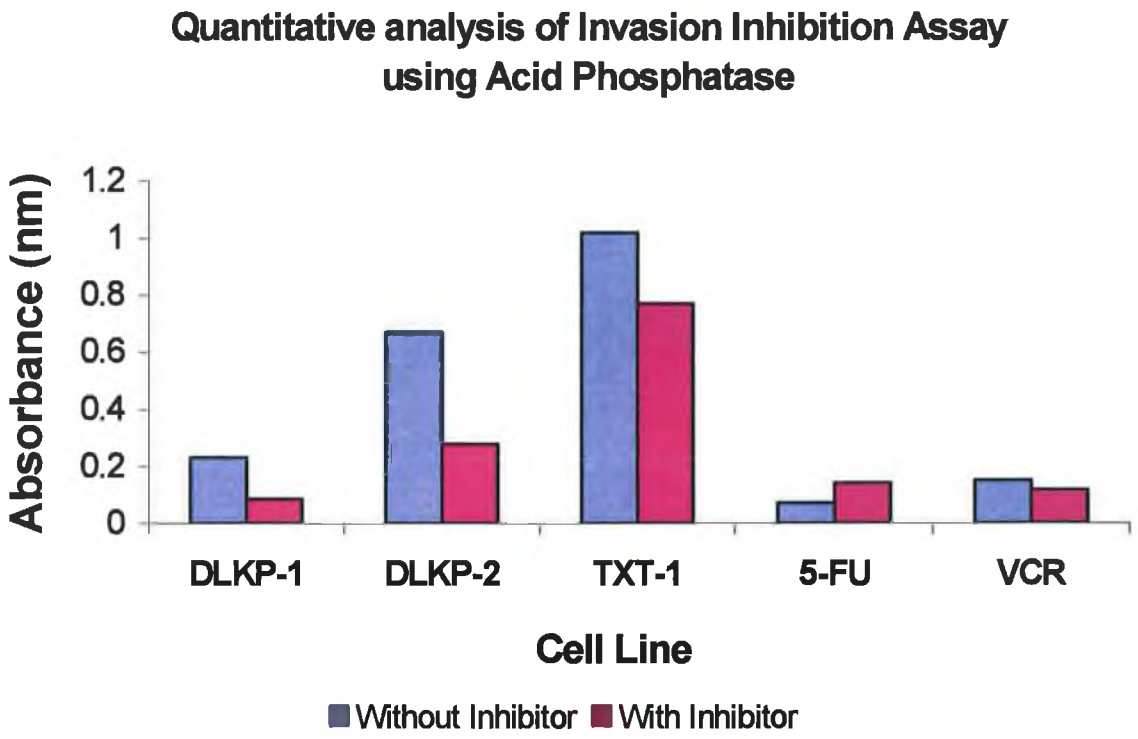


Figure 3.3.4.3 Absorbance values of MMP inhibition assay results (Figure 3.3.4.2) using acid phosphatase and reading at 405nm (n=1).

3.3.5 Analysis of DLKP resistant variants using *in vitro* Motility assays

Motility assays were carried out to compare the locomotive ability of DLKP parental cell line and its selected variants. The procedure used to demonstrate cell motility was similar to that used for the invasion assays except that the inserts were not coated with ECM prior to the addition of the cells. The assays was taken down at three time points: 12 hours, 24 hours and 48 hours. The results obtained demonstrated that DLKP taxotere-1 showed significantly higher motility than the DLKP-TXT-2, at all time points. It also showed significantly higher motility than DLKP-5-FU and DLKP-VCR at all the time points. In addition, DLKP-TXT-1 showed significantly higher motility than DLKP parental cells from both batches, in particular, DLKP-1. However, at 48 hours the difference between DLKP-TXT-2 and DLKP-2 was not as obvious. The results for each time point are shown in Figure 3.3.5.1a, b, and c. RPMI-2650 parent was used as a negative control for all motility assays (Figure 3.3.5.1d)

The motility assays, in general, correlate with the invasion assay results. i.e. cells that tend to be more invasive display an increase in motility and *vice versa*. A quantitative representation of the results is shown in Figure 3.3.5.2. The graphs show two quantitative methods of analysis as described in section 2.5.1. The acid phosphatase method (Figure 3.3.5.2A) seems to be more accurate than the crystal violet elution method (Figure 3.3.5.2B). Both graphs support the photographic analysis presented in Figures 3.3.5.1a, b, c and d, illustrating that DLKP-2 and DLKP-TXT-1 are the most motile. Motility increases dramatically after 48 hours in DLKP-1 and DLKP-2 and the four variants (Figure 3.3.5.2A). In Figure 3.3.5.2B the difference between 24 hours and 48 hours is not as dramatic as in Figure 3.3.5.2A.

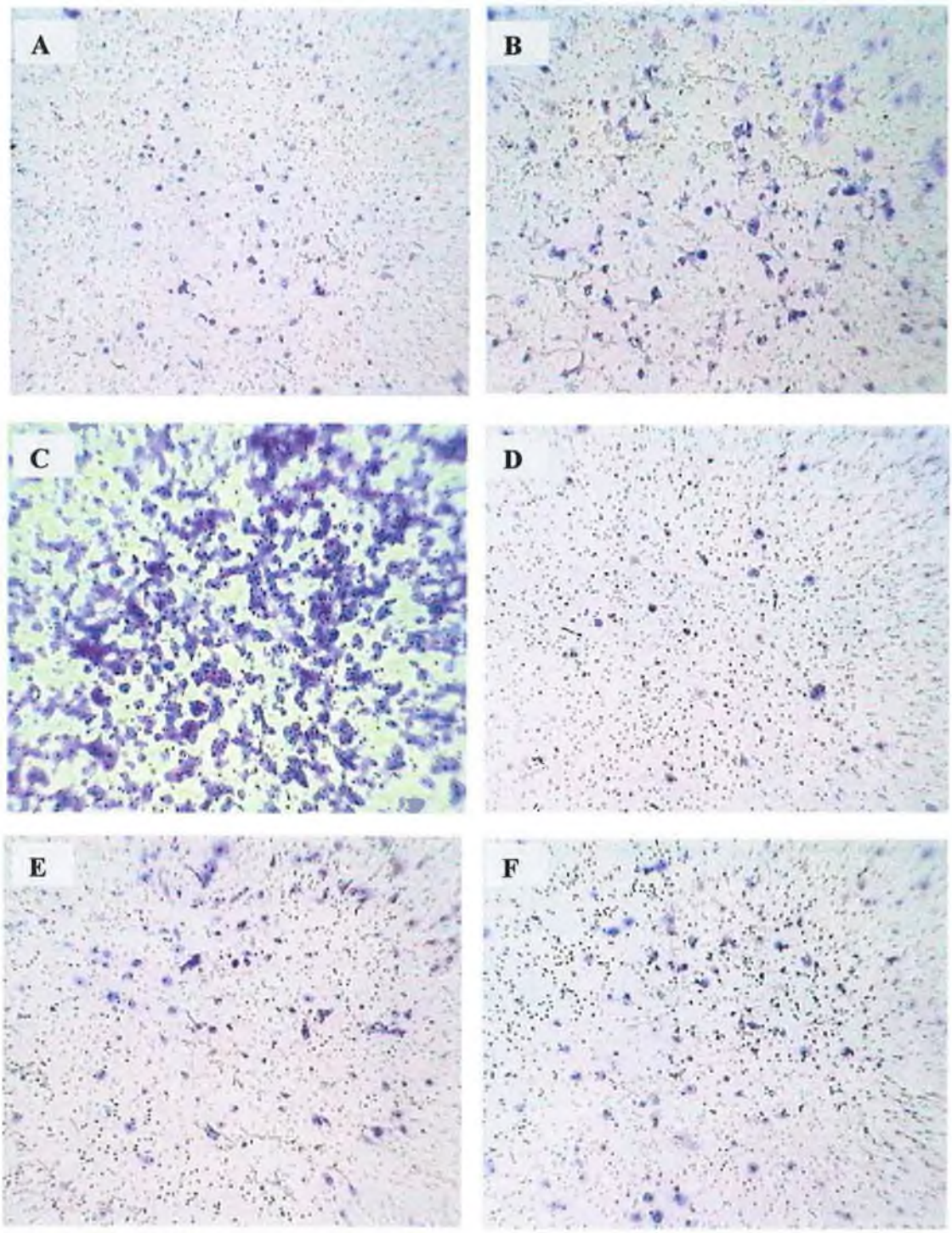


Figure 3.3.5.1a Motility assays of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-TXT-2, **(E)** DLKP-5-FU, **(F)** DLKP-VCR at 12 hours (4X) (n=3)

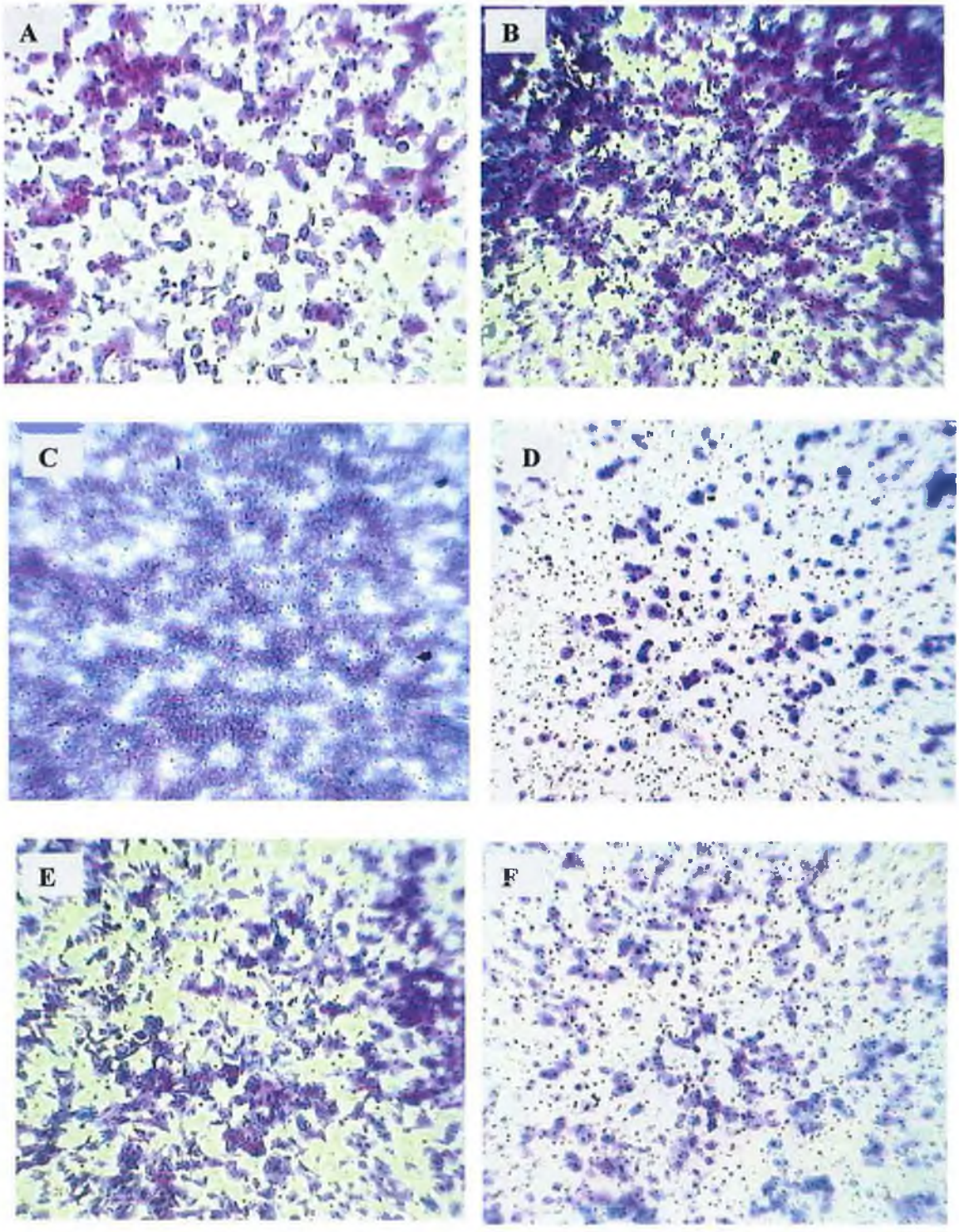


Figure 3.3.5.1b Motility assays of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-TXT-2, **(E)** DLKP-5-FU, **(F)** DLKP-VCR at 24 hours (4X) (n=3).

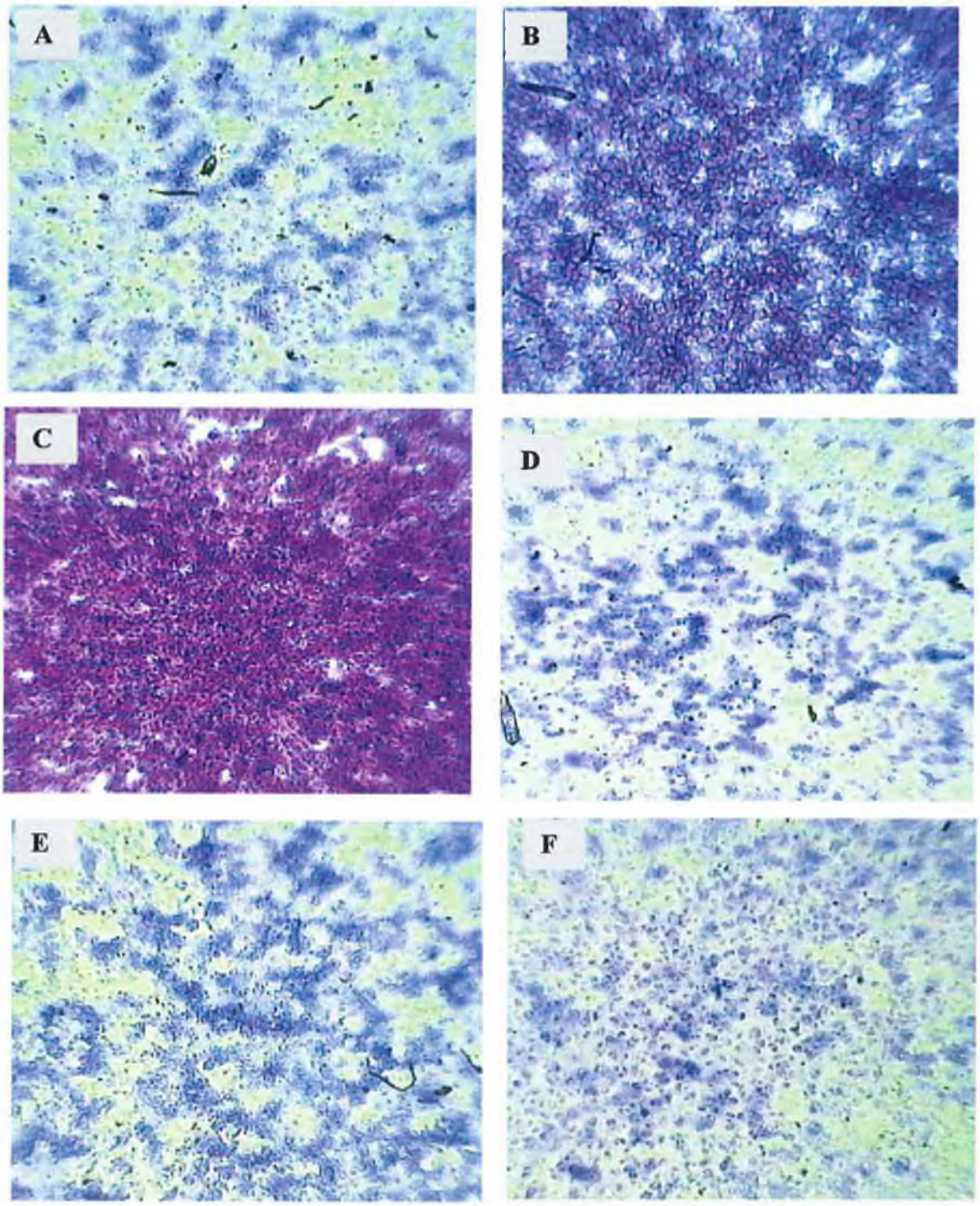


Figure 3.3.5.1c Motility assays of **(A)** DLKP-1, **(B)** DLKP-2, **(C)** DLKP-TXT-1, **(D)** DLKP-TXT-2, **(E)** DLKP-5-FU, **(F)** DLKP-VCR at 48 hours (4X) (n=3).

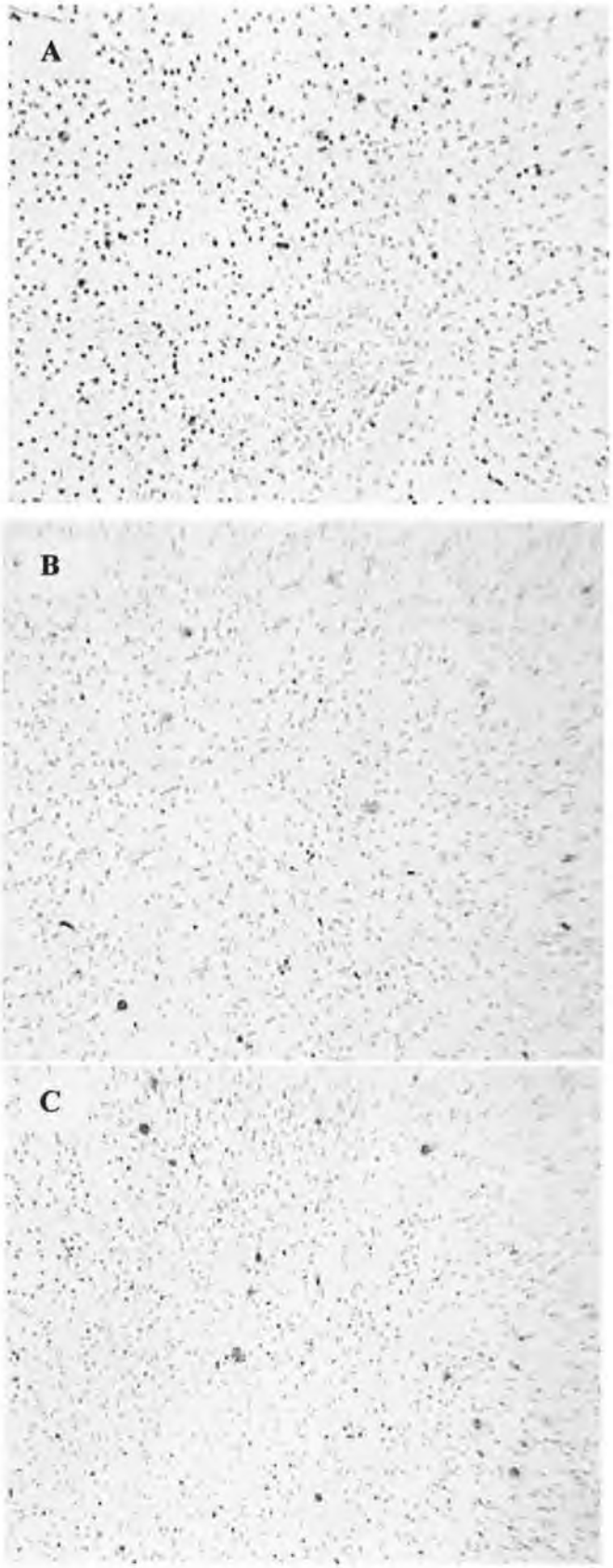
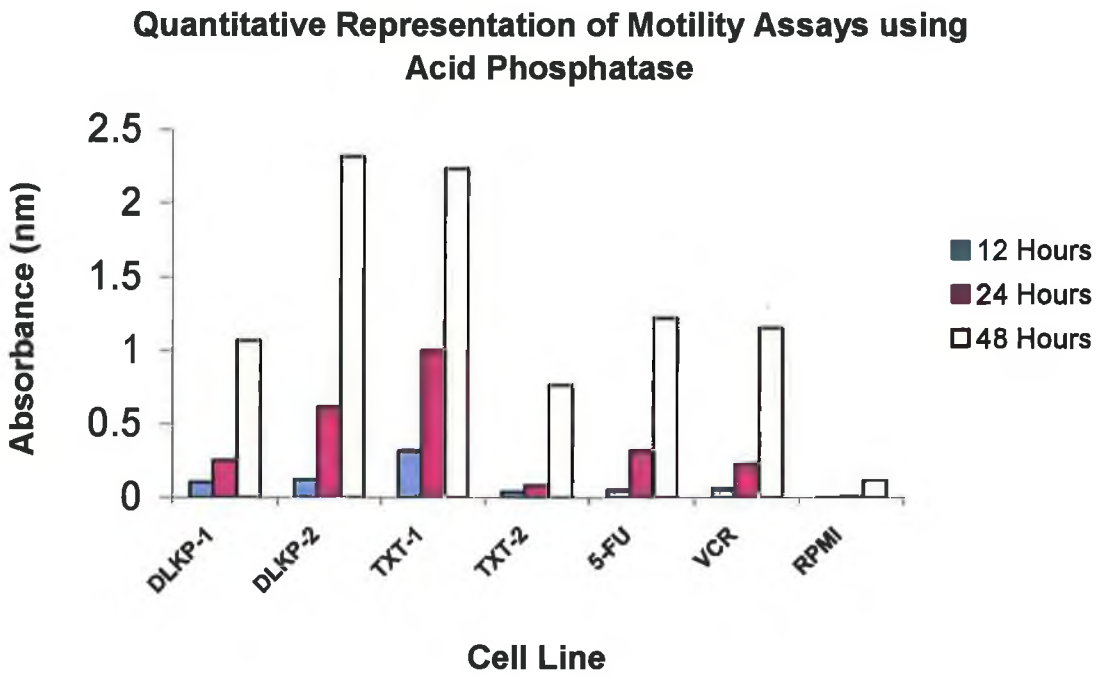


Figure 3.3.5.1d Motility assays of **(A)** RPMI-2650 at 12 hours, **(B)** RPMI-2650 at 24 hours, **(C)** RPMI-2650 at 48 hours (4X) (n=3).

(A)



(B)

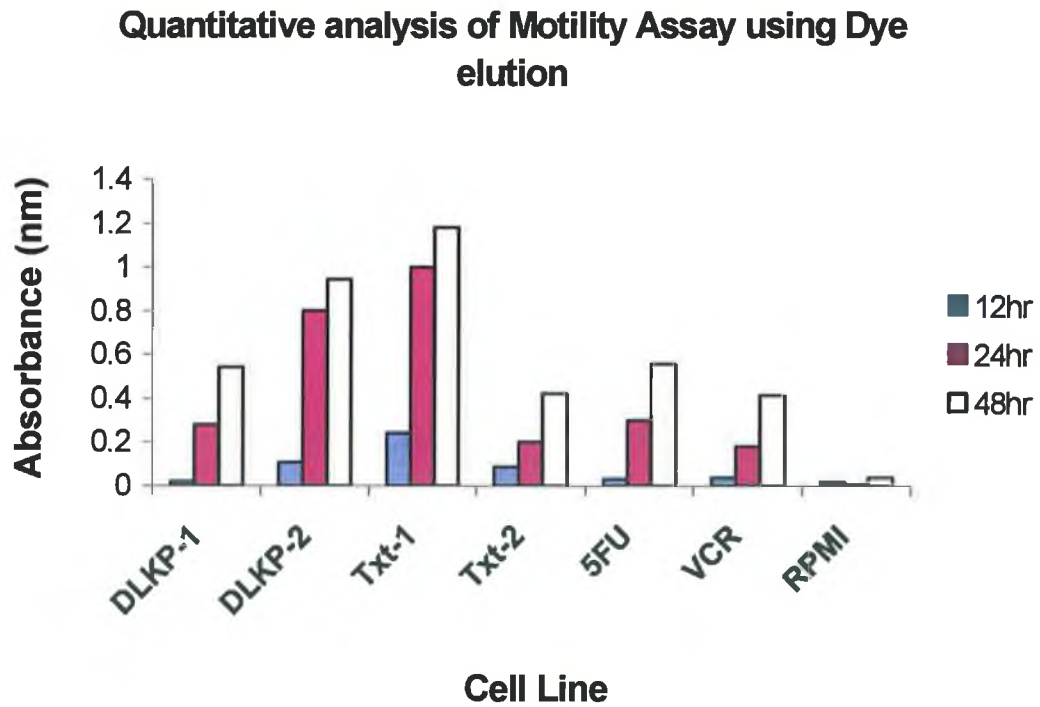


Figure 3.3.5.2 **(A)** Absorbance values of motility assay results using acid phosphatase and reading at 405nm, **(B)** Absorbance values of each eluted insert from motility assays at 12 hour, 24 hour and 48 hour time points and reading at 570 nm.

3.3.6 Studies of MMPs in the DLKP selected variants

Matrix metalloproteinases (MMPs), which may be secreted by cells, play an important role in degrading extracellular martix. To investigate the mechanisms underlying the invasive phenotype of the DLKP parental cell line and its variants, studies of MMPs were carried out as described in section 2.5.

3.3.6.1 Zymography of MMPs in the DLKP variants

Zymography gels have been carried out on concentrated supernatants collected from the 3 variants. Figure 3.3.6.1 shows that DLKP parent and its three variants showed bands representing MMP-2, MMP-9 and a third band which may be MMP-13.

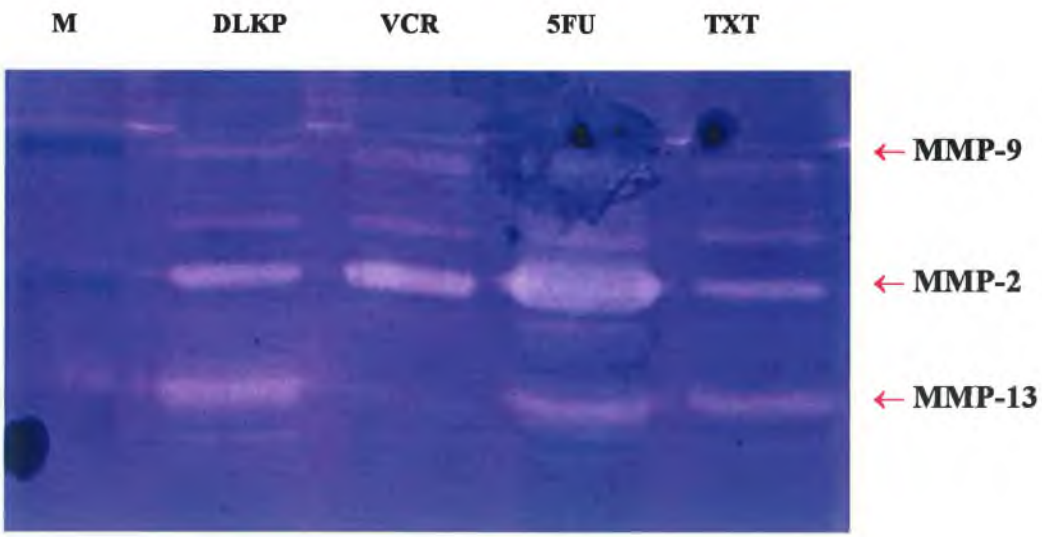


Figure 3.3.6.1 Zymograph of proteinases in the DLKP parent cell line and its variants. M=size marker (n=3)

3.3.6.2 Zymography with proteinase inhibitors or enhancer

To confirm that the three bands are MMPs, proteinase inhibitors were added to inactivate their corresponding substrates.

3.3.6.2.1 The effect of EDTA

EDTA is a chelating agent which can bind the zinc and calcium which are needed for the activation of MMPs. After the gel was incubated with EDTA, the three bands in all cell lines dissappeared. Therefore, indicating that the three bands are MMPs.

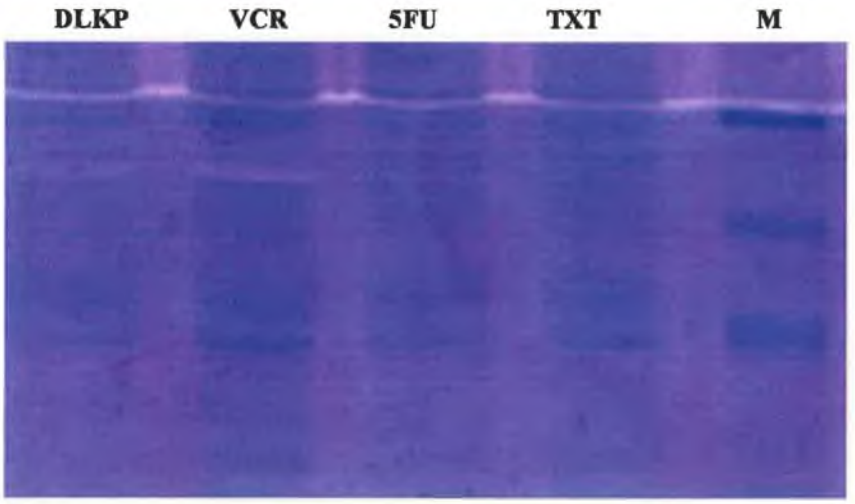
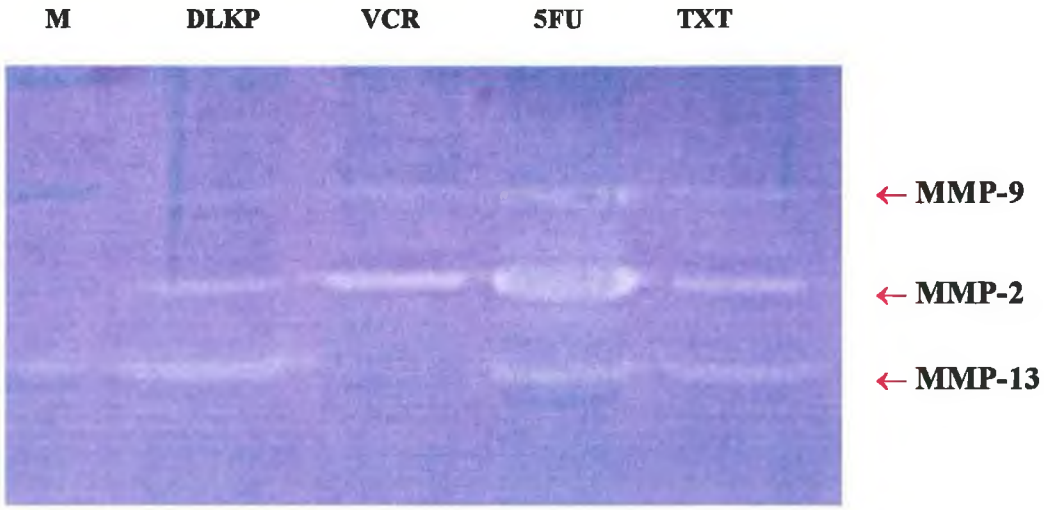


Fig. 3.3.6.2.1 Zymograph of proteinases in DLKP parent and its variants when incubated with EDTA. M=size marker (n=2).

3.3.6.2.2 The effect of PMSF

PMSF is a serine protease inhibitor, which does not inhibit MMP secretion. PMSF was used to establish that the bands observed are due to MMP secretion, not serine protease secretion. After the gel was incubated with PMSF, all bands were still visible, suggesting that all three are MMP bands.



3.3.6.2.2 Zymograph of proteinases in the DLKP parent cell line and its variants incubated with PMSF. M=size marker (n=2).

3.3.7 Analysis of DLKP resistant variants using RT-PCR analysis

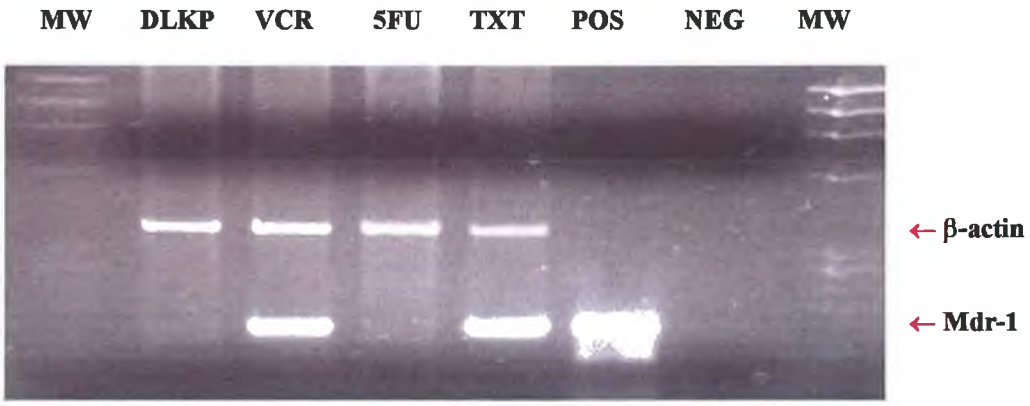
To detect the expression of MDR- and apoptosis-related genes at the RNA level, RT-PCR was carried out as described in section 2.2. All RT-PCR reactions were carried out on 3 independent RNA isolations on the DLKP variants. 10 genes were studied. The results are presented below.

In all RT-PCR gels (Figures 3.3.7.1-3.3.7.10), MW represent molecular weight marker, HinfI of ϕ-X174 DNA. Negative control is ultra pure water, and positive control, where included, is a purified plasmid of the gene in question. Except in the case of survivin RT-PCR, where positive control was a cell line transfected with survivin cDNA (Figure 3.3.7.10). All RT-PCRs were carried out in triplicate and were reproducible, with the exception of E-cadherin RT-PCR (see section 3.3.7.8).

3.3.7.1 Mdr-1 expression in DLKP cell line and its selected variants by RT-PCR

RT-PCR analysis showed that mdr-1 expression was undetectable in the DLKP parental cell line as well as the DLKP-5FU variant. Mdr-1 was highly over-expressed in the two variants, DLKP-vincristine and DLKP-taxotere.

(a)



(b)

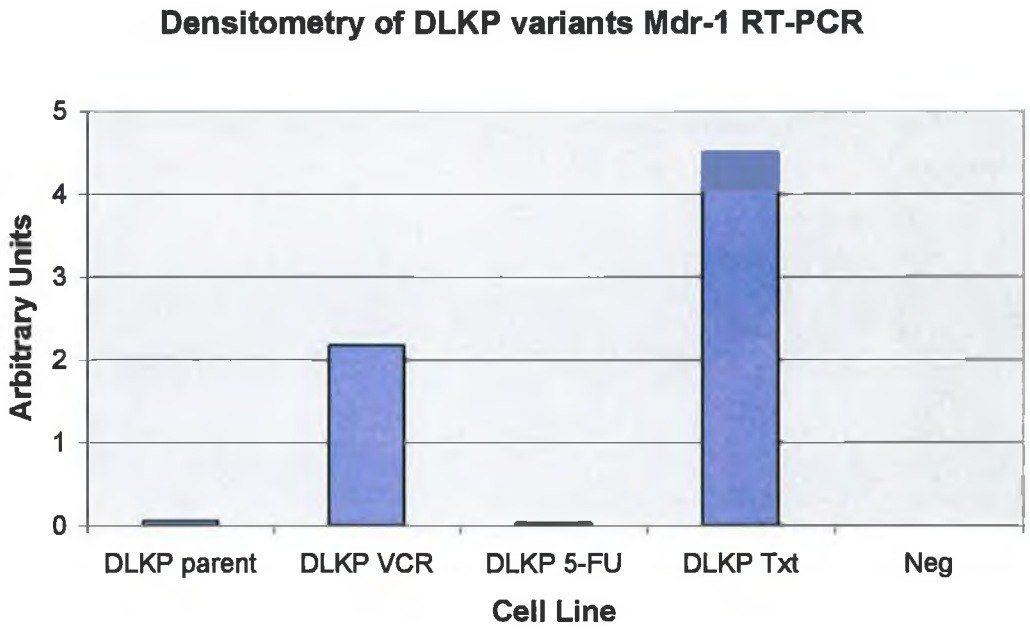
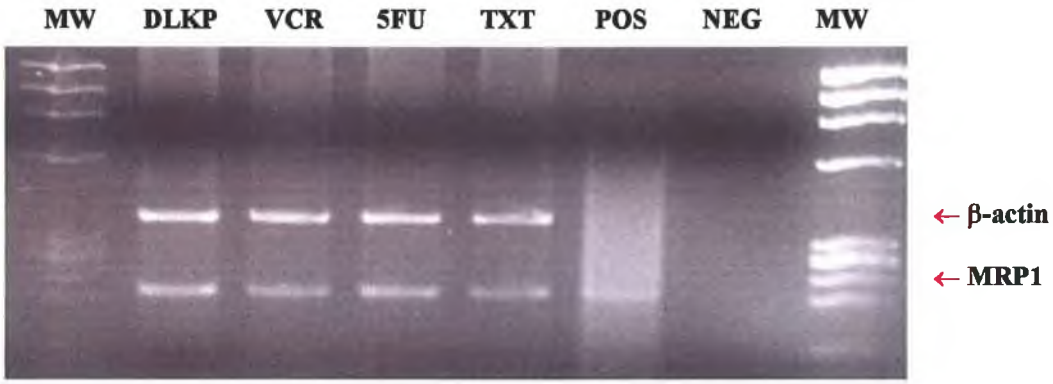


Figure 3.3.7.1 **(a)** RT-PCR analysis of mdr-1 expression in the DLKP parent cell line and its variants. **(b)** Densitometry of RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.2 MRP-1 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on MRP-1 shows that there was no change in MRP-1 mRNA expression in the DLKP parent variants compared to the parent cell line.

(a)



(b)

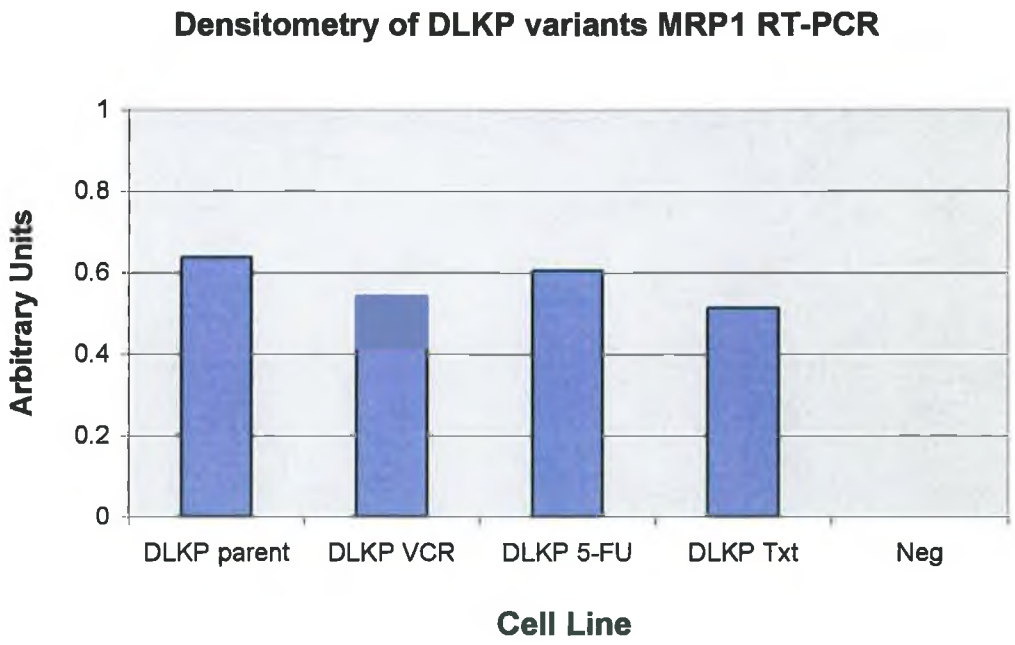


Figure 3.3.7.2 **(a)** RT-PCR analysis of MRP-1 expression in the DLKP parent cell line and its variants. **(b)** Densitometry on RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.3 MRP-2 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on MRP-2 (cMOAT) shows that there was no detectable expression of MRP-2 mRNA in DLKP parent or DLKP variants.

(a)

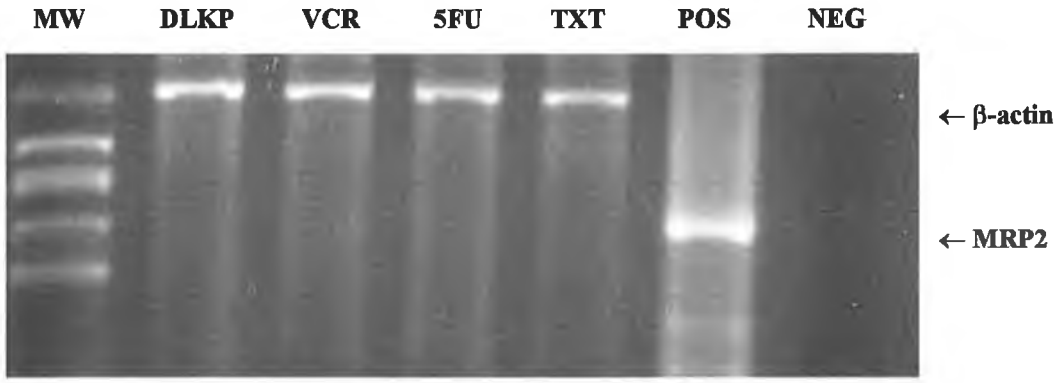
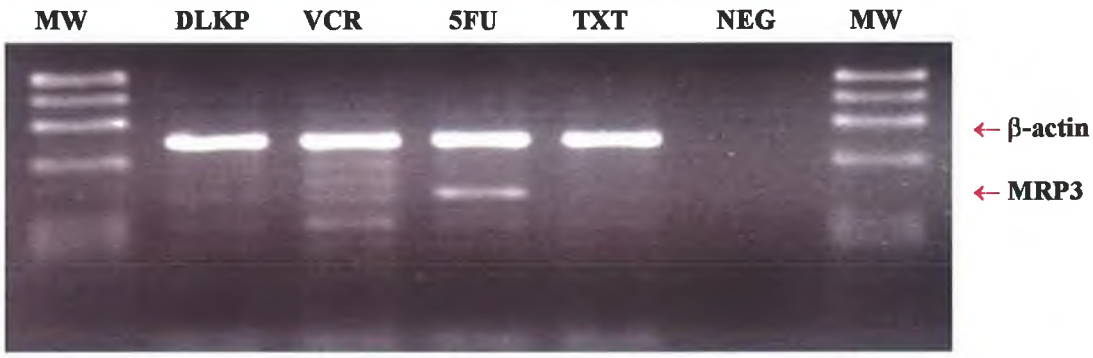


Figure 3.6.7.3 **(a)** RT-PCR analysis of MRP-2 expression in the DLKP parent cell line and its variants. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.4 MRP-3 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on MRP-3 shows that there was no detectable expression of MRP-3 mRNA in DLKP parent, DLKP-vincristine or DLKP-taxotere. There was, however, a band representing MRP-3 mRNA expression in the DLKP-5FU variant.

(a)



(b)

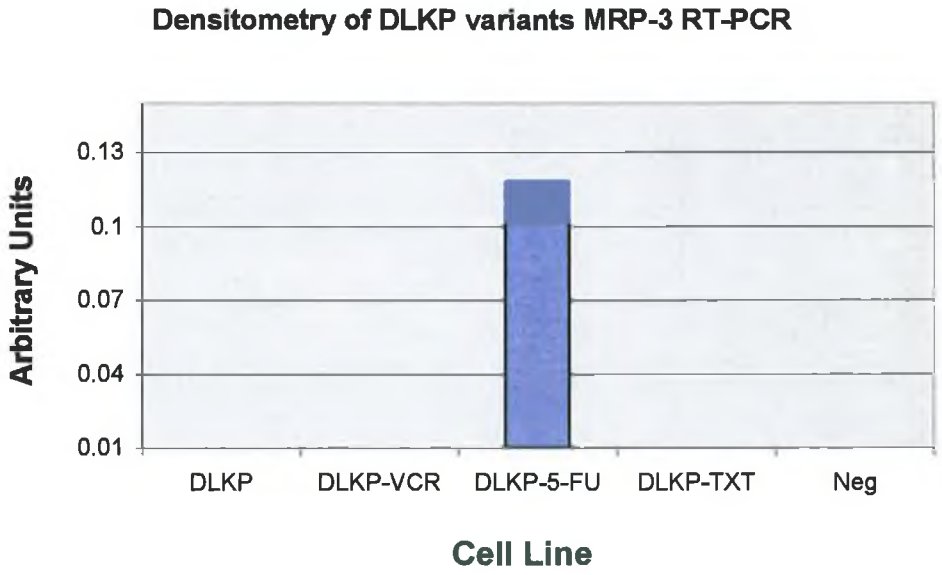
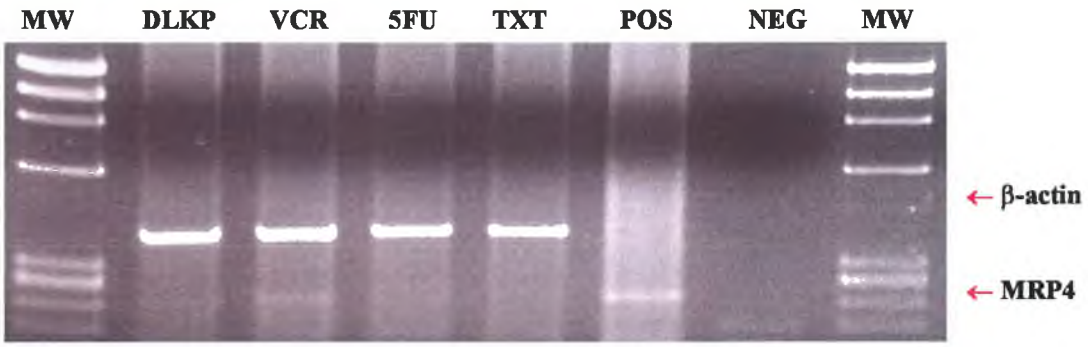


Figure 3.3.7.4 **(a)** RT-PCR analysis of MRP-3 expression in the DLKP parent cell line and its DLKP variants. **(b)** Densitometry on RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.5 MRP-4 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on MRP-4 shows a very faint band (almost un-detectable) for MRP-4 mRNA expression in DLKP parent and its three variants. The band is strongest in DLKP-vincristine.

(a)



(b)

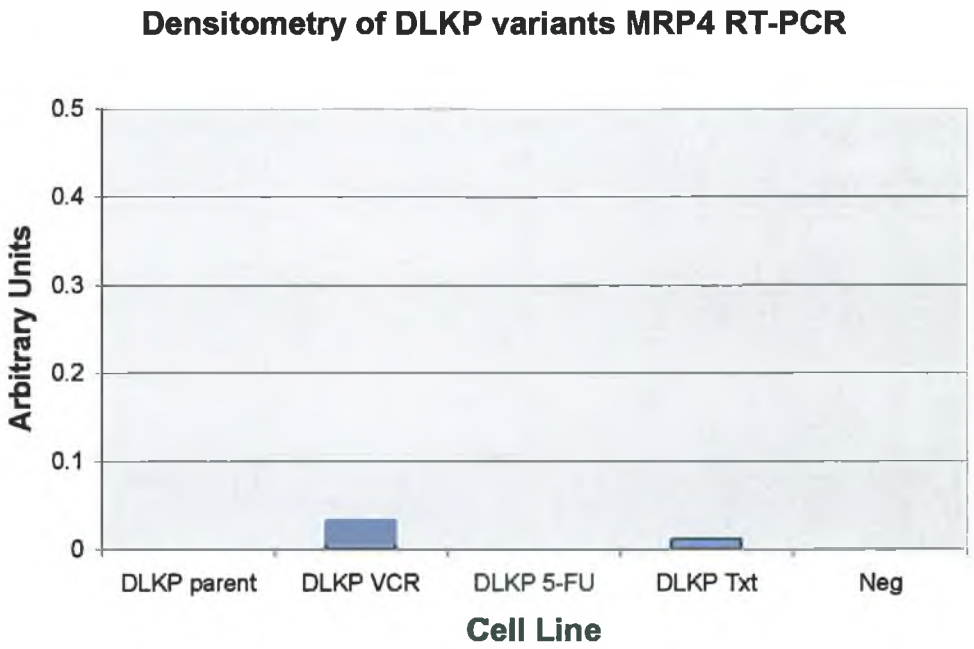


Figure 3.3.7.5 **(a)** RT-PCR analysis of MRP-4 expression in the DLKP parent cell line and its variants. **(b)** Densitometry on RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.6 MRP-5 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on MRP-5 shows undetectable levels of MRP-5 mRNA expression in DLKP parent and its three variants.

(a)

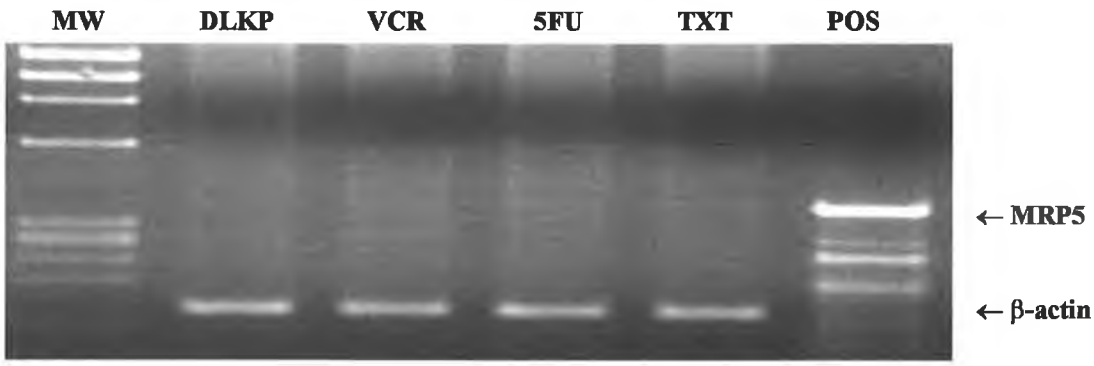
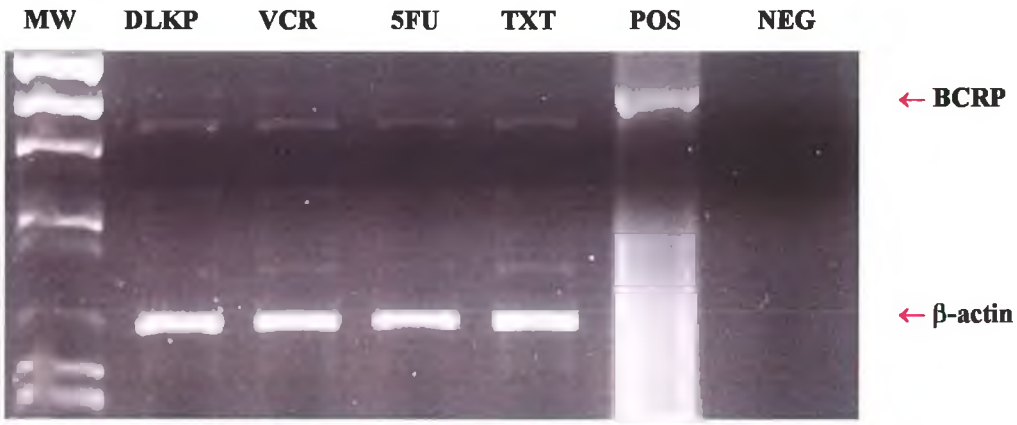


Figure 3.3.7.6 **(a)** RT-PCR analysis of MRP-5 expression in the DLKP parent cell line and its variants. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.7 BCRP expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on BCRP shows very low levels of BCRP mRNA expression in DLKP parent and its three variants.

(a)



(b)

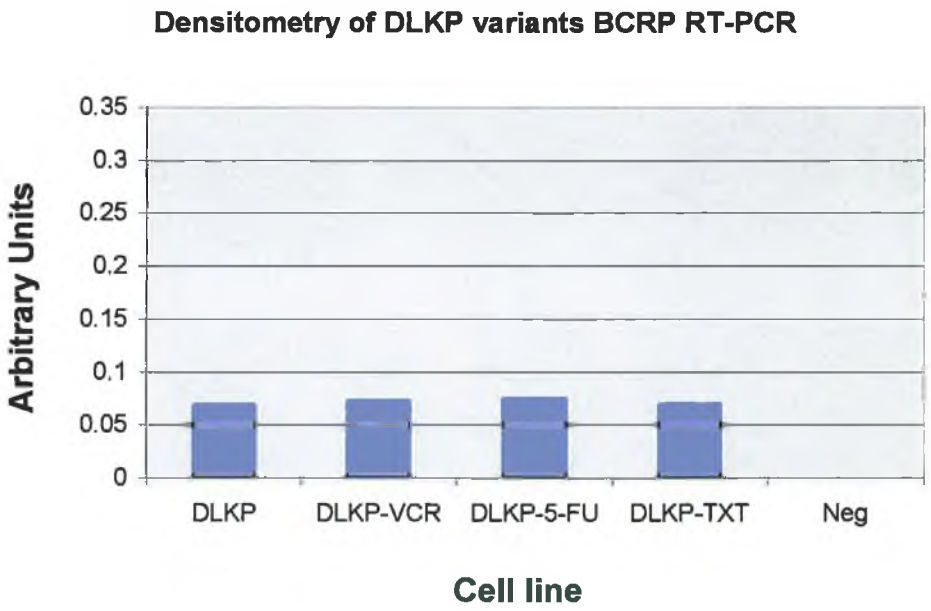
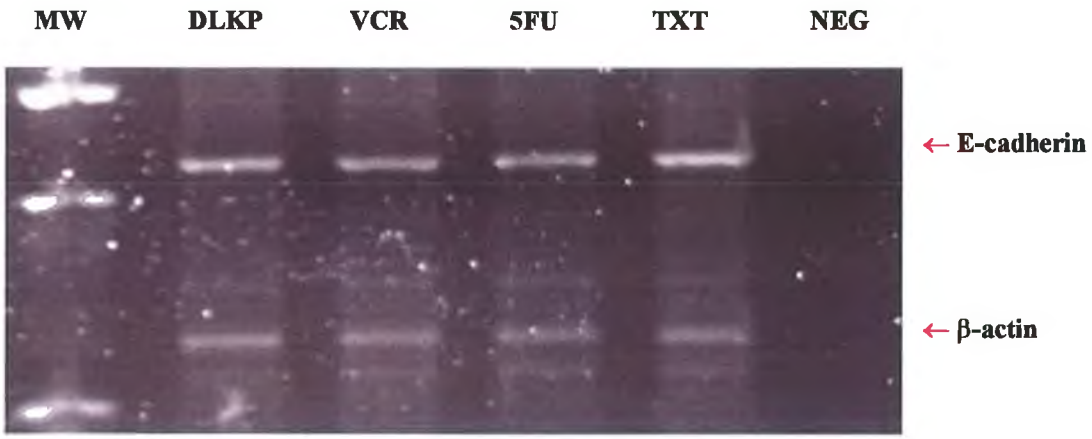


Figure 3.3.7.7 **(a)** RT-PCR analysis of BCRP expression in the DLKP parent cell line and its variants. **(b)** Densitometry of RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.8 E-cadherin expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis shows expression of E-cadherin mRNA all cell lines studied. DLKP-taxotere displays the strongest expression of E-cadherin mRNA compared to DLKP parent and the other two variants. This was only observed in one experiment, however, and could be a random result. Repeat experiments showed no change.

(a)



(b)

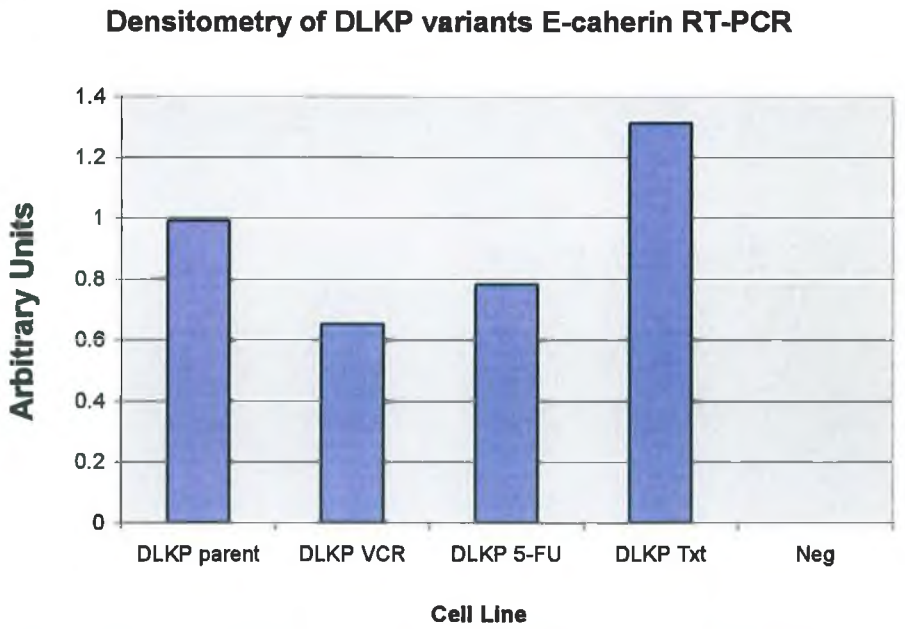
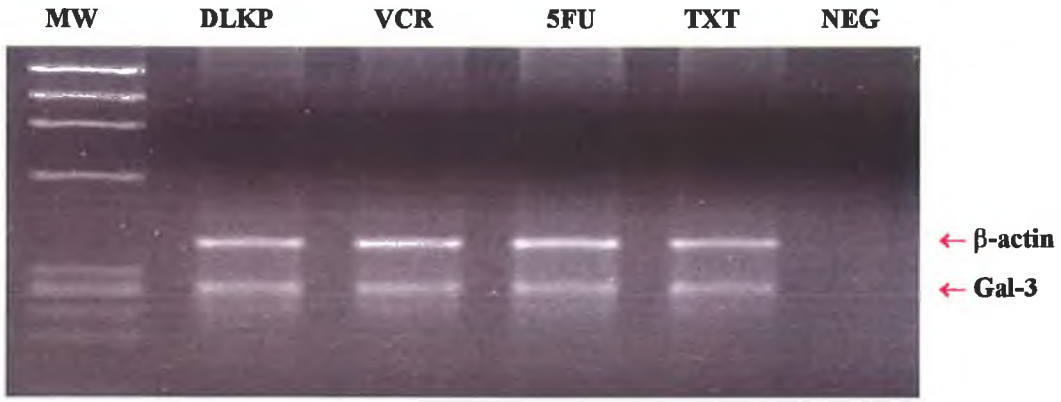


Figure 3.3.7.8 **(a)** RT-PCR analysis of E-cadherin expression in the DLKP parent cell line and its variants. **(b)** Densitometry of RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3)

3.3.7.9 Galectin-3 expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis on galectin-3 (Gal-3) shows that there was no change in galectin-3 mRNA expression in the DLKP variants compared to the parent cell line.

(a)



(b)

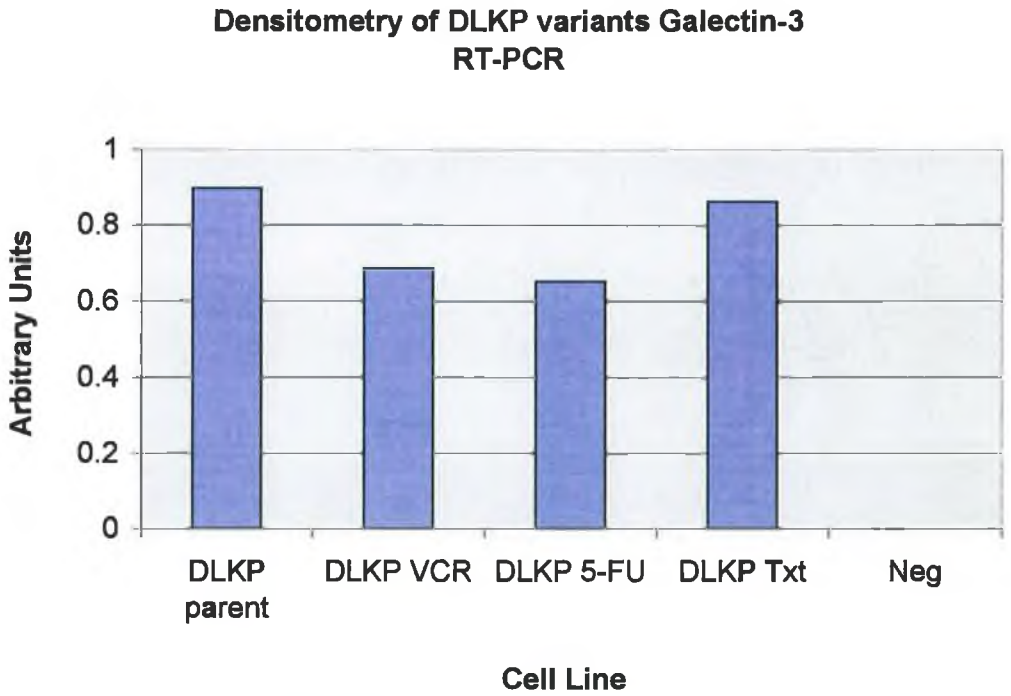
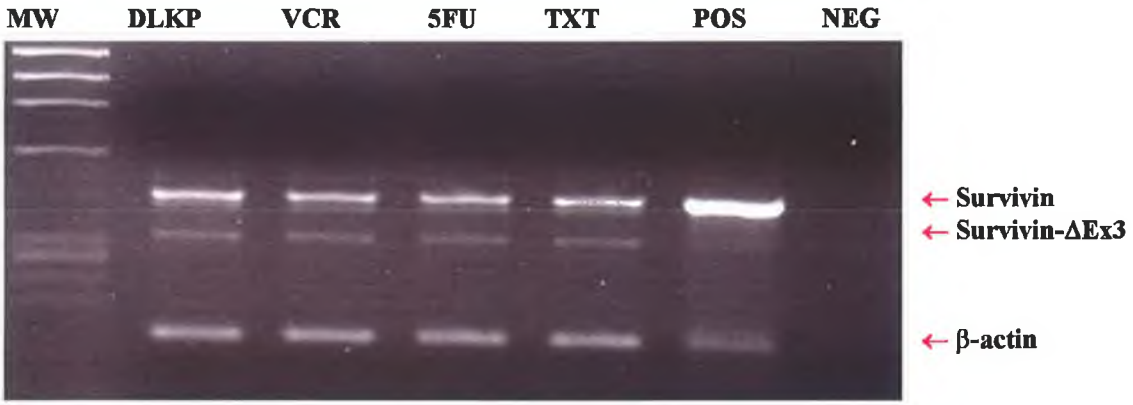


Figure 3.3.7.9 **(a)** RT-PCR analysis of galectin-3 expression in the DLKP parent cell line and its variants. **(b)** Densitometry on RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.7.10 Survivin expression in DLKP cell line and its variants by RT-PCR

RT-PCR analysis of survivin indicates that survivin mRNA expression is not greatly changed in the DLKP variants compared to DLKP parental cell line. Similarly, survivin-delta Ex3 mRNA expression was unaltered, while Survivin-2B mRNA expression was undetectable in all of the cell lines.

(a)



(b)

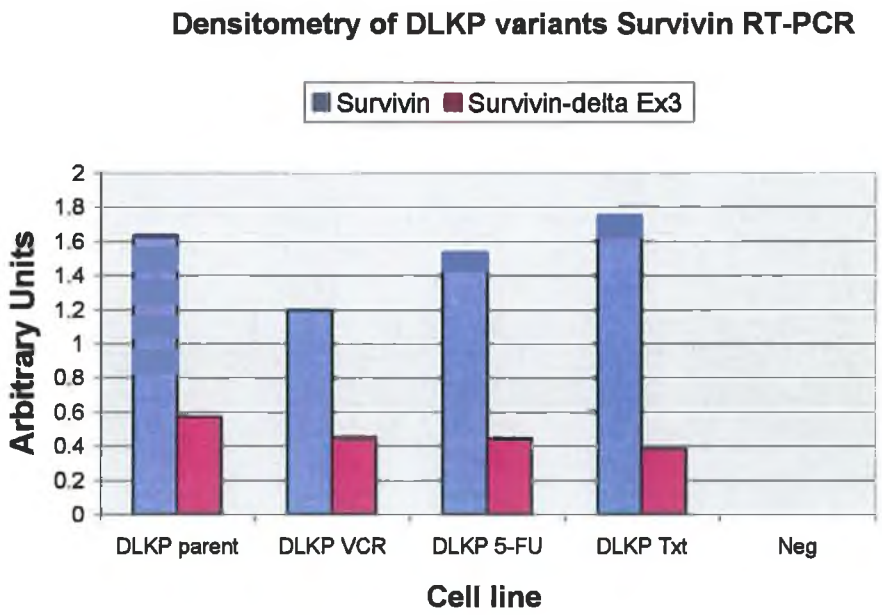


Figure 3.3.7.10 **(a)** RT-PCR analysis of survivin expression in the DLKP parent cell line and its variants. **(b)** Densitometry of RT-PCR. MW=Molecular weight marker; Neg=negative control (n=3).

3.3.8 Analysis of DLKP resistant variants using Western blot analysis

To detect the expression of relevant markers at the protein level, Western blotting was carried out as described in section 2.3. Western blots were carried out on 3 independent protein extractions from the DLKP variants. Four proteins were analysed (MDR1/P-170), MRP1, E-cadherin and survivin). The results are presented below.

3.3.8.1 P-170 expression in the DLKP cell line and its variants by western blot analysis

Western blot analysis of Mdr-1, demonstrates a dramatic increase in P-170 protein in the two MDR variants, DLKP-VCR and DLKP-TXT compared to the DLKP parent cell line and the DLKP-5-FU variant. This result was reproducible when western blot analysis was carried out in triplicate on three separate protein extractions. α-Tubulin was used as an endogenous control to ensure equal protein loading.

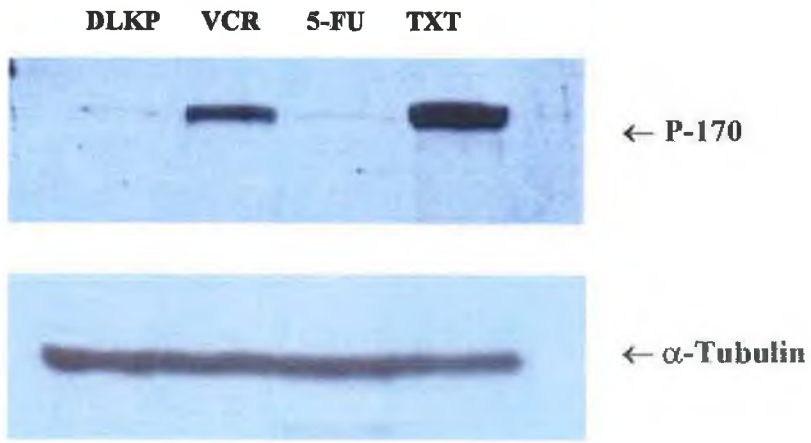


Figure 3.3.8.1 Western blot analysis of P-170 protein expression in DLKP parent and its selected variants, using α-Tubulin as an internal control to demonstrate equal loading (n=3).

3.3.8.2 MRP-1 expression in the DLKP cell line and its variants by western blot analysis

Western blot analysis of MRP-1 demonstrates an increase in MRP-1 protein expression in the DLKP-TXT variant compared to the DLKP parent cell line and the DLKP-VCR and DLKP-5FU variants. This was successfully carried out twice and the results were reproducible. An RPMI-2650 drug-selected variant was used as a positive control as has been previously shown to express MRP-1 protein by other researchers in the NICB. α-Tubulin was used as an internal control to ensure equal protein loading.

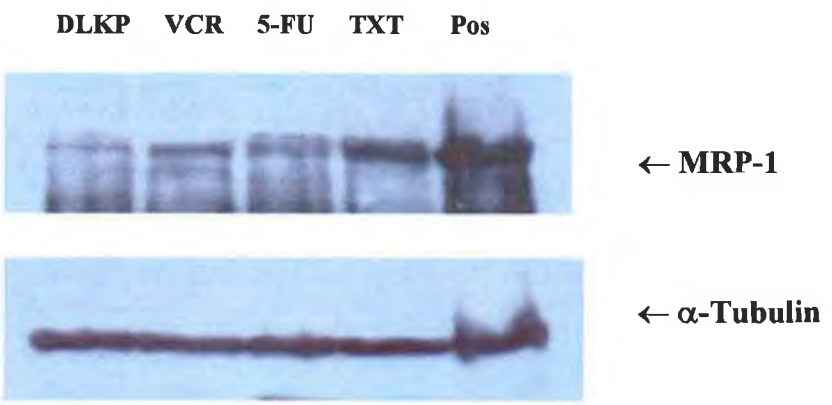


Figure 3.3.8.2 Western blot analysis of MRP1 protein expression in DLKP parent and its selected variants, using α-Tubulin as an internal control to demonstrate equal loading (n=2).

3.3.8.3 E-cadherion expression in the DLKP cell line and its variants by western blot analysis

E-cadherin protein expression was analysed by western blot analysis in the DLKP selected variants. Results indicate a slight increase in E-cadherin protein expression in the DLKP-TXT variants compared to the DLKP parent cell line and the DLKP-VCR and DLKP-5-FU variants. This experiments was only carried out once and may need to repeated to confirm this finding. α-Tubulin was used as an internal control.

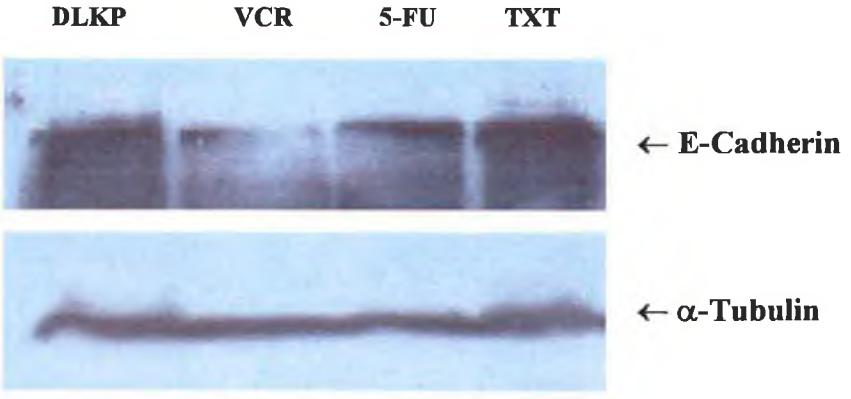


Figure 3.3.8.3 Western blot analysis of E-cadherin protein expression in DLKP parent and its selected variants, using α-Tubulin as an internal control to demonstrate equal loading (n=1).

3.3.8.4 Survivin expression in the DLKP cell line and its variants by western blot analysis

Western blot analysis was carried out on the DLKP parent cell line and its variants to study the expression of survivin protein. Results demonstrate the survivin protein is dramatically down-regulated in the two MDR variants, DLKP-VCR and DLKP-TXT compared to the DLKP parent cell line and the DLKP-5-FU variant. This result was reproducible when the western blot analysis was repeated three times on three separate protein extractions. All three western blots are shown in Figure 3.3.8.4 (A, B and C). α-Tubulin was used as an internal control to ensure equal protein loading in all experiments.

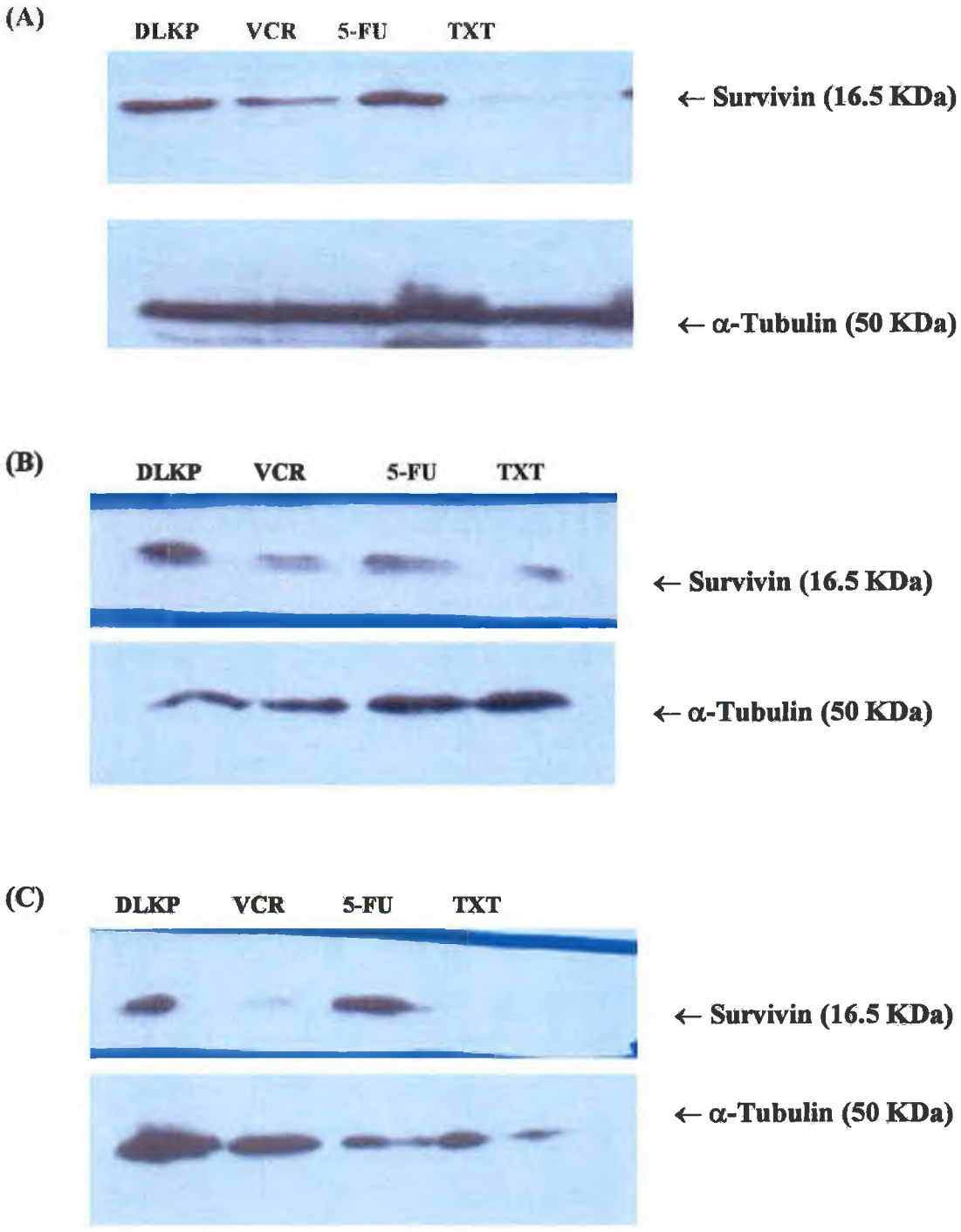


Figure 3.3.8.5 Western blot analysis of survivin protein expression in DLKP parent and its selected variants, using α-Tubulin as an internal control to demonstrate equal loading (n=3).

3.3.9 DNA Microarray Analysis

DNA microarray technique was used to identify changes in gene expression may have occurred due to drug exposure of DLKP cells. The cell lines included in this study were DLKP-1, DLKP-2, DLKP-VCR, DLKP-TXT-1 and DLKP-TXT-2.

The apoptosis- and MDR-related genes analysed by RT-PCR and/or western blot analysis were selected for preliminary DNA array analysis. These are listed in Table 3.3.9.1 and the fold changes in the three resistant cell lines, DLKP-TXT-1, DLKP-TXT-2 and DLKP-VCR is given. A fold-change of less than 2 was not considered to be a 'real' change. Table 3.3.9.1 also indicates (where relevant) if the findings agree with the RT-PCR results.

Gene of interest	DLKP-1	DLKP-VCR	DLKP-Txt-1	DLKP-Txt-2
Mdr-1	1	92.5 (Y)	300 (Y)	44
MRP1	1	0.9 (Y)	1 (Y)	0.9
***MRP2**	1	4.2 (N)	1.8 (N)	1.4
***MRP3**	1	0.7 (Y)	1.4 (Y)	1.1
MRP4	1	1 (Y)	0.3 (N)	1.1
MRP5	1	1.1 (Y)	1 (Y)	0.7
***BCRP**	1	1.1 (Y)	0.6 (Y)	2
Galectin-3	1	0.9 (Y)	0.6 (Y)	0.7
Survivin	1	1 (Y)	0.6 (Y)	1
***E-cadherin**	1	0.4 (N)	0.5 (N)	0.2

* Genes gave a very low reading and should have been recorded as 'absent'.

Table 3.3.9.1 Genes of interest and their fold changes as detected by DNA Microarray analysis (Y=Result agreed with RT-PCR; N=Result did not agree with RT-PCR).

3.4 Establishment of RPMI-2650 variants

RPMI-2650 is nasal carcinoma cell line found to have a very high level of sensitivity to cancer chemotherapeutic drugs. The aim of this section of the thesis was to establish drug resistant variants of this cell line through sequential pulsing of the cells. The RPMI-2650 cells were pulse selected with a range of chemotherapy drugs over a period of 10 to 14 weeks. This was carried out by exposing the cells to sequential pulses of an increasing concentration of drug once a week. Depending on the conditions of the cells, however, this period was extended until the cells regained confluency.

Initial concentrations of drug, which were chosen based on IC_{50} values for the RPMI-2650 cell line as determined by previous studies in the NICB, did not result in much cell death (see Table 3.4.1 for starting concentrations). After 3 pulses, this concentration was increased and cells started to die (see Table 3.4.1 for these intermediate concentrations which caused cell death). Drug concentration was gradually increased until 10, 12 or 14 pulses were reached (see Table 3.4.1 for highest concentrations of drug reached).

Seven drugs were used in this study, resulting in 5 viable cell lines. The selections were carried out in duplicate and treated as separate cell lines throughout the procedure, but not all selections survived the process to the end. Cells were frozen down at different stages of pulsing for future analysis (these included cells at 2, 4, 8 and 10 pulses).

Selection Drug	Starting Conc. of drug (μg/ml)	Intermediate Conc of Drug (μg/ml)	Highest conc. of drug (μg/ml)
Carboplatin	1	20	120
5FU	10	15	45
Vincristine	0.004	0.02	0.08
Epirubicin	0.05	0.2	1
CCNU	10	30	35

Table 3.4.1 Concentrations of Drug used for the selection of RPMI-2650 cells.

3.4.1 Morphology of the RPMI-2650 variants

During the selection procedure the morphology of the cells changed dramatically. The cells became elongated and spindle-shaped. A number of week after the selection was finished, however, the majority of the cells reverted back to their original morphology. Figure 3.4.1.1 illustrates the morphology of the parental cells and the morphology of 2 of the variants after receiving 10, 12 and 14 pulses of 5-Fluorouracil or Vincristine. The RPMI-2650 parental cell are small cells which grow in isolated clusters. The morphological change of the variants is less noticeable at 10 pulses, but increases with further drug exposure, as they become elongated and spread-out (Figure 3.4.1.1).

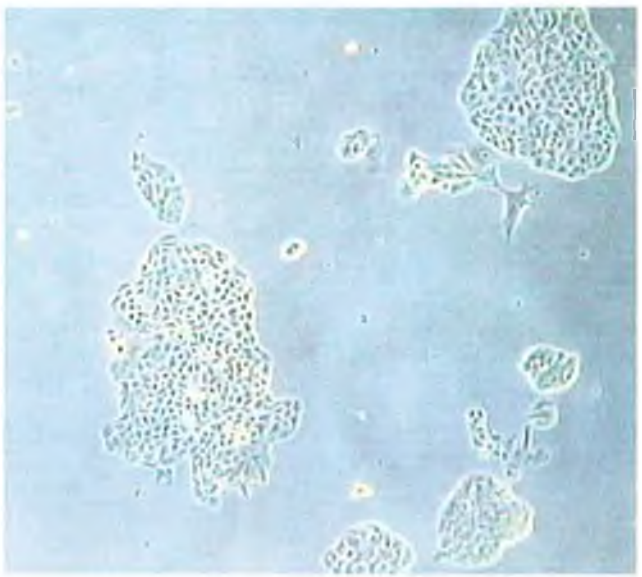


Figure 3.4.1.1a The morphology of the RPMI-2650 parental cell line (10X)

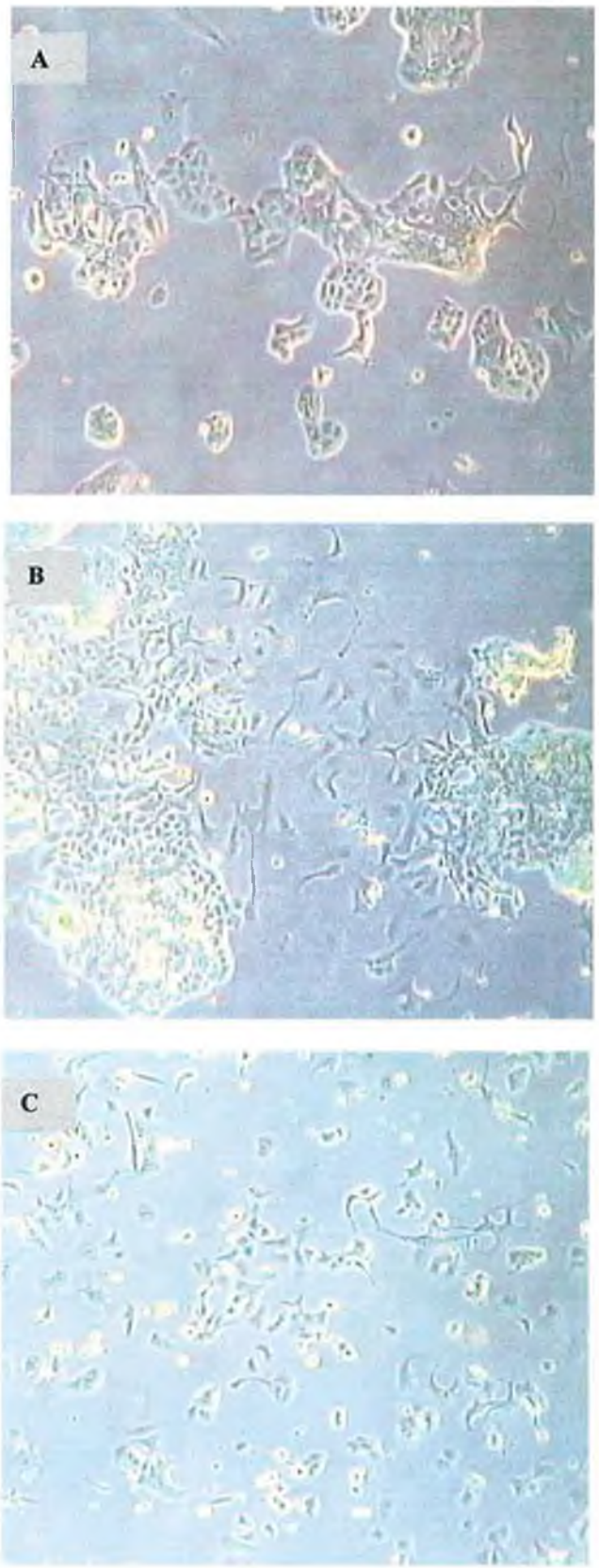


Figure 3.4.1.1b The morphology of the RPMI-2650 5-FU selected variant **(A)** after 10 pulses, **(B)** after 12 pulses, **(C)** after 14 pulses (10X).

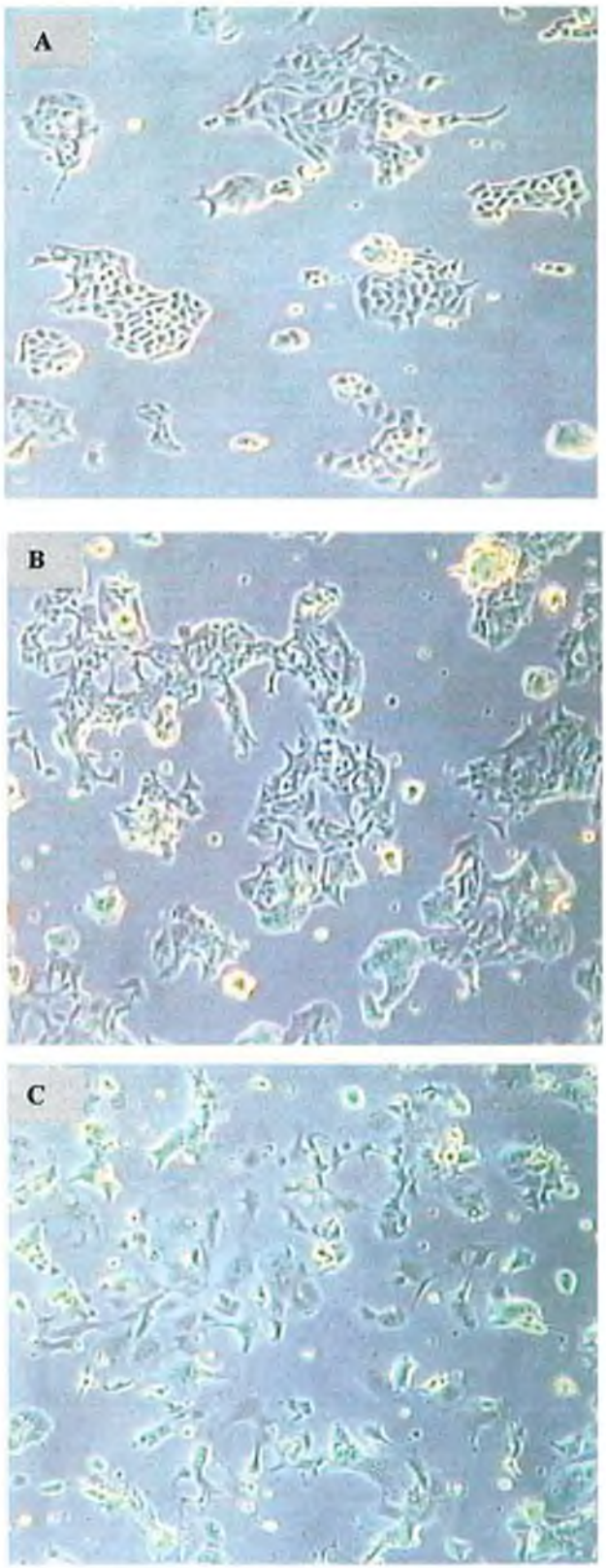


Figure 3.4.1.1c The morphology of the RPMI-2650 Vincristine-selected variant (A) after 10 pulses, (B) after 12 pulses, (C) after 14 pulses (10X).

3.4.2 Cross resistance profile of RPMI-2650 variants to a range of chemotherapy drugs

To investigate the resistance profiles of the RPMI-2650 variants, toxicity assays were carried out as described in section 2.3.1. The chemotherapeutic agents used included the selection drug, as well as other chemotherapy drugs to determine whether the variants have become multiple drug resistant. Initial results on a number of the variants revealed some level of resistance, however, when experiments were repeated, the cells seem to have to have lost their resistance. Variants that received additional pulses developed increased resistance again, however, this seems to be lost again with time (with the exception of VCR_2-12p (Table 3.4.2b)). Tables 3.4.2a, b and c represents the fold differences of each variant to each drug tested on three separate repeat experiments. The duration from one experiment to the next is included in italics in Tables 3.4.2b and c. In addition, the passage number of each variant is included in brackets to show the number of subcultures that took place from first to second and third experiments. Interesting variants which showed initial/continued resistance are highlighted in bold text and toxicity assays were carried out a third time on these variants (Tables 3.4.2c and 3.4.3c).

To determine whether the loss in resistance was due to continuous sub-culturing of the cells, frozen stocks of 3 of the variants (which were frozen before toxicity testing started) were thawed and toxicity assays were carried out again. The results are illustrated in Table 3.4.4. Ten days had elapsed between thawing the cells and carrying out the toxicity assays. Cells were thawed a third time and toxicity assays were carried out 5 days later. The results were different in these variants, and the reason for this is unclear. This is illustrated in Table 3.4.5.

In addition, two variants, RPMI-5-FU and RPMI-VCR, that had received 12 pulses were chosen for further pulsing, bringing the total number of pulses to 14. Toxicity assays were carried out twice, over a nine day period, on these 2 variants and the results are presented in Table 3.4.6 (a and b). These results were slightly different and it may be due to the density at which the cells were set up at. It was observed that these cells grow extremely slowly, and when toxicity assays were being repeated, the cells were set up at a higher density ($2x10^3$ cell/well) to allow the cells to reach 80 % grow in a period of 6-7 days. IC_{50} values of parent RPMI-2650 and variants are shown in Tables 3.4.3a, b, c and d.

(a)

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI (p37)	1	1	1	1
RPMI-5FU1 (p40)	**3.1**	**1.1**	**1.6**	**3.6**
RPMI-5FU1-12p (p43)	**14.5**	**32**	**1.1**	**7.7**
RPMI-5FU2 (p42)	1.9	0.88	1.1	0.92
RPMI-Carboplatin1 (p46)	3.1	1	0.8	2
RPMI-Carboplatin2 (p40)	2	1.14	1.3	2.8
RPMI-VCR1 (p42)	**3.7**	**1.1**	**2.4**	**8.7**
RPMI-VCR1-12p (p45)	1.6	1.6	2.1	0.4
RPMI-VCR2 (p39)	**4**	**1.3**	**1.3**	**8**
RPMI-VCR2-12p (p42)	**5.6**	**1.5**	**1.7**	**14.2**
RPMI-CCNU1 (p44)	0.4	1.3	1.1	1.8
RPMI-CCNU2 (p41)	**14.4**	**15.7**	**3.5**	**9.1**
RPMI-Epirubicin1 (p42)	1.6	1	-	1.5
RPMI-Epirubicin2 (p40)	2.2	0.85	1.1	2

Table 3.4.2a Fold resistance of RPMI variants to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line. **First experiment**.

(b)

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI (p39)	1	1	1	1
RPMI-5FU1 (p41) *19 days after 1st assay*	**0.9**	**1**	**1.4**	**0.3**
RPMI-5FU1-12p (p44) *10 days after 1st assay*	**1.2**	**1.1**	**1.4**	**2.1**
RPMI-5FU2 (p43) *6 days after 1st assay*	1.5	0.9	0.97	1.4
RPMI-Carboplatin1 (p47) *10 days after 1st assay*	0.9	1.1	1.8	3.9
RPMI-Carboplatin2 (p41) *6 days after 1st assay*	0.9	1	1.2	3.4
RPMI-VCR1 (p46) *19 days after 1st assay*	**1.8**	**1.7**	**1.1**	**1.9**
RPMI-VCR1-12p (p47) *10 days after 1st assay*	1.4	1.1	1.7	2.1
RPMI-VCR2 (p41) *19 days after 1st assay*	**1.4**	**1**	**0.6**	**1**
RPMI-VCR2-12p (p43) *10 days after 1st assay*	**14.5**	**3.2**	**2.5**	**9.7**
RPMI-CCNU1 (p46) *10 days after 1st assay*	0.3	1.1	1.6	1.6
RPMI-CCNU2 (p43) *19 days after 1st assay*	**4**	**2.2**	**0.9**	**3**
RPMI-Epirubicin1 (p43) *10 days after 1st assay*	1.3	1.4	1.4	2.6
RPMI-Epirubicin2 (p41) *6 days after 1st assay*	0.4	1.1	1	0.8

Table 3.4.2b Fold resistance of RPMI variants to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line. **Second experiment**.

(c)

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI (p40)	1	1	1	1
RPMI-5FU1 (p43) *10 days after 2nd assay*	**0.89**	**0.98**	**1.46**	**1**
RPMI-5FU1-12p (p47) *5 weeks after 2nd assay*	**1.26**	**1.11**	**0.87**	**0.2**
RPMI-VCR1 (p47) *10 days after 2nd assay*	**1.6**	**1**	**1.8**	**2.4**
RPMI-VCR2 (p42) *10 days after 2nd assay*	**1.1**	**1**	**1.1**	**1**
RPMI-VCR2-12p (p47) *5 weeks after 2nd assay*	**2.4**	**2.6**	**1.1**	**3.4**
RPMI-CCNU2 (p44) *10 days after 2nd assay*	**1.5**	**3.3**	**2.5**	**9.7**

Table 3.4.2c Fold resistance of RPMI variants to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line. **Third experiment.**

Cell line	5FU (μg/ml)	VCR (ng/ml)	CCNU (μg/ml)	Carboplatin (μg/ml)
RPMI-5FU1 (p40)	**0.23±0.04**	**0.7±.02**	**3.73±0.03**	**2.78±0.03**
RPMI-5FU1-12p (p43)	**2.61±0.03**	**20.54±0.01**	**7.52±0.02**	**13.57±0.03**
RPMI-5FU2 (p42)	0.16±0.11	0.65±0.17	5.35±0.44	0.81±0.13
RPMI-Carboplatin1 (p46)	0.56±0.04	0.64±0.08	5.6±0.05	3.5±0.06
RPMI-Carboplatin2 (p40)	0.17±0.05	0.84±0.03	6.17±0.03	2.46±0.04
RPMI-VCR1 (p42)	**0.27±0.12**	**0.67±0.19**	**5.4±0.16**	**6.73±0.11**
RPMI-VCR1-12p (p45)	0.29±0.03	1.01±0.02	14.33±0.04	0.68±0.01
RPMI-VCR2 (p39)	**0.3±0.07**	**0.81±0.06**	**3.02±0.11**	**6.15±0.06**
RPMI-VCR2-12p (p42)	**1.01±0.06**	**0.95±0.06**	**11.23±0.01**	**25.4±0.01**
RPMI-CCNU1 (p44)	0.07±0.05	0.82±0.02	7.65±0.04	3.16±0.08
RPMI-CCNU2 (p41)	**1.07±.005**	**9.66±0.007**	**7.8±0.01**	**7.05±.0.009**
RPMI-Epirubicin1 (p42)	0.28±0.01	0.65±0.04	-	2.65±0.01
RPMI-Epirubicin2 (p40)	0.17±0.01	0.71±0.009	5.24±0.01	1.8±0.02
RPMI-5FU2-14p (p47)	1.04±0.003	3.24±0.005	8.18±0.003	14.12±0.004
RPMI-VCR2-14p (p48)	18.58±0.008	-	14.4±0.01	36.5±0.009

*The Standard deviation presented is a standard deviation calculated from the IC_{50} values of eight replicate wells in each experiment (- = out of range).

Table 3.4.3a IC_{50} values of RPMI variants to a range of chemotherapy drugs (1st Exp)

Cell line	5FU (μg/ml)	VCR (ng/ml)	CCNU (μg/ml)	Carboplatin (μg/ml)
RPMI-5FU1 (p41) ***19 days after 1st assay***	**0.16±0.03**	**061±0.2**	**9.37±0.07**	**0.6±0.24**
RPMI-5FU1-12p (p44) ***10 days after 1st assay***	**0.23±0.03**	**0.76±0.01**	**4.22±0.05**	**2.2±0.02**
RPMI-5FU2 (p43) ***6 days after 1st assay***	0.25±0.12	0.65±0.09	6.02±0.11	1.6±0.14
RPMI-Carboplatin1 (p47) ***10 days after 1st assay***	0.16±0.03	0.8±0.026	5.5±0.06	4.04±0.02
RPMI-Carboplatin2 (p41) ***6 days after 1st assay***	0.15±0.1	0.74±0.09	7.45±0.21	1.36±0.03
RPMI-VCR1 (p46) ***19 days after 1st assay***	**0.33±0.06**	**1.04±0.06**	**7.2±0.03**	**3.42±0.03**
RPMI-VCR1-12p (p47) ***10 days after 1st assay***	0.25±0.01	0.8±0.05	5.07±0.3	2.18±0.3
RPMI-VCR2 (p41) ***19 days after 1st assay***	**0.25±0.08**	**0.64±0.056**	**4.1±0.09**	**1.7±0.08**
RPMI-VCR2-12p (p43) ***10 days after 1st assay***	**2.68±0.1**	**2.28±0.21**	**7.6±0.25**	**10.13±0.6**
RPMI-CCNU1 (p46) ***10 days after 1st assay***	0.06±0.02	0.8±0.04	4.76±0.04	1.71±0.03
RPMI-CCNU2 (p43) ***19 days after 1st assay***	**0.72±0006**	**1.38±0.006**	**5.81±0.005**	**5.32±0.007**
RPMI-Epirubicin1 (p43) ***10 days after 1st assay***	0.25±0.03	0.98±0.07	4.22±0.01	2.77±.03
RPMI-Epirubicin2 (p41) ***6 days after 1st assay***	0.06±0.04	0.76±0.03	6.16±0.02	0.95±0.03
RPMI-5FU2-14p (p47)	2.27±0.004	25.2±0.08	9±0.01	12.5±0.005
RPMI-VCR2-14p (p48)	-	-	20±0.01	10±0.02

*The Standard deviation presented is a standard deviation calculated from the IC_{50} values of eight replicate wells in each experiment (- = out of range).

Figure 3.4.3b IC_{50} values of RPMI variants to a range of chemotherapy drugs (2nd Exp)

Cell line	5FU (μg/ml)	VCR (ng/ml)	CCNU (μg/ml)	Carboplatin (μg/ml)
RPMI-5FU1 (p43) *10 days after 2nd assay*	**0.15±0.55**	**0.75±0.07**	**7.6±0.05**	**1.06±0.03**
RPMI-5FU1-12p (p47) *5 weeks after 2nd assay*	**0.21±0.08**	**0.8±0.03**	**5.43±0.07**	**0.22±0.03**
RPMI-VCR1 (p47) *10 days after 2nd assay*	**0.3±0.05**	**0.75±0.04**	**5.47±0.03**	**2.53±0.07**
RPMI-VCR2 (p42) *10 days after 2nd assay*	**0.08±0.1**	**0.77±0.1**	**5.77±0.06**	**1.09±0.1**
RPMI-VCR2-12p (p47) *5 weeks after 2nd assay*	**0.4±0.04**	**1.87±0.03**	**6.7±0.07**	**3.76±0.05**
RPMI-CCNU2 (p44) *10 days after 2nd assay*	**0.28±0.01**	**1.3±0.01**	**5.07±0.02**	**0.51±0.06**

*The Standard deviation presented is a standard deviation calculated from the IC_{50} values of eight replicate wells in each experiment.

Table 3.4.3c IC_{50} values of RPMI variants to a range of chemotherapy drugs (3rd Exp)

Cell line	5FU (μg/ml)	VCR (ng/ml)	CCNU (μg/ml)	Carboplatin (μg/ml)
RPMI-2650	0.13±0.04	0.68±0.05	6.41±1.32	1.21±0.4

*The results presented in Table 7.3.4 are an average of six separate experiments.

Table 3.4.3d IC_{50} values of RPMI-2650 parent cells to a range of chemotherapy drugs.

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI	1	1	1	1
RPMI-5FU1	0.9	1	1.23	1
RPMI-VCR2	0.5	1	0.9	1
RPMI-CCNU2	1.66	1.75	0.8	0.5

Table 3.4.4 Fold resistance of RPMI variants to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line, when variants were thawed first time.

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI	1	1	1	1
RPMI-5FU1	2.16	-	1.1	1.5
RPMI-VCR2	2.14	1.1	1.1	3
RPMI-CCNU2	**19.5**	**9.5**	**2**	**4.2**

Table 3.4.5 Fold resistance of RPMI variants to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line, when variants were thawed second time.

Cell line	5FU	VCR	CCNU	Carboplatin
RPMI	1	1	1	1
RPMI-5FU1-14p	4.3	3.3	1	4.3
RPMI-VCR2-14p	76.5	>300	1.7	11

Table 3.4.6a Fold resistance of RPMI-5-FU-14p and RPMI-VCR-14p to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line. **Run 1**

Cell line	5FU	VCR	CCNU	Carboplatin	Adriamycin
RPMI	1	1	1	1	1
RPMI-5FU1-14p	18	23	1.55	7.3	23
RPMI-VCR2-14p	>150	>500	3.47	5.8	77

Table 3.4.6b Fold resistance of RPMI-5-FU-14p and RPMI-VCR-14p to a range of chemotherapy drugs relative to the parental RPMI-2650 cell line. **Run 2**

3.4.3 Analysis of RPMI-2650 variants using in vitro Invasion assay.

To investigate the invasive ability of RPMI-2650 variants compared to the parental cell line, invasion assays were carried out as described in section 2.4. Cell culture inserts were coated with ECM before cells were added. After a 48 hour incubation period, invasive cells were stained with crystal violet and observed under a microscope. RPMI-2650 melphalan-resistant, a highly invasive cell line previously generated in the NICB, was used as a positive control. RPMI-2650 parental cell line is a non-invasive cell line. As shown in Figure 3.4.3.1, 3.4.3.2 and 3.4.3.3, the RPMI-2650 parent cells and all variants were non-invasive compared to the positive control. Invasion assays were carried out in duplicate

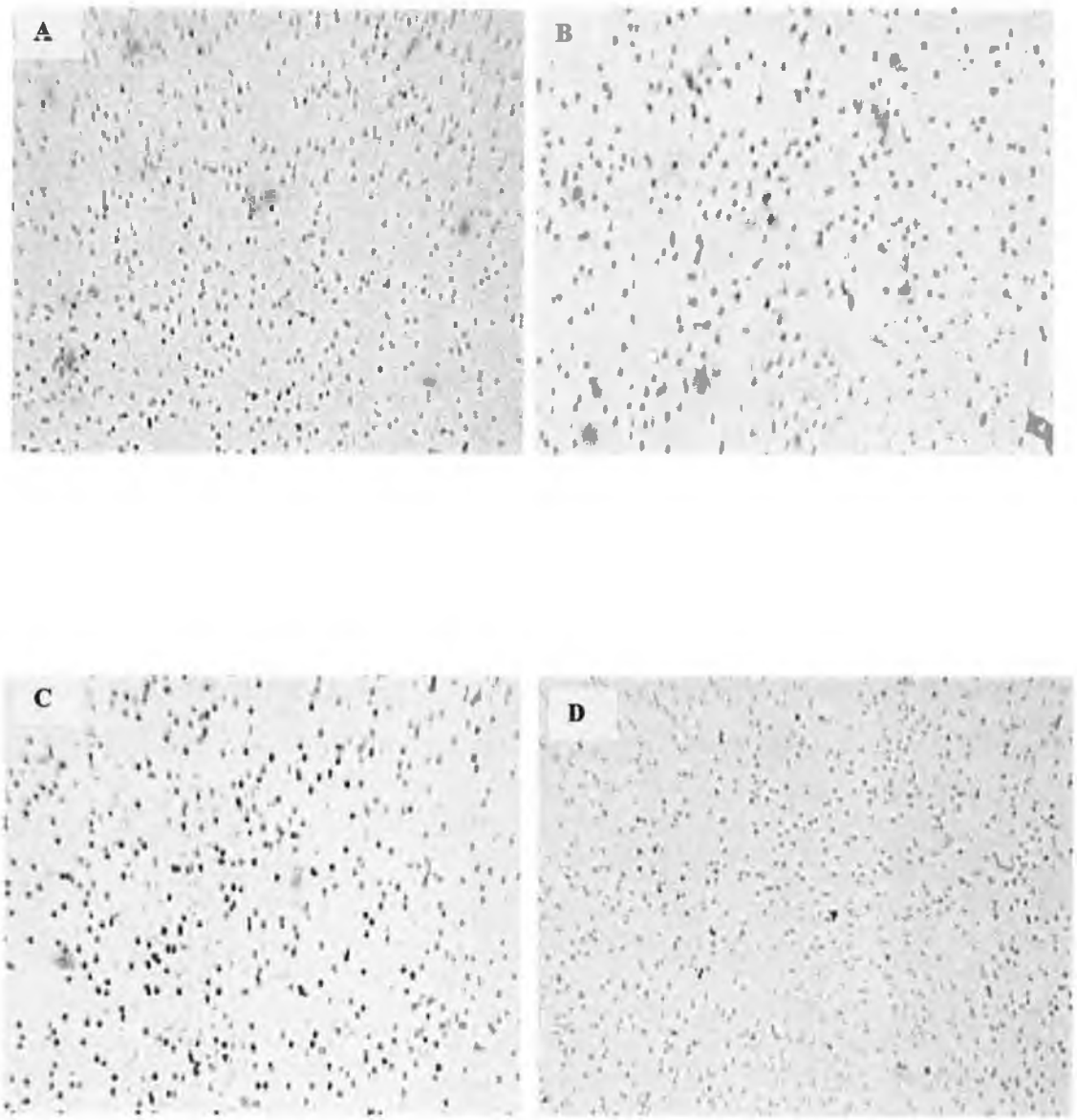


Figure 3.4.3.1a Invasion assays of **(A)** RPMI-2650-5FU1, **(B)** RPMI-2650-5FU1-12p, **(C)** RPMI-2650-VCR1, **(D)** RPMI-2650-VCR1-12p (4X) (n=2).

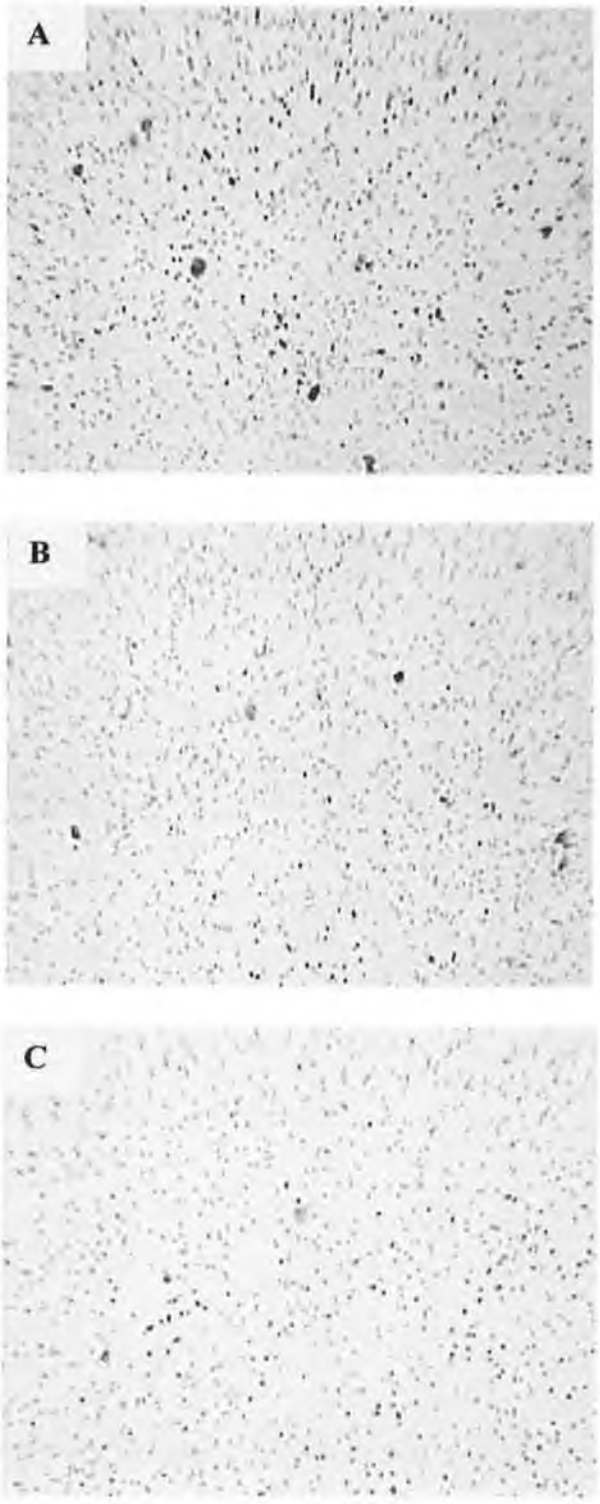


Figure 3.4.3.1b Invasion assays of **(A)** RPMI-2650-Carboplatin1, **(B)** RPMI-2650-CCNU1, **(C)** RPMI-2650-Epirubicin1 (4X) (n=2).

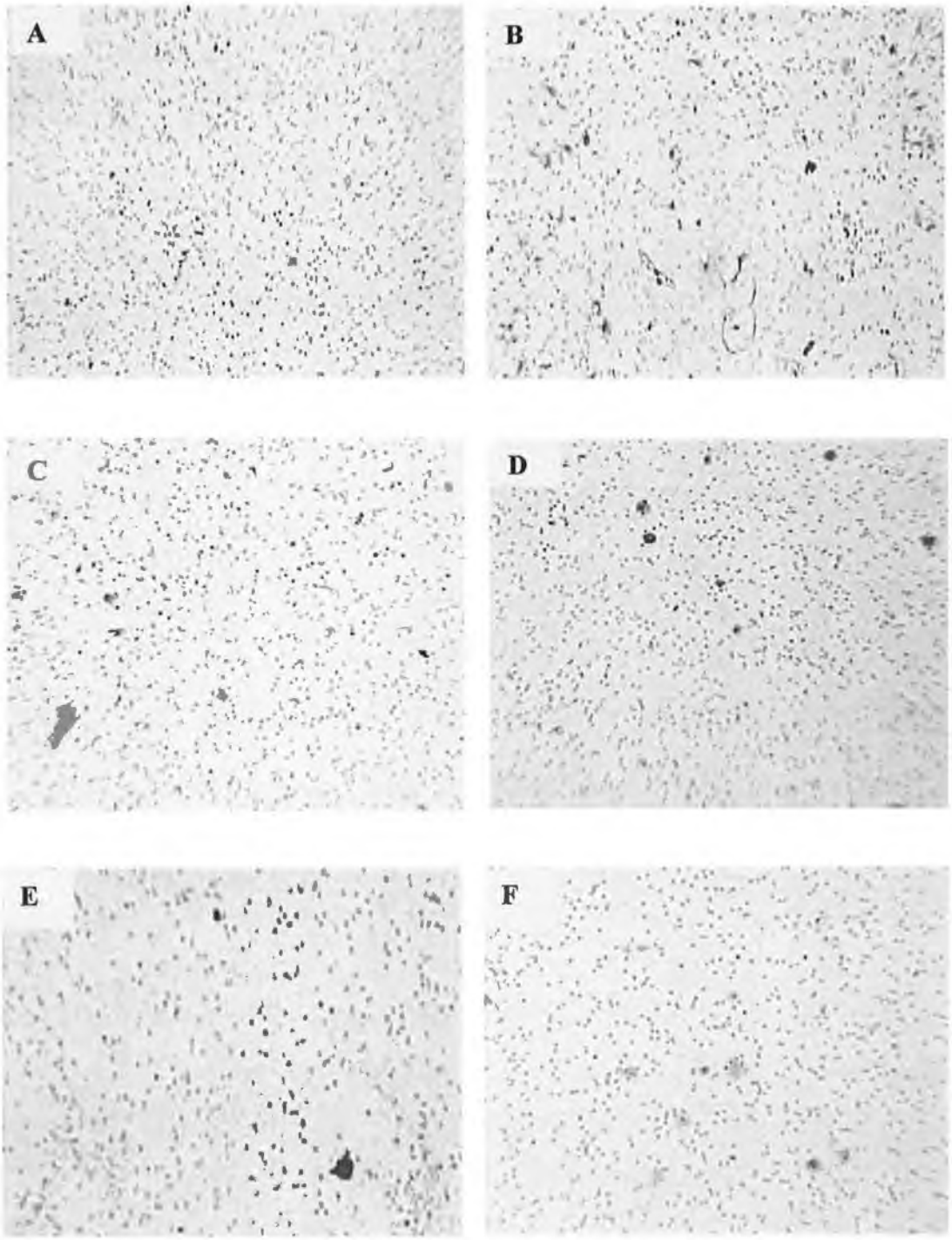
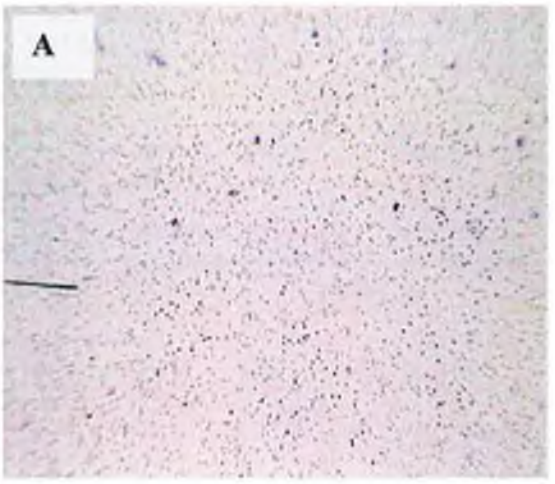


Figure 3.4.3.2 Invasion assays of **(A)** RPMI-2650-VCR2-10p, **(B)** RPMI-2650-VCR2-12p, **(C)** RPMI-2650-5FU2, **(D)** RPMI-2650-Carboplatin2, **(E)** RPMI-2650-CCNU2, **(F)** RPMI-2650-Epirubicin2 (4X) (n=2).



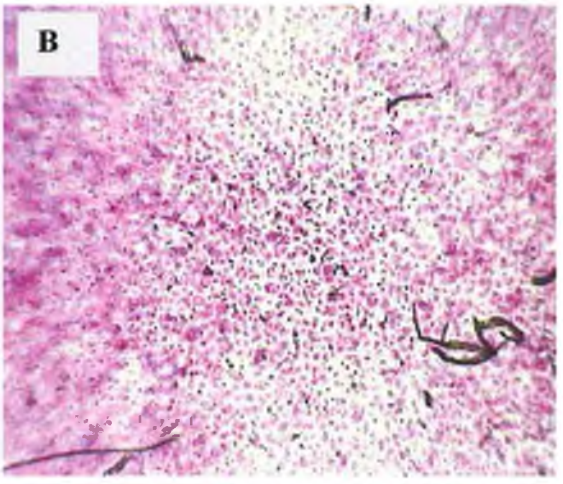


Figure 3.4.3.3 Invasion assays of **(A)** RPMI-2650 parent, **(B)** RPMI-2650-Melphalan (4X) (n=2).

3.4.4 Analysis of RPMI-2650 variants using *in vitro* Motility assays

Motility assays were carried out to compare the locomotive ability of the RPMI-2650 parental cell line and two of its variants. Similar to the invasion assays, motility assays were incubated for 48 hours, before being stained with crystal violet. Motility assays were not carried out on all RPMI-2650 variants as there was no increase in invasiveness due to drug selections. However, motility assays were carried out on the two variants that were pulsed 14 times. The results shown in Figure 3.4.4.1 demonstrate that despite the large increase in drug resistance of RPMI-5FU1-14p and RPMI-VCR2-14p, there was no change in their motility profile. The motility assay was set up on the same day as the second toxicity assay which is shown in Figure3.4.5b. Motility assays were carried out once.

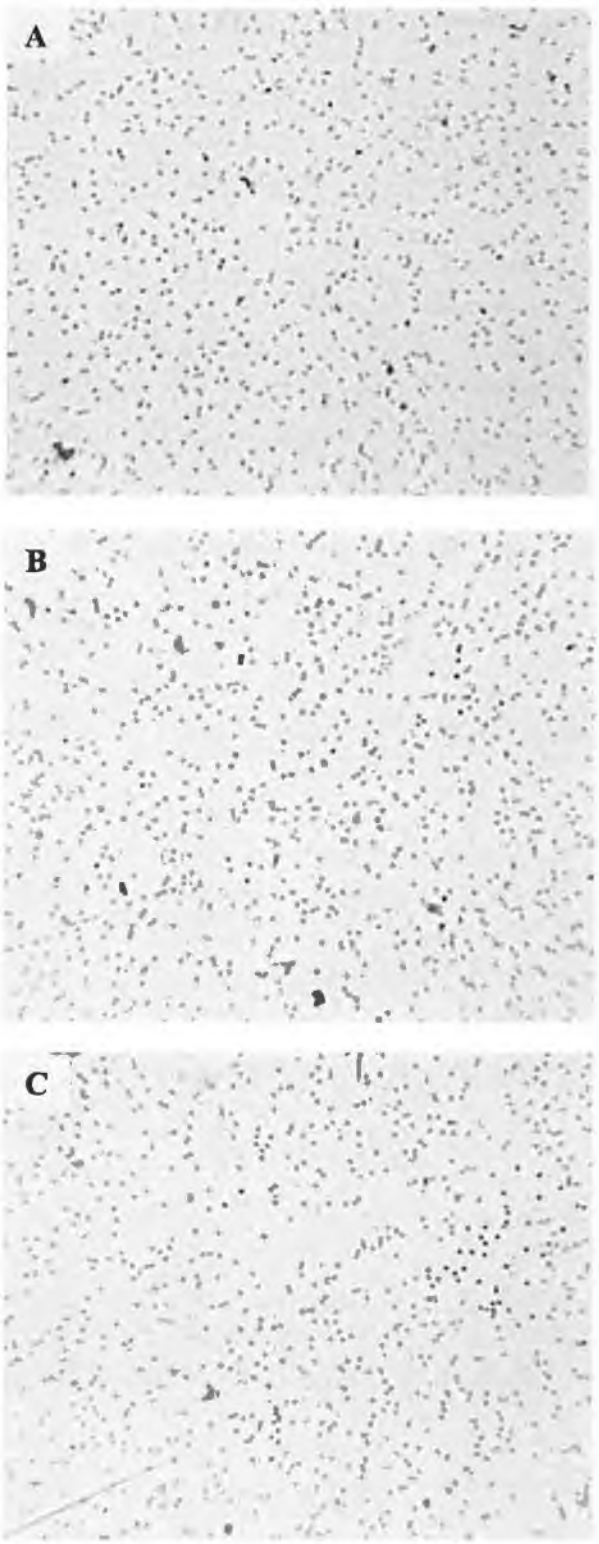


Figure 3.4.4.1 Motility assays of **(A)** RPMI-2650 parent cells, **(B)** RPMI-2650-5FU1-14p, **(C)** RPMI-2650-VCR2-14p (4X) (n=1)

3.4.5 Analysis of RPMI-2650 variants using RT-PCR

To detect the expression of relevant markers at the RNA level, RT-PCR was carried out as described in section 2.2. Eight genes were analysed. These are: MDR-1 (Pgp), MRP1, MRP2 (cMOAT), MRP3, MRP4, MRP5, Galectin-3 and Survivin and its splice variant. All RT-PCRs were carried out twice and the results were very similar in most cases, unless otherwise stated. Densitometry analysis was carried out using β-actin to normalise the results. A plasmid for each gene was used as a positive control, with the exception of Galectin-3 and Survivin. Sterile water was used as a negative control in each PCR.

3.4.5.1 MDR-1 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis (Figure 3.4.5.1) showed that MDR-1 was expressed in the RPMI-2650 parental cell line as well as all the variants, with the exception of RPMI-2650-VCR2-12p, however, this may be due to low levels of expression as this was not the case in a previous experiment. There was no change in expression levels between the variants and the parental cell line.

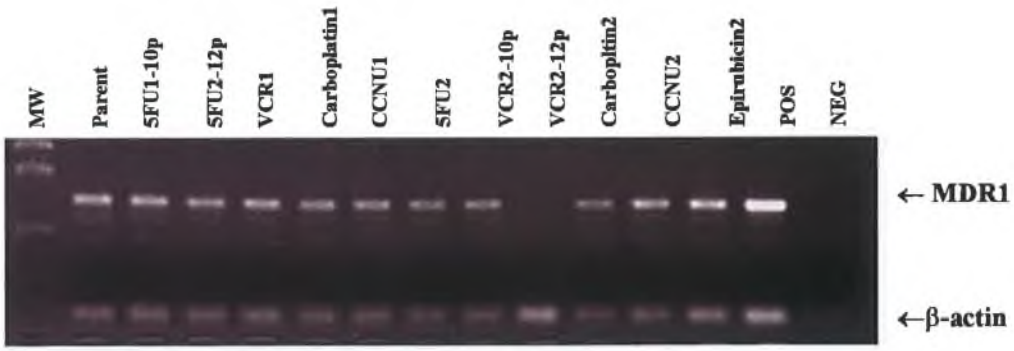
3.4.5.2 MRP1 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis (Figure 3.4.5.2) on MRP1 showed that MRP1 was expressed in all cell lines, including the parental cells. There seems to be an increase in expression in most of the variants. This increase is greatest in RPMI-VCR1, RPMI-carboplatin1, and RPMI-CCNU2.

3.7.5.3 MRP2 (cMOAT) mRNA expression in RPMI-2650 and its variants

RT-PCR analysis (Figure 3.4.5.3) on MRP2 (cMOAT) shows that there was no detectable expression of MRP2 mRNA in RPMI-2650 or RPMI-2650 variants.

(a)



(b)

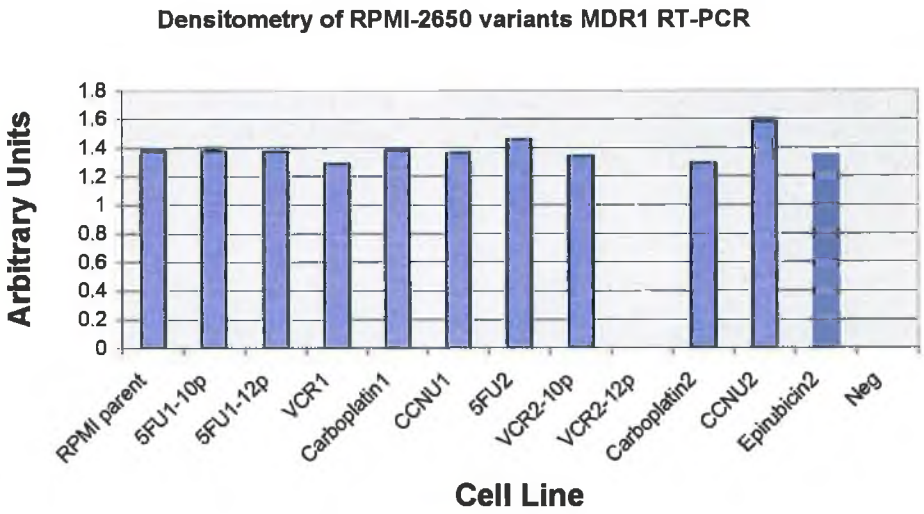
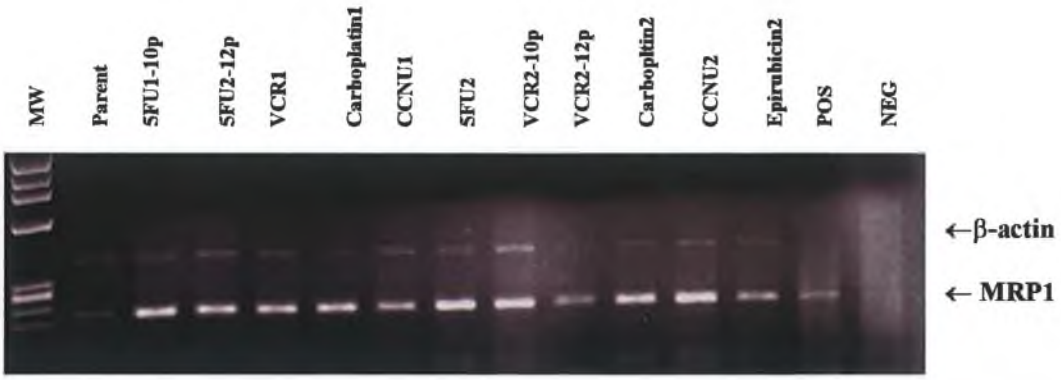


Figure 3.4.5.1 **(a)** RT-PCR analysis of MDR-1 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2).

(a)



(b)

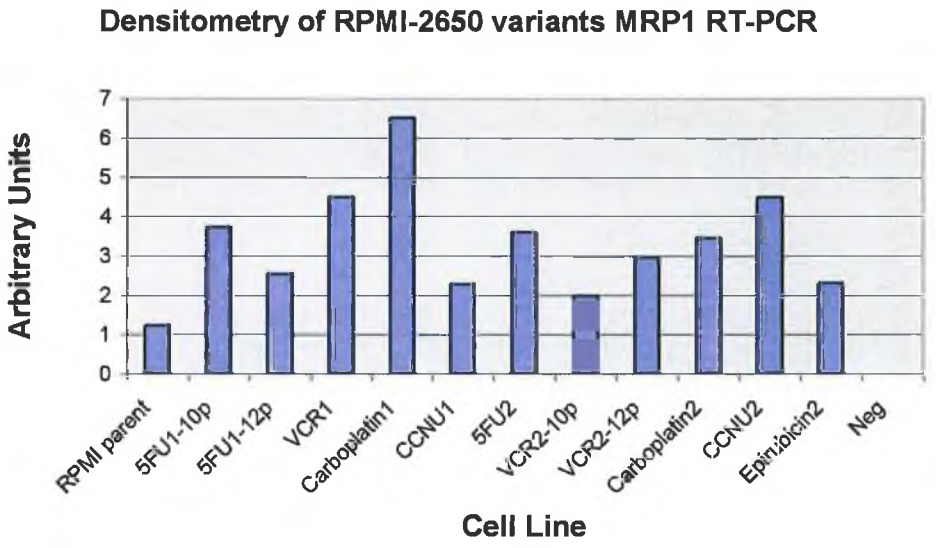


Figure 3.4.5.2 **(a)** RT-PCR analysis of MRP1 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2).

(a)

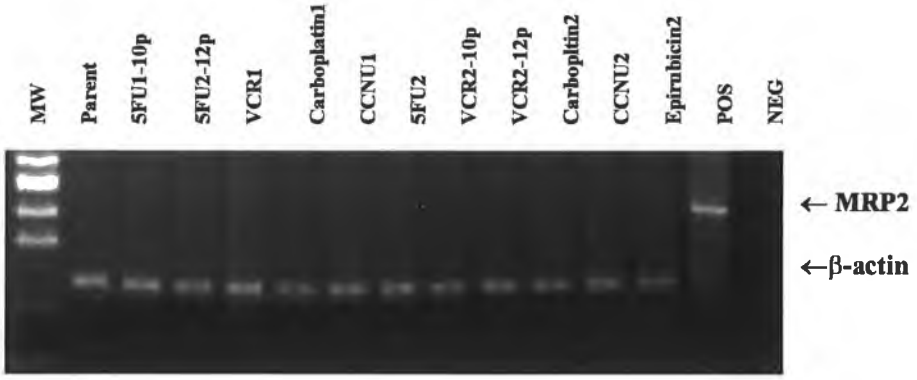


Figure 3.4.5.3 **(a)** RT-PCR analysis of MRP2 expression in the RPMI-2650 parent cell line and its variants (n=2).

3.4.5.4 MRP3 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis of MRP3 (Figure 3.4.5.4) shows that MRP3 is detected in RPMI-2650 parental cells and the RPMI-2650 variants. RPMI-VCR2-12p shows an over-expression of MRP3 compared to the parental cells. This PCR has proven problematic due to the many background bands present and it was successfully carried out once.

3.4.5.5 MRP4 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis of MRP4 (Figure 3.4.5.5) shows it is expressed in RPMI-2650 parental cells and the RPMI-2650 variants, RPMI-VCR2-10p, RPMI-VCR2-12p, RPMI-CCNU2 and RPMI-Epirubicin2 show an over-expression of MRP4 compared to the parental cells.

3.4.5.6 MRP5 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis of MRP5 (Figure 3.4.5.6) shows it is detected in RPMI-2650 parental cells and the RPMI-2650 variants. RPMI-CCNU2 and RPMI-Epirubicin2 show an over-expression of MRP5 compared to the parental cells.

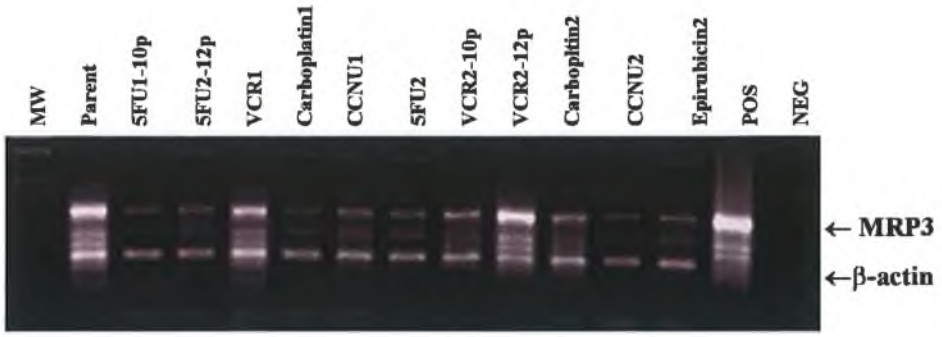
3.4.5.7 Galectin-3 mRNA expression in RPMI-2650 and its variants

RT-PCR analysis of galectin-3 (Figure 3.4.5.7) shows it is detected in RPMI-2650 parental cells and the RPMI-2650 variants. There is no dramatic change in expression levels of the variants.

3.4.5.8 Survivin expression mRNA in RPMI-2650 and its variants

RT-PCR analysis of Survivin (Figure 3.4.5.8) shows it is detected in RPMI-2650 parental cells and the RPMI-2650 variants. RPMI-5FU2, RPMI-VCR2-10p, RPMI-VCR2-12p, and RPMI-Carboplatin2 show an up-regulation of survivin. However, the expression of the two splice variants, survivin-2B and survivin-ΔEx3, remained unchanged. A survivin cDNA-transfected cell line was used as a positive control.

(a)



(b)

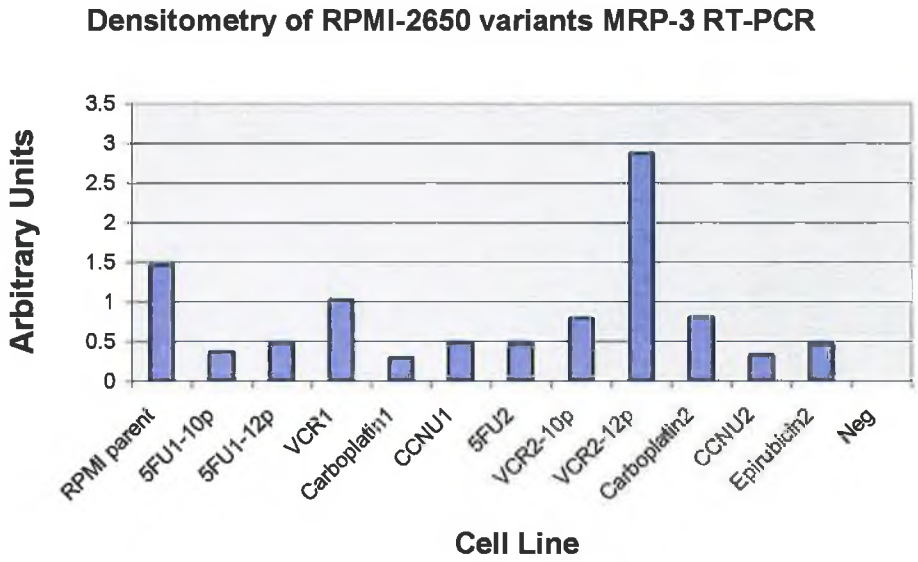
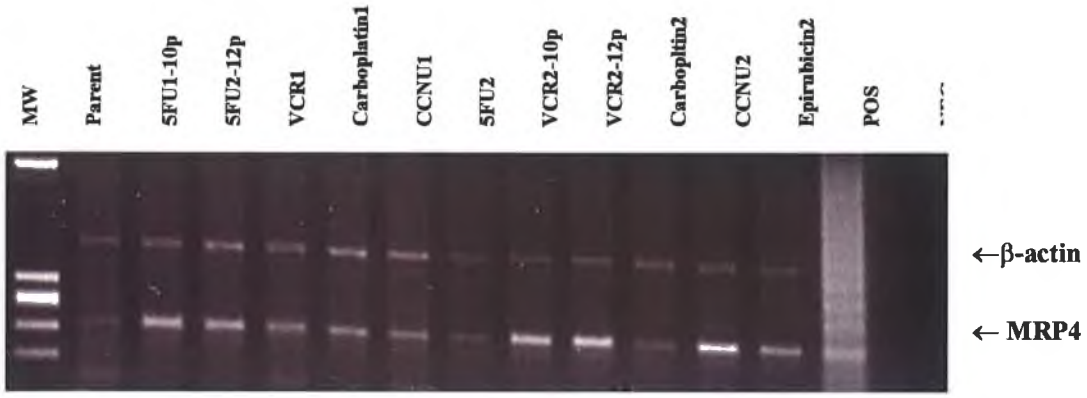


Figure 3.4.5.4 **(a)** RT-PCR analysis of MRP3 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=1).

(a)



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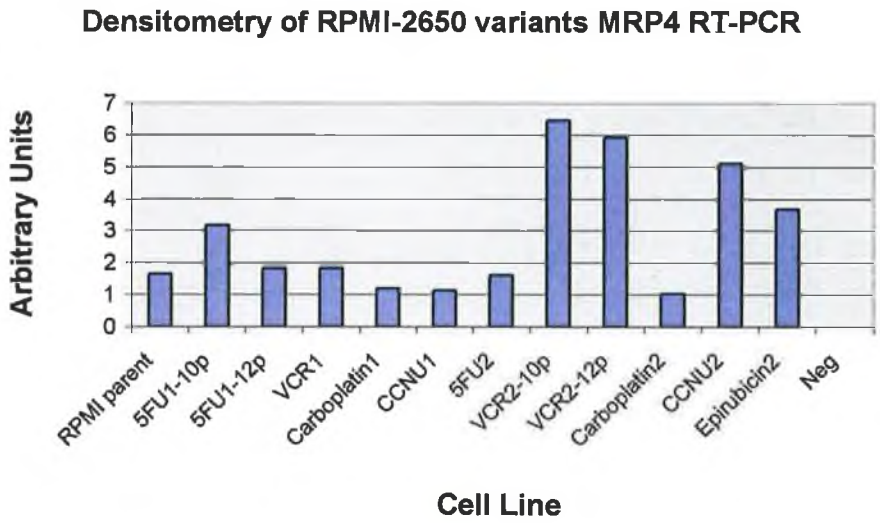
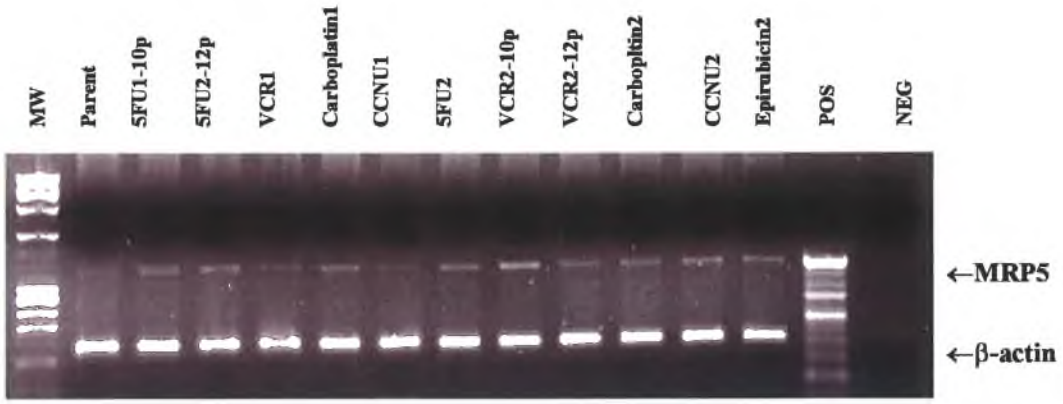


Figure 3.4.5.5 **(a)** RT-PCR analysis of MRP4 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2).

(a)



(b)

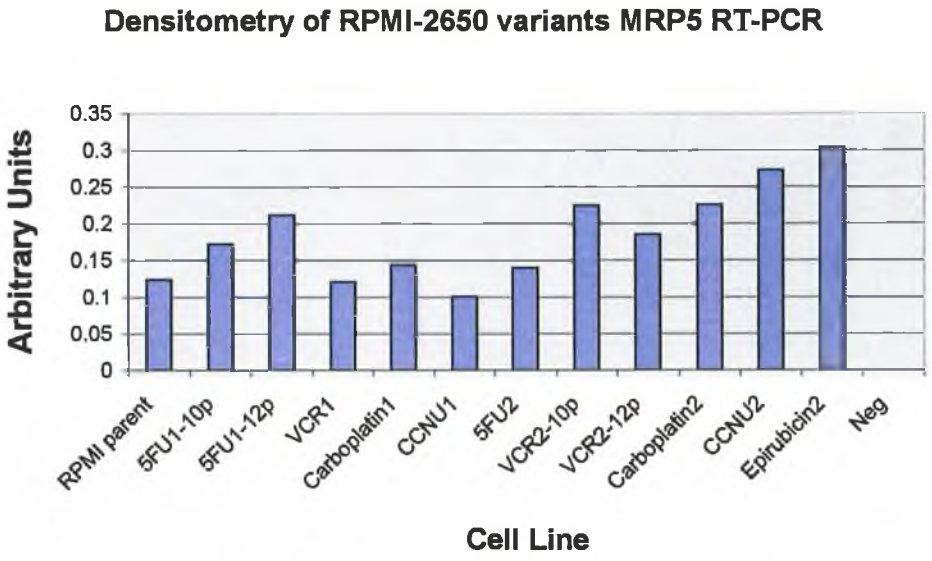
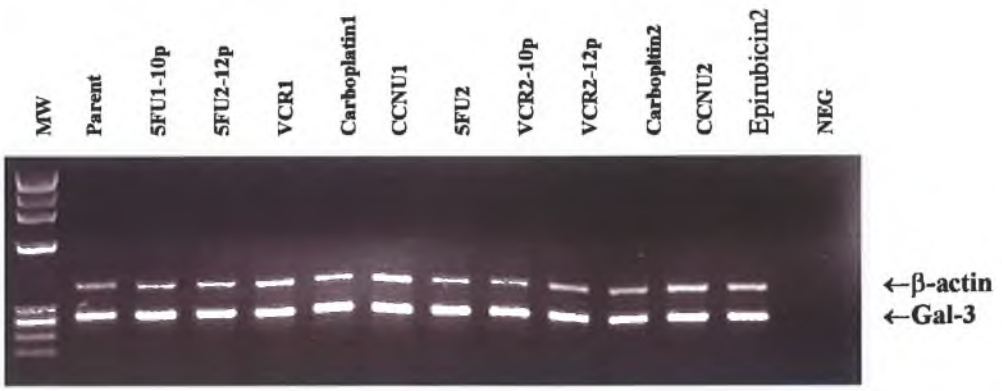


Figure 3.4.5.6 **(a)** RT-PCR analysis of MRP5 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2)

(a)



(b)

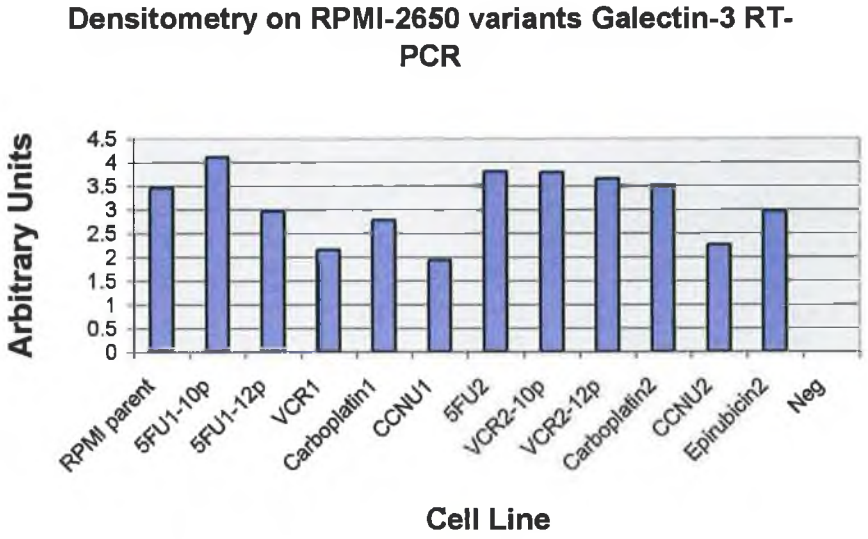
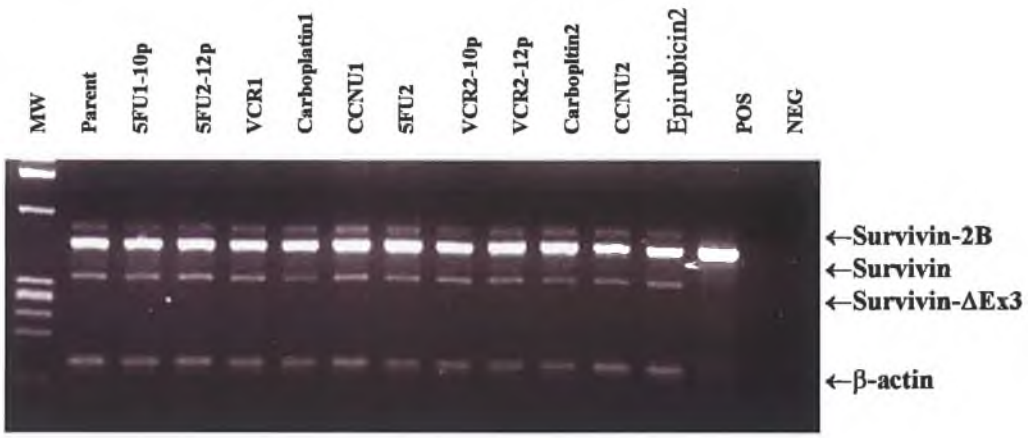


Figure 3.4.5.7 **(a)** RT-PCR analysis of Galectin-3 expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2)

(a)



(b)

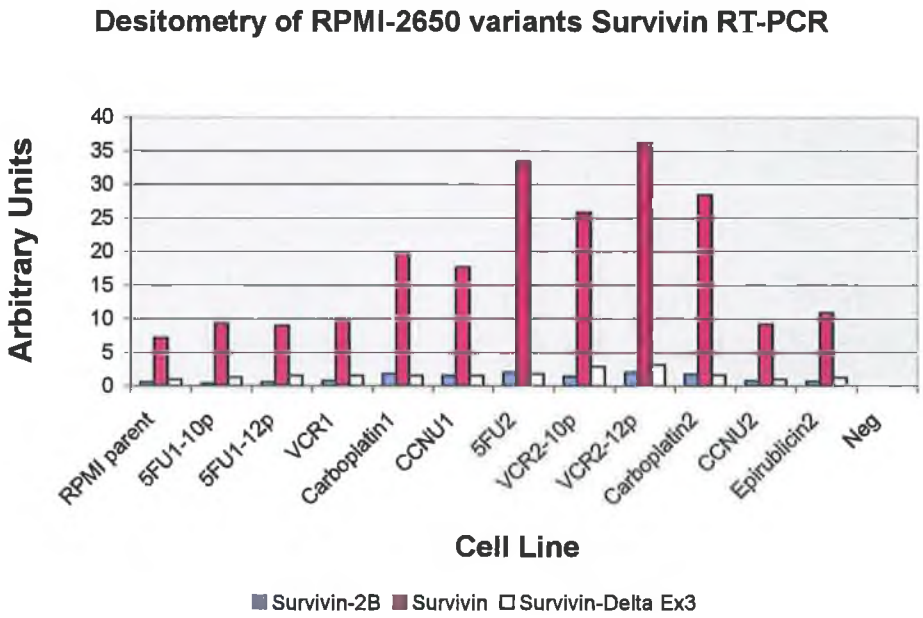


Figure 3.4.5.8 **(a)** RT-PCR analysis of Survivin expression in the RPMI-2650 parent cell line and its variants, **(b)** Densitometry of RT-PCR (n=2).

3.5 Analysis of Gene expression in Breast Tumour specimens

A clinical study was carried out on breast tumour specimens to identify genes that may be useful as prognostic markers for breast cancer. Genes included in this study were the apoptosis-related genes, galectin-3 and survivin and the MDR-related gene, MRP1.

RNA was extracted (see section 2.4.3.8 for procedure) and RT-PCR analysis was carried out on 165 breast tumour specimens. Statistical analysis was carried out on 106 of these tumour specimens, by Dr. Lorraine O'Driscoll, using the SPSS 10.1 software package. The association of two of the three genes (survivin and MRP1) with patient clinicopathological characteristics was evaluated using Chi-square test. Tumour characteristics included, tumours size and grade, diagnosis, treatment, ER status, lymph node involvement, age of patient at diagnosis, relapse-free and overall survival. Survival analysis (5 year relapse-free and overall survival probabilities) was performed using the Kaplan-Meier method and Chi-square analysis. The log rank test was carried out to assess the statistical differences between categories of each potential prognostic factor. Multivariate and univariate analysis using Cox's proportional hazards models were performed, as relevant, to test independent significance of parameters of interest. A P value below 0.05 was considered to be statistically significant. Six normal tissue samples were also analysed by RT-PCR for the expression of the three genes mentioned above.

3.5.1 Survivin mRNA Expression in Breast Tumour Biopsies

RT-PCR analysis was carried out on the tumour specimens using the survivin primers which amplify the three survivin splice variants (see Appendix A). A survivin-over-expressing cell line or a purified plasmid containing survivin cDNA were used as positive controls in some cases (Figure 3.5.1.5A, 3.5.1.6B and 3.5.1.8) and sterile water was used as a negative control. β-Actin was used as an endogenous control and densitometry was carried out on the majority of results by normalising the survivin bands to the β-actin bands (see Figures 3.5.1.1-3.5.1.8).

Results of survivin RT-PCR demonstrate survivin mRNA expression in 68% of cases. Survivin-2B mRNA was detected in 9.4% and survivin-ΔEx3 mRNA was detected in

54.7% of cases. Analysis of normal tissue samples indicated no survivin mRNA expression. Statistical results indicate a significant correlation between the mRNA expression of the survivin variants, i.e. between survivin and survivin-ΔEx3 ($p<0.0001$), survivin and survivin-2B ($p=0.022$) and survivin-2B and survivin-ΔEx3($p=0.003$) as shown in Table 3.5.1.1

Chi-squared analysis revealed that survivin mRNA expression in the breast specimens did not correlate with prognosis or any of the clinicopatholigical parameters, including, tumour size, type and grade, chemotherapy treatment or tamoxifen, lymph node or ER status or age at diagnosis (see Table 3.5.1.2).

Kaplan-Meier analysis indicated no correlation between disease-free survival or overall survival (survival rate) and survivin, survivin-ΔEx3 or survivin-2B mRNA expression as shown in Figure 3.5.1.

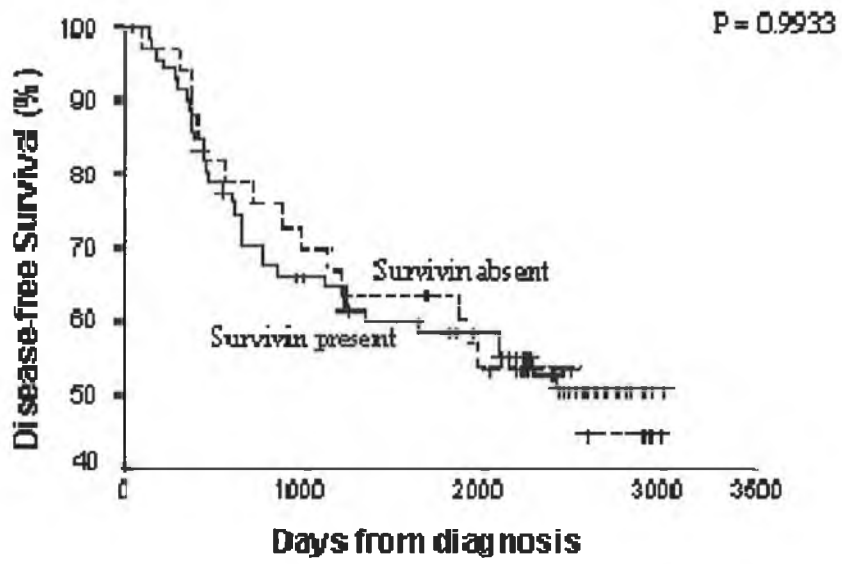
mRNA	**Survivin**		**Survivin-ΔEx3**		**Survivin-2B**	
	+	**P**	+	**P**	+	**P**
Survivin (-) **Survivin (+)**	———	———	1/34 57/72	<0.0001 *	0/34 10/72	0.022*
Sur-ΔEx3 (-) **Sur-ΔEx3 (+)**	15/48 57/58	<0.0001 *	———	———	0/48 10/58	0.003*
Surv-2B (-) **Surv-2B (+)**	62/69 10/10	0.022*	48/96 10/10	0.003*	———	———

Table 3.5.1.1 Correlation between the survivin splice variants. P value from X^2 analysis; * indicates significant parameter.

Characteristics	No. of cases	Sur (%)	P	No. of cases	S-ΔEx3 (%)	P	No. of cases	S-2B (%)	P
Age (yr.)									
<50	19/29	65.5	0.745	12/29	41.4	0.090	3/29	10.3	0.844
≥50	53/77	68.8		46/77	59.7		7/77	9.1	
Tumour size									
T1 (<2 cm)	12/18	66.7		8/18	44.4		1/18	5.6	
T2 (2-5 cm)	58/84	69.0	0.722	48/84	57.1	0.606	9/84	10.7	0.639
T3 (> 5cm)	2/4	50.0		2/4	50.0		0/4	0.0	
Lymph node metastasis									
Negative	34/45	75.6	0.148	28/45	62.2	0.182	4/45	8.9	0.869
Positive	38/61	62.3		30/61	49.2		6/61	9.8	
Histology grade									
I	6/11	54.5		5/11	45.5		1/11	9.1	
II	29/41	70.7	0.588	22/41	53.7	0.757	4/41	9.8	0.996
III	37/54	68.5		31/54	57.4		5/54	9.3	
Histology type									
IDC	59/84	70.2		50/84	59.5		9/84	10.7	
ILC	10/17	58.8	0.608	5/17	29.4	0.073	1/17	5.9	0.627
Special	3/5	60.0		3/5	60.0		0/5	0.0	
ER status									
Negative	24/34	70.6	0.763	21/34	61.8	0.398	3/34	8.8	0.814
Positive	46/68	67.6		36/68	52.9		7/68	10.3	
Chemotherapy									
No	31/45	68.9	0.971	23/45	51.1	0.301	3/45	6.7	0.410
Yes	36/52	69.2		32/52	61.5		6/52	11.5	
Tamoxifen									
No	19/26	73.1	0.768	15/26	57.7	0.938	2/26	7.7	0.594
Yes	49/70	70.0		41/70	58.6		8/70	11.4	

Table 3.5.1.2 Correlation between the survivin (Sur), survivin-2B (S-2B) and survivin-ΔEx3 (S-ΔEx3) expression with clinicopathological parameters using Chi-squared test. P value from X^2 analysis.

(A)



(B)

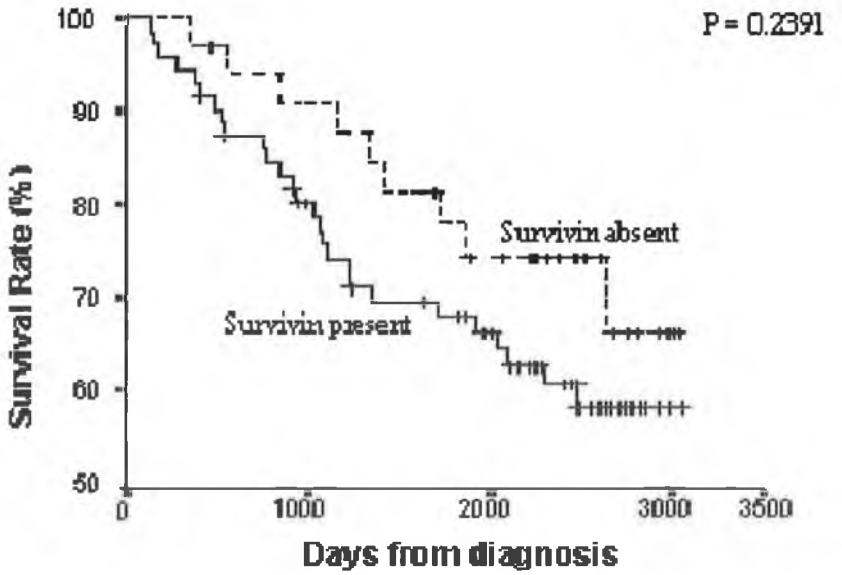
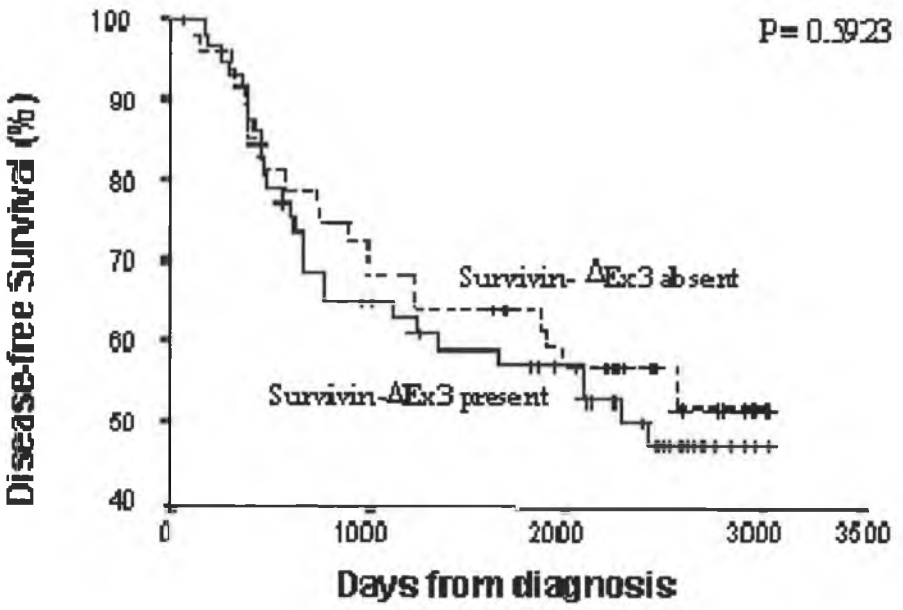


Figure 3.5.1 **(A)** and **(B)** Kaplan-Meier survival curves showing survivin mRNA did not correlate with disease free survival (P=0.9933) or overall survival (P=0.2391).

(C)



(D)

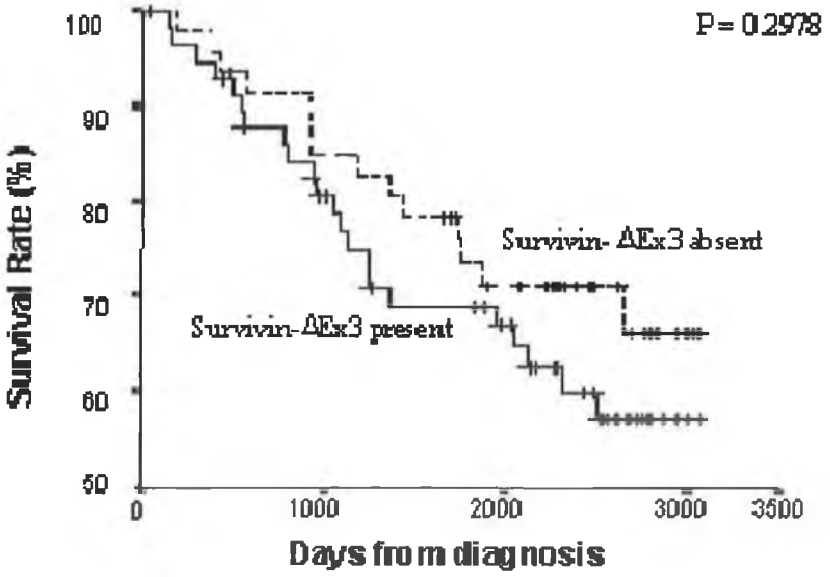
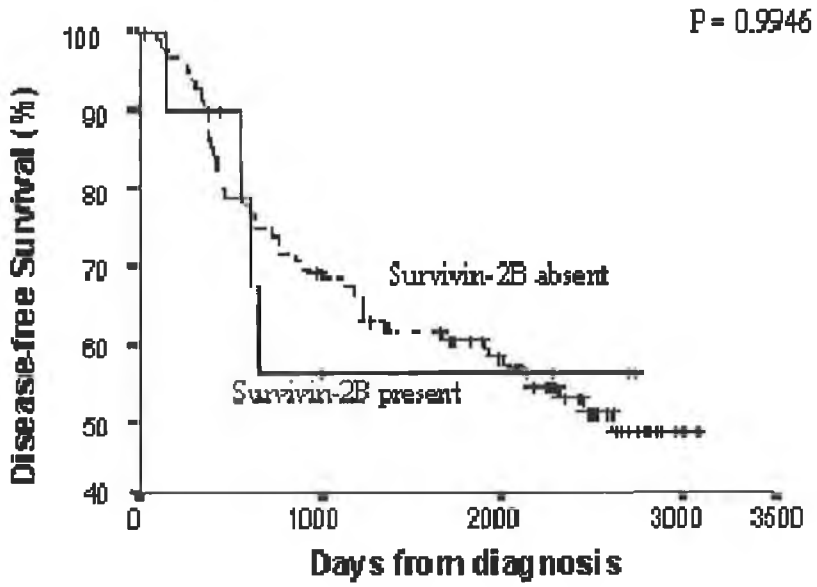


Figure 3.5.1 **(C)** and **(D)** Kaplan-Meier survival curves showing survivin-ΔEx3 mRNA did not correlate with disease free survival (P=0.5923) or overall survival (P=0.2978).

(E)



(F)

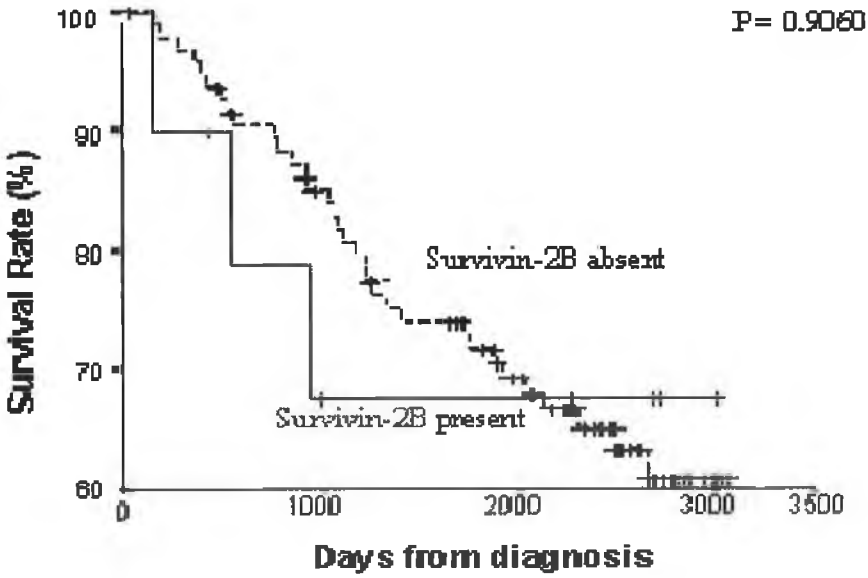
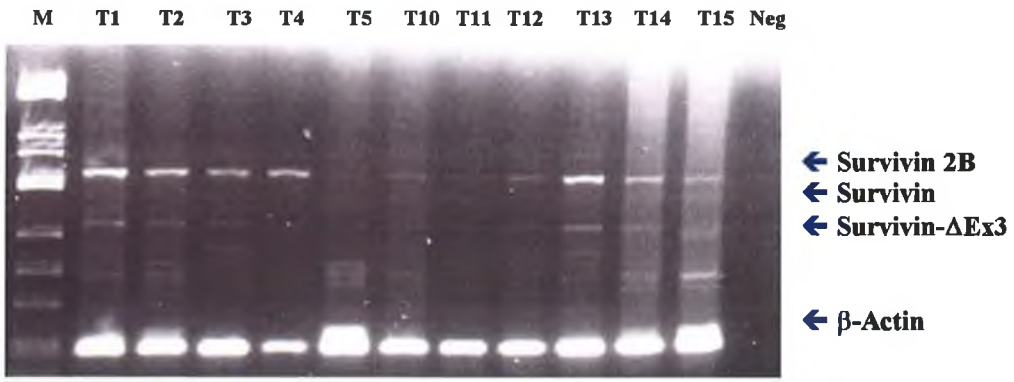
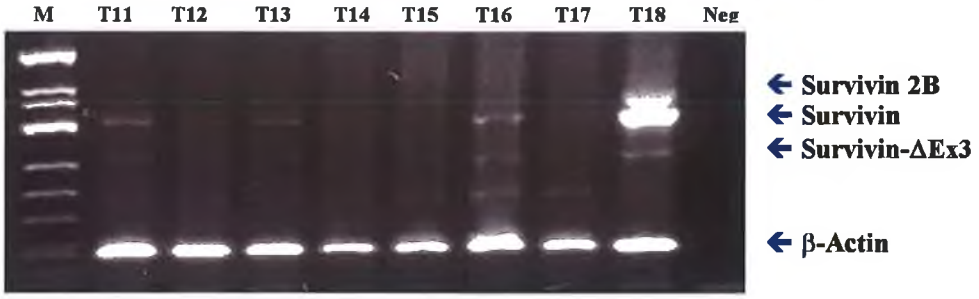


Figure 3.5.1 **(E)** and **(F)** Kaplan-Meier survival curves showing survivin-ΔEx3 mRNA did not correlate with disease free survival (P=0.9946) or overall survival (P=0.9060).

(A)



(B)



(C)

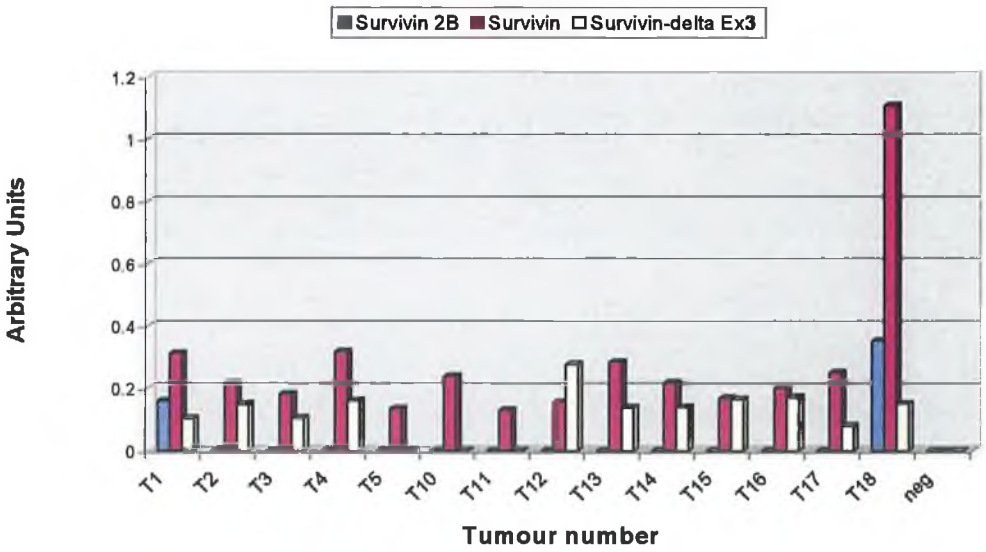
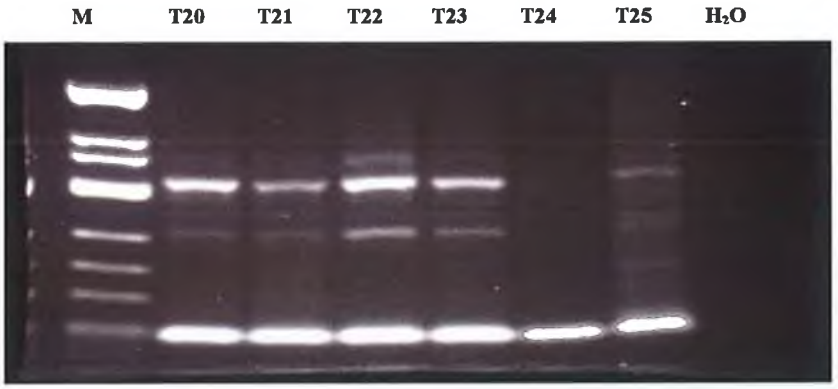
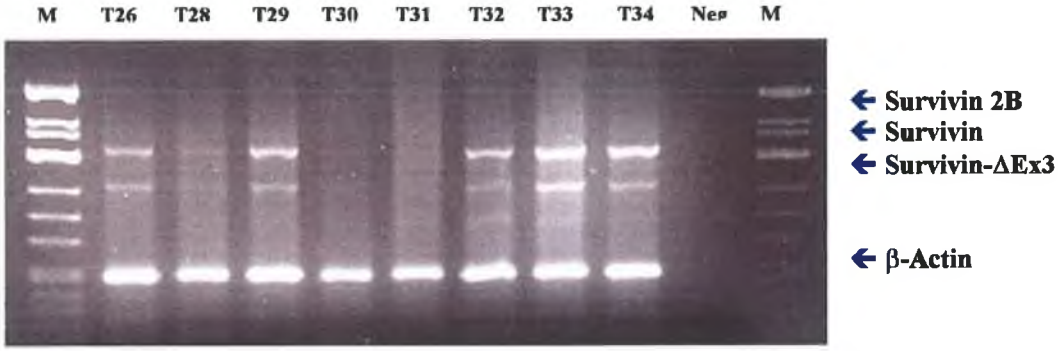


Fig. 3.5.1.1 **(A) and (B)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.1.1** **(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

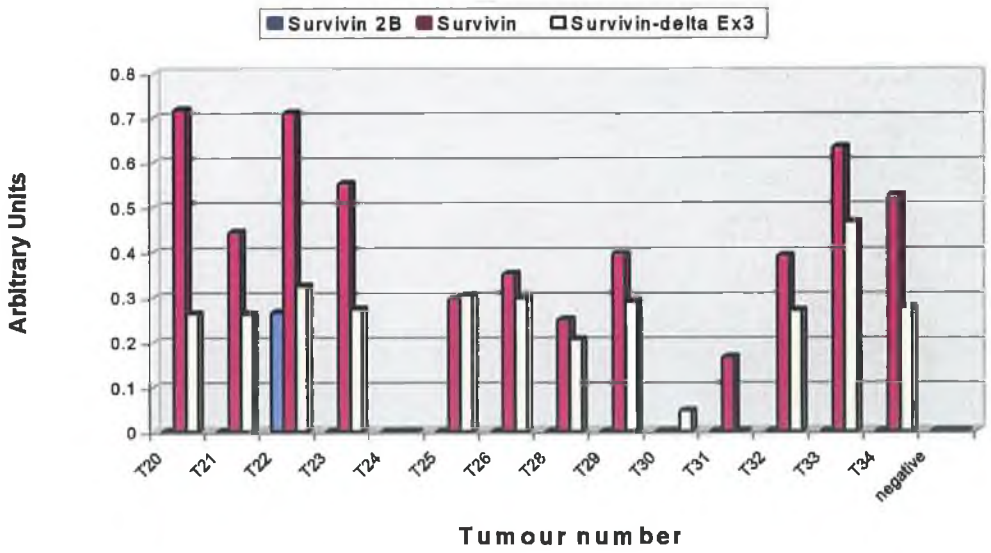
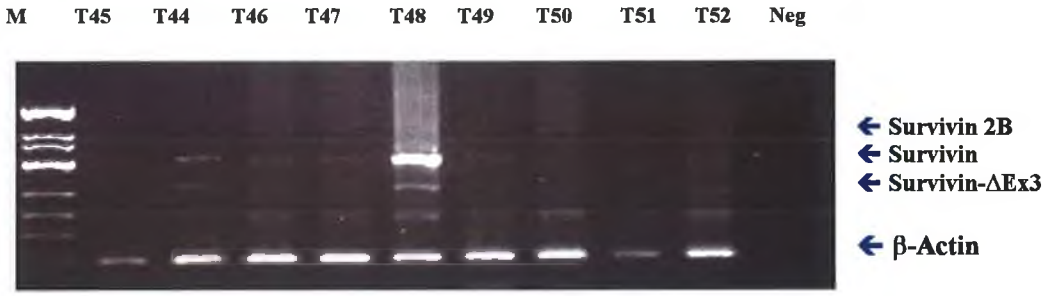
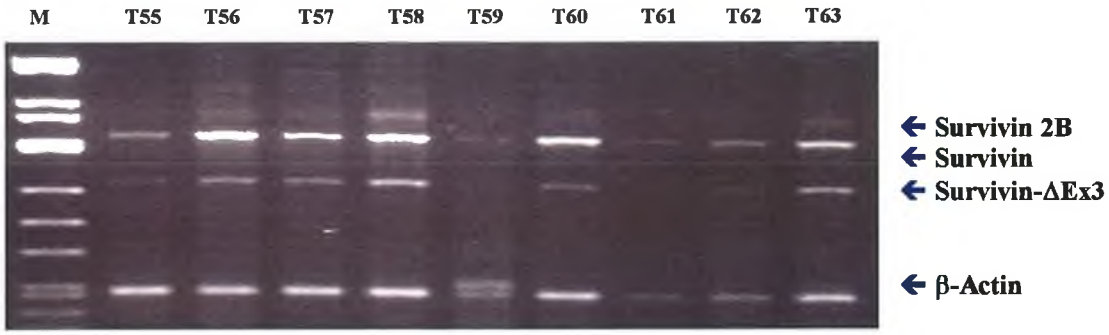


Fig. 3.5.1.2 **(A) and (B)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.1.2** **(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

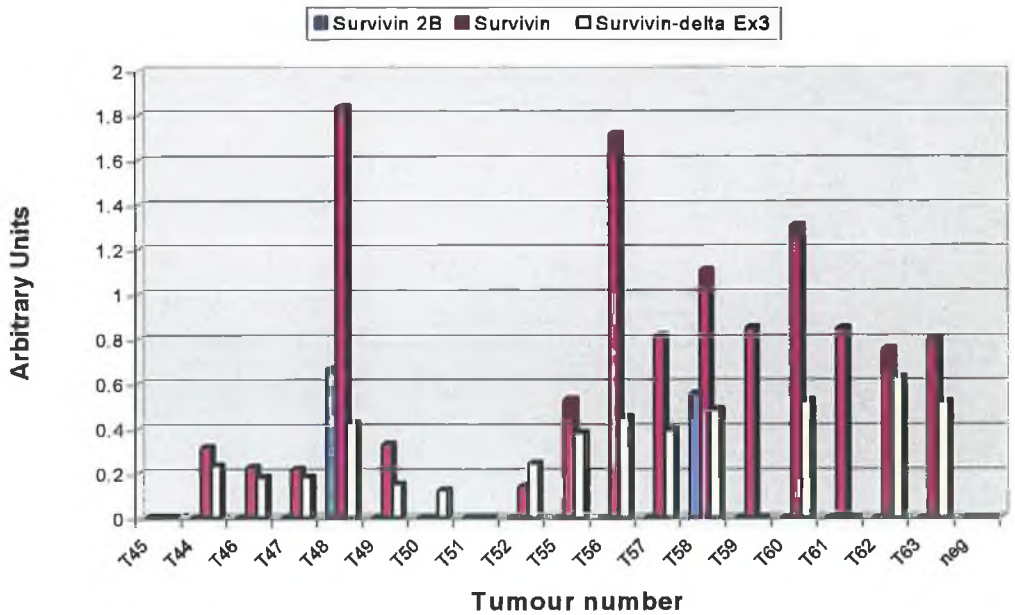
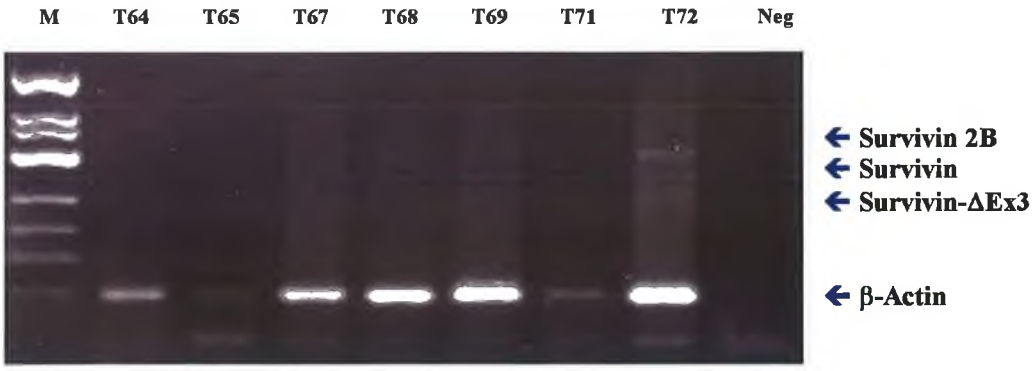
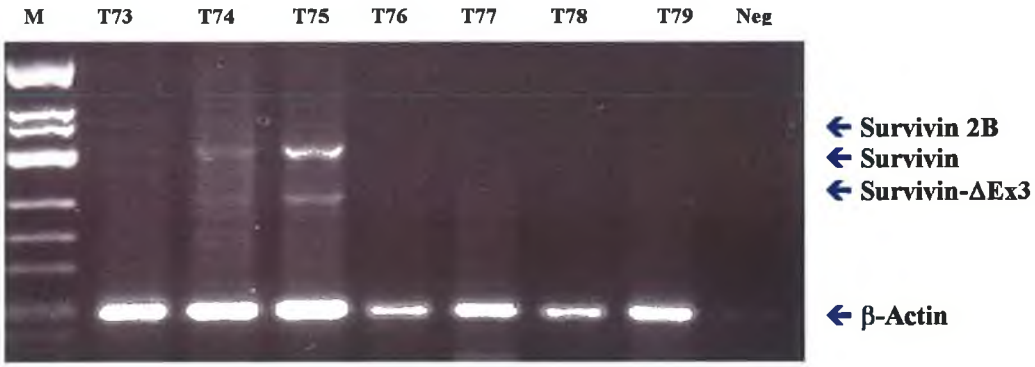


Fig. 3.5.1.3 **(A) and (B)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.1.3** **(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

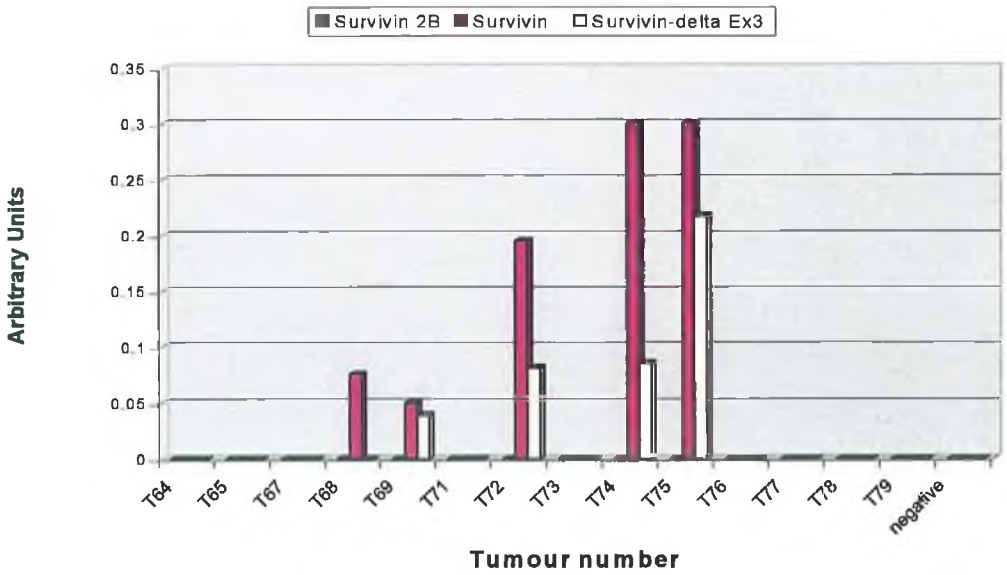
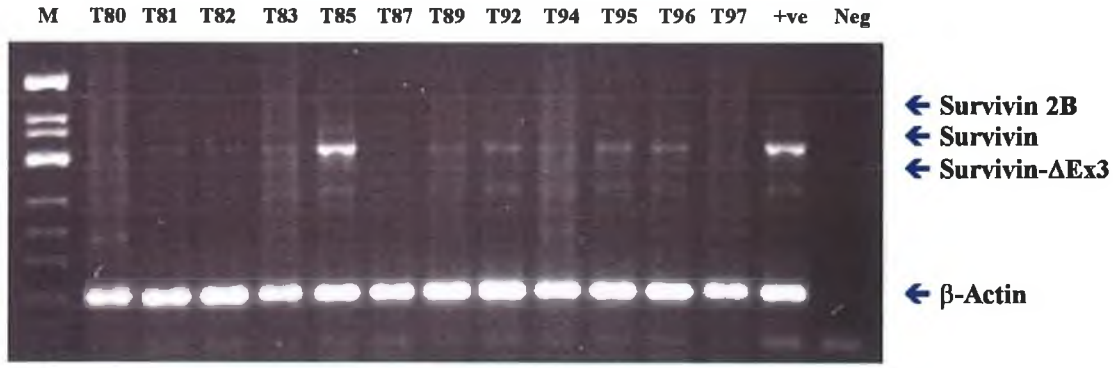
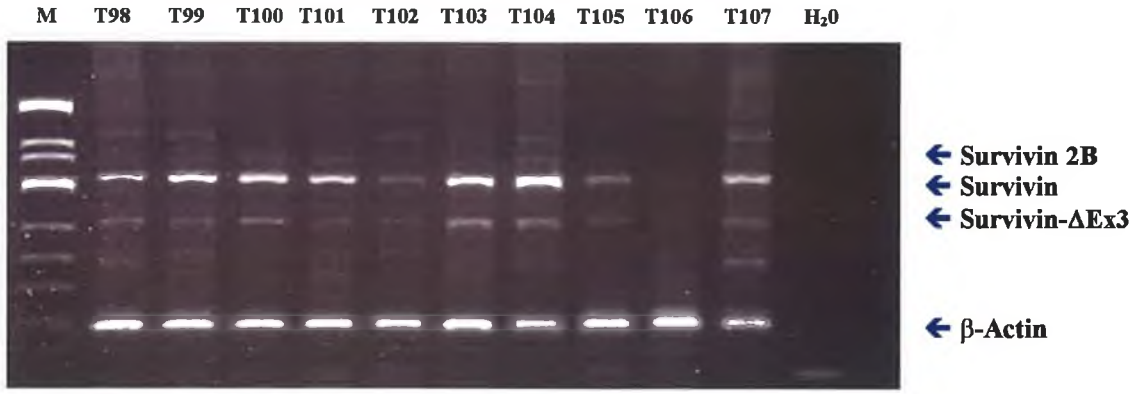


Fig. 3.5.1.4 **(A) and (B)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.1.4** **(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

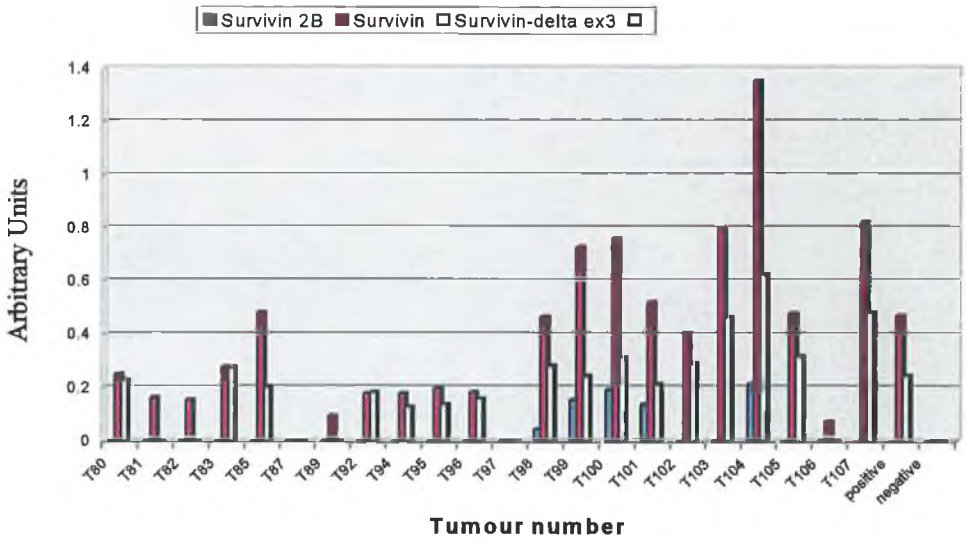
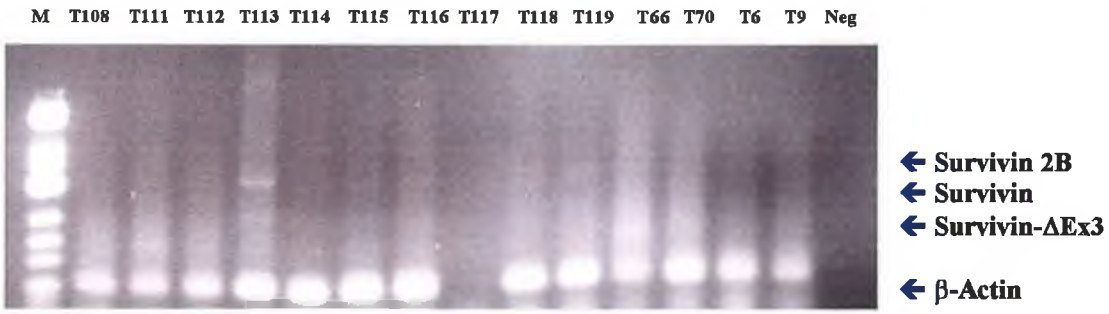
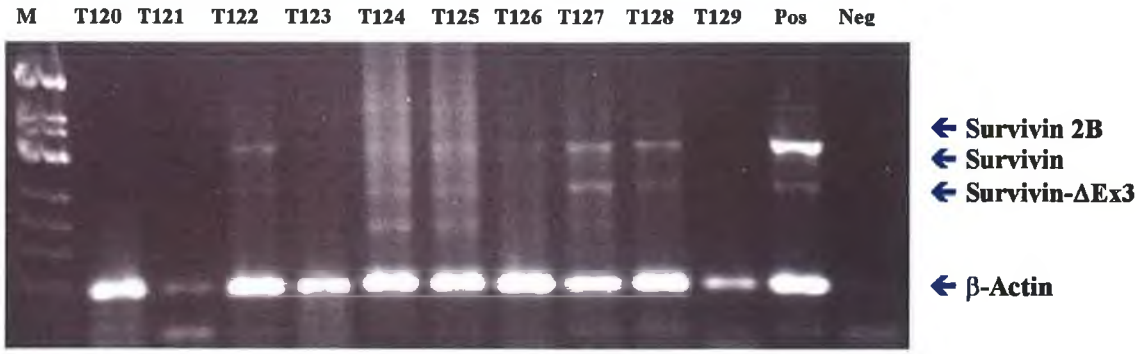


Fig. 3.5.1.5 **(A) and (B)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.1.5** **(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

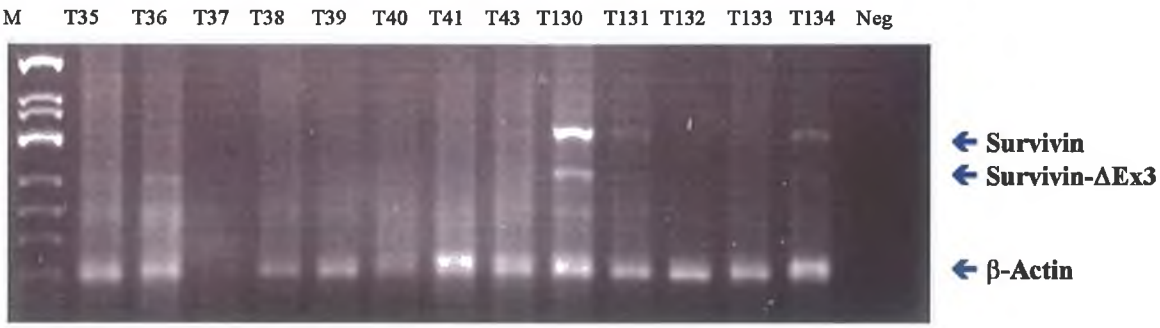


Fig. 3.5.1.6 **(A), (B)** and **(C)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens; M=Molecular weight marker; Pos=positive control; Neg=negative control.

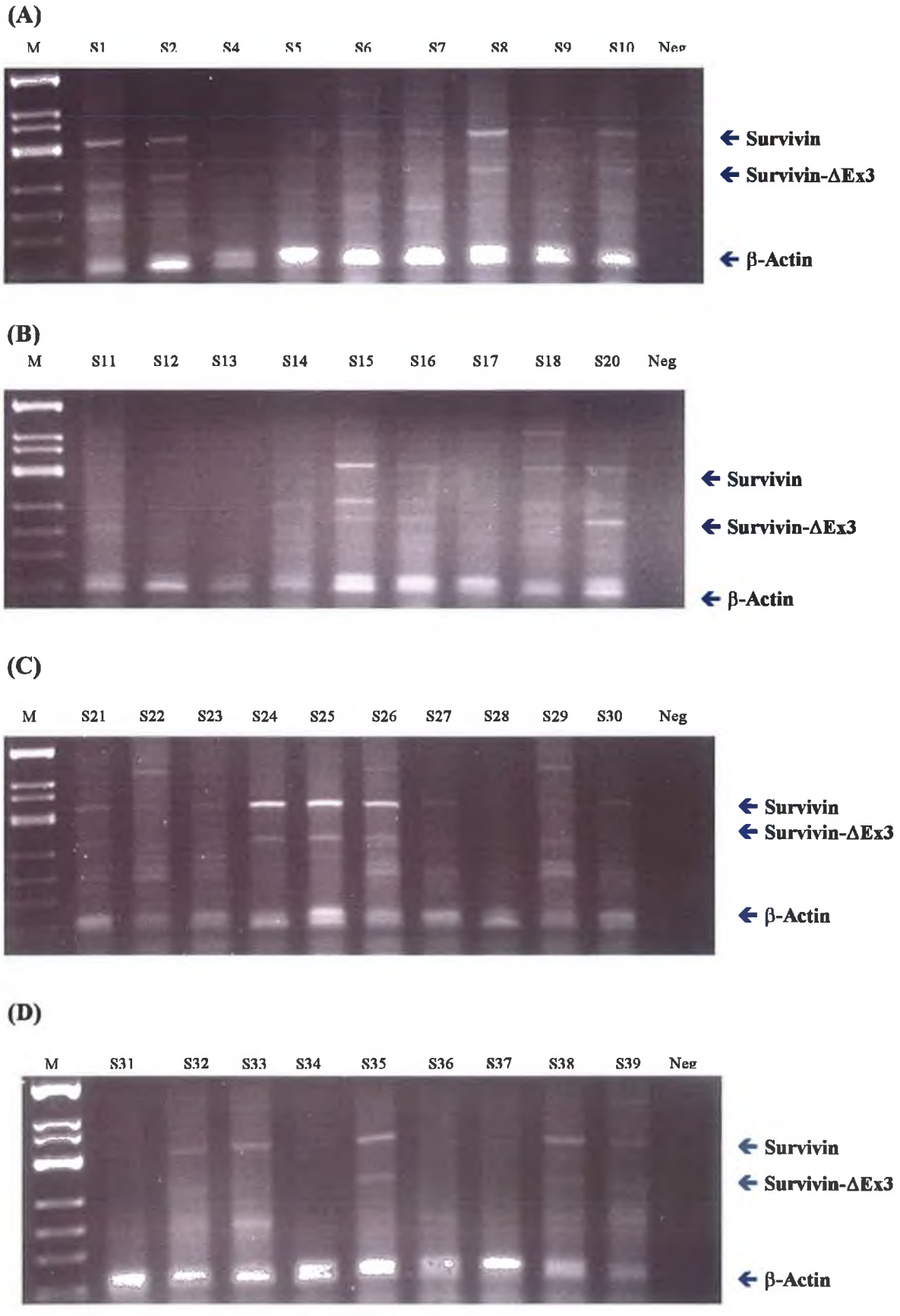
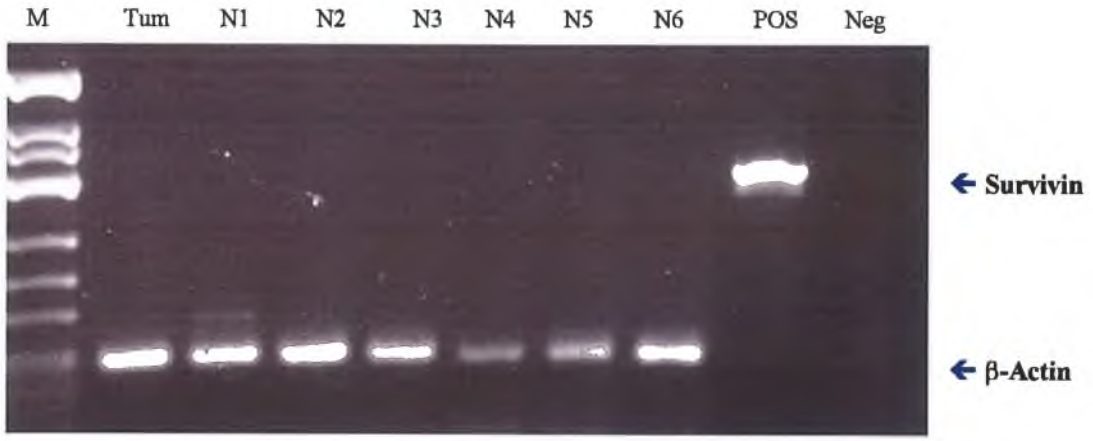


Fig. 3.5.1.7 **(A), (B), (C)** and **(D)** Gel electrophoresis of survivin RT-PCR results on breast tumour tissue specimens. M=Molecular weight marker; Neg=negative control.

(A)



(B)

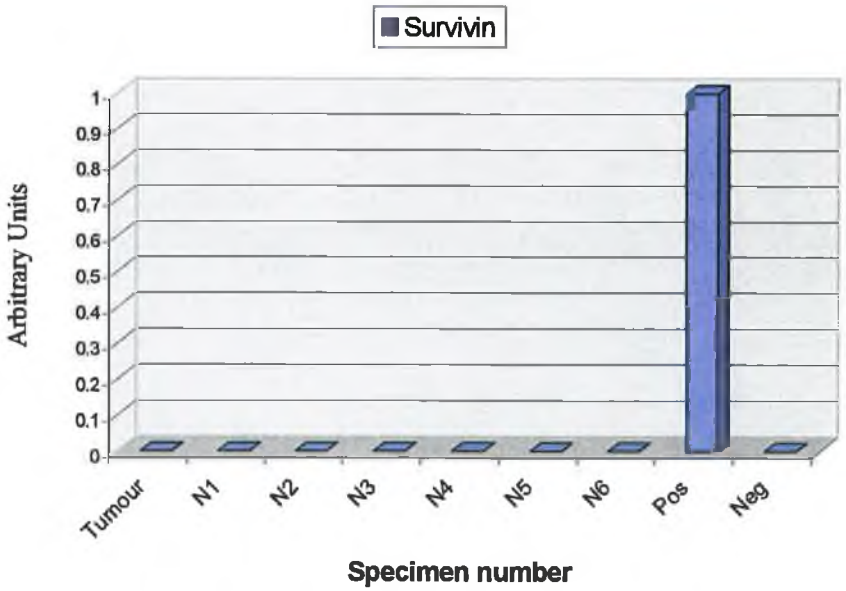
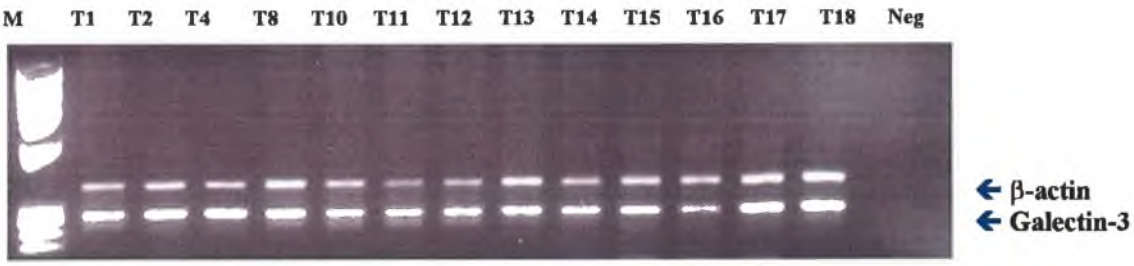


Fig. 3.5.1.8 **(A)** Gel electrophoresis of survivin RT-PCR results on normal breast tissue specimens (N1-N6) and one tumour sample (Tumour); **Fig. 3.5.1.8 (B)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Pos= positive control; Neg=negative control. (n=1).

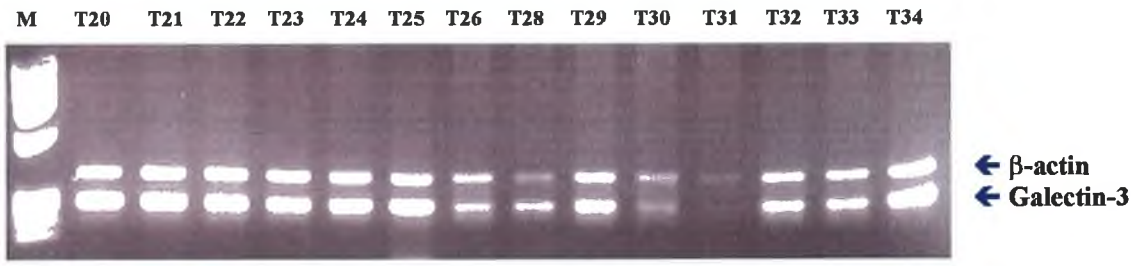
3.5.2 Galectin-3 mRNA Expression in Breast Tumour Biopsies

RT-PCR analysis was carried out to detect galectin-3 mRNA expression in the breast tumour specimens. Galectin-3 mRNA expression was detected in 99% of cases. Galectin-3 mRNA expression was detected in 83% of cases of normal tissue specimens. Due to its expression in almost all the tumour specimens analysed, galectin-3 mRNA expression showed no correlation with prognosis or any of the clinicopathological parameter. No statistical analysis was carried out on these results. RT-PCR results are presented in this section (Figures 3.5.2.1-3.5.2.8). β-actin was used as an endogenous control and, as before, sterile water was used as a negative control.

(A)



(B)



(C)

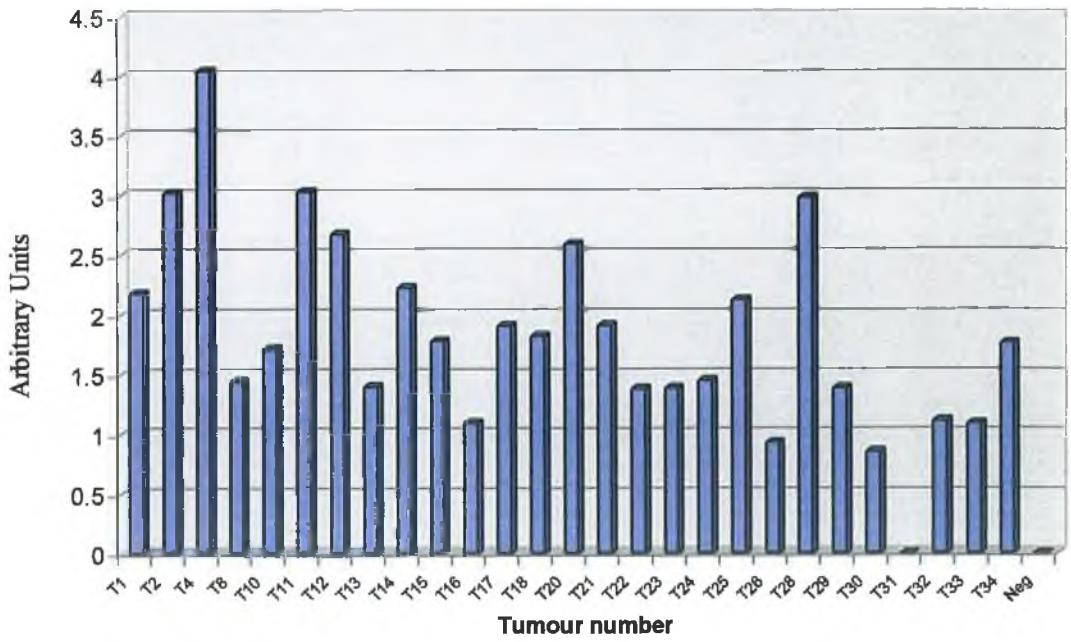
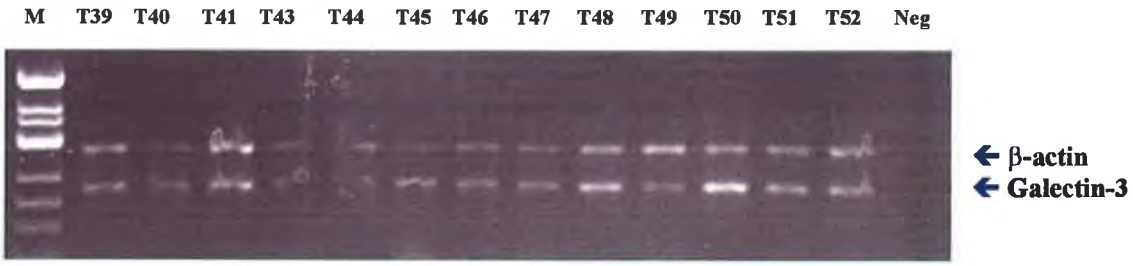
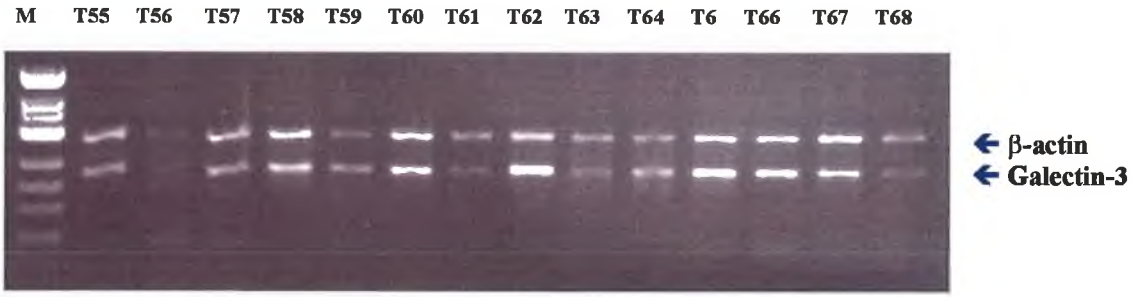


Fig. 3.5.2.1 (A) and (B) Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.1(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

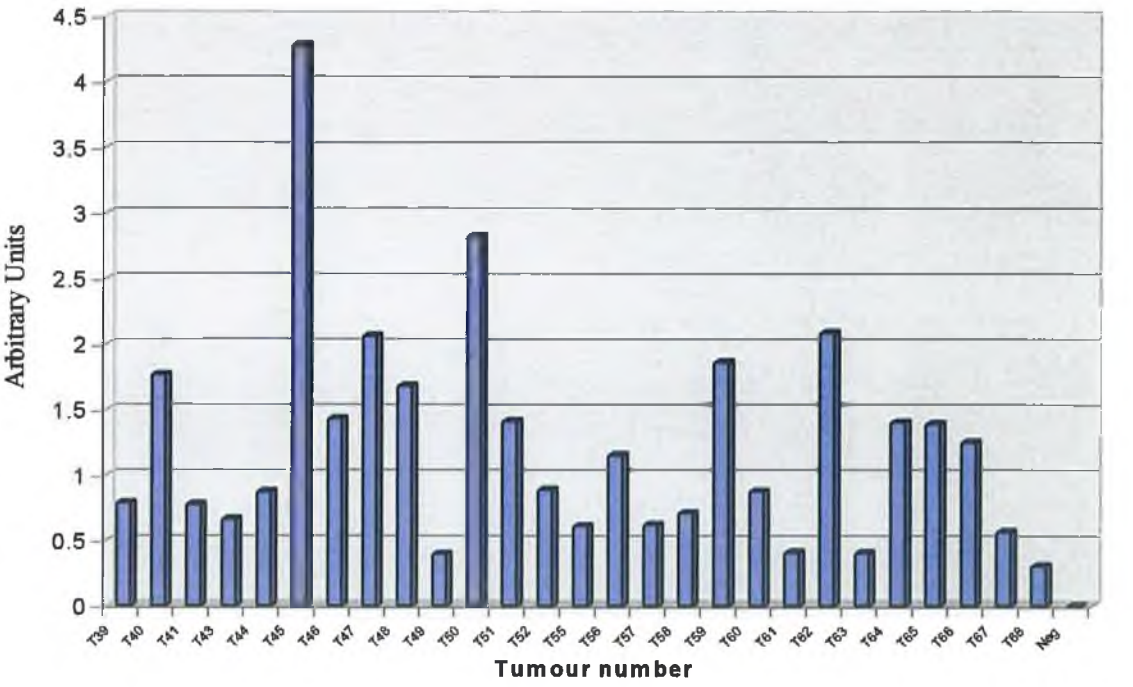


Fig. 3.5.2.2 **(A) and (B)** Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.2(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

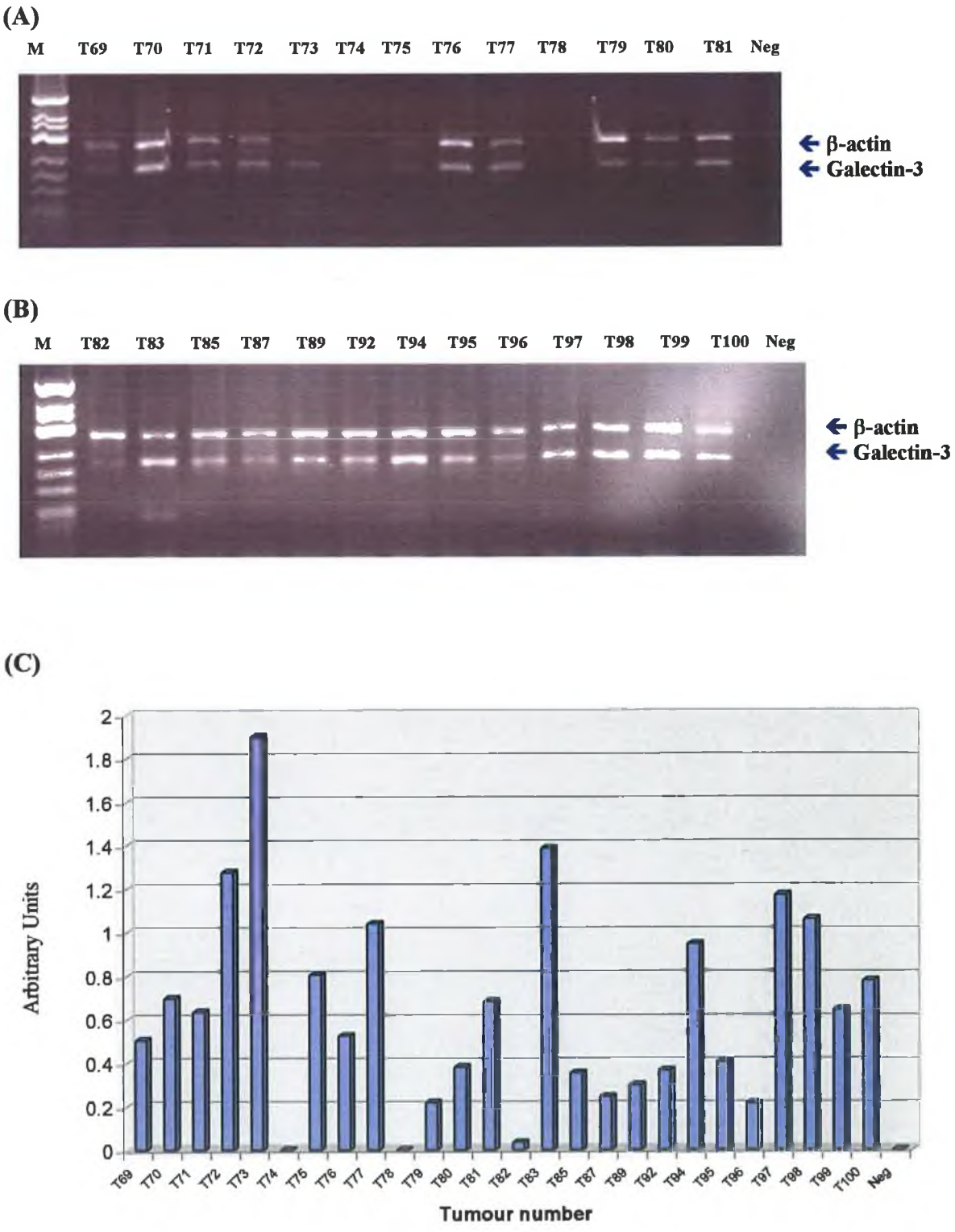
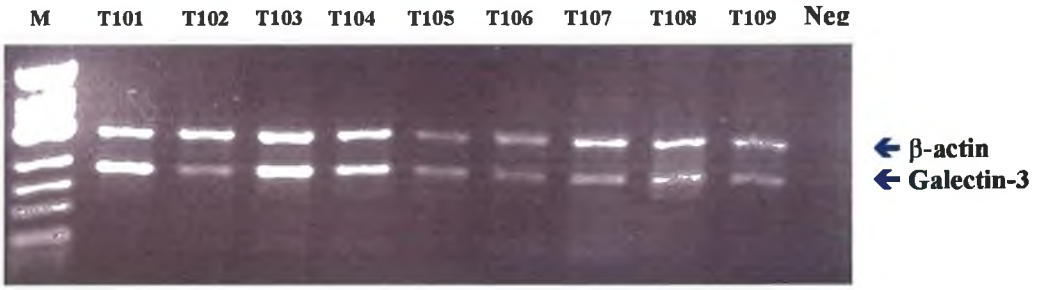
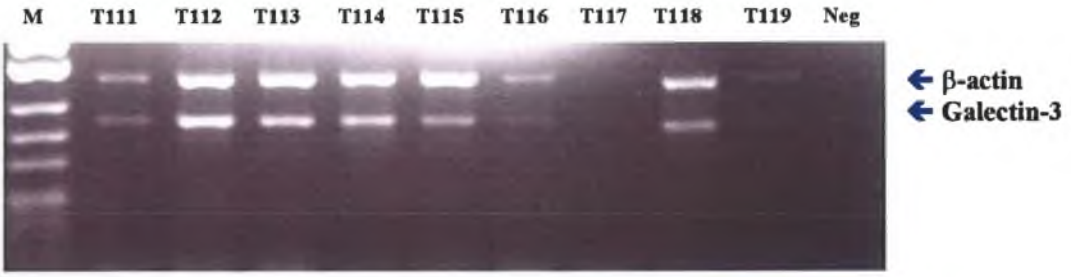


Fig. 3.5.2.3 **(A) and (B)** Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.3(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

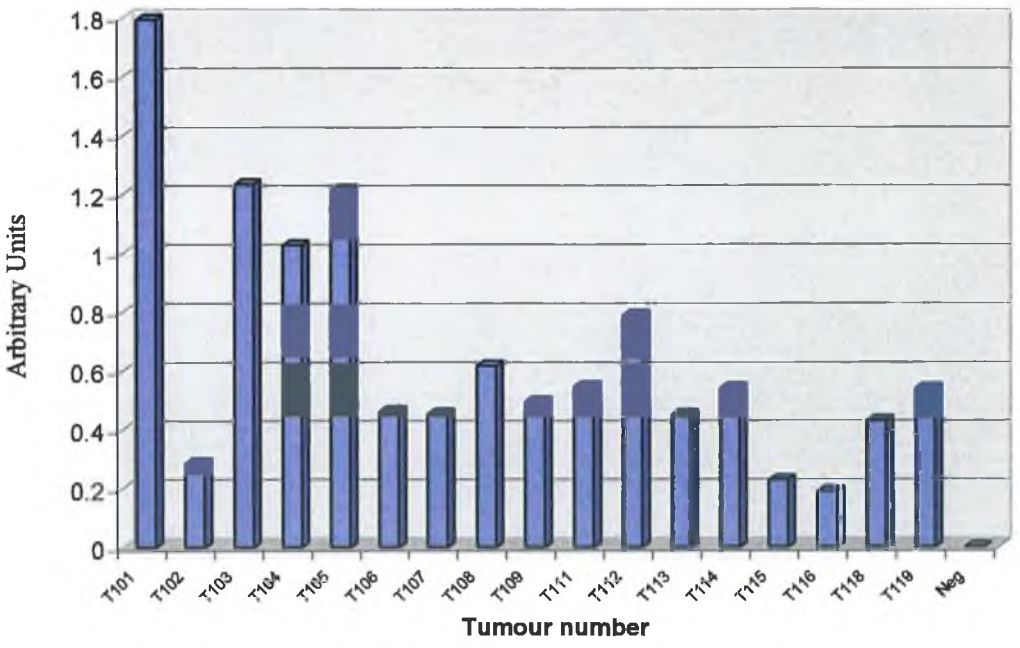


Fig. 3.5.2.4 **(A) and (B)** Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.4(C)** Densitometric analysis of RT-PCR results. M=Molecular weight; Neg=negative control. (n=1).

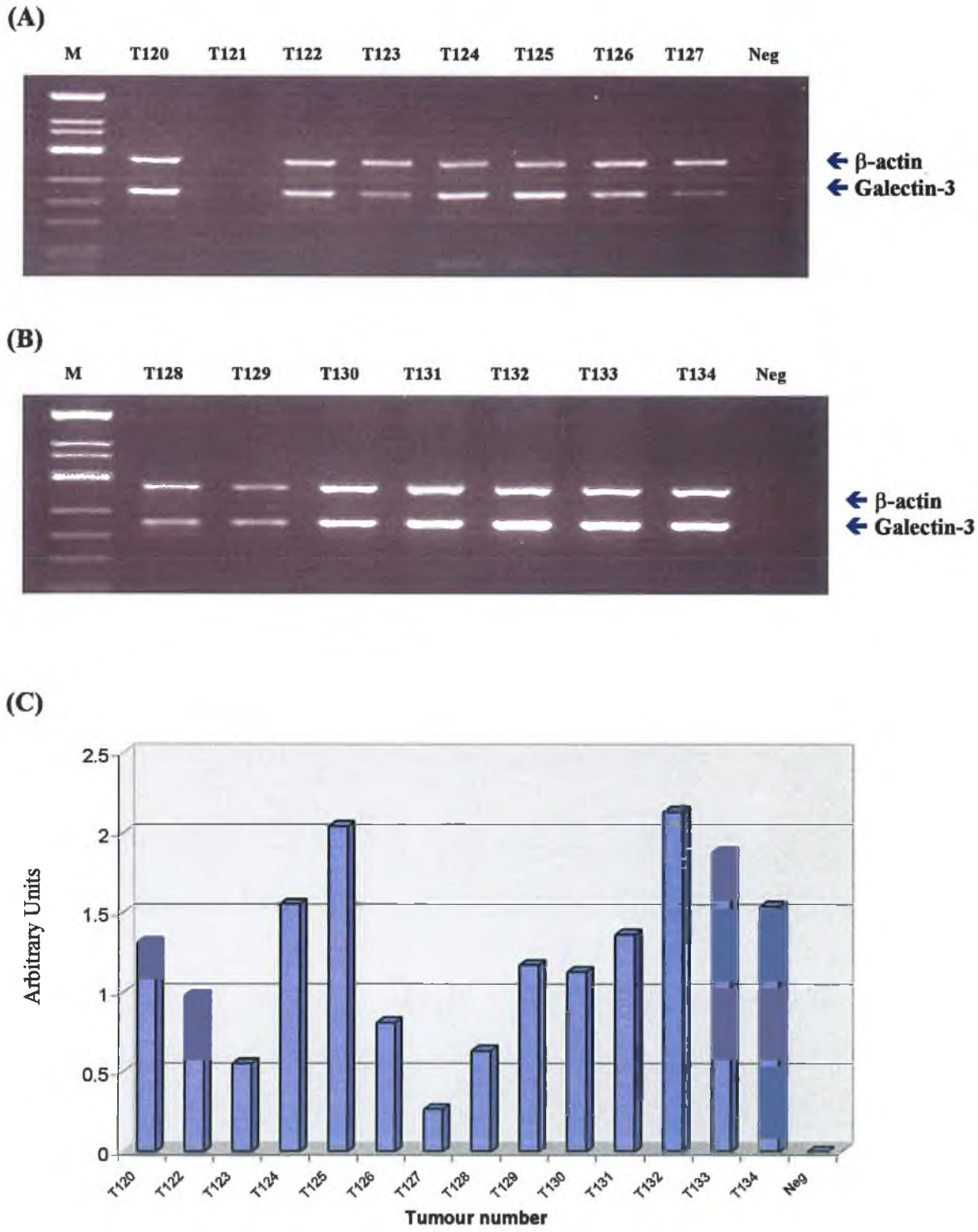
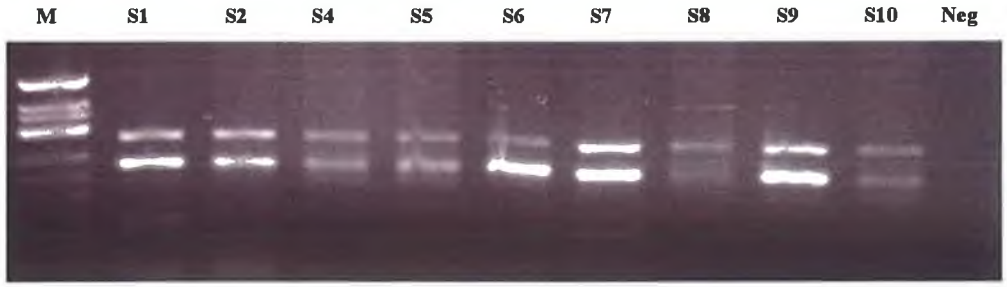
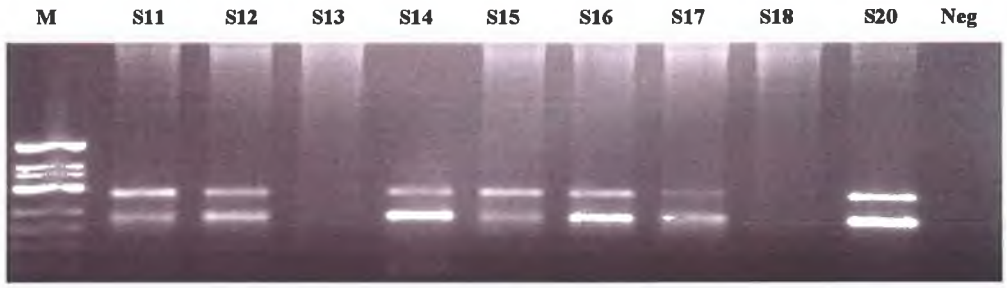


Fig. 3.5.2.5 (A) and (B) Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.5(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

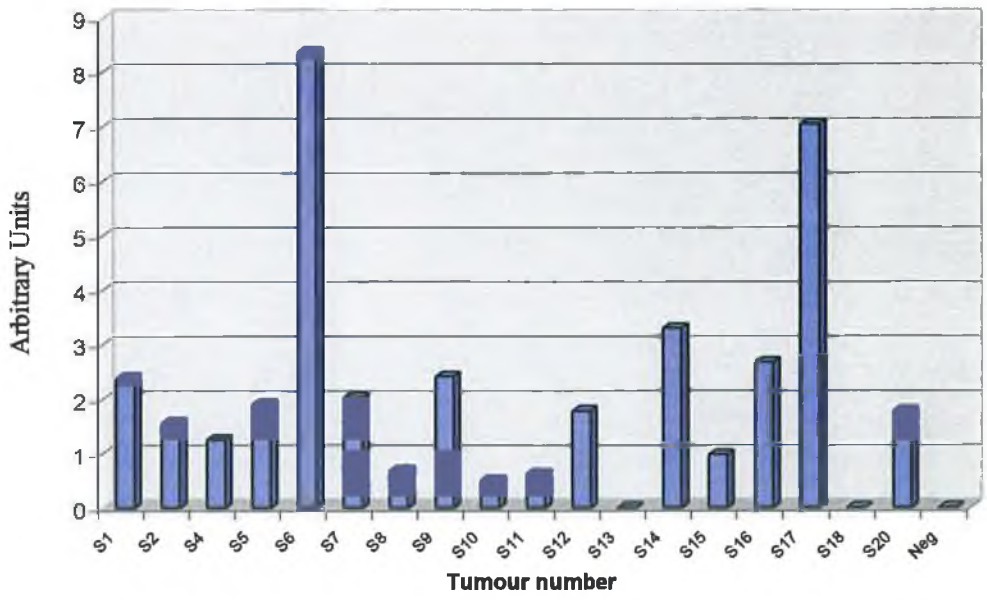


Fig. 3.5.2.6 (A) and (B) Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.6(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

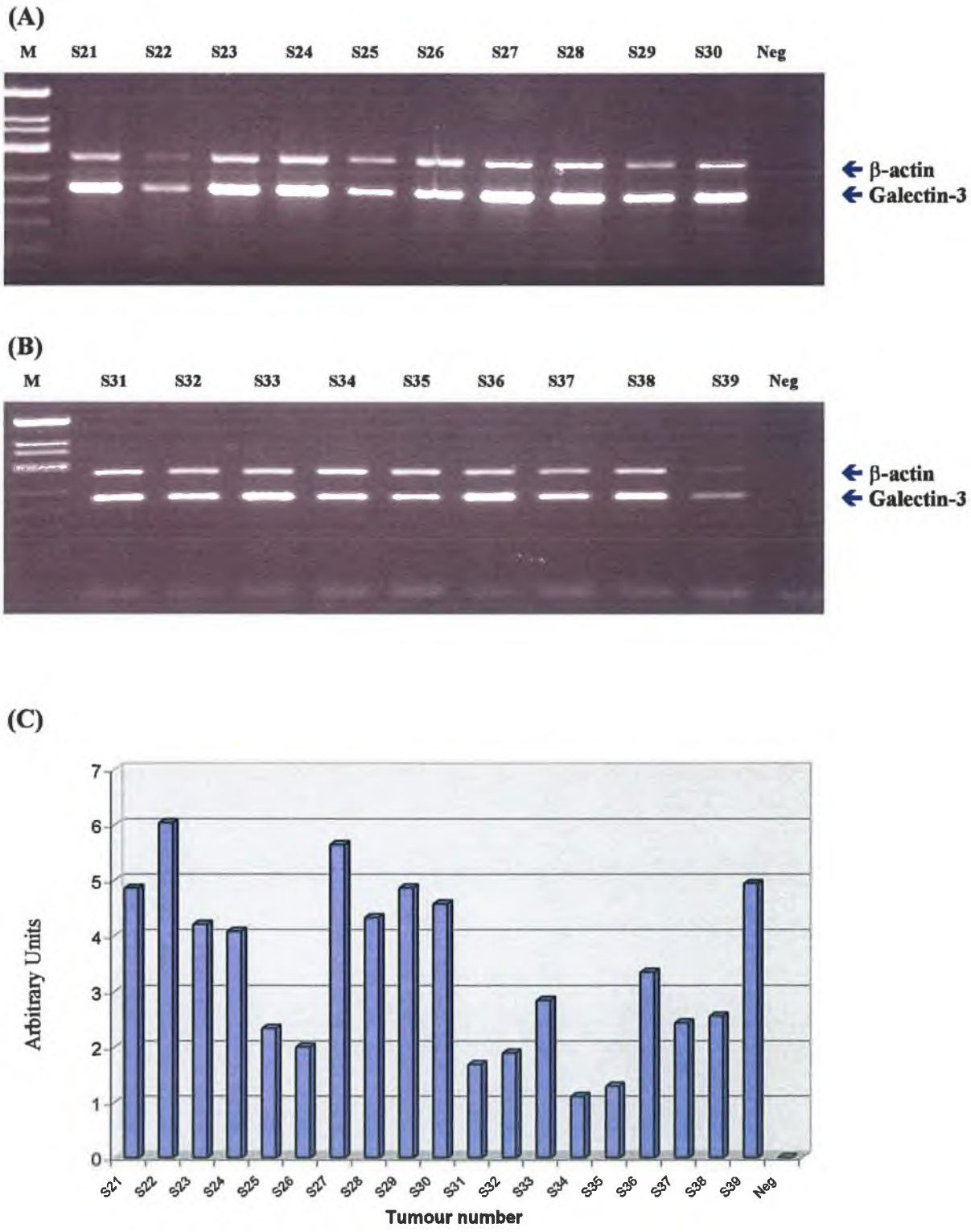
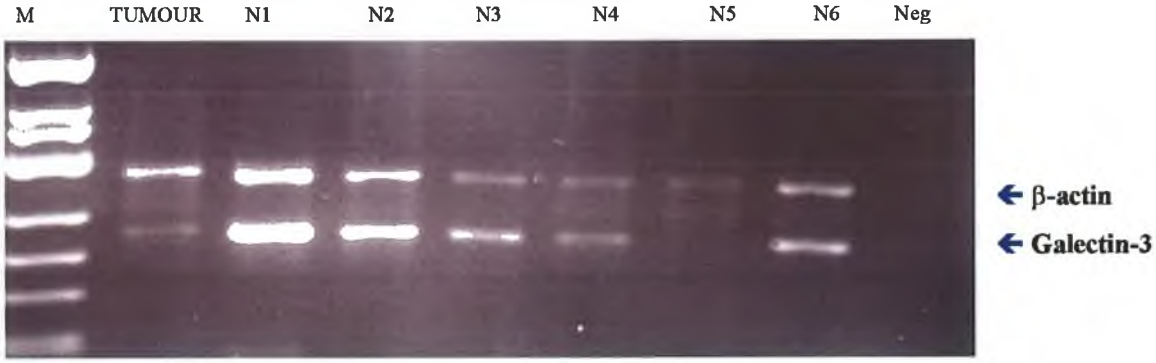


Fig. 3.5.2.7 **(A) and (B)** Gel electrophoresis of Gal-3 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.2.7(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)

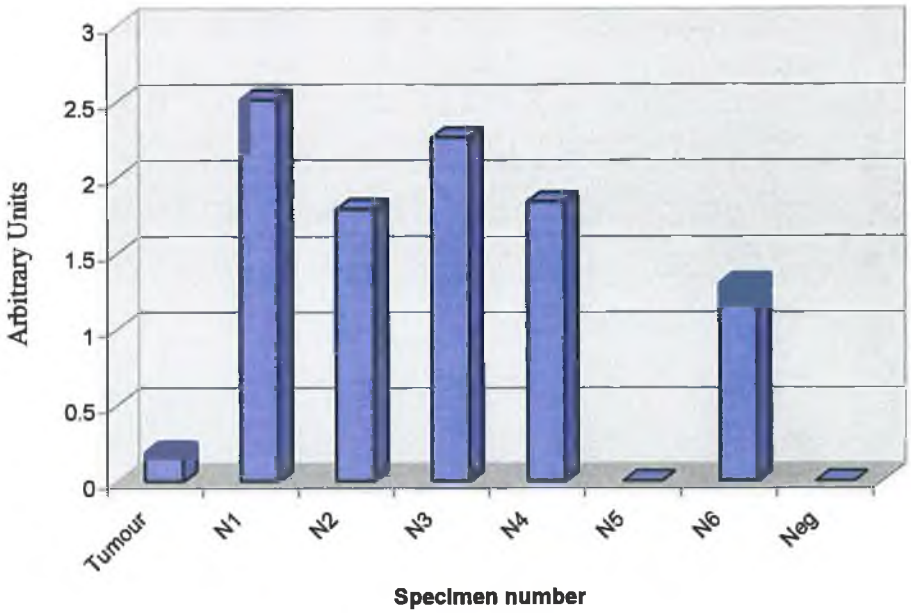


Fig. 3.5.2.8 **(A)** Gel electrophoresis of Gal-3 RT-PCR results on normal breast tissue specimens (N1-N6) and one tumour; **Fig. 3.6.2.8 (B)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

3.5.3 MRP-1 mRNA Expression in Breast Tumour Biopsies

RT-PCR analysis was carried out for to detect MRP1 mRNA expression in the breast tumour specimens. Results indicate that 72.8% of tumour specimens express MRP1 mRNA, while 66% of normal samples express MRP1 mRNA.

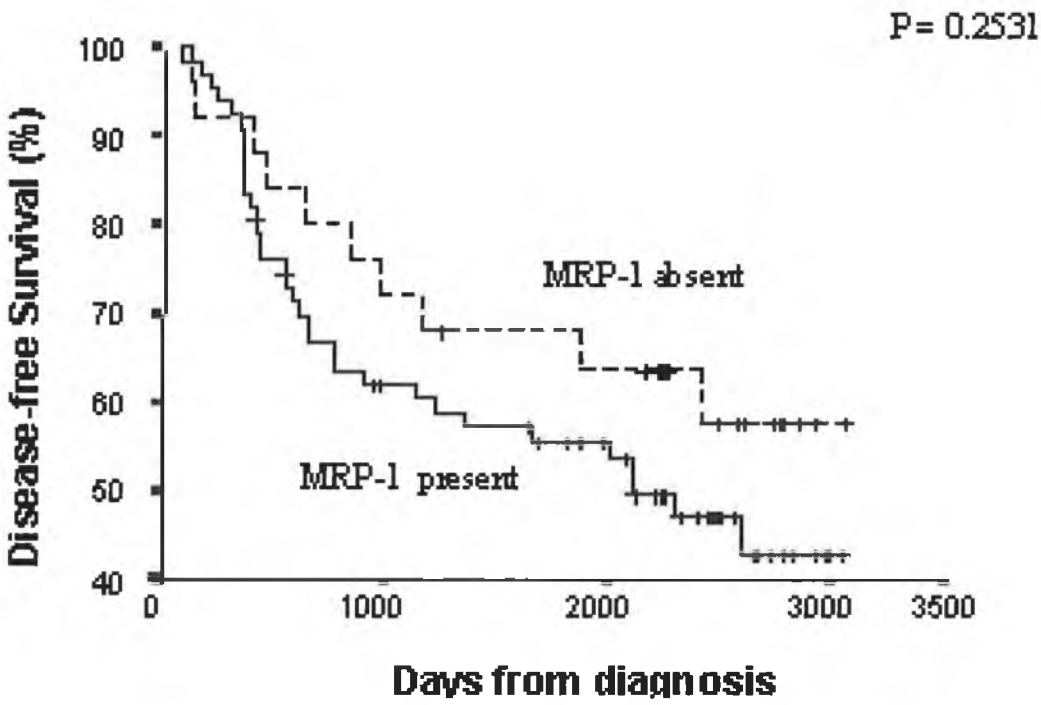
Chi-squared analysis revealed that MRP1 mRNA expression in the breast specimens did not correlate with prognosis or with the clinicopatholigical parameters, including, tumour size, type and grade, chemotherapy treatment or tamoxifen, lymph node or ER status or age at diagnosis (see Table 3.5.3.1). Kaplan-Meier analysis indicated no correlation between disease-free survival (P=0.2531) or overall survival (survival rate) (P=0.3942) and MRP1 mRNA expression as shown in Figure 3.5.3.

β-actin was used as an endogenous control in the RT-PCR analysis and sterile water was used as a negative control (see Figures 3.5.3.1-3.5.3.8).

Characteristics	No. of cases	MRP-1 (%)	P
Age (yr.)			
<50	17/22	77.3	0.591
≥50	50/70	71.4	
Tumour size			
T1 (<2 cm)	11/15	73.3	
T2 (2-5 cm)	55/74	74.3	0.294
T3 (> 5cm)	1/3	33.3	
Lymph node metastasis			
Negative	29/39	74.4	0.777
Positive	38/53	71.7	
Histology grade			
I	10/11	90.9	
II	24/33	72.7	0.329
III	33/48	68.8	
Histology type			
IDC	53/73	72.6	
ILC	10/14	71.4	0.930
Special	4/5	80.0	
ER status			
Negative	26/31	83.9	0.092
Positive	39/58	67.2	
Chemotherapy			
No	27/40	67.5	0.376
Yes	35/46	76.1	
Tamoxifen			
No	17/24	70.8	0.784
Yes	45/61	73.8	

Table 3.5.3.1 Correlation between MRP1 expression with clinicopathological parameters using Chi-squared test. P value from X^2 analysis.

(A)



(B)

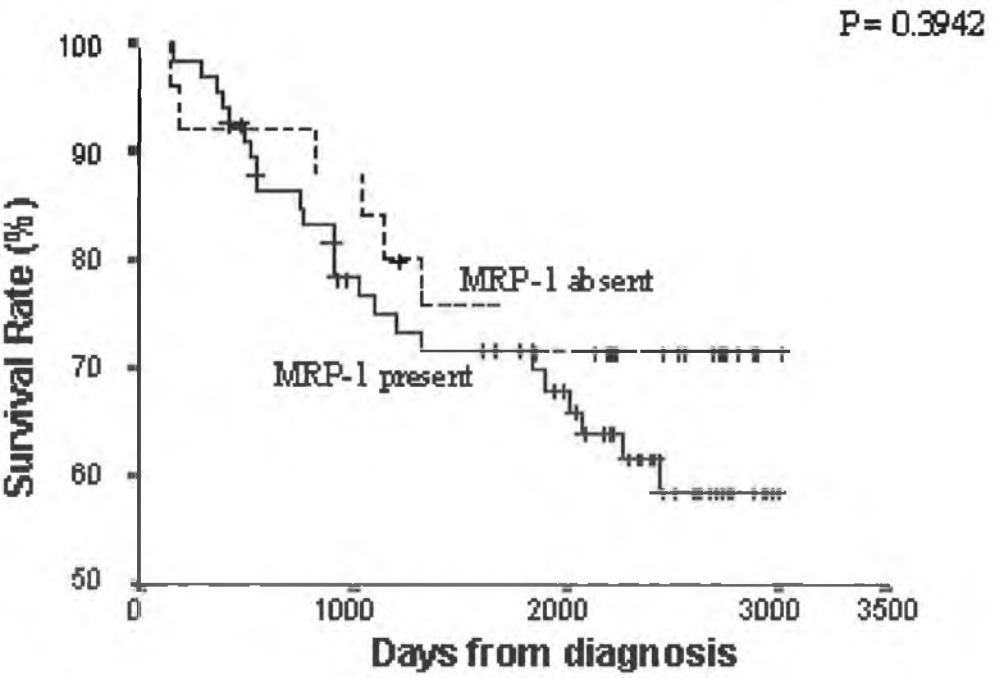
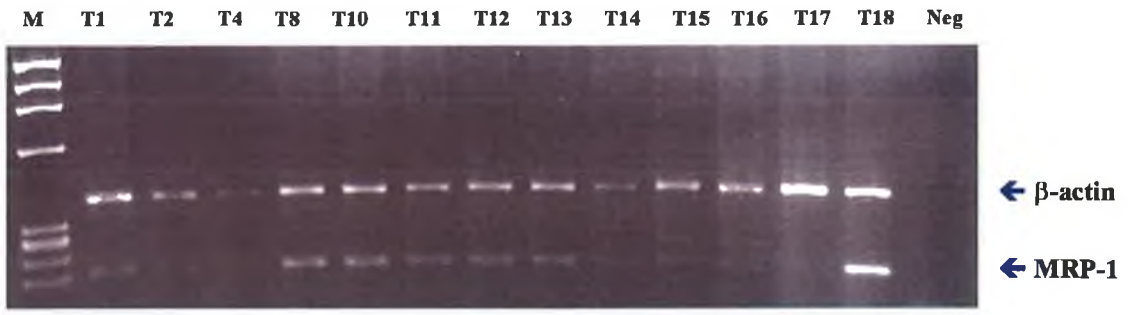
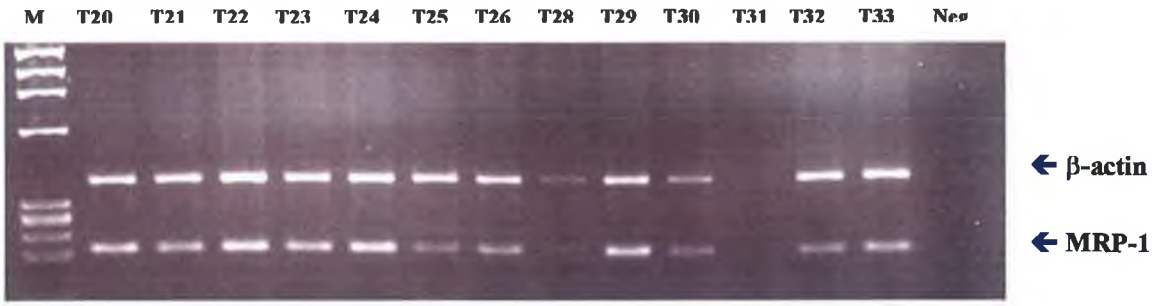


Figure 3.5.3 **(A)** and **(B)** Kaplan-Meier survival curves showing MRP1 mRNA did not correlate with disease free survival (P=0.2531) or overall survival (P=0.3942).

(A)



(B)



(C)

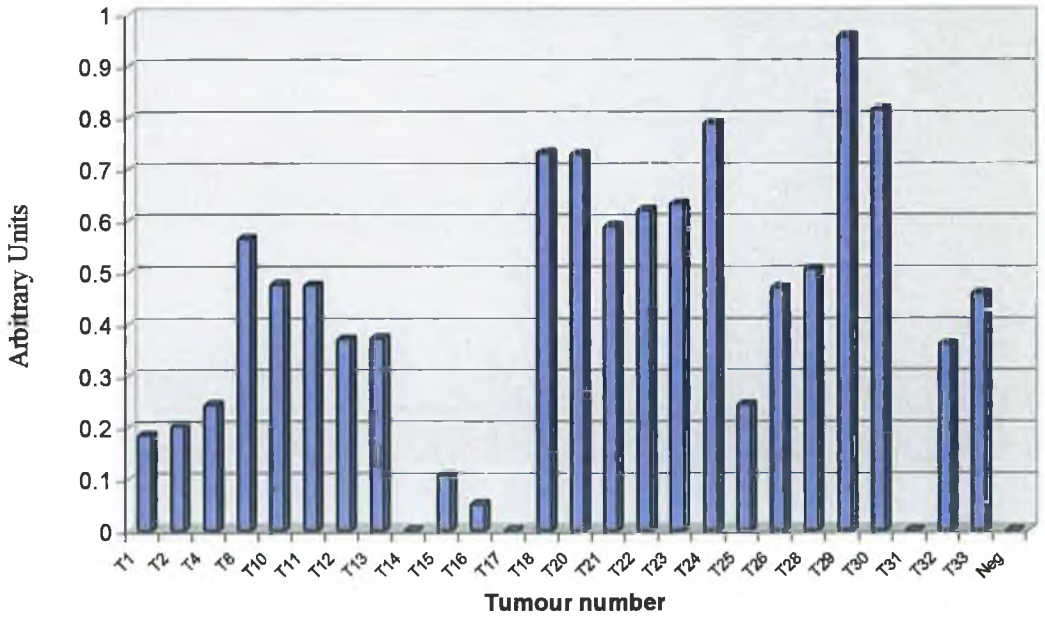
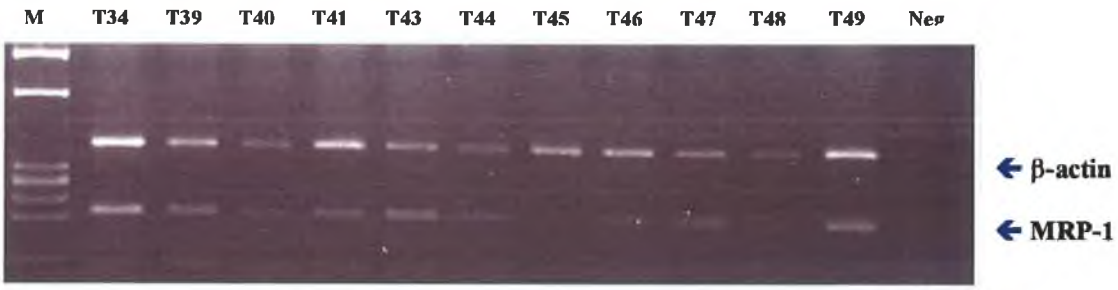
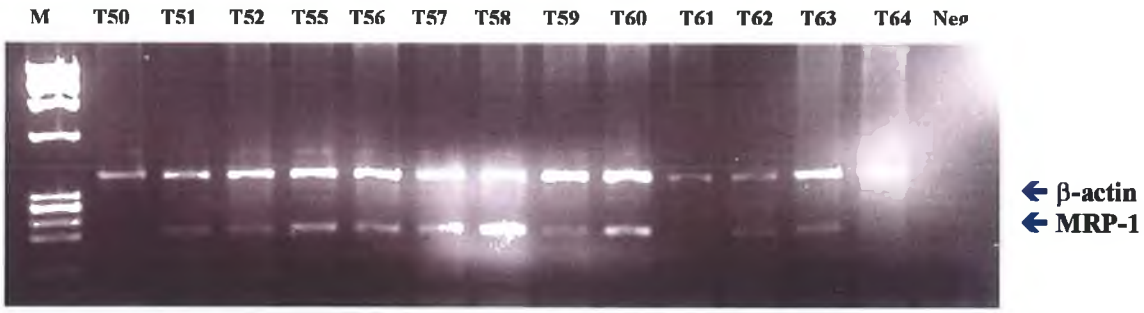


Fig. 3.5.3.1 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.1(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=Negative control. (n=1).

(A)



(B)



(C)

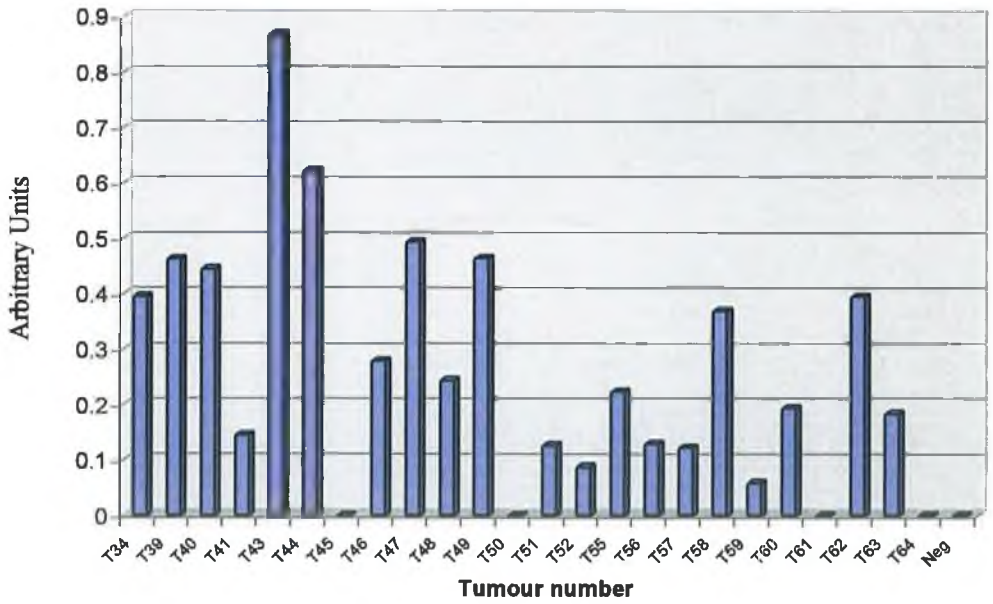
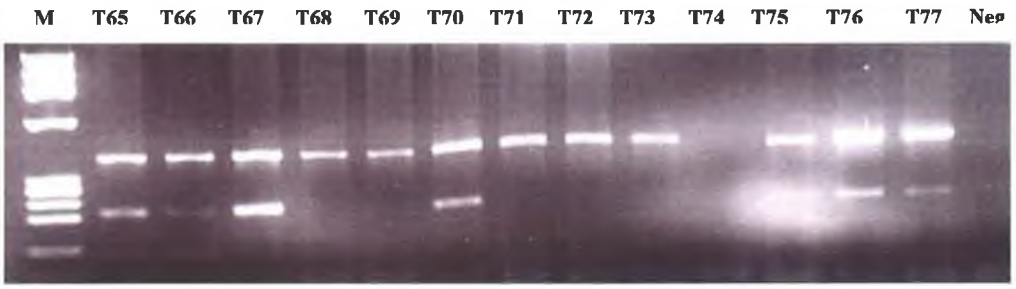
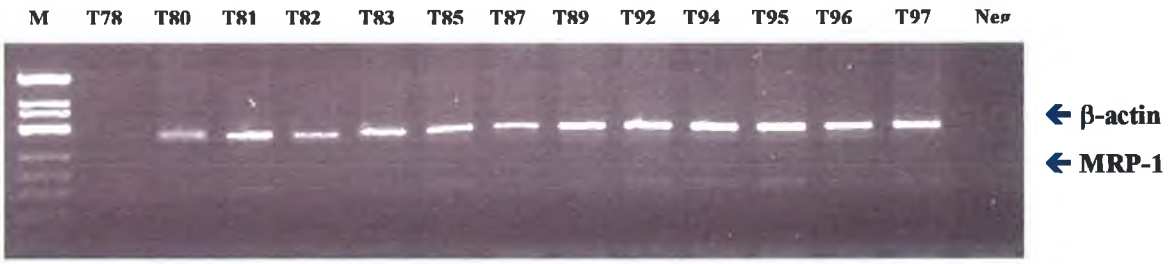


Fig. 3.5.3.2 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.2(C)** Densitometric analysis of RT-PCR results. M=Molecular weight; Neg=negative control. (n=1).

(A)



(B)



(C)

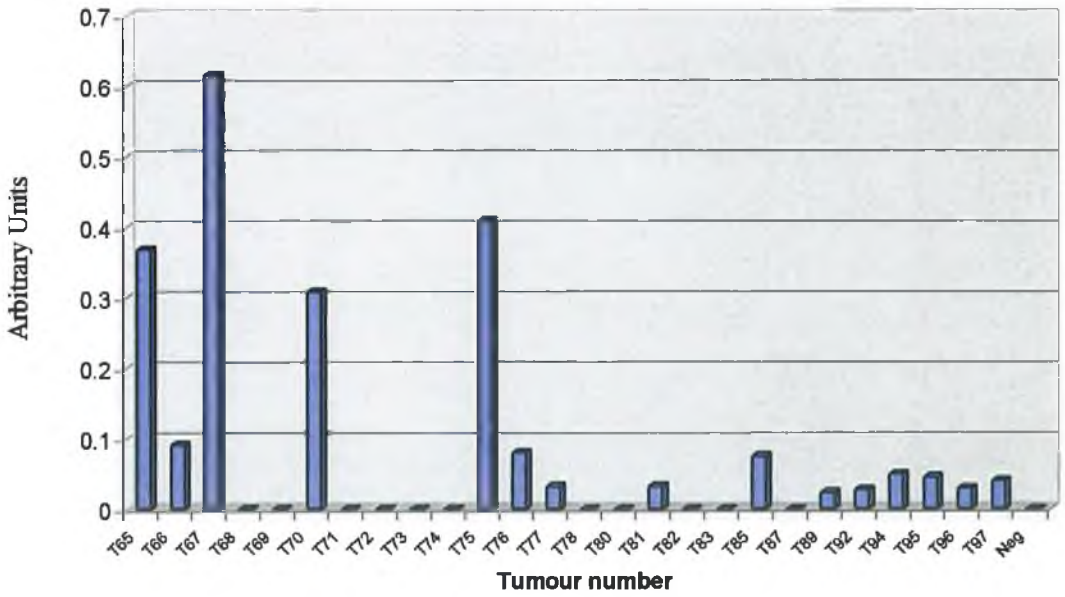
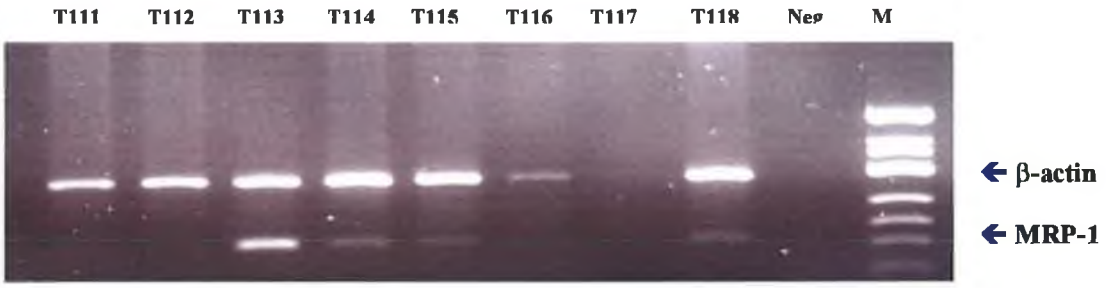


Fig. 3.5.3.3 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.3(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)

(B)



(C)

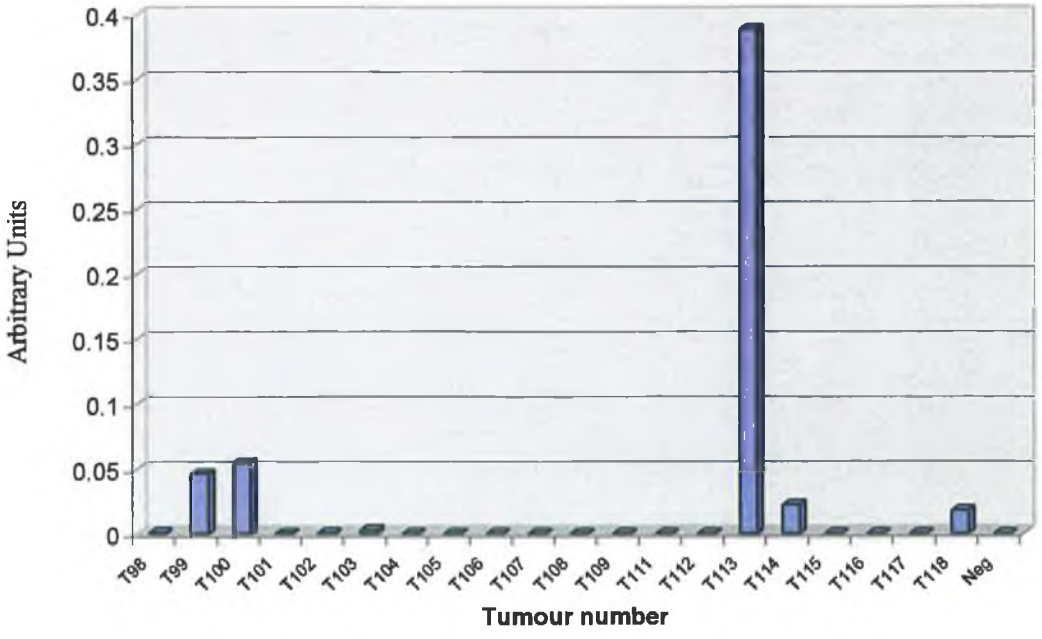
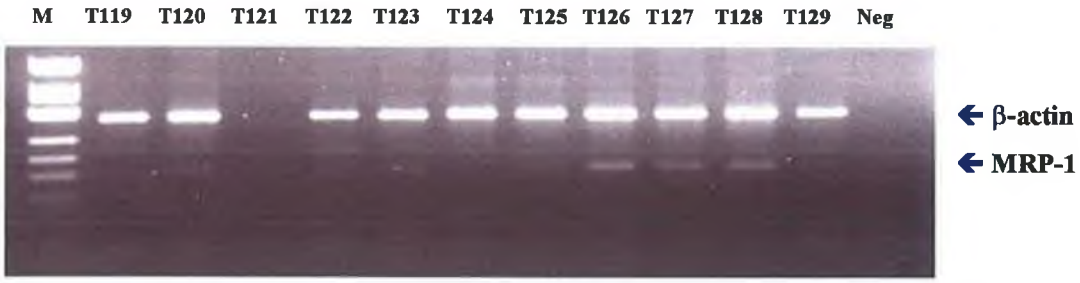
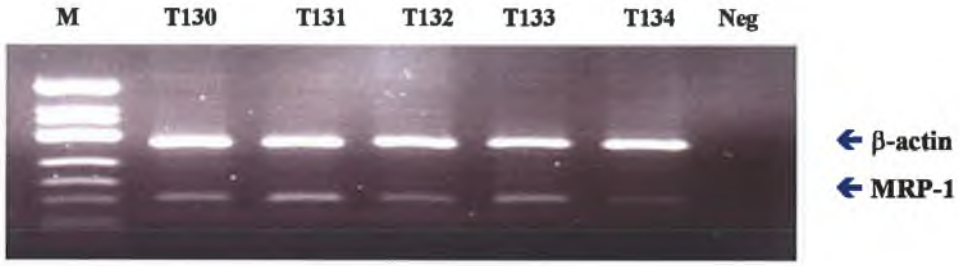


Fig. 3.5.3.4 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.4(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

(A)



(B)



(C)

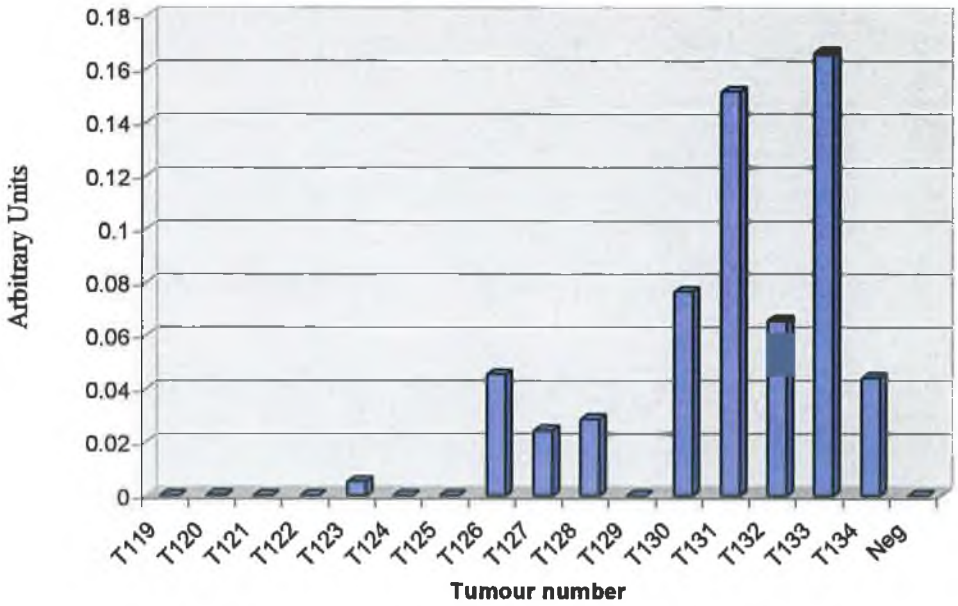


Fig. 3.5.3.5 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.5(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

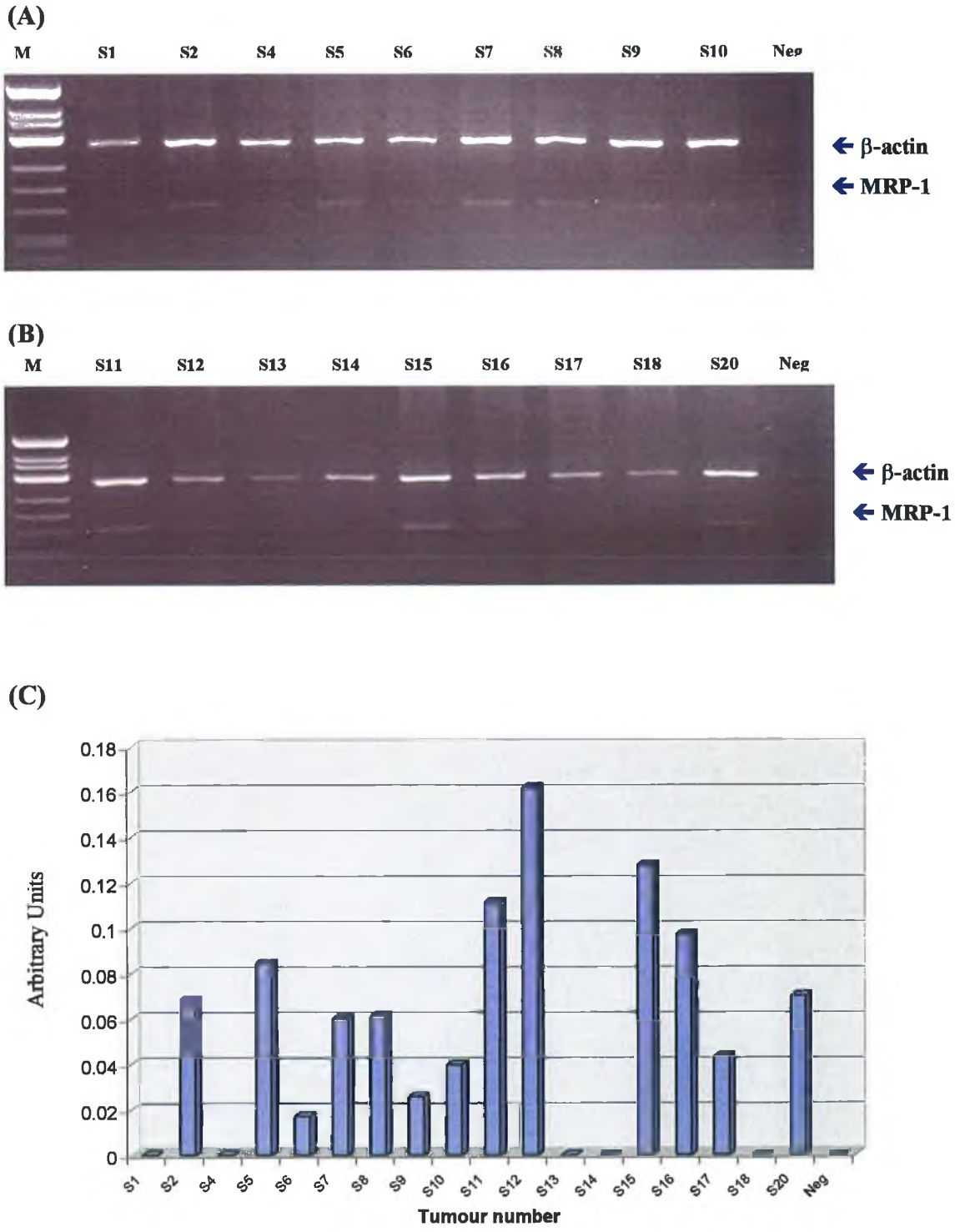
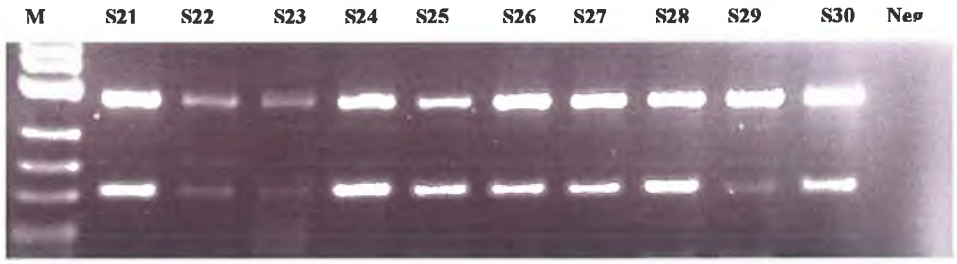
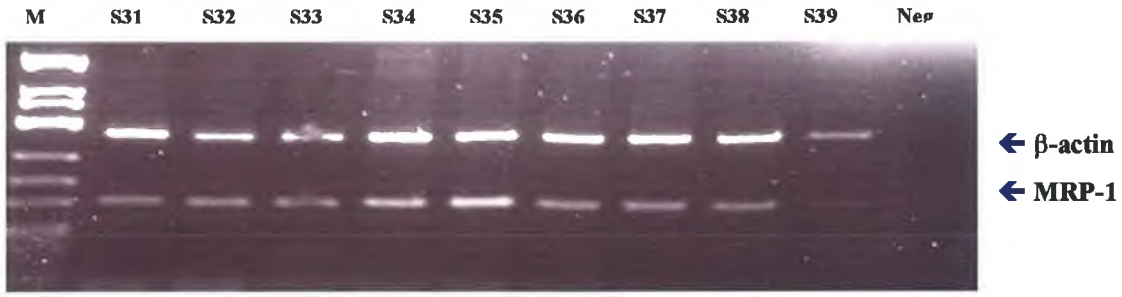


Fig. 3.5.3.6 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.6(C)** Densitometric analysis of RT-PCR results. M=Molecular weight; Neg=negative control. (n=1).

(A)



(B)



(C)

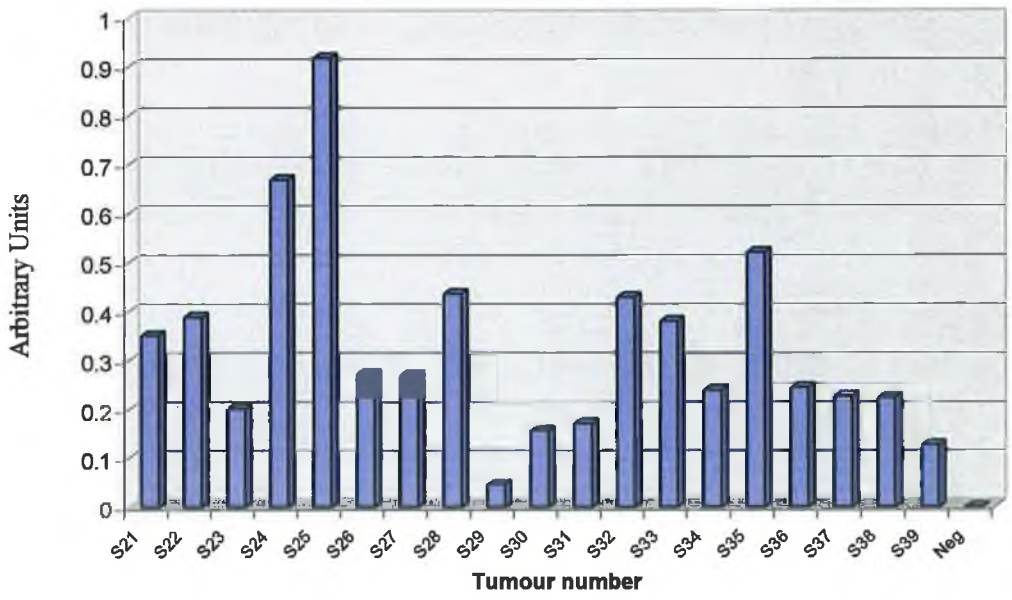
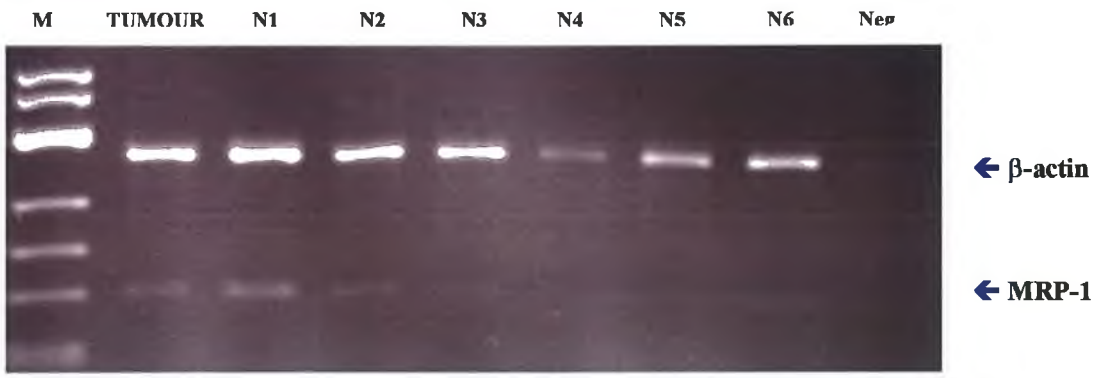


Fig. 3.5.3.7 **(A) and (B)** Gel electrophoresis of MRP-1 RT-PCR results on breast tumour tissue specimens; **Fig. 3.5.3.7(C)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=Negative control. (n=1).

(A)



(B)

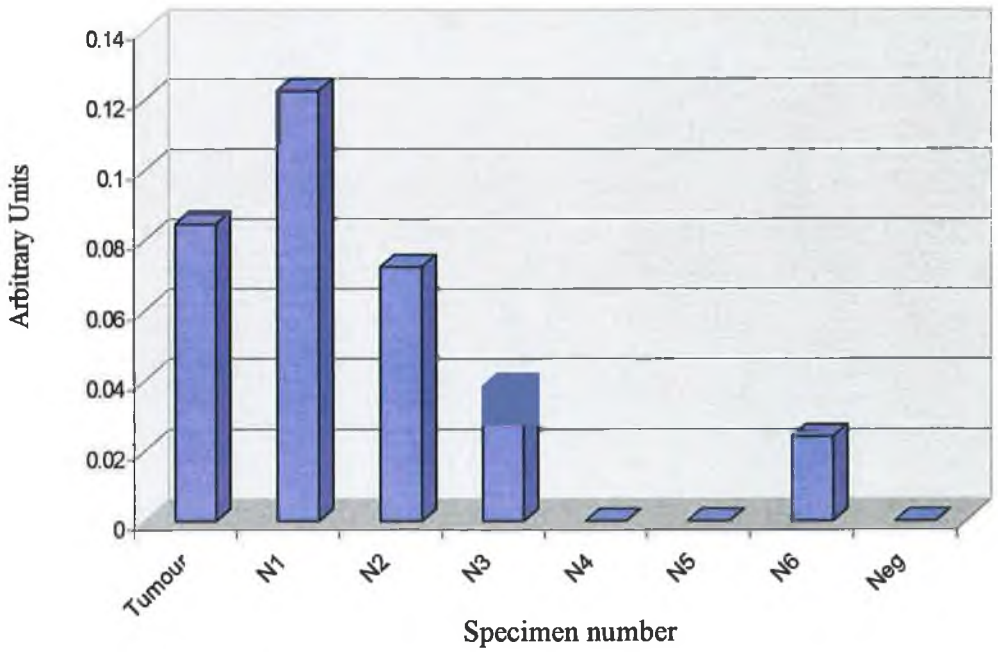


Fig. 3.5.3.8 **(A)** Gel electrophoresis of MRP-1 RT-PCR results on normal breast tissue specimens; **Fig. 3.5.3.8 (B)** Densitometric analysis of RT-PCR results. M=Molecular weight marker; Neg=negative control. (n=1).

4.0 Discussion

4.1 Analysis of apoptosis-related gene expression in MDR and invasiveness

Apoptosis is a genetically regulated biological process that is fundamental to the development of organisms and to homeostasis of tissues (Kerr *et al.*, 1972). It is regulated by many genes in normal tissues. The protein products of these genes can influence cell viability, either by promoting or inhibiting cell death. Cancer cells develop gene mutations enabling them to escape the normal tendency to die (Vaux *et al.*, 1988). When the expression of pro-apoptotic and anti-apoptotic genes is imbalanced, cells are predisposed to tumorigenic conversion and resistance to chemotherapeutic drugs (Roy *et al.*, 2000; Schmitt *et al.*, 1997). Previous studies at the NICB have shown that chemotherapeutic drug treatment alters the expression of apoptosis-related genes, Bcl and caspase family genes.

This thesis investigates the role of two apoptosis-related genes, galectin-3 and survivin, in drug resistance and invasiveness which may be associated with such resistance. In addition, the impact of increasing the expression of these genes in drug sensitive cell lines is investigated through transfection experiments.

4.2 Analysis of Galectin-3

One of the most devastating aspects of cancer is the emergence of metastases in organs distant from the primary tumor and most deaths from cancer are related to metastases (see section 1.3) (Keleg *et al.*, 2003). Most deaths from breast cancer, for example, result from metastasis to the bone, lung, brain, and liver (Moon *et al.*, 2001). It is therefore vital to identify the mechanisms underlying the processes of invasion and metastasis. One of the genes that has been linked to tumour cell invasion and metastasis is galectin-3 (see section 1.5.3) (Matarrese *et al.*, 2000). Galectin-3 has been shown to induce *in vitro* invasiveness, in addition to inhibiting apoptosis and accelerating cell growth and proliferation. Although there are conflicting reports regarding the association of galectin-3 with metastasis and invasion, with reports of it being down-regulated (Irimura *et al.*, 1991; Infusa *et al.*, 2001)

or up-regulated (Castronovo *et al.*, 1992) in tumour metastasis, there are many reports supporting its involvement in metastasis.

4.2.1 Expression of Galectin-3 in RPMI-2650 and its MDR variants

The expression of galectin-3 in the non-invasive nasal carcinoma cell line, RPMI-2650, was investigated and found to be expressed at the mRNA and protein level, by RT-PCR and western blot analysis, respectively. In addition galectin-3 expression was also investigated in two MDR-variants of RPMI-2650, RPMI-taxol resistant and RPMI-melphalan resistant cells lines, developed at the NICB by continuous exposure to taxol and melphalan, respectively (Dr. Y. Liang, PhD 1999). The RPMI-taxol resistant variant exhibits an MDR phenotype, compared to the RPMI-2650 parent cell line, with a large fold-resistance to adriamycin, vincristine, vinblastine, taxol and 5-fluorouracil. The RPMI-melphalan-resistant variant exhibits an invasive, as well as an MDR phenotype, with a large fold-increase to VP-16, melphalan, cisplatin and cadmium chloride (Liang *et al.*, 2001).

In agreement with the literature on the involvement of galectin-3 in metastasis (see section 1.5.3) (Bresalier *et al.*, 1998; Choi Kim *et al.*, 1999 and Matarrese *et al.*, 2000), it was found, by western blot analysis (section 2.4.1) that galectin-3 protein was highly over-expressed in RPMI-melphalan, an MDR variant with an increased invasive, motile and adhesive phenotype. Galectin-3 protein was not, however, over-expressed in RPMI-taxol, an MDR variant with an unaltered invasive, motile and adhesive phenotype (see section 3.1.1.2), suggesting that galectin-3 over-expression is associated with *in vitro* invasiveness and metastasis in RPMI-2650 nasal carcinoma cell line. These results were carried out in triplicate and proved to be reproducible. RT-PCR analysis (see section 2.4.3.5) results indicate a small increase of galectin-3 mRNA (in contrast to the large increase in protein level) in the RPMI-2650 melphalan-resistant cells, suggesting the involvement of post-transcriptional regulation of galectin-3 protein in this cell line. Galectin-3 mRNA expression was down-regulated in the RPMI-2650 taxol resistant variant (see section 3.1.1.1), however, direct evaluation of any possible galectin-3 association with drug resistance in the RPMI variants cannot be made from these experiments.

To further analyse the role of galectin-3 in drug resistance and invasiveness, up-regulation of galectin-3 in a non-invasive cancer cell line was carried out. By over-expressing galectin-3 in a non-invasive, drug-sensitive, cell line, we can study the phenotypic changes that may be associated with the up-regulation of this protein, and thereby gain further insight into its function.

4.2.2 Galectin-3 transfection and up-regulation in DLKP cells

Galectin-3 up-regulation alters the invasive potential of certain cell lines, including colon (Bresalier *et al.*, 1998), breast (Choi Kim *et al.*, 1999 and Matarrese *et al.*, 2000), and thyroid follicular cells (Takenaka *et al.*, 2003). Galectin-3 also plays a role in apoptosis and has been shown to protect cells from apoptosis induced by anti-Fas and staurosporine (Yang *et al.*, 1996), cisplatin (Akahini *et al.*, 1997) and nitric oxide (Moon *et al.*, 2001) (see section 1.5.2).

In an attempt to establish the significance of the increased galectin-3 expression in the invasive RPMI-melphalan resistance cell line and to further elucidate the role of galectin-3 in invasion and metastasis, as well as its role in inhibiting apoptosis, galectin-3 cDNA was transfected into the non-invasive, drug-sensitive, RPMI-2650 parental cells. Due to the nature of this cell line, however, and its poor growth rate at low cell density, it was not possible to carry out the transfection successfully. RPMI-2650 cells fail to grow from single cells, making the cloning procedure very difficult.

The non-invasive, drug-sensitive, lung carcinoma cell line, DLKP was therefore chosen to over-express galectin-3, by carrying out a stable transfection of galectin-3 cDNA and successfully selecting mixed population and three clonal populations resistant to geneticin at a concentration of 1mg/ml (see section 2.4.5). Three clones (C2, C12 and C13), in addition to the mixed population (MP) were established and analysed for galectin-3 mRNA and protein expression by RT-PCR and western blot analysis, respectively. A control transfection was also carried out in which DLKP cells were transfected with the empty vector PREP9.

Results from RT-PCR analysis revealed an up-regulation of galectin-3 by 1.6 fold in C12, by 2.2 fold in C13 and by 3.5 fold in the MP. There was, however, no up-regulation of galectin-3 in C2 or the mock transfectant (Vec), indicating that the galectin-3 mRNA over-expression is due to galectin-3 cDNA transfection (section 3.1.2.1). Western blot analysis results supported the RT-PCR findings, revealing an up-regulation of galectin-3 by 2 fold in C12, 1.9 fold in C13 and by 5.7 fold in the MP. Galectin-3 protein was dramatically down-regulated in C2, for unknown reasons (section 3.1.2.2). These results were reproducible when the RT-PCR and western blot analysis were carried out in triplicate.

4.2.3 Galectin-3 up-regulation and drug resistance in DLKP

The effect of galectin-3 over-expression on resistance to chemotherapeutic drugs has been determined in the breast cancer cell line, BT549, by exposing cells transfected with plasmid cDNA containing inserts, in either the sense or the antisense orientation, encoding human galectin-3, as well as parental cells, to cisplatin (Akahani *et al.*, 1997). The findings indicated that only galectin-3 expressing cells were protected against apoptosis induced by cisplatin (> 60 % viability), while the parental cells and the antisense control cells were not (< 30 % viability) (Akahani *et al.*, 1997), proving that galectin-3 functions as an anti-apoptotic gene (see section 1.5.2). In a separate study, galectin-3 over-expression in human leukemia T-cells was found to enhance resistance to apoptosis induced by anti-Fas antibody and staurosporine (Yang *et al.*, 1996).

In our experiments, the effect of galectin-3 over-expression on resistance to three chemotherapy drugs, taxol, carboplatin and adriamycin, was determined by carrying out *in vitro* toxicity assays (section 2.3.1). These chemotherapeutic drugs were selected to include a range of structurally and mechanistically different classes of drugs, i.e., the taxanes, the platinum compounds and the anthracyclines, respectively (for detail on these classes of chemotherapy drugs see section 1.1.1). It was found, however, that galectin-3 over-expressing clones did not show changes in drug resistance or sensitivity, suggesting that galectin-3 may not play a role in drug resistance in this cell line. It is interesting to note, however, that DLKP-MP cells were associated with increased resistance to carboplatin,

showing a 2.4-fold resistance compared to the parental cells lines and the mock transfection (see section 3.1.2.3). This may suggest resistant populations may be present in the mixed population and it may be useful to clone out further populations from this cell line, to identify those with drug resistance, for further analysis. In conclusion, however, galectin-3 over-expression failed to protect the cells from drug-induced death and therefore failed to induce an MDR phenotype in the over-expressing clones.

4.2.4 Galectin-3 up-regulation in DLKP is associated with induction of an invasive phenotype

As previously mentioned (section 4.2.2), galectin-3 over-expression has been found to induce metastasis in colon, breast and thyroid follicular cell lines. In a study by Bresalier *et al.* (1998), it was shown that the down-regulation of galectin-3, using antisense methods, resulted in marked decrease in the metastatic potential of a highly metastatic colon cell line, HM7, while the up-regulation of galectin-3 enhanced the metastatic potential of a low metastatic colon cell line, LS174T. Similar results were obtained by another group in 2002 using a human breast cancer cell line, BT549, which was transfected with galectin-3 cDNA and injected into the spleen of nude mice. The mice developed tumours in both the spleen and the liver, indicating that galectin-3 can enhance the metastatic potential of BT549 cells (Song *et al.*, 2002). Furthermore, the over-expression of galectin-3 also confers a malignant phenotype on TAD-2 thyroid follicular cells (Takenaka *et al.*, 2003). TAD-2 cells transfected with galectin-3 cDNA showed anchorage-independent growth and loss of contact inhibition, suggesting that galectin-3 plays an important role in malignant transformation in thyroid follicular cells (Takenaka *et al.*, 2003).

In agreement with these studies, our experiments show that galectin-3 over-expression in lung tumour cells is also associated with induced invasion. DLKP parent has been shown to be non-invasive (Y. Liang, PhD, 1999). Transfection with the control vector did not alter that (Figure 3.1.2.4). However, the DLKP transfectants over-expressing galectin-3, i.e. C12, C13 and MP, demonstrated a large increase in invasiveness compared to the parental cells (see section 3.1.2.4). C2, the only clone with no galectin-3 mRNA or protein over-expression, had the same non-invasive properties as DLKP-parent, correlating with the fact

that it has basal levels of galectin-3 expression. The same was true for motility (see section 3.1.2.5), suggesting that galectin-3 plays a role in invasion and motility. These experiments were carried out in triplicate and were reproducible, suggesting that galectin-3 over-expression induces a stable invasive and motile phenotype in the transfected cells. In addition the invasion and motility assays were quantitatively assessed by dye elution and the colormetric analysis was supportive of the microscopic analysis (see Figures 3.1.2.4 and 3.1.2.5). Galectin-3 was also found to be associated with increased adhesion to a range of extra cellular matrix proteins, namely, ECM, fibronectin and laminin (see section 4.2.4.1).

4.2.4.1 Galectin-3 up-regulation in DLKP is associated with increase adhesion to ECM proteins.

Extracellular matrix (ECM), as purchased from sigma, is an artificial reconstituted basement membrane. It contains components of the natural basement membrane. The adhesion assays with ECM, demonstrates that DLKP-gal-3 C12, C13 and MP were the most adhesive to ECM, therefore suggesting that galectin-3 over-expression supports DLKP adhesion to ECM. DLKP parent, DLKP-vec and DLKP-gal-3 C2 were all less adhesive. RPMI-2650 was least adhesive to ECM and was used as a negative control, while HT-1080 was as adhesive as MP and was used as a positive control in these experiments (as well as in the invasion and motility assays). Fibronectin is a major component of the extra cellular matrix (see section 1.3.1). As with ECM, galectin-3 over-expression correlates with DLKP adhesion to fibronectin. DLKP-gal-3 C12, C13 and MP were the most adhesive to fibronectin. Laminin is another major component of the basement membrane. Result of the adhesion assays show that DLKP-gal-3 C12, C13 and MP were slightly more adhesive to laminin than the parental cell line, DLKP-vec and DLKP C2. Collagen type IV is another major component of the basement membrane. All the DLKP cell lines were equally adhesive to collagen IV, suggesting that galectin-3 over-expression does not correlate with DLKP adhesion to collagen IV. These results were reproducible when carried out in triplicate, as can be seen from the standard deviation bars on the graph representing the finding of these assays (see Figure 3.1.2.6).

Our results on adhesion studies support some of those of Warfield *et al.* (1997), where a study was carried out on the breast carcinoma cell line, BT549, and galectin-3 over-expressing clones were found to adhere more rapidly to laminin and fibronectin. In addition, the cells were also able to invade through matrigel-coated filters at approx. three times the rate of parental cells. Contrary to our results, however, the results of Warfield *et al.*, suggest that galectin-3 is essential for adhesion of breast carcinoma cells to laminin and collagen IV, but not to fibronectin. Taken together, our results indicate galectin-3 involvement in promoting cell adhesion to ECM, fibronectin and laminin in the DLKP cell line.

4.2.5 Galectin-3 up-regulation in DLKP is not associated with increased cell proliferation rate

In addition to its association with invasion, metastasis and apoptosis control, galectin-3 has also been associated with changes in growth rates of certain cancer cell types. Growth properties of human leukemia T cells transfected with galectin-3 cDNA, and over-expressing galectin-3 protein, were examined by Yang *et al.* (1996). It was found that cells over-expressing galectin-3 displayed higher growth rates than cells that do not over-express this protein. This suggests that galectin-3 is a cell growth regulator and appears to confer cell survival by inhibiting apoptosis.

In our experiments, however, we found no evidence of changing growth rates of DLKP clones over-expressing galectin-3, compared to parental cells or mock transfectants, and therefore conclude that galectin-3 up-regulation in DLKP is not associated with an increased proliferation rate in these cells (see section 3.1.2.7).

4.3 Analysis of Survivin levels in drug resistant cell lines

Survivin is a protein that inhibits apoptosis and regulates cell division (Altieri *et al.*, 1999). Recently there has been great interest in survivin as a diagnostic marker and potential drug target because of its predominantly cancer-specific expression in adult human organ tissues. Survivin is expressed in most human cancers, but not in the majority of normal, adult tissues (Ambrosini *et al.*, 1997, Adida *et al.*, 1998a and O'Driscoll *et al.*, 2003) (see section 1.6.3 for more detail).

Studies have shown that survivin expression is up-regulated in association with the development of drug resistance (Notarbartolo *et al.*, 2002) and in response to apoptotic stimuli (Ikeguchi *et al.*, 2002, Muenchen *et al.*, 2001 and Kennedy *et al.*, 2000), thereby protecting cells from apoptosis and promoting the development of cancerous tissue. The aim of this section of the thesis was to investigate the role of survivin in drug resistance. The RPMI-2650 drug resistant variants (Liang *et al.*, 2001) were used as a model for the initial part of the analysis as they were for the initial galectin-3 study (section 4.2.1).

4.3.1 Expression of Survivin in RPMI-2650 and its MDR variants

Survivin up-regulation correlation with drug resistance was demonstrated in a study by Notarbartolo *et al.* (2002), where the human leukemia cell line, HL60, was compared with its multidrug resistant, P-gp overexpressing, variant HL60R. In HL60R cells, there was an increase in survivin mRNA compared to the parental cells. This suggests that the selection of the MDR HL60R cell line, by exposure to Doxycycline hydrochloride, had induced the expression of factors opposing apoptosis, including IAPs. This, in turn, suggests that the inability of cancer cells to undergo apoptosis may contribute to the complex phenomenon of drug resistance.

In our experiments, survivin expression was studied in the nasal carcinoma cells, RPMI-2650 and it two MDR variants, RPMI-taxol and RPMI-melphalan resistant cell lines. Contrary to findings by Notarbartolo *et al.*, we found survivin mRNA and protein to be

down-regulated in the RPMI-taxol resistant variant compared to both the parental cells and the RPMI-melphalan resistant variant (see sections 3.2.1.1 and 3.2.1.2). The results were reproducible in repeat experiments. RPMI-taxol resistant cells are highly resistant to their selection drug, taxol (226-fold) and are cross-resistant to doxorubicin, vincristine, vinblastine and VP-16, but not to melphalan, cadmium chloride, cisplatin or 5-FU, indicating the involvement of P-gp in its MDR phenotype (Liang *et al.*, 2001). The role of survivin down-regulation in this taxol-resistant cell line, if any, is unknown at present.

4.3.2 Survivin cDNA stable transfection into DLKP cells

In an attempt to establish the significance of the down regulation of survivin mRNA and protein in RPMI-taxol resistance cells, as discussed in the previous section, survivin cDNA was stably transfected into DLKP cells. It was hoped that by over-expressing survivin in a drug-sensitive cell line, we could gain further insight into its role in drug-resistance. Survivin expression was previously analysed in three cell lines; DLKP, a lung carcinoma cell line, SKOV-3, an ovarian carcinoma cell line, and MCF-7, a breast carcinoma cell line. It was found that DLKP expressed intermediate levels of survivin protein compared to SKOV-3 and MCF-7 and was therefore selected for the cDNA transfection.

Survivin cDNA was sequenced and it was found that the correct survivin coding sequence was inserted into pTarget plasmid. This plasmid was transfected into DLKP using lipofectin, and five clones, resistant to geneticin at a concentration of 1mg/ml, were successfully established (section 2.4.5). The clones were analysed for changes in survivin mRNA and protein expression by RT-PCR and western blot analysis, respectively. Despite their resistance to geneticin, none of the clones showed a consistent change in survivin mRNA or protein expression (see Figures 3.2.2.1 and 3.2.2.2). This may suggest that levels of survivin mRNA and protein may not be altered in DLKP cells due to mechanisms within the cells preventing over-expression. This may be linked to expression of EPR-1 (see section 1.6.4 in the introduction), which may be acting as a natural antisense to survivin, keeping its level of expression constant.

Despite the lack of detectable survivin over-expression in the DLKP clones, *in vitro* toxicity assays were carried out on three of the five clones to determine whether survivin cDNA transfection was perhaps causing small increases in survivin level, thereby having any effect on protecting the cells from drug-induced death. Two chemotherapy drugs were used in this experiment: adriamycin and carboplatin. However, none of the three DLKP-survivin clones appeared to have any increased resistance to these drugs compared to DLKP parent cells (see section 3.2.2.3). This may be due to the lack of up-regulation of survivin as seen by RT-PCR and western blot analysis. The toxicity assays were carried out once using adriamycin and twice using carboplatin and were not reproducible, however, a mean of the two assays using carboplatin is shown in Figure 3.2.2.3B.

Other groups have, however, successfully transfected survivin cDNA into different cell lines (Tamm *et al.* 1998; Li *et al.*, 1998; Islam *et al.*, 2000a; Kasof *et al.*, 2001a; Tran *et al.*, 2002). Islam *et al.* (2000a) stably transfected a survivin cDNA construct into CHP 134, a human neuroblastoma cell line. Similar to our technique, lipofectamine was also used and geneticin was used to select two clones. The clones over-expressed survivin mRNA as demonstrated by northern blot analysis. Furthermore, one of the survivin over-expressing clones was protected from apoptosis induced by retinoic acid, highlighting the role of survivin in apoptosis inhibition (Islam *et al.*, 2000). A second attempt was, therefore, made at over-expressing survivin in a different cell line. The cell line selected was SKOV-3 'Tet off', a human ovarian carcinoma cells line, containing a tetracycline inducible plasmid (see section 4.3.3).

4.3.3 Survivin cDNA stable transfection into SKOV-3 'Tet off' Cells

The SKOV-3 'Tet off' (T.O.) cell line was chosen for the survivin cDNA transfection as it allows gene expression to be turned on when tetracycline or doxycycline (Dox; a tetracycline derivative), is removed from the culture media, or turned off when they are added. This theoretically allows the expression of genes to be tightly regulated in response to varying concentrations of tetracycline or doxycycline and thereby allows loss or gain of gene function, as required.

The system consists of two plasmids, one encoding the tetracycline controllable transactivator protein (tTA) under control of a cytomegalovirus (CMV) promoter, and the second, pTRE containing the tet operator (tetO) minimal promoter driving the gene of interest. It involves constitutive expression of the tet transactivator protein (tTA) in response to human CMV. tTA is a fusion protein composed of the tet repressor of *Escherichia coli* and the transcriptional activation domain of the VP16 protein of herpes simplex virus. In the absence of tet, the tet repressor portion of tTA mediates high affinity, specific binding to sequences from the tet resistance operator of Tn10 (tetO), resulting in gene expression. In the presence of tet, however, a conformational change in tet repressor prevents tTA from binding to its operator, inhibting gene expression as shown in Figure 4.1.

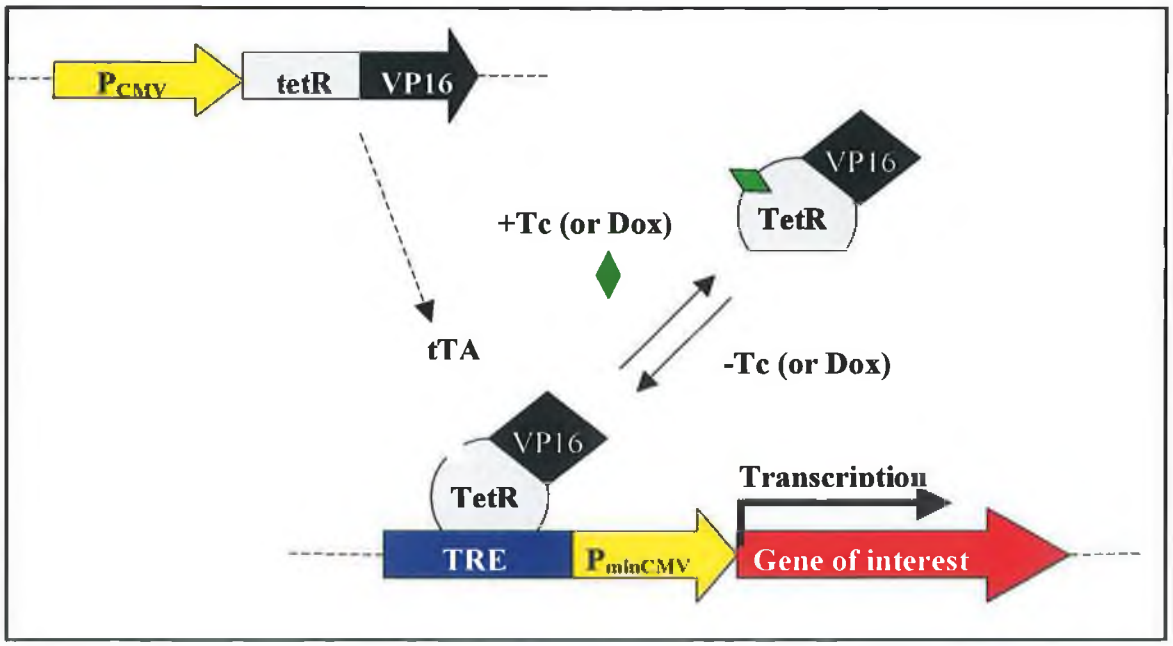


Figure 4.1 Schematic representation of gene regulation in the Tet-Off system. The tetracycline-controlled transactivator (tTA) is a fusion of the wild-type Tet repressor (TetR) to the VP16 activation domain of the herpes simplex virus. The tet-responsive element (TRE) is located upstream of the minimal immediate early promoter of the cytomegalovirus (P_{minCMV}), which is silent in the absence of activation. tTA binds the TRE, and thereby activates transcription of gene of interest, in the absence of tetracycline (Tc) or doxycycline (Dox) (Figure adapted from Clontech Laboratories user manual).

Survivin cDNA, originally cloned into pTarget plasmid, was subcloned into pTRE plasmid, and was stably transfected into SKOV-3 'T.O.' using Fugene 6 as the transfection agent (this technique was first optimized by carrying out a luciferase assay to achieve maximum transfection efficiency (see section 3.2.3)). A second plasmid, PTK-Hyg, was co-transfected with survivin cDNA-containing pTRE and clones were selected in hygromycin (see section 2.4.5.2).

Twelve SKOV-3 'T.O' survivin clones and a mixed population (MP) were established and characterised by RT-PCR and western blot analysis initially (Figures 3.2.3.1.1 and 3.2.3.1.2), to determine if survivin expression was up-regulated in these cell lines. However, as with the DLKP stable transfection, there was no consistent change in survivin mRNA or protein expression. RT-PCR analysis was carried out in triplicate, and no up-regulation of survivin mRNA was observed in the repeat experiments. Western blot analysis was also carried out in triplicate and no up-regulation of survivin protein was observed. Toxicity profiles of the clones were analysed using carboplatin and taxol, to determine if survivin cDNA transfection had any effect on the drug resistance profile of the clones. Again, survivin cDNA transfection played no role in protecting the cells from drug-induced death (see Figure 3.2.3.1.3). This was probably due to the fact that there was no apparent survivin mRNA or protein up-regulation detected by RT-PCR or western blot analysis. Since the clones established were all resistant to hygromycin, it was assumed that the transfection experiment was successful and no further analysis was carried out to check if survivin cDNA was actually transfected into the cells. It is possible that the cells developed resistance to the selection antibiotic, hygromycin; therefore, PCR analysis needs to be carried out to determine whether transfection of survivin cDNA was successful.

Furthermore, two of the clones, C2 and C5, along with the mixed population were selected for further analysis, using the inducible system to switch survivin expression on and off, by sub-culturing the cells in the absence and presence of doxycycline. RT-PCR and western blot analysis was carried out on the clones and the MP, however, there was no change at the mRNA level between the 'on' state and the 'off' state (see Figure 3.2.3.1.1C and D). This experiment was repeated three times and no change in survivin mRNA expression was apparent in any of the repeated RT-PCR experiments.

There was only a very slight change at the protein level between survivin expression in the 'on' and the 'off' states, with C2, displaying the most variation, a 1.35-fold increase in survivin protein in the 'on' state, compared to the 'off' state (see Figure 3.2.3.1.2E and F). This was, however, observed in one experiment and was not evident in the repeated western blot. Toxicity assays also showed a very slight difference between the 'on' and 'off' states of survivin expression, with C5 showing slightly more resistance in its 'on' state to carboplatin (1.5-fold) and to taxol (1.66-fold) (see Figure 3.2.3.1.3C and D). Results represent a mean of two separate experiments, which were reproducible.

Overall, it is concluded that survivin cDNA stable transfection into SKOV-3 'T.O.' showed no change in survivin mRNA or survivin protein expression and did not induce a drug resistance profile in these cells. This leads us to believe that, if survivin cDNA has been successfully transfected, EPR-1 may be acting as a natural antisense to survivin in the SKOV-3 'Tet off' cell line, as well as in the DLKP cell line (as mentioned in section 4.3.2). It was believed, therefore, that the only way to overcome this difficulty was to carry out transient transfections of survivin cDNA into these cell lines in order to see a more immediate affect on survivin expression without giving the cells a chance to adjust their intracellular levels of survivin/EPR-1 expression.

4.3.4 Survivin cDNA transient transfection into SKOV-3 'Tet off' cells

The majority of studies involving survivin cDNA transfections into cancer cell lines have been through transient transfections. The reason for this may be that it is not always possible to create stable transfections over-expressing survivin. This may be due to processes within the cell that prevent the up-regulation of survivin mRNA and protein, perhaps due to effector cell protease receptor-1 (EPR-1) acting as a natural antisense to survivin, and thereby keeping survivin's levels constant in the cell. Survivin was reported to be highly complementary to EPR-1 (Ambrosini *et al.*, 1997) (see section 1.6.4). Due to their complementary sequences, it has been suggested that there may be functional intereactions between survivin and EPR-1 proteins and that EPR-1 may act as a natural antisense to survivin (Ambrosini *et al.*, 1997).

Transient transfection allows rapid analysis of a gene function. The use of transient transfections in apoptosis research has been widely reported (Wrone-Smith *et al.*, 2001; Kuo *et al.*, 2001; Townsend *et al.*, 1999; Katoh *et al.*, 1998; Los *et al.*, 1997).

Our attention was, therefore, turned to the use of transient transfection techniques to over-express survivin cDNA in the SKOV-3 'Tet off' cell line (section 2.4.5.4). The transient transfection technique was first optimized to achieve maximum transfection efficiency. This was carried out using the 5-bromo-4-chloro-3-indolyl-β-D-galactopy-ranoside (X-Gal) staining method (section 2.4.5.3). Previous studies in this lab by Dr. Deirdre Cronin showed that various cell lines gave different transfection efficiencies, such as Hela cells, where up to 40% transfection efficiency was achieved and DLKP cells, where up to 35% transfection efficiency was achieved (D. Cronin, PhD, 2002). For our current cell line of interest, SKOV-3 'Tet off', a transfection efficiency of approximately 30% was achieved using Fugene 6 (see section 3.2.4.1). This apparently allowed sufficient expression of survivin cDNA to show increases in survivin at the mRNA. No increase in survivin protein levels was detected (see sections 4.3.4.1 and 4.3.4.2).

4.3.4.1 Survivin cDNA transient transfection caused survivin mRNA up-regulation in SKOV-3 'Tet off'

RT-PCR analysis was carried out on RNA from cells harvested at a range of time intervals. These were 24, 48, 72 hours and 1 week post transfection. There was a dramatic increase of survivin mRNA levels after 24 hours (approx. 18-fold, see Figure 3.2.4.2.1B). This increase was maintained at 48 and 72 hours, but in some repeat experiments, survivin mRNA expression dropped to almost basal levels after 5 days as shown in Figures 3.2.4.2.1A and 3.2.4.2.1B. This is to be expected at the survivin cDNA-containing plasmid would be degraded in the cells; stable transfections involve drug selection of the very small proportion of cells, which have incorporated the plasmid into their chromosomal DNA. Furthermore, the function of the inducible system was tested in the transient transfection by carrying out the transfection procedure in the presence and absence of doxycycline to turn survivin expression 'off' and 'on', as discussed in section 4.3.3. This proved to work successfully and the effect of this inducibility was most dramatic at 24 hours, with a 50 %

inhibition of survivin mRNA expression in the presence of dox, i.e. in the 'off' state compared to the 'on' state. The effect is still obvious at 48 and 72 hours, but not to the same extent (see Figure 3.2.4.2.1A and B). Surprisingly, the survivin mRNA expression after 5 days seems to be lower in the 'on' state than the 'off' state (see Figure 3.2.4.2.1A and B). This has been found in repeat experiments, and the reason for this is not clear. To prove that the effects seen were due to survivin over-expression, a control transfection containing the empty pTRE vector was carried out and the level of survivin mRNA remained constant at all time points (time points included on this experiment are 24, 48, and 72 hours) as shown in Figure 3.2.4.2.1C and D. These experiments were carried out in triplicate and the results were reproducible. It is important to mention that in all RT-PCR analysis using the survivin primers which pick up the three splice variants of survivin, suvivin-2B isoform was undetectable and in some, but not all, cases survivin-ΔEx3 was detected. The reason for this variation is unknown.

4.3.4.2 Survivin cDNA transient transfection caused no survivin protein up-regulation in SKOV-3 'Tet off'

Western blot analysis was carried out on protein extracted at 24, 48, 72 hours and 5 days post-transfection. However, there was no survivin protein up-regulation seen at any of the time points as shown in Figures 3.2.4.2.2A, B, C and D. Numerous attempts were made to over-express the survivin protein in the cells, but this was not possible. This was also carried out using different concentrations of protein ranging from 10 to 30 μg, in an attempt to avoid protein saturation, but no survivin protein over-expression was detected in any of the western blots carried out. Immunofluorescence was also carried as another means of detecting survivin protein over-expression. Survivin protein was found to be expressed in the cytoplasmic region of the cells (see Figure 3.2.4.2.3), in agreement with similar findings by several groups (Ambrosini *et al.*, 1997; Kawasaki *et al.*, 1998; Lu *et al.*, 1998; Adida *et al.*, 1998; Tanaka *et al.*, 2000; Muzio *et al.*, 2001; Satoh *et al.*, 2001). Despite numerous experiments, survivin protein over-expression could not be obtained. The reason for this is unclear and may be due to some translational block by these cells.

In addition, survivin expression is cell cycle dependent with up-regulation of survivin in the cells during the G2/M phase of the cell cycle, followed by a rapid decline in the G1 phase (Li *et al.*, 1998) (see section 1.6.6). Zhoa *et al.* (2000) carried out a study showing that the ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner and that structural changes destabilise the survivin protein. The group found that proteosome inhibitors prolonged the half-life of survivin, thereby proving that proteosomes are involved in survivin degradation (Zhoa *et al.*, 2000) (see section 4.7). This may help to explain why we were unable to detect an increase in survivin protein by transient transfection equivalent to the increase in survivin mRNA that was observed.

Some research papers carrying out survivin transfection experiments do not present changes in survivin protein expression, but proceed to show functional changes in the cells caused by survivin up-regulation (Islam *et al.*, 2000; Kasof *et al.* 2001) or down-regulation by antisense (Olie *et al.*, 2001). We, therefore, proceeded to carry out experiments to detect changes in the cells that may be caused by the transfection of survivin cDNA.

4.3.4.3 Does Survivin cDNA transient transfection protect the cells from drug-induced apoptosis?

Despite the lack of survivin protein over-expression following the SKOV-3 'Tet off' transient transfection, we took the analysis of this experiment further and went on to try and determine whether the transfection resulted in any functional changes within the cells and whether the survivin was protecting the cells from drug-induced apoptosis. This was carried out first, by standard *in vitro* toxicity assays (see Figure 3.2.4.2.4), whereby drug (taxol and cisplatin) was added at different time points (24, 48 and 72 hours) post transfection, to include all periods of survivin mRNA up-regulation detected by RT-PCR, and cells were incubated for the 5-7 days before being assessed for cell viability. However, despite numerous attempts to optimize this technique, a consistent result showing increased resistance of the cells, could not be achieved. The inducible system was also tested as well as the mock transfection and in all cases the IC_{50} values remained unchanged.

Technically, the toxicity assay was difficult to optimize as cells were being monitored over five to seven days when the survivin mRNA level was reverting back to that of parental cells. Further optimization of *in vitro* toxicity testing is needed, perhaps by carrying out short-term toxicity assays. Taxol and cisplatin were chosen because they have been used by other research groups when studying the effect of survivin up- or down-regulation on drug resistance in various cell line models. Taxol has previously been used to induce apoptosis in cell transfected with survivin cDNA (Li *et al.*, 1998) or with survivin dominant-negative mutant (Tran *et al.*, 2002). Cisplatin has been used to induce apoptosis in cells transfected with survivin ribozyme (Pennati *et al.*, 2002) or with survivin dominant-negative mutant (Grossman *et al.*, 2001 and Tran *et al.*, 2002).

The role of survivin in drug resistance was examined in endothelial cells (ECs). EC chemoprotection can be induced by VEGF (vascular endothelial growth factor). Recent reports suggest that in addition to EC protection by VEGF, survivin over-expression can also protect ECs from drug-induced cell death by preserving the microtubule network (Tran *et al.*, 2002). This was demonstrated through the transfection of survivin cDNA in HUVEC (human umbilical vein endothelial cells), followed by the use of the chemotherapy drugs, CDDP and taxol to induce apoptosis. Survivin over-expression protected ECs from apoptosis induced by these drugs. When survivin wild-type transfection was replaced by a survivin mutant, this protection against cisplatin and taxol-induced apoptosis was lost (Tran *et al.*, 2002). Zaffaroni *et al.* (2002) studied the effects of survivin on drug resistance and found that stable transfection of human ovarian carcinoma with survivin cDNA caused a 4-6-fold increase in cell resistance to taxol and taxotere (Zaffaroni *et al.*, 2002).

The second method we used to determine whether survivin transfection is protecting the cells from drug induced apoptosis was the TUNEL (terminal deoxynucleotide transferase-mediated deoxyuridine phosphate nick end-labelling) assay. This is an assay designed to detect apoptosis in cells, by labeling extensive DNA strand breaks, a process which occurs during apoptosis (see section 2.3.2.1). Before the assay was carried out, transfected and parental cells were treated with taxol, a microtubule-stabilising drug previously used in survivin studies to induce apoptosis, as survivin-microtubule interaction is required for apoptosis inhibition (Li *et al.*, 1998). No changes were detected in the levels of apoptosis in the survivin transfectants compared to the control cells as shown in Figure 3.2.4.2.5. The

experiment was not always successful. As non-apoptotic cells undergoing cell division, also fluoresced, this made it difficult to distinguish apoptotic cells from dividing cells.

This method of apoptosis detection (i.e. TUNEL) was also used by Shankar *et al.* (2001) to assess the level of apoptosis induction caused by survivin expression inhibition, when survivin antisense oligonucleotides were added to a human neuroblastoma and an oligodendroglioma resulting in reduction in survivin protein levels and spontaneous apoptosis (Shankar *et al.*, 2001). TUNEL assay was also used in detecting apoptosis induced by transfection of survivin antisense oligonucleotide into human choriocarcinoma cell lines (JEG-3 and BeWo) and a human trophoblast cell line (tPA30-1). Results indicated up to 9-fold increase in TUNEL-positive cells (apoptotic cells), when these cells were transfected with survivin antisense (Shiozaki *et al.*, 2003). Other groups have used transient transfection techniques to over-express or down-regulate survivin in different cell lines and were able to show functional changes within the cells as a result of survivin over-expression.

In a study by Kasof *et al.* (2001b), Hela cells were transfected with apoptotic genes in the Ptracer vector containing a green fluorescence protein (GFP) marker for accessing viability. Transfection with pro-apoptotic genes, e.g. Bax, led to approx. 90% reduction in viability. However, co-transfection of Bax with survivin, or livin, a novel member of the IAP family and closely related to survivin (Vucic *et al.*, 2000; Kasof *et al.*, 2001b) (see section 1.6.5.2), resulted in a 4-6-fold increase in viability. A similar study was carried out by Tamm *et al.* (1998), where survivin cDNA transient transfection in 293 cells was able to partially inhibit apoptosis induced by the co-transfection of Bax or Fas. Survivin expression also protected 293 cells from apoptosis induced by over-expression of pro-caspase-3 and -7, as well as inhibiting the processing of these two zymogens into active caspases (Tamm *et al.*, 1998).

4.3.4.4 Does Survivin cDNA transient transfection induce an invasive phenotype?

To date there has not yet been a direct correlation between survivin expression in cancer cells and the induction of an invasive and metastatic phenotype. Survivin expression in tumours has, however, been associated with a more metastatic tumour, with poor prognosis and that is more resistant to therapy (Yoshida *et al.*, 2003; Ikehara *et al.*, 2002). The clinical aspect of survivin will be discussed in further detail in section 4.5.
Here we attempted to establish whether or not there is a correlation between survivin over-expression in SKOV-3 'tet off' and invasiveness by examining the *in vitro* invasiveness of the SKOV-3 'Tet off'-survivin transfected cells compared to the parental cells and the control transfections.

Cells were set up in invasion chambers 24 hours post-transfection and the results were obtained 48 hours later (see section 2.5.1). Initial experiments suggested a decrease in invasiveness between the transfected cells and the parental cells or the mock transfection. There also seemed to be a decrease in invasiveness between the transfected cells in their 'on' state of expression compared to their 'off' state. RPMI-2650 parent cells were used as a negative control and RPMI-melphalan-resistant cells were used as a positive control for the invasion assay (see section 3.2.4.2.6). When the experiment was repeated, however, this decrease in invasiveness was no longer apparent. Experiment was repeated several times and the change in invasiveness was not reproducible, suggesting that the effect seen the first time was not a real effect and that survivin was having no effect on *in vitro* invasiveness of the transfected cells. The reason behind the lack of any functional change caused by survivin transient transfection may be due to the lack of protein over-expression in the transfected cells. No further analysis was carried out in these cell lines.

4.3.5 Survivin cDNA transient transfection into DLKP cells

To establish whether the lack of survivin protein over-expression in SKOV-3 'Tet off' cells is cell line specific, DLKP was chosen for transient transfection experiments. Survivin cDNA in pTarget was transiently transfected into DLKP cells using the same technique as in section 4.3.4 (also see section 2.4.5.4). As in section 4.3.4, RT-PCR analysis was carried

out on RNA from cells harvested at different time intervals, i.e. 24, 48, 72 hours and 5 days post transfection. There was a dramatic increase of survivin mRNA after 24 hours as shown by RT-PCR analysis. This increase was maintained even after 72 hours, after which it started to drop to almost basal levels at 5 days as shown in Figure 3.2.5.1.

Western blot analysis, on the other hand, did not show any survivin protein up-regulation at 24, 48 or 72 hour time points (see Figure 3.2.5.2), suggesting that lack of survivin protein in transient transfections may not be cell line specific. However, to further investigate this finding, a third cells line was selected for further analysis.

4.3.6 Survivin cDNA transient transfection into MCF-7 'Tet off' cells

The human breast carcinoma cell line, MCF-7 'Tet off', is similar to the ovarian carcinoma cell line, SKOV-3 'Tet off' previously used in survivin transfection experiments, in that it contains the 'Tet off' plasmid which allows gene expression to be turned on when tetracycline or doxycycline is removed from the culture media, or turned off when they are added, allowing the expression of clones genes to be tightly regulated. This cell line, as with the SKOV-3 'Tet off' cell line, was developed in our lab. by Helena Joyce, M.Sc.

Survivin cDNA (which, as previously mentioned, was subcloned into the pTRE plasmid) was transiently transfected into MCF-7 'Tet off', and as in sections 4.3.4 and 4.3.5, RT-PCR analysis was carried out on RNA from cells harvested at different time intervals, i.e. 24, 48, 72 hours and 5 days post transfection. There was a dramatic increase in survivin mRNA levels after 24 hours, as demonstrated by RT-PCR analysis. This increase was maintained even after 72 hours but dropped to almost basal levels after 5 days (see Figure 3.2.6.1A). Transfection of the empty pTRE plasmid into the cells did not show any change in survivin mRNA expression (Figure 3.2.6.1C). Furthermore, the function of the inducible system was tested in the MCF-7 'Tet off' transiently transfected cells by carrying out the transfection procedure in the presence and absence of doxocycline to turn survivin expression 'off' and 'on', respectively, as discussed in section 4.3.3 and 4.3.4. RT-PCR analysis showed no difference between the two states of survivin expression, i.e. the over-expression of survivin mRNA in the transfected cells was not down-regulated or inhibited

by the addition of doxocycline as shown in Figure 3.2.6.1B. This suggests that the inducible, Tet off plasmid does not function in the MCF-7 cell line as it did in the SKOV-3 cells. As with all survivin RT-PCR analysis presented in this thesis, the survivin primers used are primers that were previously used by Mahotka *et al.* (1999), which pick up the three splice variants of survivin; survivin-2B, survivin and survivin-delta Ex3, however, only the original survivin variant was detected in these cells.

Western blot analysis was carried out on protein extracted at the same time points as before. Interestingly, we observed survivin protein up-regulation for the first time in our experiments at 24 hours post-transfection. As with all survivin western blots presented in this thesis, α-tubulin was used as an endogenous control to detect equal loading of protein samples as shown in Figure 3.2.6.2A. When the transfection as repeated a second time, we were unable to reproduce the initial result of survivin protein up-regulation (Figure 3.2.6.2B). The experiment was repeated several times in an attempt to reproduce the initial results, but without success. The reason for this is unknown and further transient transfection studies in a wider range of cell lines may be needed. This may help identify cell lines that, for unknown reasons at present, have the ability to over-express survivin protein.

The objective of the survivin studies was to establish a role for survivin in drug resistance and determine whether or not survivin played a role in *in vitro* invasiveness. This was to be achieved through over-expression of survivin in drug-sensitive cancer cells. Despite several attempts, however, we were unsuccessful in identifying a cell line which over-expresses both survivin mRNA and protein, simultaneously. As described above, neither survivin mRNA nor protein over-expression was attainable through stable transfection experiments. Survivin mRNA, but not protein, was over-expressed through transient transfection of survivin cDNA in three cell line models (SKOV-3 'Tet off', DLKP and MCF-7 'Tet off'). Survivin mRNA over-expression alone was not enough to induce any phenotypic (functional) changes in the cells. We were not able, therefore, to establish a role for survivin in any of the cell lines.

4.4 Survivin expression in MDR cells

Our findings that survivin mRNA and protein was reduced in the RPMI-2650 taxol-resistant variant, as discussed in section 4.3 and the finding that galectin-3 was over-expressed in RPMI-2650 melphalan-resistant variant as discussed in section 4.2, led to the establishment of DLKP and RPMI-2650 drug resistant variants, which were to provide a model for studying the expression of survivin and galectin-3 to further elucidate their role in drug resistance and invasiveness. In addition, studies were designed so as to help us establish a relationship between drug resistance and invasiveness. It has previously been shown in our laboratories that induction of an MDR phenotype, through drug exposure, has been accompanied by induction of an invasive phenotype in RPMI-2560 cells (Liang *et al.*, 2001) and DLKP cells (Liang *et al.*, in prep), both 'normally' non-invasive.

4.4.1 Establishment of MDR variants of DLKP cell line

DLKP is a poorly differentiated squamous lung cancer cell line, which was established from a lymph node metastasis biopsy by bronchoscopy in this Centre (Law *et al.*, 1992). There has been much characterisation carried out on this cell line since it was established. Toxicity assay results indicate that this cell line is very sensitive to adriamycin, vincristine, VP-16 and cisplatin (Clynes *et al.*, 1992). RT-PCR analysis shows that DLKP cells express low levels of MRP-1 mRNA, but lacks MDR-1 mRNA expression (NicAmhlaoibh *et al.*, 1999). DLKP was derived from a secondary site of the primary tumour. However, invasion assays demonstrate a low level of invasiveness in this cell line, suggesting that although DLKP cells are apparently metastatic *in vivo*, this is not the case *in vitro* (Y. Liang, PhD, 1999; O'Driscoll et al., 2002).

In order to study phenotypic and genotypic features related to acquired chemoresistance, Liang *et al.* (manuscript in prep.) established ten MDR variants of the DLKP cell line by sequential pulse exposure to increasing concentrations of ten commonly used chemotherapy drugs. The drugs chosen were: etoposide, vincristine, taxotere, mitoxantrone, 5-fluorouracil, methotrexate, CCNU, BCNU, cisplatin and chlorambucil. Results revealed

that all ten drugs induced drug resistance and over-expression of P-glycoprotein (P-gp) in DLKP cells.

Multiple drug resistance, MDR, is the mechanism developed by tumour cells upon treatment with chemotherapy drugs, whereby the tumour develops cross-resistance to functionally and mechanistically unrelated drugs (see section 1.2). It constitutes one of the main problems in cancer therapy and is cause of chemotherapy failure in cancer treatment (Clynes *et al.*, 1992). The first insight into this phenomenon was revealed in the 1960s, and much attention has been drawn to the study of MDR since then (Royal *et al.*, 1968). Many cell lines have been used as models to elucidate the mechanism involved in chemoresistance (Benard *et al.*, 1989). It has been widely reported that the induction of MDR is strongly associated with the over-expression of P-gp and MRP1 (multiple drug resistance protein) (Clynes *et al.*, 1990; Goldstein *et al.*, 1989; Lum *et al.*, 1993; Harrison *et al.*, 1995; El-Deiry *et al.*, 1997). Several structurally and functionally unrelated agents, including the anthracyclines, the vinca alkoids, actinomycin D, the epipodophyllotoxins and the taxanes are substrates for P-gp and are strongly associated with MDR.

In the DLKP selection studies carried out by Liang *et al.* (in prep), taxotere appeared to induce the highest level of MDR and was associated with the highest level of over-expression of P-gp and MRP-1 in this variant, demonstrated by RT-PCR and western blot analysis. Furthermore, findings by Liang *et al.* indicate that DLKP exposure to certain drugs, namely, mitoxantrone, BCNU, chlorambucil, 5-FU, methotrexate and cisplatin, induced an invasive phenotype in the resulting variants, whereas etoposide, vincristine, taxotere and CCNU did not induce such invasiveness. These findings, along with previous findings in this Center indicating that melphalan causes an induction of invasiveness in the nasal carcinoma cell line, RPMI-2650 (Liang *et al.*, 2001), suggest a strong link between drug resistance and cancer invasion/metastasis.

To further elucidate the role of cancer chemotherapeutic drugs in the enhancement of invasion and metastasis in the DLKP cell line and to determine whether the effects caused, by certain drugs, on *in vitro* invasiveness, is a random affect on cells or whether induction of *in vitro* invasiveness is cell type and drug specific, we repeated the work carried out by Liang *et al.* (in prep.) using three of the ten drugs previously included. The drugs selected

for the purpose of this thesis were taxotere, vincristine and 5-FU. These drugs were selected with the RPMI-2650 drug selection study in mind. It was intended to choose drugs that over-lap in order to compare their effects on the two cell lines (DLKP and RPMI-2650) (see section 4.5). The cells were exposed to sequential pulses of a stepwise increased concentration of drug for approx. ten weeks, after which they were analysed by toxicity assays, *in vitro* invasion assays, gelatin zymography studies, and gene expression was examined by RT-PCR and, in some cases, by western blot analysis. The findings from this study are summarised in Table 4.1.

DLKP selection variants were developed by pulse exposure of cells to sub-lethal concentrations of drug, following weekly 4-hour exposure to the drug for approx. 3 months. (Part of this work was carried out by Mr. John Cahill, NICB). The method of pulse-selection, instead of continuous selection, which is occasionally used in *in vitro* studies (Liang *et al.*, 2001) was used in this thesis, to try to mimic clinical drug resistance, where drug is administered to patients on a weekly basis for a period of 2-3 months, depending on the type of cancer in question.

Resistant variants of the human colon tumour cell line, LoVo, has been developed by this method of pulse-selection with adriamycin and have been shown to possess a more stable and different toxicity profile to a variant developed by continuous exposure to the same drug (Yang and Trujillo, 1990). Similarly, intermittent exposure of the human myeloidleukemic cell line, K562, to epirubicin or vinblastine over a 3-month period resulted in the establishment of resistant variants which were maintained as resistant with the re-exposure to the selecting agent for a period of 4 days every 4-5 weeks (Marks *et al.*, 1993).

4.4.1.1 Induction of a multiple drug resistance phenotype in DLKP selected variants

The MDR phenotype has been developed in animal and human cell lines in tissue culture through exposure of sensitive cells to increasing sublethal concentrations of a particular selecting agent, i.e. a cytotoxic drug. High degrees of resistance can be achieved by this method of selection. *In vitro*, broad resistance to structurally and functionally unrelated drugs is observed intrinsically or after intermittent or prolonged exposure of tumour cells to a single agent. This classical multidrug resistance is typically characterised by cross-resistance to four classes of commonly used natural product drugs, the anthracylines, vinca alkaloids, taxanes and epipdophyllotoxins (Hipfner *et al.*, 1999).

In our studies, exposure of DLKP cells to two of the three drugs, taxotere and vincristine, induced drug resistance to their selection agents, i.e. taxotere and vincristine, respectively. Cross-resistance to a range of structurally and functionally related (taxotere, taxol, vincristine) and un-related (adriamycin) chemotherapy drugs was also induced. Exposure to 5-FU, on the other hand, did not induce drug resistance, but did induce a dramatic change in the morphology of the cells, as discussed in section 4.4.3. Results from all the DLKP variants are summarised in Table 4.1.

Variant	Drug resistance	Gene expression (RT-PCR/Western blot)	Morphology change	Invasive/motile phenotype	MMP secretion
DLKP-TXT	TXT, TAX, VCR, ADR	↑ Pgp mRNA+protein ↑ MRP1 protein ↑ E-cad mRNA+protein ↓Survivin protein	Cells became very slightly stretched	Yes	MMP-2, 9 & -13 ↑ ↓
DLKP-VCR	TXT, TAX, VCR, ADR	↑ Pgp mRNA+protein ↑ MRP4 mRNA ↓Survivin protein	Cells became stretched and slightly elongated	No	MMP-2, 9 & -13 ↑ ↓
DLKP-5-FU	None	↑ MRP3 mRNA	Cell became very stretched, elongated and branch-like	No	MMP-2, 9 & -13 ↑

TXT= Taxotere; TAX= Taxol; VCR= Vincristine; ADR= Adriamycin; E-cad=E-cadherin

Table 4.1 Summary of phenotypic and genotypic changes induced by DLKP drug-selection.

4.4.2.1 Taxotere and Cross-resistance

The DLKP-taxotere resistant variant was found to be approximately 420-fold resistant to taxotere and 210-fold resistant to taxol. Given the similarities of these two taxanes, it is not surprising that the taxotere resistant variant is also resistant to taxol. DLKP-taxotere was found to be approximately 6650-fold resistant to vincristine, a vinca alkaloid, which, similarly to the taxanes, functions by disrupting the tubulin/microtubule equilibrium, resulting in cell cycle arrest at metaphase (see section 1.1.1.1 and 1.1.1.2) (Wilson *et al.*, 1975). DLKP-taxotere is 52-fold resistant to adriamycin, an anthracycline with different structural and functional properties to the taxanes and the vinca alkaloids, but also associated with MDR. The DLKP-taxotere MDR variant was not found to be cross resistant to 5-FU, CCNU or cisplatin (section 3.3.1). The toxicity assays were all repeated at least three times and the majority were reproducible. However, in some cases the standard deviation values were quite high (see Table 3.6.1.2a). This may be due to a small loss of resistance resulting from prolonged growth of cells in the absence of selection pressure. In future experiments it may be beneficial to expose cell to their selecting agent occasionally to avoid loss of resistance in this heterogenous cell line. The need to re-expose drug-resistant cell line to their selection agent occasionally to maintain their resistance levels has previously been found in DLKP-10p, an adriamycin-resistant cell line variant established in the NICB (Cleary, PhD, 1995). Taken together, the findings from the *in vitro* toxicity testing demonstrate a pattern of multiple drug resistance similar to findings by many other groups.

To investigate the phenomenon of development of resistance to chemotherapeutic agents, osteosarcoma cells, TE-85, were exposed to increasing doses of taxol or taxotere over a period of nine months (Burns *et al.*, 2001). Variants that were highly resistant to taxol and taxotere, respectively, were developed. The taxol resistant cell line, showed >1000-fold resistance to taxol and taxotere and 60-fold resistance to adriamycin. Similarly, the taxotere-resistant cell line, showed >1000 to taxol, 800-fold resistance to taxotere and 90-fold resistance to adriamycin. There was little cross-resistance to topotecan and enhanced sensitivity to cisplatin. This is in agreement with our findings in the DLKP-TXT variant with regard to cross-resistance to taxol and lack of resistance to cisplatin. Similarly, a

recent study was carried out by Liu *et al* (2001b), where the pancreatic adenocarcinoma cell line, SUIT-2, was selected by exposure to taxotere and was found to be significantly resistant to taxotere, as well as showing cross-resistance to adriamycin, but no resistance to 5-FU (Liu *et al.*, 2001b). Furthermore, Ikubo *et al.*, (1999) studied the cytotoxicity of anti-microtubule agents to human small-cell lung cancer cell lines including, a primary cell line from an untreated patient, cell lines from treated patients as representatives of intrinsic drug-resistance and cell lines selected by continuous exposure to increasing concentrations of adriamycin, etoposide or cisplatin, as representative of acquired drug resistance. Results showed that the cell lines treated with adriamycin or etoposide were highly resistant to taxol, taxotere, vincristine, vindesine and vinblastine as well being resistant to their selection agents. The cell line treated with cisplatin, however, showed no cross-resistance to the anti-microtubule agents (Ikubo *et al.*, 1999). In a study by Bhalla *et al.* (1993), the human myeloid leukemia cell line, HL-60, cell line was exposure to progressively higher concentrations of taxol, which resulted in two taxol-resistant cell lines. The cells also displayed a variable degree of cross-resistance to taxotere, vincristine and adriamycin, but were sensitive to the antimetabolite, cytosine arabinoside (Ara-C) (Bhalla *et al.*, 1993).

Taken together, these findings support the theory that some forms of taxotere resistance are typical of a classic MDR phenotype. There are, however, conflicting reports suggesting this not to be the case. In a study by Hill *et al.* (1994), cross-resistance to taxotere was not apparent in four epipodophyllotoxin-selected cell lines with alterations in topoisomerase II, suggesting that taxotere cross-resistance is not automatically expressed by classic MDR tumour cells.

Despite the discrepancies in the literature, our current finding correlate with a previous study carried out by Liang *et al.* (2003; in prep) in so far as, although the fold differences varied greatly in some cases, the drug resistance profiles were similar. Liang et al. (2003, in prep), found that the DLKP-taxotere resistant variant was approx. 36-fold resistant to taxotere, 262-fold resistant to vincristine and 33-fold resistant to adriamycin. Although cross-resistance of this variant to 5-FU and CCNU was not tested, DLKP-taxotere MDR variant was not resistance to cisplatin, in agreement with our findings. Our findings also correlate with a previous study carried out in our lab. (Liang *et al.*, 2001), on the nasal carcinoma cell line, RPMI-2650, where it was found that an RPMI-2650 taxol-resistant

variant was found to be resistant to taxol (226-fold) and cross-resistant to adriamycin, vincristine, vinblastine, and VP-16. However, there was no significant resistance to melphalan, cisplatin or 5-FU.

4.4.2.2 Changes in expression of apoptosis and MDR-related genes associated with taxotere resistance

To date, some specific mechanisms of drug resistance have been elucidated, among which the best understood are increased expression of mdr-1 encoded Pgp (Lu *et al.*, 2000; Liu *et al.*, 1999; Zhang *et al.*, 1999; Xu *et al.*, 1998), MRP1 (Kornmann *et al.*, 1999; Liang *et al.*, 2001; Yu *et al.*, 2001) and LRP (Aszalos *et al.*, 1998; Liu *et al.*, 2001). These membrane transporter proteins play an important role in multiple drug resistance involving drug efflux (section 1.2).

As discussed in section 1.2.1, P-gp was identified as a membrane efflux pump that results in decreased drug accumulation and diminished cytotoxicity (Lum *et al.*, 1993). P-gp is widely expressed in tumour specimens of patients with a variety of hematological and solid tumours, and has been reported to increase in expression after the development of clinical resistance to chemotherapy (Pastan *et al.*, 1988; Beck *et al.*, 1991; Ling *et al.*, 1992). MRP1 is also associated with the MDR phenotype and has been identified in non-Pgp multidrug resistant cell lines from a variety of tumour types, including leukemias, fibrosacrcoma, non-small cell lung, small cell lung, breast, cervix, prostate and bladder carcinomas (Izquierdo *et al.* 1996), as well as in many normal human tissues (Sugawara *et al.*, 1997; Loe *et al.*, 1996a; Zaman *et al.*, 1993; Cole *et al.*, 1992; Kool *et al.*, 1997).

In an attempt to identify the mechanisms of MDR in the DLKP-taxotere resistant MDR variant, alterations in the expression levels of a number of MDR markers were investigated. In agreement with the cross-resistance pattern of this variant (i.e., its cross-resistance to taxol, vincristine and adriamycin and its lack of resistance to cisplatin, 5-FU and CCNU, all indicators of the involvement of Pgp in its MDR phenotype), dramatic over-expression of P-gp mRNA and protein were detected in the DLKP-taxotere resistant variant by RT-PCR and western blot analysis when compared to the parental cells (section 3.3.7.1 and 3.3.8.1).

This is also in agreement with findings by other groups when studying the mechanisms of drug resistance with respect to taxane-related-resistance. In a study by Liu *et al.* (2001a), intrinsic and acquired taxotere resistant cell lines have shown the expression of mdr-1 gene.

Other groups that found similar cross-resistance patterns as we did in taxotere-resistant cells, found that MDR was mainly mediated by Pgp (Van Ark-Otte *et al.*, 1998; Burns *et al*, 2001; Liu *et al.*, 2001a and 2001b; Bhalla *et al.*, 1994; Liang *et al.*, 2001; Shirawaka *et al.*, 1999) but not by MRP (Liu *et al.*, 2001a and 2001b; Liang *et al.*, 2001). As well as proving that taxotere resistance in the pancreatic adenocarcinoma cell line, SUIT-2, was mediated by Pgp, Liu *et al.* (2001a) also confirmed that taxotere resistance in these cells was not mediated by MRP, by RT-PCR, and also by using a specific inhibitor of MRP. The MRP inhibitor indomethacin, sensitised the taxotere-resistant cells to taxotere, but had no sensitising effect to taxotere cytotoxicity, indicating that the taxotere-related drug resistance in these cells is mainly mediated by Pgp and not MRP.

In agreement with this, we found no over-expression of MRP1 mRNA as seen by RT-PCR analysis (section 3.3.7.2) in the DLKP taxotere-resistant variant. However, it was surprising to find over-expression in MRP1 protein in the DLKP taxotere-resistant variant by western blot analysis (section 3.3.8.2). However, this is in agreement with previous findings by Liang *et al.* (manuscript in prep), in the DLKP taxotere-resistant variant previously established, suggesting a possible involvement of post-transcriptional regulation in expression of MRP1 protein. In addition, these findings support previous findings suggesting that cancer cells that over-express Pgp or MRP1 do not show cross-resistance to platinum-containing compounds (e.g. cisplatin), alkylating agents and anti-metabolites (e.g. 5-FU) (Taniguchi *et al.*, 1996; Koike *et al.*, 1997). MRP2 (cMOAT) (Figure 3.6.7.3), MRP3 (Figure 3.3.7.4) and MRP5 (Figure 3.3.7.6) were undetectable by RT-PCR in the DLKP-taxotere MDR variant, suggesting that they play no role in drug resistance of this variant, while low traces of MRP4 and BCRP mRNA were present (Figures 3.3.7.5 and 3.3.7.7). These experiments were carried out in triplicate and found to be reproducible.

Galectin-3 mRNA expression and survivin mRNA and protein expression were analysed in the DLKP taxotere-resistant variant. Based on the previous findings in the RPMI-2650 variants, where galectin-3 was over-expressed in the RPM-2650 melphalan-resistant and

invasive cell line (see sections 3.1.1.2 and 4.2.1), indicating a role for galectin-3 in *in vitro* invasiveness, we expected to detected galectin-3 over-expression in the DLKP taxotere-resistant variant. As will be discussed in the next section, this is a variant, which exhibited an elevated invasive phenotype. This was not, however, found to be the case. Galectin-3 mRNA was un-changed in the DLKP taxotere-resistant cell line when compared to the parental cells as shown in Figure 3.3.7.9. This was carried out in triplicate and the results were reproducible. It is important to remember, however, that galectin-3 mRNA was not dramatically over-expressed in the RPMI-2650 melphalan-resistant variant (see section 3.1.1.1) and the over-expression was only observed at the protein level as shown by western blot analysis. In our current experiments, galectin-3 protein expression was not analysed and it will be interesting to carry out western blot analysis for this protein in future studies.

As previously mentioned, survivin mRNA was found to be down regulated in previous studies on the RPMI-2650 taxol-resistant cell line (see section 3.2.1.1. and 4.3.1). This was an unexpected result as previous studies have reported survivin up-regulation in response to chemotherapy treatment *in vitro* (Notarbartolo *et al.*, 2002; Kennedy *et al.*, 2000; Muenchen *et al.*, 2001; Ikeguchi *et al.*, 2002). Survivin mRNA expression in the DLKP taxotere-resistant variant remained unchanged, as shown by RT-PCR analysis (See Figure 3.3.7.10). Survivin protein, however, was dramatically down regulated and its expression was almost completely inhibited in the DLKP taxotere resistant variant, as shown by western blot analysis. This was consistent in the repeat experiments, where the western blot analysis was carried out on three different protein extractions and α-tubulin was used as an internal control to ensure equal loading of protein, as shown in Figure 3.3.8.4. Change at the protein but not at the mRNA levels, indicates the involvement of post-transcriptional regulation in the expression of survivin protein (see section 4.7).

DNA micorarray analysis was carried out on the DLKP parent and DLKP taxotere-resistant cell lines. Analysis of all the genes mentioned above (i.e. Mdr-1, MRP-1, -2, -3, -4, and 5, BCRP, galectin-3 and survivin) matched RT-PCR results. Mdr-1 expression was up regulated by 300-fold in the DLKP-taxotere variant when compared to the parental cell line. The remaining genes were unchanged (see Table 3.3.9.1). Changes lower than 2-fold were not considered to be 'real' results. The DLKP taxotere-resistant variant (DLKP-

TXT2) established previously by Liang *et al.* (manuscript in prep.) was included in the array analysis for comparison with our current DLKP-taxotere MDR variant (DLKP-TXT1) and the results from the two variants were in agreement. With Mdr-1 showing a much smaller fold increase (44-fold) (Table 3.3.9.1). The DNA microarray needs to be repeated to obtain reliable results.

4.4.2.3 *In vitro* invasiveness and motility in DLKP Taxotere-resistant cell line

Invasion and metastasis of tumour cells, as discussed in section 4.2 is a major cause of death for cancer patients. Statistics show that approximately 50% of all cancer patients may develop metastasis (Fidler *et al.*, 1994). Invasion is one of the most critical steps of metastasis in cancer. Studies of drug resistance and cancer invasion and metastasis have not always been studied in tandem. However, more studies have started to focus on a possible correlation between cancer invasion/metastasis and drug resistance in cancer, as increasing evidence is proving that the drug resistance phenotype may induce the invasive phenotype in cancer cells (Kondo *et al.*, 1961; Kerbel *et al.*, 1982; Takenaga *et al.*, 1986; McMillan *et al.*, 1987).

The aim of this part of the thesis was to determine whether the induction of an invasive phenotype in cancer cells is drug-specific or a random process. Previous findings show that taxol does not induce an invasive phenotype in RPMI-2650 (Liang *et al.*, 2001). In the study by Liang *et al.* (2001), RPMI-2650Ml (melphalan resistant) and RPMI-2650Tx (Taxol resistant) cell lines were established through continuous selection with melphalan and taxol, respectively. Both sublines exhibited a multiple drug resistant phenotype. However, RPMI-2650Ml, but not RPMI-2650Tx, exhibited a highly invasive phenotype, with increased expression of MMP-2 and MMP-9 as well as increased adhesion to collagen type IV, laminin, fibronectin and matrigel (Liang *et al.*, 2001).

Through exposure to increasing concentration of taxotere, the pancreatic adenocarcinoma cell line, SUIT-2/TXT acquired resistance to taxotere and increased Mdr-1 mRNA expression. In addition taxotere exposure had a large inhibitory effect on tumour cell invasion (Liu *et al.*, 2001c). It is postulated that the reason may be due to taxotere

interference with the function of the fundamental part of the cyoskeleton by inducing changes in the microtubules, which are important components of cell motility and intracellular transport (Eckert *et al.*, 1997). In a study by Belotti *et al.* (1996), the effect of taxol on the adhesive and motility properties of human ovarian carcinoma cell lines, OVCAR 5, SK-OV-3, and HOC-1OTC, was investigated. Taxol was found to significantly inhibit the motility of these cell lines, but it did not effect their adhesion to the subendothelial matrix, proving that taxol is a potent inhibitor of ovarian carcinoma cell motility and that this activity is independent of its cytotoxic activity. In a similar, but separate study by the same group, results indicated that taxol has a strong antiangiogenic activity, a property that might contribute to its antineoplastic activity *in vivo* (Belotti *et al.*, 1996).

In breast cancer, taxol and taxotere, in combination with bisphosphonates (which are often used in combination with standard chemotherapy or hormonal therapy for the treatment of cancer-associated osteolytic metastases), may be useful for the treatment of patients with cancer types that are known to metastasize to bone. The bisphosphonate, ibandronate, was found to enhance the anti-tumour activity of taxoids against invasion and cell adhesion to bone (Magnetto *et al.*, 1999), confirming the anti-invasive properties of taxanes. These are all important findings regarding the taxanes' ability to inhibit metastasis and should be taken into account in clinical trials where tumour angiogenesis is being targeted. Grant *et al.* (2003) compared the effect of the two taxanes, taxol and taxotere, on angiogenic processes *in vitro*, in human umbilical vein endothelial cells (HUVEC) and *in vivo* in HT1080 tumour growth. They found both taxanes to block angiogenesis mainly by inhibition of proliferation and also by induction of cell death, with taxotere proving more potent at inhibiting angiogenesis than taxol.

In the DLKP studies previously carried out by Liang *et al.* (manuscript in prep) pulse selection with taxotere had no effect on the invasiveness of DLKP cells. Our current results, however, contradict this finding in DLKP cells and the findings by other groups in other taxol and taxotere-induced resistant cell line, as discussed above. We found selection with taxotere to greatly increase the invasiveness of DLKP cells (see section 3.3.3). Invasion assays were repeated numerous times and results were always conclusive for this DLKP variant, showing increased invasiveness in every experiment. The invasion assays

were carried out in two types of invasion chambers. This included inserts with no matrigel coating and this was carried out before the assay was set up and inserts that were purchased with a matrigel already coating the inserts. The results from both of these types of invasion chambers were compared and proved to very similar as shown in Figure 3.3.3.1 and 3.3.3.3.

In addition taxotere-resistance caused a huge increase in the motility of the cells as noted over three time points, 12, 24 and 48 hours after motility assays were initially set up. The difference in motility between the parental cell line and the taxotere-resistant variant is most dramatic at 12 and 24 hours where the fold increase is 3- and 4-fold, respectively (section 3.3.5). Invasion assays were carried out in triplicate and were reproducible.

There are a small number of published reports supporting our current findings, indicating that taxol increases motility and invasion. Silbergeld *et al* (1995) found taxol to increase *in vitro* motility in glioblastoma cells. This motility was increased with increasing concentration of taxol. Welch *et al.* (1989) found that some cytoskeleton disrupting agents, such as vincristine, colcemid and colchicines, inhibited invasion, whereas taxol did not (Welch *et al.*, 1989).

Furthermore, this invasiveness of DLKP-taxotere resistant cells was inhibited using MMP inhibitors. The MMP inhibitors used were: MMP inhibitor I, which inhibits MMP-1, -3, -8 and MMP-9; MMP inhibitor III, which inhibits MMP-1, -2, -3, -7 and MMP-13; and MMP-2 inhibitor I, which inhibits MMP-2. The most effective agent at inhibiting invasion in DLKP-taxotere was MMP inhibitor III (see Figures 3.3.4.1 and 3.3.4.2). These experiments were carried out once and need to be repeated for confirmation of results.

MMPs are thought to play a very important role in cancer invasion and metastasis as they are used by many types of cancer to break down matrix barriers, and thereby allowing tumour cells to penetrate tissues, gain access to blood vessels, exit blood vessels and metastasis to a distant sites (Denis and Verweij, 1997). The development of matrix metalloproteinases inhibitors (MMPIs) as an anti-cancer agent is therefore very important. The challenge however, in the development of MMPIs is to identify which types of cancer involve MMPs and therefore might respond to these inhibitors (Denis and Verweij, 1997).

In the early 1980s, pharmaceutical companies became involved in the search for synthetic MMPIs, and a series of highly effective MMPIs have been successful in restricting tumour growth and inhibiting tumour metastasis. Many of these are being studied in clinical trials (Yu *et al.*, 1997).

The invasion and motility assays for the DLKP selected variants presented in this thesis include DLKP-taxotere resistant cells previously established by Liang *et al.* (2003 in prep) for comparison purposes. This is denoted DLKP-Taxotere-2. The DLKP-taxotere resistant cell line established for the purpose of these studies is denoted DLKP-Taxotere-1 (sections 3.3.3 and 3.3.5). In addition, the invasion and motility assays (and the second invasion inhibition assay (Figure 3.3.4.2)) presented in the results section include two DLKP parent cell lines (sections 3.3.3, 3.3.4 and 3.3.5). The reason for this is that some batches of this cell line were found to be more invasive (DLKP-2) than other batches (DLKP-1). As previously mentioned, DLKP is a non-invasive cell line and the DLKP variants were thus established from a non-invasive DLKP cell line (section 4.3). The finding that different batches of the same cell line may exhibit two different invasive phenotypes is surprising and the reason for this is unknown. However, it was brought to our attention after the experiments were carried out that DLKP-2 may have been *Mycoplasma* contaminated and this may have caused the increased invasive phenotype. This is not certain, however, as there were other batches of DLKP parent cell line tested that were found to be more invasive than DLKP-1. In all other analysis of the DLKP MDR variants, including toxicity assays (section 3.3.1), RT-PCR (section 3.3.7), western blot analysis (section 3.3.8) and zymography analysis (section 3.3.6), DLKP-1 and DLKP-taxotere-1 were analysed.

Colormetric analysis was also carried out on the invasion and motility assays to obtain a quantitative method of analysis. Initially this was carried out by using acetic acid to elute the crystal violet dye from the bottom of the invasion chambers, following staining of the cells, which have invaded and attached to the other side of the invasion chamber. The absorbance of the eluted dye was read at 570nm. This method was found to be unsatisfactory due to high background staining from the invasion chambers and this resulted in inaccurate readings. This high background staining was, however, not found with previous studies using such inserts (Liang *et al.*, 2001; O'Driscoll *et al.*, 2002) and may be due to batch-to-batch variation of inserts. A second method of quantitation was

used, whereby cell that are attached to the underside of the invasion chambers were not stained with crystal violet as per usual, but instead were incubated in acid phosphatase substrate-containing buffer at 37°C for 2 hours, after which the reaction was stopped and samples were read on a plate reader at 405nm. This method resembles the *in vitro* toxicity assays discussed in section 2.3. For this method, invasion, inhibition or motility assays were carried out in duplicate to allow for the staining and the photography of the cells that were attached to the underside of the invasion chamber. RPMI-2650 parent cell line was used as a negative control for invasion and motility assays.

To further assess the role of MMPs in the invasiveness of the DLKP taxotere-resistant cell line, we examined the secretion of MMPs in the resistant cells compared to the parental, sensitive cells using gelatin zymography. Results demonstrate that the DLKP taxotere-resistant variant had slightly weaker of MMP-13 activity than the parental cells. MMP-9 and MMP-2 secretion was unchanged (See section 3.3.6). This doesn't fully agree with previous finding in the DLKP selections by Liang *et al.* (in prep.), where DLKP taxotere-resistant cells had the weakest activity of MMP-9 and MMP-13 compared to the other, highly invasive variants, such as DLKP-mitoxantrone. However, whereas this result was not surprising for DLKP-taxotere-2, due to its lack of invasive properties, it is surprising in our current DLKP-taxotere variant (DLKP-taxotere-1), given its very high invasive and metastatic properties. Further analysis of MMP secretion, including western blot analysis, would be beneficial to help explain these results.

E-cadherin is involved in cancer invasion and metastasis (see section 1.3.1). It is a member of a family of transmembrane glycoproteins that appear to function by connecting cells to each other by homophilic interactions (Liang *et al.*, 2002). The expression of the cell adhesion molecule, E-cadherin, was analysed by RT-PCR and western blot analysis in the DLKP taxotere-resistant variant. E-cadherin mRNA expression seemed to be slightly over-expressed in the DLKP taxotere-resistant variant (see Figure 3.3.7.8), which would correlate with the invasive phenotype of this cell line. However, in repeat experiments this was not observed. E-cadherin mRNA expression was un-changed in the DLKP taxotere-resistant variant that was developed by Liang *et al.* (2003, in prep).

E-cadherin protein was over-expressed in the DLKP-taxotere variant, as shown by western blot analysis in Figure 3.3.8.3. This experiment was only carried out once and it needs to be repeated for confirmation of this result. It is surprising that E-cadherin protein was detected by western blot in the DLKP cell line and its drug resistant variants, as Liang *et al.* found no E-cadherin protein expression in any of the DLKP cell lines (Liang *et al.*, 2003, in prep.). The reasons for this discrepancy is not known and RT-PCR and western blot analyses need to be repeated for confirmation of these findings

DNA microarray results were not, however, in agreement with the RT-PCR results. E-cadherin was down regulated in our current DLKP-taxotere MDR variant (DLKP-TXT1) by 2-fold (see Table 3.3.9.1). The DLKP-taxotere MDR variant established by Liang *et al.* (manuscript in prep.) (DLKP-TXT2), showed a 5-fold down-regulation of E-cadherin expression. A repeat of the DNA microarray analysis is needed for accurate results.

4.4.3.1 Cross resistance pattern of Vincristine-selected DLKP cells

The DLKP-vincristine resistant variant was approximately 5330-fold resistant to vincristine, 233-fold resistant to taxotere, 124-fold resistant to taxol and 26-fold resistant to adriamycin - all MDR associated drugs. Similar to DLKP-taxotere, the DLKP-vincristine MDR variant was not resistant to 5-FU, CCNU or cisplatin, therefore exhibiting a typical MDR profile (see section 3.6.1.2). Toxicity assays were carried out in triplicate and as mentioned in section 4.4.2.1, while the majority of results were reproducible, the standard deviation for a minority of the drugs, namely taxotere and vincristine were quite high and cells may need to be exposed to their selecting agent again to avoid changes in resistance levels. In general these findings show similar trends, to some extent, with the findings from the previous study on DLKP selection, where the DLKP-vincristine resistant variant was only 1.9-fold resistant to vincristine, and 1.7-fold resistant to adriamycin, with no resistance to cisplatin. Similar patterns of resistance were observed by many other research groups (Gupta *et al.*, 1985; Haber *et al.*, 1989; Toffoli *et al.*, 1992; Sekiya *et al.*, 1992; Zhang *et al.*, 1994).

This type of MDR-related cross-resistance has been shown by other groups to be typical of the vinca alkaloids. Gupta *et al.* (1985) established resistant variants of Chinese hamster ovary (CHO) cells, after selection using vinblastine. The cross-resistance pattern of the resistant variant to a wide variety of anti-cancer drugs was determined. The vinblastine-resistant cells were found to exhibit patterns of cross-resistance to a number of anti-mitotic inhibitors, namely, vinblastine, vincristine, vindesine, taxol, podophyllotoxin and colchicines, as well as a large number of other compounds (Gupta *et al.*, 1985). Zhang *et al.* (1994) established a vincristine-resistant clone of human epidermal carcinoma, KB cells, by a stepwise selection of cells through exposure to increasing doses of vincristine. The resistant cells were found to be 175-fold resistant to vincristine and cross-resistant to taxol, colchicine and adriamycin. Verapmil, which is a calcium channel blocker that reverses drug resistance, caused an increase in vincristine accumulation in the cells and reversed vincristine resistance (Zhang *et al.*, 1994). Similar studies were carried out on a hepatocellular carcinoma (HCC) cell line. Hepatocellular carcinoma is one of the malignant tumours with poor chemosensitivity to anticancer drugs (Huang *et al.*, 1999). The human HCC cell line, Bel $_{7402}$, was found, by Huang *et al.* to have innate resistance to adriamycin,

the most frequently used drug for HCC therapy. This led to further investigation of this cells line with respect to its cross-resistance properties. As previously discussed, adriamycin, vincristine and taxol have been proven to be transported by Pgp. Cross-resistance of the adriamycin-resistant cell line, Bel $_{7402}$, to these (vincristine and taxol) and other drugs (5-FU), was examined. Huang *et al.* (1999), found Bel $_{7402}$ to be resistant to taxol and highly resistant to vincristine, when compared to the drug sensitive human epidermal carcinoma cell line, KB. The adriamycin-resistant cell line was only slightly resistant to 5-FU. This innate resistance to the three Pgp transporting drugs was dramatically reversed by verapamil, while verapamil had no effect on 5-FU resistance, indicating that innate resistance in this HCC cell line may be associated with an MDR phenotype.

Studies in murine leukemic T cell line showed that vincristine resistant variants exhibit cross-resistance to adriamycin, VP-16, dexamethasone and methotrexate (Lopes *et al.*, 2001). Kotechetov *et al.* (2003) established two variants of the neuroblastoma cell line, UKF-NB, through the treatment with vincristine or adriamycin. Both variants exhibited high resistance to vincristine and adriamycin, but did not show any cross-resistance to cisplatin.

Taken together, all these results, support our findings and show that vincristine resistance in this variant, similar to taxotere resistance, follows a typical MDR pattern of resistance, showing cross resistance to several structurally and functionally unrelated agents, including the anthracyclines, the vinca alkoids, actinomycin D, the epipodophyllotoxins and the taxanes, which are all substrates for P-gp.

4.4.3.2 Changes in expression of apoptosis and MDR-related genes associated with vincristine resistance

The P-glycoprotein is a receptor for vinca alkaloids and tumour cell drug resistance is proportional to the amount of Pgp (Zhou *et al.*, 1992). To investigate whether mdr1 gene products are involved in conferring the chemoresistant phenotype in the DLKP vincristine-resistant variants, we examined the expression profile of a number of MDR-related genes, including Pgp and the MRPs.

We found Pgp mRNA and protein expression to be dramatically over-expressed in DLKP-vincristine compared to DLKP parent, by RT-PCR and western blot analysis (see Figures 3.3.7.1 and 3.3.8.1). Results were carried out in triplicate and were reproducible. As discussed in section 4.3.2.2, Pgp over-expression is an indicator of an MDR phenotype and since vincristine resistance is typical of an MDR phenotype, it is not surprising to find over-expression of Pgp in the DLKP vincristine-resistant variant. Pgp was found to be over-expressed by western blot analysis in the previous DLKP vincristine-resistant variant established by Liang *et al.* (in prep.). Liang *et al.*, however, found no change in Pgp mRNA, suggesting the involvement of post-transcriptional regulation in the expression of this protein. MRP-1 mRNA and protein expression were unchanged in the DLKP vincristine-resistant variant in the present study (see Figure 3.3.7.2 and 3.3.8.2) in agreement with the previous study by Liang *et al* (2003; in prep.), suggesting that MRP-1 does not play a role in vincristine resistance.

These results correlated with findings by other research groups. The innate resistance to adriamycin in the HCC cell line, Bel $_{7402}$, discussed in the previous section, caused an MDR phenotype in the cells, as seen by the pattern of cross resistance (see section 4.4.3.1). To further illustrate that resistance seen in this cell line was associated with classical MDR mechanisms, the expression of Pgp protein was investigated in the cells by immunocytochemistry. Pgp staining was present in a greater proportion of Bel $_{7402}$ cells and staining was more intense than that in the sensitive KB cells, indicating that Pgp plays an important role in the intrinsic resistance of Bel $_{7402}$ cells to adriamycin, vincristine and taxol (Huang *et al.*, 1999). In 1997, this group had also demonstrated that vincristine or taxol resistant HL60 cells express high levels of Pgp, while adriamycin-resistant HL-60 cells

possess high levels of MRP-1. Pgp, but not MRP-1, over-expression inhibited taxol accumulation and taxol-induced apoptosis, indicating that MRP-1 over-expression is not responsible for taxol resistance in HL-60 cells (Huang *et al.*, 1997).

Real-time PCR analysis of a rhabdomyosarcoma cell line and its vincristine-resistant variant revealed an increase in Pgp mRNA expression, which led to resistance to vincristine, etoposide, and doxorubicin but not cisplatin, which is not a substrate for P-gp. This resistance was inhibited in the presence of MDR modulators, whereas MRP-1 modulators were unable to prevent the development of resistance to vincristine. This indicates that the development of vincristine resistance in this cell line is caused by Pgp and not MRP-1. Furthermore, MRP-1 mRNA expression was unaltered in the vincristine-resistant cell lines, as demonstrated by real-time PCR (Cocker *et al.*, 2001).

In our DLKP vincristine resistant variant, the expression of MRP2 (cMOAT), MRP3 and MRP5 were undetectable by RT-PCR in the DLKP-vincristine MDR variant (see Figures 3.3.7.3, 3.3.7.4 and 3.3.7.6), while traces of MRP4 and BCRP mRNA were present (see Figures 3.3.7.5 and 3.3.7.7).

Galectin-3 mRNA expression was analysed in the DLKP vincristine-resistant variant and there was no change in its expression compared to the DLKP parent cells (see Figure 3.3.7.9). The studies were carried out in triplicate and were found to be reproducible. This result is not surprising since DLKP-vincristine is an MDR variant, which did not develop an invasive phenotype. This may suggest that galectin-3 does not play a role in drug resistance to vincristine in the DLKP cells. Survivin mRNA and protein expression were also analysed by RT-PCR and western blot analysis, to determine the role of survivin in vincristine drug resistance.

Survivin mRNA expression was not greatly altered in the DLKP vincristine-resistant variant as shown in Figure 3.3.7.10. Survivin protein, however, was dramatically down regulated in this variant (see Figure 3.3.8.4). As in section 4.4.2.2, this survivin down-regulation at the protein level, but not at the mRNA level, suggests the involvement of post-transcriptional regulation in expression of survivin protein in this MDR variant (see

section 4.7). These results were reproducible as seen in the three experiments as shown in Figure 3.3.8.5.

As mentioned in section 4.4.2.2 DNA micorarray analysis was carried out on the DLKP parent and DLKP taxotere-resistant cell lines, as well as the DLKP vincristine-resistant cell line. Analysis of all the genes mentioned above (i.e. Mdr-1, MRP-1, -2, -3, -4, and 5, BCRP, galectin-3 and survivin) matched RT-PCR results, with the exception of MRP2; which was up-regulated by 4.2-fold in the DLKP-vincristine variant. Mdr-1 expression was up regulated by 92.5-fold in the DLKP-vincrisitne variant when compared to the parental cell line. The remaining genes were unchanged (see Table 3.3.9.1). As mentioned in section 4.4.2.2 changes lower than 2-fold were not considered to be 'real' results. The DNA microarray needs to be repeated to obtain reliable results.

4.4.3.3 *In vitro* invasiveness and motility of the Vincristine-resistant variant

As discussed in section 4.4.2.3, the aim of this part of the thesis was to determine whether the effect of drug resistance on the induction of an invasive phenotype in the cells was a drug-specific feature or a random event. Previous findings in the DLKP selections carried out by Liang *et al.*, showed that the DLKP vincristine-resistant variant did not show an increase in invasiveness. This is in agreement with out current findings, where, compared to the parental DLKP cells, the DLKP-vincristine MDR variant was non-invasive (see figure 3.3.3.1 and 3.3.3.2) and showed no increase in motility at any of the three time points analysed, including 12, 24 and 48 hours (see Figures 3.3.5.1 and 3.3.5.2), suggesting that vincristine-resistance does not induce an invasive or motile phenotype in DLKP cells. As with the invasion and motility assays discussed in section 4.4.2.3, the assays were carried out in triplicate and were reproducible. In addition, the two types of invasion chambers were used and the same colormetric analysis was carried out to establish a quantitative method of invasion and motility analysis.

This trend was also found to be the case in a murine T-cell lymphoid leukemia cell line, selected in increasing concentrations of vincristine. In fact this vincristine-resistant cell line was less invasive than the parental cell line (Lopes *et al.*, 2002). Similarly, a study carried

out by Hikawa *et al.* (2000) on a human glioma cell line, illustrated that when this cell line is selected with certain drugs (etoposide, vincristine and adriamycin), and becomes drug-resistant, it expresses higher levels of integrins which, in turn, leads to an increase in the adhesive ability of the cells in a drug concentration-dependent manner. This increase in adhesion was not, however, linked to an increase in cell invasion of the cells. In fact, the cells invasion of the drug-resistant cell line was dramatically lower than the cell invasion of the parental cells line (Hikawa *et al.*, 2000).

On the contrary, a study by Kotchetkov *et al.* (2003) on a vincristine-resistant neuroblastoma cell line, revealed that vincristine resistance resulted in an approximately 2-fold increase in cell growth *in vitro*, as well as enhanced adhesion, trans-endothelial penetration and higher tumorigenicity *in vivo*, indicating that drug resistance in this cell line may contribute to the poor prognosis in advanced forms of neuroblastoma, due to increased malignancy (Kotchetkov *et al.*, 2003).

Furthermore, to assess the role of MMPs in the invasiveness of the DLKP vincristine-resistant cell line, we examined the secretion of MMPs in the resistant cells compared to the parental, sensitive, cells using gelatin zymography. These studies indicate that the DLKP-vincristine variant showed a similar level of MMP-9 and MMP-2 secretion to the parental cells. However, the secretion of MMP-13 seems to have been almost completely inhibited in the DLKP-vincristine MDR variant. Finding by Liang *et al.* on MMP secretion, by gelatin zymography in the DLKP-vincristine variant, show that MMP secretion of MMP-2, -9 and –13 were unchanged compared to the parental cells.

Finally, our findings, as with the finding of Liang *et al.*, show an unchanged level of expression of E-cadherin in the DLKP vincristine-resistant cell line, by both RT-PCR (see Figure 3.3.7.8) and western blot analysis (see Figure 3.3.8.3). This further supports the finding that vincristine resistance has no effect on the invasive ability of DLKP cells. DNA Microarray results, however, contradict this result. E-cadherin expression is down regulated by 2-fold in the DLKP-vincristine-resistant variant (Table 3.3.9.1). The reason for this discrepancy is not known and further analysis of E-cadherin expression in this variant is needed.

4.4.4.1 5-Fluorouracil Selection

5-fluorouracil (5-FU) is a member of a class of antimetabolite drugs called the antipyrimidines. The mechanism of action of 5-FU is complicated and may consist of several steps. These can be summerised as follows: incorporation into DNA, followed by incorporation into RNA, leading to thymidylate synthase inhibition and thereby DNA synthesis inhibition due to lack of thymidylate formation (Van Triest *et al.*, 1997). 5-FU has been found to act through two different pathways, depending on the does of the drug. In human colorectal cell lines, a low dose exposure to 5-FU induced G_2-M phase arrest, while at higher doses, 5-FU induced G_1-S phase arrest of the cell cycle (Yoshikawa *et al.*, 2001). As with the two drugs already discussed in this section of the thesis, i.e. vincristine and taxotere, the problem of inherent or acquired resistance of certain tumours to cytotoxic drug therapy remains a problem even with non Pgp-transported drugs, like the antimetabolites.

In our current studies (section 3.3) on the DLKP 5-FU-selected variant, we found no increase in drug resistance to 5-FU, or any of the other drugs tested (taxotere, taxol, vincristine, adriamycin, cisplatin or CCNU). On the contrary, we found slight sensitivity of DLKP 5-FU-selected variant to taxol (0.6-fold), adriamycin (0.5-fold) and cisplatin (0.6-fold). In the previous DLKP selections, this was not the case, however. Liang *et al.* (2003; in prep) reported the DLKP-5FU variant to be 2-fold resistance to 5-FU, and cross-resistance to vincristine. There was no resistance to adriamycin (1.2-fold), taxotere (1.3-fold) or methotrexate (1.39-fold) and there was slight sensitivity to cisplatin (0.56-fold) (see section 3.3.1.1). This shows that the MDR phenotype is lacking in this selected variant.

On the contrary, Chung *et al.* (2000) have found 5-FU-resistant, gastric cancer cell lines to be highly resistant to 5-FU (800-fold), with cross-resistance to taxol, cisplatin and adriamycin. Furthermore, an increased expression of thymidine kinase (TK) was observed in the 5-FU-resistant cells (Chung *et al.*, 2000). Another 5-FU-resistant gastric carcinoma cell line was approx. 140-fold more resistant to 5-FU than parental cells, as well as exhibiting cross-resistance to mitomycin C. The cells were morphologically different to the parent cell and had developed a more malignant phenotype than that of the parental cell line (Yue *et al.*, 1993).

In addition, we analysed the expression of MDR-related gene, mdr-1, and MRPs in the 5-FU-selected variant and found no over-expression of Pgp by RT-PCR (see section 3.3.7.1) and western blot analysis (see section 3.3.8.1). Similarly, we found no over-expression of MRP-1 by RT-PCR and western blot analysis. No MRP-2 (cMOAT), MRP-4 or MRP-5 expression was detected in this variant. MRP-3 mRNA expression was up regulated, however in this variant as shown in Figure 3.3.7.4. In contras to results from this thesis, Liang *et al.* (2003 in prep) found Pgp to be over-expressed the DLKP-5FU variant by western blot analysis, but not by RT-PCR. However, none of the other genes mentioned above were found to be up regulated in this variant.

To assess whether selection with 5-FU had an effect on the invasive phenotype of the cells, invasion assays and motility assays were carried out on the DLKP-5-FU selected variant. Compared to the parental cell line, DLKP, DLKP-5-FU had no increased invasive phenotype (see section 3.3.3.1 and 3.3.3.2). This is in contrast with DLKP-5-FU cells selected previously by Liang *et al.*, where the DLKP-5-FU variant was approximately 3-fold more invasive than the parent cells DLKP-5FU selected variant showed no increased motility at any of the three time points studied, including 12, 24, and 48 hours (see Figure 3.3.5.1 and 3.3.5.2). The experiments were carried out in triplicate and as with the taxotere-resistant and vincristine-resistant variants, two types of invasion chambers were used and the assays were analysed quantitatively. We noted, however, a dramatic change in the physical morphology of the 5-FU selected cells. DLKP cells, which normally grow in the shape of 'cobble-stone', became a lot more elongated when exposed to 5-FU (Figure 3.3.2.1). This change in morphology was not noted in the more invasive DLKP-5-FU variants previously established. The reason for the discrepancy between the two variants is unclear.

The analysis of MMP secretion by gelatin zymography, showed a large increase in MMP-2 secretion in the 5-FU selected variant when compared to DLKP parent (see section 3.3.6.1). This was not observed by Liang *et al.*, but an increase in MMP-9 secretion was observed in the 5-FU variant. Finally, the expression of E-cadherin remained unchanged in this variants as seen by RT-PCR (see Figure 3.3.7.8) and western blot analysis (see Figure 3.3.8.3), supporting the finding that 5-FU does not induce an invasive phenotype in the DLKP cells in this study.

4.5 Establishment of MDR variants of RPMI-2650 cell line

RPMI-2650 is a nasal carcinoma cell line established by Moore and Sandberg (1963). This cell line was derived from a 52-year-old male who had an extensive malignant tumour of the nasal septum removed. The pathological diagnosis was anaplastic squamous cell carcinoma. The patient subsequently developed bilateral cervical lymph node metastases. RPMI-2650 cell line is a heterogenous population with many sub-clones exhibiting different characteristics (Moore and Sandberg, 1963).

This cell line has been previously characterised in this laboratory by Dr. Yizheng Liang (Y. Liang, PhD, 1999). Characterisation included analysis of gene expression (mainly MDR-related genes) and cytotoxicity analysis, including a number of chemotherapy drugs. Results revealed Pgp and MRP1 expression, demonstrated by RT-PCR, western blot and immunocytochemistry analysis. In addition, the cells were found to be very sensitive to chemotherapeutic drugs. Chemotherapy drugs tested included adriamycin, vincristine, vinblastine, etoposide, taxol, melphalan, 5-FU and cisplatin, suggesting that Pgp and/or MRP1 may be non-functional in this cell line. This was further confirmed by adriamycin distribution studies, where strong adriamycin accumulation was noted in the nuclei of RPMI-2650, indicating that very little drug efflux was occurring, and hence that Pgp and/or MRP1 may not be functioning as drug efflux pumps. Further characterisation, using invasion assays, was carried out to determine whether RPMI-2650 expressed an invasive phenotype. Although the patient from whom this cell line was derived developed cervical lymph node metastases, invasion assays revealed that RPMI-2650 is non-invasive *in vitro*, suggesting that the final outcome of invasion/metastasis depends on the interaction of the host and the tumour (Y. Liang, PhD, 1999).

In an attempt to establish if MDR could be induced in the RPMI-2650 cell line and to subsequently identify the mechanisms of drug resistance in established variants, as well as to investigate the effect of chemotherapy drugs on cell invasion, drug selection studies were carried out by Liang *et al.* (2001) on the RPMI-2650 cell line. Two commonly used chemotherapeutic drugs were selected for this study, namely, melphalan (an alkylating agent) and taxol (see section 1.1.1.2) (an anti-mitotic anticancer drug).

Through continuous exposure of the parental cells to increasing concentrations of melphalan over a period of nine months or to taxol over a period of six months, two RPMI-2650 drug resistant variants were established. These were RPMI-2650 melphalan resistant (RPMI-2650Ml) and RPMI-taxol resistant (RPMI-2650-Tx) cell lines (Liang *et al.*, 2001). Alterations in a number of MDR-related genes were investigated, as well as the toxicity profile of the selected variants.

Results of RT-PCR and western blot analysis revealed over-expression of MRP1, MRP2 and Pgp, leading to decreased accumulation of drug in the RPMI-2650Ml variant, as demonstrated by circumvention studies. Furthermore, RPMI-2650-Ml variant was found to be 11-fold more resistant to melphalan compared to the parental cells, in addition to exhibiting cross-resistance to a number of other drugs, showing a cross-resistance profile that is consistent with the involvement of MRP family members and Pgp. RPMI-2650-Tx variant was found to be 226-fold more resistant to taxol compared to the parental cells, and also exhibited cross-resistance to a number of other chemotherapeutic drugs, showing a cross-resistance profile that is consistent with the involvement of Pgp. In addition to a multiple drug resistant phenotype, melphalan selection caused a dramatic induction of an invasive phenotype in the RPMI-2650Ml resistant MDR variant. Taxol selection, however, had no effect on invasiveness. The induction of a MDR phenotype in the RPMI-2650 cells (and in the case of RPMI-2650-Ml, an invasive phenotype) was accompanied by a dramatic change in the morphology compared to the parent cell line (Liang *et al.*, 2001).

These results, along with our findings in the DLKP-taxotere-resistant cell line, as well as many other reports (Giavazzi *et al.*, 1983; Scaddan *et al.*, 1993; Lucke *et al.*, 1994; Haga *et al.*, 1997; Staroselsky *et al.*, 1996, Choi *et al.*, 1999 and Liu *et al.*, 2001c), suggest that invasion and metastasis may sometimes be associated with an MDR phenotype.

In this part of the thesis, we aimed to investigate this phenomenon further and try to establish which other drugs/family of drugs may have a similar effect on *in vitro* invasiveness of RPMI-2650 cells, by carrying out further drug selections on this cell line. This would also provide us with drug-resistant RPMI-2650 cell lines for analysing the expression of galectin-3 and survivin.

Pulse selections, using seven drugs, were carried out on RPMI-2650 parent cell line, by exposing the cells to an increasing concentration of drug for 4 hours, once a week, for a period of approx. 3 months. The chemotherapy drugs selected for this study were: taxotere, vincristine, 5-FU, epirubicin, CCNU, carboplatin and melphalan. Melphalan and taxotere were selected to act as 'control drugs' to reproduce the findings by Liang *et al.* through continuous exposure to melphalan and taxol (Y. Liang, PhD, 1999; Liang *et al.*, 2001). However, for reasons that are not clear, the selection procedure was not successful for these two drugs. The remaining five drugs were used for the pulse selection procedure. This work was carried out in duplicate to assess reproducibility. All selected variants were given a number (1 or 2) for each replicated drug.

RPMI-2650 nasal carcinoma, cells are very small, with a morphology similar to that of normal nasal epithelial cells (Moore and Sandberg, 1963). They grow as tightly packed cell clumps, forming a dense sheet on the base of a culture flask or plate. Throughout the selection procedure with all drugs, the cells underwent a dramatic change in morphology. They became elongated and spindle-shaped. However, a number of weeks after the cells were removed from selection pressure, the majority of cells reverted back to their original morphology (Figure 3.4.1.1 a, b and c).

Preliminary analysis was carried out on the 10 selected variants to investigate their toxicity profile (see section 3.4.2), their *in vitro* invasiveness (see section 3.4.3.) and motility (see section 3.4.4), as well as alterations in a number of MDR and apoptosis-related genes (see section 3.4.5), which may have occurred.

4.5.1 The effect of Pulse selection on the toxicity Profile of RPMI-2650

Initial investigation of the toxicity profile of the RPMI-2650 pulse-selected variants after ten pulses revealed an increase in drug resistance of some, but not all the variants to their selecting agent and to other unrelated drugs. For example, RPMI-2650 5-FU_1-selected variant, showed a 3-fold increase in resistance to 5-FU, 1.6-fold increase in resistance to CCNU and a 3.6-fold increase in resistance to carboplatin. RPMI-2650 VCR_1-selected variants, showed no increase in resistance to vincristine, but did show cross-resistance to 5-FU (3.7-fold), CCNU (2.4-fold) and carboplatin (8.7-fold). RPMI-2650 $CCNU_2$-selected variant showed the highest level of cross-resistance, with a 3.5-fold increase in resistance to CCNU, a 14.4-fold increase in resistance to 5-FU, a 15.7-fold increase in resistance to vincristine and a 9.1-fold increase in resistance to carboplatin. Furthermore, cells that were given extra pulses had further increased in resistance (section 3.4.2).

When toxicity assays were repeated a number of weeks later, however, this resistance was lost, suggesting that the resistance initially seen was a transient effect. To establish why the resistance initially observed was gone after a period of time, two of most the variants which were initially drug resistant, namely, RPMI 2650-$5FU_1$ and RPMI-2650 VCR_2 were pulsed further, with 5-FU and vincristine, respectively, bringing the total number of pulses to 14. This caused a dramatic increase in resistance, especially in RPMI-VCR_2, which became over 300-fold more resistant to vincristine than the parental cells (see Table 3.4.6a). When toxicity assays were repeated on these two variants a second time, drug resistance was maintained, although the levels differed greatly between the experiments (see Table 3.4.6b). Both variants showed cross-resistance to 5-FU, vincristine, adriamycin and to a much lesser extent CCNU and carboplatin. Adriamycin was included in the drugs tested to try to establish the mechanism of drug resistance that may be taking place. RPMI-2650 $5FU_1$ was 23-fold resistant to adriamycin, while RPMI-2650 VCR_2 showed a 77-fold increase in resistance to adriamycin, suggesting a typical MDR phenotype in the two variants. Further toxicity testing needs to be carried out on these variants, to determine whether the extra drug pulsing is causing transient or permanent resistance.

Taken together, however, the result from the RPMI-2650 drug-selected variants suggest that unstable multiple drug resistance is taking place. This resistance is lost during

subculturing and this may be due to the decline or the loss of expression of some MDR-related genes. Similar findings have been reported in the adriamycin-resistant Chinese hamster V79 cell line (Howell *et al.*, 1984), where is was found that an adriamycin-resistance in this cell line can be increased after a multi-step selection process involving continuous growth of the cells to adriamycin, making the cells 3000 times more resistant to adriamycin than is the parental V79 line. This high-level resistance phenotype, however, was unstable and was lost upon culture in the absence of drug. Similar findings were observed by Tohda *et al.* (1997), where a Chinese hamster ovary cell line resistant to okadaic acid expressed an MDR phenotype and increased expression of Pgp and topoisomerase II (topoII). In the absence of okadaic acid, however, the MDR was decreased and this was accompanied by a decrease in Pgp and topoII expression.

In our studies, as in the studies by Tohda et al., the MDR phenotype seems to be reversible. This may help identify novel MDR-related genes that may be useful as therapeutic drug targets.

4.5.2 The effect of pulse selection on the Invasive/Motility Phenotype of RPMI-2560

To investigate the effect of chemotherapy drugs on cell invasion and motility, invasion assays were carried out on all ten variants, including three of the variants at 12 pulses. However, unlike the finding by Liang *et al.* (2001) in the RPMI-melphalan resistant variant, no increase in invasiveness was seen in any of the variants, compared to the parental cells (see section 3.4.3). This may be due to the difference in selection procedure used. Whereas Liang *et al.* (2001) used continuous selection to induce an invasive phenotype, we used pulse selection method and this may have inhibited invasiveness developing in this cell line. Motility assays were carried out once on some, but not all, the RPMI-2650 variants. None of the variants showed an increase in motility compared to the parent cells. Furthermore, motility assays were carried out on the two variants at 14 pulses, RPMI-2650 $5FU_1$ and RPMI-2650 VCR_2, however, there was no increase in motility, as shown in Figure 3.4.4.1. This was only carried out once. Invasion assays have not been carried out on these two variants and will need to be performed in future to determine if the extra drug pulses induce an invasive phenotype in these variants.

4.5.3 The effect of Pulse selection on gene Expression of RPMI-2650.

MDR is usually accompanied by an up-regulation of Pgp expression and/or expression of MRP genes. In our current study, preliminary RT-PCR analysis revealed no up-regulation in Mdr-1 mRNA expression compared to the parent cells (see section 3.4.5.1). There was a small increase in MRP-1 expression in the RPMI variants (see section 3.4.5.2). MRP2 expression was lacking (Figure 3.4.5.3), while MRP3 expression was up regulated in RPMI-2650 VCR2-12p (see Figure 3.4.5.4). RPMI-2650 VCR2-10p and VCR2-12p over-express MRP4 (see Figure 3.4.5.5), while MRP5 and galectin-3 mRNA expression was unchanged (see Figures 3.4.5.6 and 3.4.5.7). Survivin mRNA expression seems to be up-regulated in a number of variants, however the bands on the gels seem to over-saturated, making it difficult to determine whether the over-expression is a real result (section 3.4.5.8).

All RT-PCR analysis was carried out twice and need to be repeated for confirmation of these findings. The RT-PCR results are difficult to explain at present since drug-resistance in the RPMI variants is unstable. Cell may need to be further pulsed to obtain stable resistance and RT-PCR analysis should be carried out again to be sure of that any changes in gene expression are stable and due to drug resistance. It is also important to determine whether the sudden increase in resistance in RPMI-2650-$5FU_1$-14p and RPMI-2650-VCR_2-14p is a stable resistance and if it is, further analysis such as RT-PCR, western blot analysis and invasion assay needs to be carried out on these variants.

4.6 Analysis of gene expression in a panel of invasive breast tumour biopsies

4.6.1 An Overview of Breast Cancer

Breast cancer is the most common malignancy affecting women worldwide. One of the strongest risk factors for breast cancer is family history (Madigan *et al.*, 1995; Dupont *et al.*, 1987), with studies showing increased breast cancer incidences in relatives of breast cancer patients compared with the general population, especially if diagnosis is at a young age (Claus *et al.*, 1990; Tulinius *et al.*, 1992). Elevated risk of ovarian cancer has also been found among relatives of breast cancer patients (Narod *et al.*, 1994). Breast and ovarian cancer susceptibility genes are estimated to account for 7% of breast cancer cases and 10% of ovarian cancer cases in the general population (Claus *et al.*, 1996). BRCA1 and BRCA2 have been identified as two important breast cancer susceptibility genes (Miki *et al.*, 1994; Wooster *et al.*, 1995) and have led to much medical interest. However, BRCA1 and BRCA2 mutations have also been identified in patients with no family history of this disease. Mammography screening for breast cancer is a very important process, especially for patients at higher risk of developing breast cancer due to family history. Randomised trials of breast screening using this method have shown a reduction, by over 30%, in mortality from breast cancer in women aged over 50 years (Nystrom *et al.*, 1993).

Chemotherapy plays a major role in the treatment of breast cancer. Adjuvant chemotherapy is therapy administered after surgery on breast cancer and it decreases the chances of recurrence by 24% (Leonessa *et al.*, 2003). Neoadjuvant chemotherapy is therapy administered before surgery to patients with advanced, but un-metastasised (local) breast cancer. This therapy can reduce the size of a breast tumour in 80-90% of patients (Kling *et al.*, 1997), allowing for a more constricted surgical approach. This also results in complete response or complete remission (CR) in approx. 20% of patients (Moll and Chumas, 1997; Sapunar and Smith, 2000). Complete remission is achieved if the disease has not returned within five years post-treatment. Metastatic breast cancer may also show response to first-line chemotherapy (in approx. 25-55% of cases) (Winer *et al.*, 2001).

The antimetabolites, 5-FU and methotrexate, as well as the alkylating agent, cyclophosphamide, have all been routinely used for breast cancer treatment. However, the anthracyclines and the taxanes are the most effective and active agents in metastatic breast cancer, with response rates of over 30% in patients who have not been previously treated with any chemotherapeutic drugs (Ellis *et al.*, 2000; Bishop *et al.*, 1997 and 1999). Although anthracyclines have been described as the most effective single agent in the treatment of breast cancer, drugs such as adriamycin do not discriminate between normal and caner cells, resulting in severe side effects for the patient. Furthermore such treatment is associated with the development of a multiple drug resistance phenotype commonly seen in relapsed breast cancer patients, resulting in poor prognosis and lack of response to second line chemotherapy with CMF (cyclophosphamide, methotrexate, 5-fluorouracil). Combination therapy is commonly used in breast cancer instead of single agent treatments. Anthracycline and taxane combinations have been found to induce over 50% response rates with complete remission occurring in approx. 15% of cases (Miller and Sledge, 1999). Although the figures may look promising, breast cancer remains an incurable disease for many patients, especially at advanced stages. One important reason for this is multiple drug resistance.

The aim of this study is to identify a panel of genes that may be clinically relevant markers to predict the outcome and possibly the drug resistance of tumours in individual patients. For the purpose of this study, mRNA levels of a number of genes that were identified, from the literature, as being potentially of relevance to breast cancer, were analysed. RT-PCR is a highly sensitive technique and small amounts of tissue are needed for analysis. The main problem, however, is that the specimens being analysed may not be pure tumour and genes in the surrounding 'normal' tissue may also be amplified, thereby giving false positive results. Therefore, one needs to bear this mind when carrying out such analysis. The ideal method of analysis of tumour tissue would be to use laser micro-dissection techniques, where the 'pure' tumour tissue is identified and selected for analysis leaving behind 'normal' tissue. However, this is not yet an option for analysis in the NICB, and standard methods of RNA extraction and RT-PCR were used throughout the clinical study.

4.6.2 The Clinical Study

The clinical study was done to investigate if a reliable method of analysis could be obtained using basic laboratory analytical procedures to analyse the mRNA expression of galectin-3, survivin and MRP-1 in a panel of breast tumour specimens and to investigate the prognostic and predictive potential of galectin-3, survivin and MRP-1.

This work was part of a collaboration study with St. Vincent's University hospital (SVUH) in Dublin. Tumour samples were donated by Dr. Susan Kennedy, Dept. of Pathology, SVUH. Tumour tissue was collected over a number of years (1993-1997) from breast cancer patients and was stored at –80°C until needed. Sufficient RNA was extracted from approximately 165 tumours using RNA extraction techniques previously optimised by Dr. Doolan (PhD, 2001). This method (discussed in more detail in section 2.4), involved dissecting the tumour very finely using a homogeniser or a sterile blade before re-suspending the tumour in Tri Reagent and freezing prior to the RNA extraction procedure. Other RNA extraction methods, from tumour samples, developed in this lab. involved RNA extraction from fresh, 'snap' frozen tissue and archival paraffin-embedded breast tissue (P. Doolan, PhD, 2001; O'Driscoll *et al.*, 1996).

A number of clinical and pathological parameters were obtained from patients' charts including details on patient age at diagnosis, tumour type, size and grade, chemotherapy and hormonal treatment received, and outcome for patients in terms of relapse-free survival and overall survival. Most patients received tamoxifen and/or CMF chemotherapy. Some received adriamycin or taxol in addition to CMF, while some patients did not receive any drug treatment. Statistical analysis was performed, by Dr. Lorraine O'Driscoll, using the SPSS 10.1 software package, on 106 of the 165 tumours analysed by RT-PCR. The reason for exclusion of 59 cases was the lack of relevant clinical information on all these specimens. The clinical database was built in Excel, compiling information from a series of smaller databases and, subsequently, crosschecking and obtaining outstanding information from clinical records, where relevant. This information was systematically converted to a format recognised for analysis by the SPSS, resulting in a database of more than 18,000 data points. Univariate and multivariate statistical analyses of the results was then performed using the SPSS 10.1 software package. Descriptive statistics was used to

summarise patient characteristics. Statistical analysis of the results was performed using Pearson's X^2 test, systematically analysing groups of parameters in pairs, to investigate relationships between reverse transcriptase-polymerase chain reaction, immunohistochemical and clinicopathological and histopathologic findings. Kaplan-Meier survival curves were established and were subsequently checked using the log-rank, Breslow and Tarone-ware tests (*p*-values represent log-rank, unless otherwise indicated) to assess the relevance of potential prognostic factors. Multivariate survival analyses were performed using the Cox regression backward stepwise likelihood ratio. The data was censored at 5 years for multivariate analysis. A value of $p < 0.05$ was considered statistically significant. Six non-cancerous (normal) breast biopsies (N1-N6) were also included in the analysis. The tumours were all given a 'T' or an 'S' number as a method of coding.

To gain insight into the genes that are perhaps likely to influence prognosis and teach us more about this disease by understanding the roles played by the different genes/family of genes, a number of gene transcripts were examined in this study (Table 4.2). The majority of transcripts analysed are of apoptosis-related genes, with the exception of one, which is related to multiple drug resistance (MRP1). This work was divided between two people, as indicated in Table 4.2.

Apoptosis is usually induced by anticancer agents as a mechanism of their death-inducing activity (Clynes *et al.*, 1998). This mechanism of cell death is similar to cell death mechanisms caused by DNA damage or deprivation of growth factors (Bottini *et al.*, 2003). Therefore, over-expression of genes that inhibit the apoptotic pathway could produce drug resistant tumours that are not being killed in response to drug. Bcl-2 has been shown to protect cells against death induced by most chemotherapeutic drugs, suggesting that Bcl-2 over-expression may play a role in resistance to chemotherapy. High expression of Bcl-2 has been associated with chemotherapy resistance in neuroblastoma and acute myeloid leukemia. Bcl-2 expression has, surprisingly, been associated with good prognosis in many cancers (Bottini *et al.*, 2003). Survivin has also been associated with resistance to chemotherapy in advanced ovarian carcinoma, where high levels of survivin protein were significantly associated with clinical resistance to a taxol/platinum-based regimen (Zaffaroni *et al.*, 2002). Furthermore, the survivin gene is generally considered to be a very

interesting diagnostic marker and potential drug target because of its predominantly cancer-specific expression in adult human organ tissues. As discussed in previous sections of the thesis, survivin is expressed in most human cancers, but not in the majority of normal, adult tissue (see section 1.6.3) (Ambrosini *et al.*, 1997 Adida *et al.*, 1998a and O'Driscoll *et al.*, 2003).

The results of the clinical study that will be discussed in this thesis are the results from the analysis of the first three genes listed in Table 4.2. The results of the analysis carried out on the remaining genes have been discussed in the PhD thesis of Dr. D. Cronin (PhD, 2002).

GeneTranscript of interest	Function	Analysed by
Survivin*	Anti-apoptotic	R. Linehan
Survivin-2B*	Pro-apoptotic	R. Linehan
Survivin-ΔEx3*	Anti-apoptotic	R. Linehan
Galectin-3	Anti-apoptotic	R. Linehan
MRP-1	MDR	R. Linehan
Bcl-2	Anti-apoptotic	D. Cronin
Bax-α	Pro-apoptotic	D. Cronin
Bag-1	Anti-apoptotic	D. Cronin
Mcl-1	Anti-apoptotic	D. Cronin

* Survivin and its two splice variants (Survivin-2B and Survivin-ΔEx3) were co-studied using one set of primers, which amplifies the three transcripts (see appendix A).

Table 4.2 Markers analysed by RT-PCR in clinical study

4.6.3 Survivin mRNA Expression in Breast tumour Biopsies

The aim of this part of the clinical study was to investigate the expression of survivin mRNA in invasive breast carcinomas by RT-PCR analysis. The mRNA expression levels of the extra two survivin splice variants, survivin 2B (retains part of intron 2 as a cryptic exon), and survivin-delta Ex 3 (lacks exon 3), identified in 1999 by Mahotka *et al.*, were co-analysed in the tumour samples.

Survivin mRNA expression was detected in 68% (72/106) of cases. Survivin was the dominant transcript in the tumour specimens. Survivin-2B mRNA expression was detected in 9.4% (10/106), while survivin-ΔEx3 mRNA was detected in 54.7% (58/106) of cases. Analysis of 'normal' tissue samples revealed no survivin, survivin-2B or survivin-ΔEx3 mRNA expression, confirming findings by many other groups that survivin is not expressed in the majority of normal adult tissue (Ambrosini *et al.*, 1997). Survivin protein was detected in 60% of breast tumours analysed by immunohistochemistry (Kennedy *et al*, 2003). This study was carried out on a larger number of tumour samples (293), but included all of the 106 tumours that were analysed by RT-PCR.

Survivin mRNA expression has been widely reported in many other types of cancers as well as breast cancer. A recent study of survivin expression in breast cancer revealed 90% of tumours expressed survivin mRNA, with 23% expression in normal tissue (Nasu *et al.*, 2002). Sarela *et al.* (2000) reported survivin mRNA expression in 63.5% of specimens of colorectal cancer and found that its expression was correlated with a poor survival rate. They also reported 29% survivin mRNA expression in normal tissue (Sarela *et al.*, 2000). Survivin mRNA was expressed in 13% of gastric tumours, with no expression reported in normal tissue, and no correlation of survivin expression with survival (Ikeguchi and Kaibara, 2001), while survivin mRNA expression in neuroblastoma (Tajira *et al.*, 2001) and esophageal cancer (Kato *et al.*, 2001) was correlated with poor prognosis and poor survival, respectively.

Not many groups have studied the expression of all three survivin splice variants. Kappler *et al.* (2001) examined the expression of the three splice variants in soft tissue sarcoma specimens and found, similar to our own finding, survivin to be the dominant transcript, while survivin-2B was the least dominant. They found survivin mRNA expression in 64% of cases, survivin-ΔEx3 mRNA expression in 42% and no survivin-2B mRNA expression in any of the samples analysed. Survivin mRNA expression was correlated with a poor survival rate (Kappler *et al.*, 2001). Krieg *et al.* (2002) also studied the expression of the three survivin spice variants. In gastric cancer all three splice variants (survivin being the dominant transcript) were detected by RT-PCR in all the specimens analysed, irrespective of their histological type, grade or stage. Survivin mRNA expression was found in normal gastric tissue also. In addition, they found that the expression of survivin-2B mRNA was dramatically decreased in advanced stages of gastric cancer, which might support the suggestion by Islam *et al.* (2000) that survivin-2B is acting as a natural antagonist to survivin and survivin-ΔEx3. Similar findings were reported by Mahotka *et al.* (2002), where renal carcinomas were found to express all three survivin variants, with survivin-2B mRNA down-regulation associated with advanced stages of the disease.

While the majority of studies of survivin expression in cancer indicate an association with poor prognosis, there are small number of cases where this is not the case. Other methods of detection of survivin expression include immunohistochemistry. This has been used by many researchers to identify localisation of survivin protein in tumour tissues and also to correlate survivin protein expression with clinical outcome. As mentioned, in a recent immunohistochemical study of 293 breast tumour specimens (including the 106 cases studied by RT-PCR), we found that survivin protein expression to be located mainly in the nuclei (but also present in the cytoplasm in a small number of tumour tissues) of tumour cells (Kennedy *et al.*, 2003). Results from this study indicate that survivin protein expression in the nucleus may be a favourable prognostic indicator (Kennedy *et al.*, 2003). This is in disagreement with studies carried out by Tanaka *et al.* (2000) on 167 cases of breast tumours, where they found that survivin expression in breast cancer resulted in poor prognosis. In the study reported by Tanaka *et al.* (2000), survivin protein staining was found in the cytoplasm of the cells, suggesting that survivin localisation in tumour cells may be correlated with disease outcome.

In our studies, statistical analysis indicated no significant association between survivin mRNA expression and disease outcome. In addition, we found no correlation between survivin mRNA expression and age of patient (P=0.745), tumour size (P=0.722), clinical stage (P=0.588), histology type (P=0.608), lymph node status (P= 0.148) or estrogen receptor status (P=0.763). There was no correlation between survivin mRNA expression and disease-free survival (P=0.993) or overall survival (P=0.243), as indicated by Kaplan-Meier analysis (The P-values presented here are for the main, dominant survivin transcript. For full results on all splice variant, see section 3.5.2). Although Nasu *et al.* (2002) did not investigate association of survivin mRNA expression and patient outcome, they did report that they found no significant association between survivin mRNA expression and age of patient at diagnosis, tumour size, clinical stage, histology type, lymph node status or estrogen receptor status (Nasu *et al.*, 2002).

Furthermore, we found no correlation between survivin mRNA expression and survivin protein expression in breast carcinoma (P=0.283). There was, however, a significant correlation between the expression of the three survivin variants, with survivin and survivin-ΔEx3 (P<0.0001), survivin and survivin-2B (P=0.022), and survivin-ΔEx3 and survivin-2B (P=0.003).

Survivin protein expression has previously been found to correlate with Bcl-2 protein expression (Tanaka *et al.*, 2000; Kawasaki *et al.*, 1998; Lu *et al.*, 1998; Sarela *et al.*, 2002). In our studies of survivin mRNA expression and survivin protein expression (Kennedy *et al.*, 2003) in breast carcinoma, there was no correlation between survivin and Bcl-2 expression. Survivin mRNA expression did not correlate with the expression of the other markers included in our study, although there was an apparent association between survivin-ΔEx3 mRNA expression and Mcl-1 mRNA expression (analysis of Mcl-1 carried out by D. Cronin, PhD, 2002).

4.6.4 Galectin-3 mRNA Expression in Breast tumour Biopsies

Galectin-3, a β-galactosidas-binding-protein, has been proposed to regulate cell growth, to mediate cell adhesion and to inhibit apoptosis (see sections 1.5.2 and 1.5.3) (Matarrese *et al.*, 2000; Akahani *et al.*, 1997). The aim of this part of the clinical study was to investigate the expression of galectin-3 mRNA in invasive breast carcinoma by RT-PCR analysis. Galectin-3 mRNA expression was detected in approximately 99% of cases analysed.

Galectin-3 protein expression has been reported in many types cancer, including breast (Castronovo *et al.*, 1996; Idikio, 1998), colorectal (Sanjuan *et al.*, 1997; Nakamura *et al.*, 1999; Legendre *et al.*, 2003; Nagy *et al.*, 2003) pancreatic (Berberat *et al.*, 2001 and Kunzli et al., 2002) non-small cell lung cancer (Gabius *et al.*, 2002; Yoshimura *et al.*, 2003), ovarian (Van der Brule *et al.*, 1994), and gastric cancers (Miyazaki *et al.*, 2002) (see section 1.5.4). The majority of the findings from these studies indicate that decreased expression of galectin-3 protein and/or mRNA is associated with progression of cancer. This was found to be the case in breast cancer, where decreased galectin-3 expression was also associated with an invasive phenotype and a more aggressive tumour. In intra-ductal and invasive carcinoma, a large down-regulation of galectin-3 was observed, while breast cancer cells metastatic to the lymph nodes exhibited very low or undetectable levels of galectin-3. Furthermore, all normal and benign breast specimens examined exhibited detectable galectin-3 expression, as seen by immunohistochemistry (Castronovo *et al.*, 1996). Similar results were reported by Idikio *et al.* (1998), where the expression of galectin-3 was determined in 27 invasive breast cancers by immunocytochemistry. Results suggested that increasing histological grade of breast cancer leads to reduced galectin-3 expression and possibly reduced matrix binding and increased cancer cell motility (Idikio *et al.*, 1998).

In our studies, galectin-3 mRNA expression was found in 83% (5/6) cases of normal tissue specimens (see section 3.5.3). Due to its expression in almost all the breast tumour tissue analysed, statistical evaluation of galectin-3 mRNA expression was not appropriate. Results from this study suggest that expression of galectin-3 at the gene transcript level cannot be considered as a prognostic indicator of disease outcome for breast cancer patients.

4.6.5 MRP1 mRNA Expression in Breast tumour Biopsies

As previously mentioned (see section 4.6.1), chemotherapy plays a major part in the treatment of breast cancer and development of a multiple drug resistance phenotype is the main set back in the treatment of breast cancer, as is the case with most common cancers. The study of MDR-related genes in cancer is, therefore, an important step in understanding the mechanisms of development of this phenotype. The expression of the membrane transporter, MRP1, which confers a MDR phenotype similar to that conferred by Pgp was investigated by RT-PCR in the breast tumour biopsies to establish whether its expression correlates with response to chemotherapy and with other clinicopathological parameters.

MRP1 mRNA expression was detected in 72.8% (77/106) of tumour specimens and in 66% (4/6) of normal tissue specimens. MRP1 protein expression was investigated by S. Kennedy's group in 176 breast tumour specimens, using immunohistochemistry technique. We found no correlation between MRP1 mRNA expression and MRP1 protein expression in breast carcinoma (P=0.333), using Chi-square univariate analysis.

The expression of MDR-related genes in breast cancer have been carried out by several other research groups (Filipits *et al.*, 1999; Zochbauer-Muller *et al.*, 2001; Burger *et al.*, 2003). mRNA expression levels of BCRP, LRP, MRP1, MRP2 and MDR1 were investigated in 59 primary breast tumours specimens from patients who received chemotherapy treatment. It was found that the expression of these MDR-related genes was higher in non-responding tumours compared with responding tumours. This was most significant for MDR1, where overall response was much lower in tumours that expressed high levels of MDR-1 mRNA, with high expression levels of MDR1 significantly associated with a poor relapse-free survival. In addition, there was a correlation between BCRP and MRP1 mRNA expression and progression-free survival in patients who received CAF (cyclophosphamide, adriamycin, 5-fluorouracil) treatment, but not in patients who received CMF (cyclophosphamide, methotrexate, 5-fluorouracil). There was no correlation between the expression of any of the five MDR-related genes and overall survival (Burger *et al.*, 2003).

In a study by Filipits *et al.* (1999), MRP1 protein expression in 100 breast tumour specimens was studied using immunohistochemistry. MRP1 was expressed in 80% of specimens and was more frequent in ER (estrogen receptor) and PR (progesterone receptor) negative carcinomas, but it was independent of tumour size and lymph node involvement. Patients who had MRP1 negative tumours had longer overall survival and disease-free survival, whereas patients who had MRP1 positive tumours had worse prognosis (Filipits *et al.*, 1999).

Taken together, these results suggest that MRP1 expression is indicative of poor prognosis in breast cancer patients. However, we found no correlation between MRP1 mRNA expression and the outcome of the disease. This is in agreement with a similar study by Kanzaki *et al.* (2001), where, MRP1, MDR1, BCRP, and LRP mRNA expression was analysed by RT-PCR in 43 untreated breast tumour patients; the majority of whom subsequently received doxorubicin-based chemotherapy after surgery. The expression of these four genes was independent of age, and estrogen or progesterone receptor status, and was also unrelated to the relapse-free or overall survival of the patients who received chemotherapy (Kanzaki *et al.*, 2001).

4.7 Possible role of Survivin in post-transcription modification

Degradation of several intracellular proteins involved in cell cycle control and tumour growth is regulated by the ubiquitin-dependent multicatalytic protease complex (proteasome) (Soligo *et al.*, 2001). The ubiquitin-proteosome pathway plays a central role in the regulation of essential processes such as cell cycle control, antigen processing, transcription and signal transduction. Two steps are involved in ubiquitin-proteosome-dependent degradation of protein: labeling of the target protein with multiple ubitquitin moieties and degradation of the tagged protein by the 26S proteosome (Zhao *et al.*, 2000).

As discussed in section 1.6.6.2, expression of survivin is cell cycle-dependent with up-regulation in the G_2/M phase and down-regulation in the G1 phase (Li *et al.*, 1997). The pattern of survivin expression suggests that the ubiquitin-proteosome pathway may be involved in the regulation of survivin. Cycloheximide, a protein synthesis inhibitor, caused a down-regulation in survivin protein level, with half of the protein degraded after 30 minutes. Proteosome inhibitors prevented this degradation, suggesting that proteosomes are involved in survivin degradation. Furthermore, results indicate that the decline of survivin protein level in the G1 phase of the cell cycle is a result of proteosome activity and demonstrate the presence of a post-transcriptional cell cycle-dependent mechanism of survivin regulation *in vivo* (Zhao *et al.*, 2000).

In our current studies, survivin protein expression was dramatically down regulated in DLKP MDR variants, DLKP-taxotere (see sections 3.3.8.4 and 4.4.2.2) and DLKP-vincristine (see sections 3.3.8.4 and 4.4.3.2). Survivin mRNA expression was unaltered in these two MDR variants (see section 3.3.7.10). These findings suggest that survivin is post-transcriptionally regulated, and that treatment of DLKP cells with the microtubule-stabilising and –destablising drugs, taxotere and vincristine, respectively, may play a role in survivin protein degradation in this cell line. Survivin interacts with microtubules of the mitotic spindle during the beginning of mitosis resulting in the counteraction of cell death in the G_2/M phase of the cell cycle. Disruption of survivin-microtubule interactions results in loss of survivin's anti-apoptosis function (Li *et al.*, 1998).

Similar findings were reported by Wall *et al.* (2003), where exposure of breast carcinoma MCF-7 or cervical carcinoma HeLa cells to anticancer agents, including adriamycin, or taxol, resulted in increased survivin protein expression with no change in survivin mRNA expression. Transcription of the survivin genes was repressed by anticancer agents, which is consistent with similar findings observed with other G_2/M-regulated genes containing cell-cycle dependent element (CDE) (Wall *et al.*, 2003).

Taken together these results may suggest a decrease in protein synthesis and may explain the lack of protein over-expression, in SKOV-3 'T.O.', DLKP and MCF-7 'T.O' survivin cDNA transient transfections, where a dramatic over-expression in survivin mRNA was obtained (see sections 4.3.4, 4.3.5 and 4.3.6, respectively).

Survivin's role in post-transcriptional modification *in vivo* may explain the discrepancies observed in the clinical study descried in section between the relevance of survivin mRNA and survivin protein in prognosis of breast cancer. As discussed in section survivin protein expression correlated with good disease prognosis (Kennedy *et al.*, 2003), whereas survivin mRNA expression did not correlate with such prognosis.

5.0 Conclusions & Future Work

5.1 Conclusions

The main objective of this thesis was to investigate the expression of apoptosis-related genes, galectin-3 and survivin, in human cancer cells and to establish their relationship with *in vitro* invasiveness and drug resistance.

1. Galectin-3 mRNA and protein expression was up-regulated in the RMPI-2650 melphalan-resistant variant - a variant which developed a highly elevated adhesive, motile and invasive phenotype due to its exposure to melphalan, in addition to an MDR phenotype - compared to the RPMI-2650 parental cell line.

2. Galectin-3 cDNA was stably transfected into the non-invasive human lung carcinoma cell line, DLKP, and three clones (C2, C12 and C13) along with the mixed population (MP) were generated under geneticin selection pressure. Different clones displayed up-regulated or down-regulated levels of galectin-3 expression, as shown by RT-PCR and Western blot analysis. Galectin-3 over-expressing clones were not resistant to the chemotherapeutic drugs, taxol, carboplatin and adriamycin, as demonstrated by *in vitro* toxicity assays. However, DLKP-MP (mixed population), which shows the highest level of galectin-3 mRNA and protein over-expression, displayed a 2-fold increase in resistance to carboplatin. Galectin-3 over-expression resulted in an increase in *in vitro* invasiveness, cell motility and adhesion to extracellular matrix, fibronectin and laminin, highlighting the association of galectin-3 over-expression with *in vitro* invasiveness and metastasis in lung cancer. Galectin-3 over-expression did not result in a change of proliferation rate of this cell line.

3. Evidence from the literature on survivin indicates that it may be responsible for an increase in resistance to some cytotoxic drugs, such as taxol, etoposide and cisplatin. Findings from analysing the expression of survivin in the RPMI-2650 drug resistant variants, by RT-PCR and western blot analysis, showed that survivin expression was down-regulated in the RPMI-2650 taxol-resistant variant, a variant which did not develop an invasive phenotype but did develop a MDR phenotype.

4. Survivin cDNA was firstly stably transfected into DLKP, generating 5 clones. The clones did not show any significant changes in survivin mRNA or protein survivin expression, as demonstrated by RT-PCR and western blot analysis. There was also no change in resistance levels to the chemotherapeutic drugs, carboplatin or adriamycin.

5. A second cell line was selected for another survivin cDNA stable transfection, namely, SKOV-3 'Tet off', an ovarian carcinoma cell line stably transfected with a tetracycline regulated plasmid, thereby allowing the expression of a transfected cDNA to be down-regulated by addition of tetracycline or doxocycline in the medium. Twelve clones were generated from the transfection; none exhibited any major change in survivin mRNA and protein expression. No change in resistance to the chemotherapeutic drugs carboplatin and taxol was observed. Further analysis was carried out on two of the SKOV-3 'Tet off' survivin clones (C2 and C5) and the mixed population (MP) in the presence and absence of doxocycline. Survivin mRNA and protein expression remained un-changed between the 'on' and 'off' states of gene expression, and no change in resistance to carboplatin and taxol was observed.

6. A transient transfection of survivin cDNA into SKOV-3 'Tet off' was carried out and survivin mRNA and protein expression was analysed at different time points post-transfection, namely, 24 hours, 48 hours, 72 hours and 5 days, by RT-PCR and western blot analysis, respectively. Results demonstrated survivin mRNA to be dramatically up regulated after 24 hours, with a 50% inhibition of this up-regulation resulting from the addition of doxocyline to the growth media, demonstrating the use of the inducible system in this cell line. This up-regulation was not observed when a control empty vector was transiently transfected into the cells. No up-regulation of survivin protein was observed by western blot analysis or by immunofluorescence. *In vitro* toxicity assays and the apoptosis detection assay, TUNEL, revealed that survivin mRNA up-regulation alone was not sufficient to induce a resistance phenotype in the cells and there was no protection from taxol or cisplatin-induced apoptosis. To determine if the up-regulation of survivin mRNA resulted in a change in the invasive phenotype of SKOV-3 cells, which are normally

invasive, *in vitro* invasion assays were carried out 24 hours prior to transfection. *In vitro* invasiveness of SKOV-3 remained unaltered.

The same experiment was carried out on two more cell lines (DLKP and MCF-7 'Tet Off') in an effort to determine whether the effect observed was unique to this cell line. Transient transfection of DLKP was carried out and similar results to those observed in the SKOV-3 'Tet Off' cell line were obtained. Survivin mRNA was dramatically over-expressed after 24 hours of transfection, as demonstrated by RT-PCR, while survivin protein was un-changed, as demonstrated by western blot analysis. Transient transfection of MCF-7 'Tet Off' demonstrated that survivin mRNA was dramatically over-expressed after 24 hours. In addition, survivin protein was initially over-expressed after 24 hours, as demonstrated by western blot analysis. However, this observation of survivin protein over-expression was not reproducible. Taken together, these results suggest that there may be some translational block preventing the over-expression of survivin protein in these transfection experiments. An alternative explanation may be that EPR-1, which has been suggested to act as a natural antisense to survivin, is playing a role in preventing the over-expression of survivin protein. This does not explain, however, why other research groups succeeded in obtaining survivin over-expressing cell lines (including MCF-7), which resulted in apoptosis inhibition.

7. To further study the effect of MDR on the expression of the anti-apoptosis genes, galectin-3 and survivin, the lung carcinoma and the nasal carcinoma cell lines, DLKP and RPMI-2650 cells, respectively, were chosen.

The DLKP pulse selections yielded two MDR variants, namely, DLKP taxotere-resistant and DLKP vincristine-resistant sublines, which displayed approx. 420-fold and 5329-fold resistance to their selection agents, respectively, and 210-fold and 124-fold resistance to taxol, respectively. A non-resistant variant, DLKP-5-FU also resulted. In addition to acquiring a MDR phenotype, DLKP taxotere-resistant (DLKP-taxotere-1) cells also acquired an invasive phenotype compared to the parental DLKP cell line. Previous results obtained by others in this laboratory,

revealed that when the DLKP cell line was pulse selected with a range of chemotherapeutic drugs, the DLKP taxotere-resistant (DLKP-taxotere-2) and the DLKP vincristine-resistant variants did not acquire an invasive phenotype, whereas, DLKP 5FU-resistant variant did. These discrepancies suggest that perhaps the relationship between MDR and *in vitro* invasiveness is a random process, and not drug-specific or cell line-specific. DLKP parental cells are normally non-invasive *in vitro* (DLKP-1), however, a strain of DLKP (DLKP-2) was identified in the course of these studies, which exhibited a highly invasive phenotype. This finding was unexpected.

Galectin-3 mRNA expression was un-changed in all three variants compared to the parental cells. Similarly, survivin mRNA was also unchanged DNA Microarray analysis supported these findings. Survivin protein expression was dramatically down regulated in DLKP taxotere-resistant and DLKP-vincristine-resistant variants compared to DLKP-5FU and DLKP parent, as demonstrated by western blot analysis, suggesting the involvement of post-transcriptional regulation in the expression of survivin protein. Survivin expression was also down regulated in the RPMI-taxol resistant cell line. Taken together, these results suggest that survivin may play a role in vincristine, taxol and taxotere drug resistance, supporting findings by other research groups.

Further gene expression analysis in the DLKP variants, by DNA microarray, RT-PCR and western blot analysis, demonstrated dramatic over-expression of Mdr-1 mRNA and protein in DLKP taxotere-resistant and DLKP vincristine-resistant variants, supporting the typical pattern of MDR resistance in there variants. RT-PCR results showed that MRP-1, -2, -4-, -5 and BCRP mRNA expression was not altered between the variants and the parental cells, while MRP-3 seems to be expressed in the DLKP-5FU variant, but not in the other two variant or the parental cells. DNA microarray analysis indicated an increase in MRP-2 expression in the DLKP-vincristine variant when compared to the parental cell line, while MRP-1, -3, -4. –5 and BCRP expression was unchanged in the two MDR variants. RT-PCR analysis indicate that E-cadherin mRNA expression was increased in the DLKP taxotere-resistant variants, supporting its role in the invasive phenotype of this

variant. DNA Microarray analysis, however, demonstrate a 2-fold down-regulation in this variant. MRP-1 protein expression was slightly up regulated in the DLKP taxotere-resistant variants, suggesting post-transcriptional regulation.

8. The RPMI-2650 pulse selections were carried out in duplicate. Preliminary findings suggest that a transient resistance was developing in the variants, which was being lost with sub-culturing. Similar finding have been reported by other researchers in hamster tumour cells. The RPMI-2650 drug-selected variants seem to regain their resistance when further pulsed. *In vitro* invasiveness and motility of the cells remained un-altered. Analysis of gene expression by RT-PCR, indicate no dramatic change in galectin-3, survivin, Mdr-1, MRP-1, -2, -3, -4, or MRP-5.

9. RT-PCR analysis on 165 breast tumour samples was carried out to examine the expression of survivin (and its two splice variants, survivin-ΔEx3, survivin 2B), galectin-3 and MRP1. This clinical tumour specimens study demonstrated the ability to extract high quality RNA and to generate reliable RT-PCR results from human tissue. Results on 106 cases indicated that none of the three genes correlated with disease outcome, suggesting that survivin, galectin-3 and MRP1 cannot be considered as prognostic indicators of disease outcome for breast cancer patients. The expression of these genes was also analysed in normal breast tissue. Survivin was undetected in these specimens, supporting many finding by other researchers that survivin is expressed in all common cancers, but not in most normal adult tissue, while both galectin-3 and MRP1 expression was detected in over 50% of normal specimens, supporting a role for these genes in normal cellular functions in human adult tissue.

5.2 Future Work

Future work arising from this thesis is summarised in the following points:

1. Cancer invasion/metastasis is one of the major causes of death in cancer patients. Targeting naturally occurring genes that promote cancer invasion and metastasis may, in combination with other cancer therapies such as chemotherapy and/or radiotherapy, be effective in slowing down cancer growth and metastasis. Future work for examining this theory *in vitro* could include down-regulation of galectin-3 expression in an invasive and MDR cancer cell line, e.g. RPMI-melphalan-resistant cell line, using antisense oligonucleotides, siRNA or ribozyme techniques. As we have shown that up-regulation of galectin-3 expression induces *in vitro* invasiveness in a lung cancer cell line, down-regulation of galectin-3 expression would be important to determine whether the inhibition of galectin-3 expression would inhibit *in vitro* invasiveness. The use of antisense oligonucleotides in down-regulating galectin-3 has already been reported in breast and colon cancer cell lines, successfully inhibiting *in vitro* invasiveness, but has not yet been used in a nasal carcinoma cell line. siRNA technique is a new method for down-regulating gene expression and has proven successful in down-regulating galectin-3 in pituitary tumours, slowing cellular proliferation rates.

2. DNA microarray technology may be used to identify novel invasion-related genes, which may be significantly altered in DLKP-galectin-3 clone 13. As previously mentioned, this is a clone which stably over-expresses galectin-3 mRNA and protein and expresses an invasive phenotype. Microarray analysis could help identify novel genes that play a role in invasion, motility, and adhesion, as well as other apoptosis-related genes that may be induced or inhibited by galectin-3 over-expression. In addition, the DLKP-galectin-3 mixed population may be cloned out further to obtain additional clonal populations, which may be useful for further analysis.

3. Survivin is an attractive target for cancer therapy due to its unique expression pattern. It is important, therefore, to obtain a cell line model where expression of survivin can be regulated and to study functional changes that may result from this regulation. We have successfully obtained a cell line where survivin mRNA expression can be regulated, using the 'Tet' regulatory system. However, we have not succeeded in developing a cell line to over-express survivin protein and, therefore, a functional change has not been observed. It would be useful to carry out survivin cDNA transient transfections in the presence of proteosome inhibitors to prevent degradation of survivin protein. This may allow survivin protein to be over-expressed and a functional change may result.

4. DNA microarray analysis indicated changes in the expression of some gene between the DLKP MDR variants and the parental line. It would be useful to repeat the array experiment to ensure that the results are reproducible, to validate these results by RT-PCR, and to investigate (by western blot analysis) if these changes occurred at the protein level. This could identify novel genes that are playing a role in drug resistance and invasion, and could allow further analysis of their role via transfection studies.

5. Future characterisation of the DLKP MDR variants should include drug accumulation assays to compare the DLKP parent cells to their drug resistance variants. These studies can be carried out along with circumvention assays using agents such as verapamil or cyclosporin A to inhibit P-glycoprotein. A ribozyme to Mdr-1 has previously been designed and used in the NICB, showing promising results in DLKP adriamycin-resistant cells. This ribozyme may be transfected into DLKP vincristine-resistant and DLKP taxotere-resistant variants, which show dramatic Mdr-1 mRNA and protein over-expression. siRNA against Mdr-1 has also been designed in the NICB recently, and this may also be used in transfection experiments to down-regulate Mdr-1 expression in the two DLKP MDR variants, to determine if it will sensitise them to chemotherapeutic agents.

7. Characterisation of the RPMI-2650 drug selected variants merits further research. Further *in vitro* toxicity testing should be carried out on cells pulsed 10 times, compared to cells pulsed 12 and 14 times. This may determine whether the initial resistance observed is a transient affect, which was lost over time. Cells may need to be further pulsed to re-gain resistance. Invasion assays need to be carried out on the RPMI-2650 VCR–14p and the RPMI-2650 5-FU-14p variants, as well as repeating motility assays on these two variants. In addition, RT-PCR and western blot analysis needs to be carried on these two variants. Further characterisation of the 10p and 12p variants should include repeating RT-PCR analysis on the MDR-related genes, in addition to galectin-3 and survivin. Western blot analysis should also be carried out on these variants. Once it has been determined whether the variants can remain resistant over a period of time, DNA microarray analysis may be carried out to identify novel genes that may play a role in drug resistance. Functional studies may then be carried out on genes of interest. These may include cDNA over-expression. Alternatively, ribozyme, antisense or siRNA for down-regulation of specific genes, identified as potentially being of interest, could be performed to elucidate their role(s).

8. Unstable drug resistance has previously been reported in a hamster tumour cell line, which is associated with changes in MDR-related gene expression. Our studies of the RPMI-2650 drug-selected variants may be useful to examine how unstable resistance is achieved. This may be important from a clinical aspect since drug resistance is a main cause of chemotherapy failure and if this can be averted it may lead to better cancer therapy. It would be useful to isolate RNA from the RPMI-2650 drug-treated variants at different intervals of sub-culturing and to study the patterns of MDR-related gene expression by DNA microarry and RT-PCR analysis. This may identify key genes, which may be down regulated or switched off through sub-culturing of initially resistant cells. Once such genes have been identified they may be targeted using antisense or siRNA techniques in stable drug-resistant cell lines and may ultimately lead to therapeutic targets.

9. The breast tumour tissue specimens available at the NICB provide a source of information that can be used for further gene expression analysis. It would be important, therefore, to extract RNA from these specimens, for real-time, quantitative PCR analysis, using laser capture micro-dissection to isolate tumour tissue which is not at risk of being contaminated with normal tissue that may be surrounding the tumour. This would provide accurate information on gene expression specific to tumour tissue, which may be compared to normal tissue. It may also be beneficial to analyse the expression and localisation of galectin-3 protein by immunohistochemistry in the tumour specimens, as galectin-3 localisation seems to be an important prognostic factor. The breast tumour samples may also be used for DNA microarray studies to identify a much larger number of genes (possibly including novel genes) that may play an important role in breast cancer and may be used as prognostic or predictive markers for this disease.

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7.0 Appendices

7.1 Appendix A

7.1.1 Primers used for RT-PCR gene expression analysis

Table 7.1.1 Primer Sequences used for RT-PCR analysis

Gene	Primers (5'-3')	Annealing Temp (°C)	Product Size (bp)
Galectin-3	F *gct ggg cca ctg att gtg cct tat*	54	281
	R *acc agt act tgt att ttg aat ggt*		
Survivin	F *gca tgg gtg ccc cga cgt tg*	62	329
	R *gct ccg gcc aga ggc ctc aa*		431
			500
Survivin RT	*agg aac ctg cag ctc aga*		
Mdr-1	F *gtt caa act tct gct cct ga*	54	157
	R *ccc atc att gca ata gca gg*		
MRP1	F *gta cat taa cat gat ctg gtc*	54	202
	R *cgt tca tca gct tga tcc gat*		
MRP2	F *ctg cct ctt cag aat ctt ag*	54	241
	R *ccc aag ttg cag gct ggc c*		
MRP3	F gat acg ctc gcc aca gtc c	63	262
	R *cag ttg gcc gtg atg tgg ctg*		
MRP4	F *cca ttg aag atc ttc ctg g*	42	239
	R *ggt gtt caa tct gtg tgc*		

Table 7.1.1 continued Primer Sequences used for RT-PCR analysis

Gene	Primers (5'-3')	Annealing Temp (°C)	Product Size (bp)
MRP5	F ***gga taa ctt ctc agt ggg***	49	381
	R ***gga atg gca atg ctc taa ag***		
BCRP	F ***aga ctt atg ttc cac ggg cc***	63	1113
	R ***caa ggc cac gtg att ctt cc***		
E-cadherin	F ***agc cat ggg ccc ttg gag***	65	653
	R ***cca gag gct ctg tca cct tc***		
β-actin (L)	F ***gaa atc gtg cgt gac att aag gag aag ct***		383
	R ***tca gga gga gca atg atc ttg a***		
β-actin (S)	F ***tgg aca tcc gca aag acc tgt ac***	55	142
	R ***tca gga gga gca atg atc ttg a***		

F=Forward primer, R=Reverse primer, β-actin (L)= β-actin(Long), β-actin (S)= β-actin(Short).

7.1.2 Coding sequences for Survivin and Galectin-3 genes

(1) Survivin Coding Sequence Accession number: NM_001168

50 a tgggtgcccc gacgttgccc cctgcctggc agccctttct caaggaccac cgcatctcta cattcaagaa ctggcccttc ttggagggct gcgcctgcac cccggagcgg atggccgagg ctggcttcat ccactgcccc actgagaacg agccagactt ggcccagtgt ttcttctgct tcaaggagct ggaaggctgg gagccagatg acgaccccat agaggaacat aaaaagcatt cgtccggttg cgctttcctt tctgtcaaga agcagtttga agaattaacc cttggtgaat ttttgaaact ggacagagaa agagccaaga acaaaattgc aaaggaaacc aacaataaga agaaagaatt tgaggaaact gcgaagaaag tgcgccgtgc catcgagcag ctggctgcca tggattga **478**

(2) Galectin-3 Coding Sequence Accession number: AB006780

54 atggcag acaatttttc gctccatgat gcgttatctg ggtctggaaa cccaaaccct caaggatggc ctggcgcatg ggggaaccag cctgctgggg caggggggcta cccaggggct tcctatcctg gggcctaccc cgggcaggca cccccagggg cttatcctgg acaggcacct ccaggcgcct accctggagc acctggagct tatcccggag cacctgcacc tggagtctac ccagggccac ccagcggccc tggggcctac ccatcttctg gacagccaag tgccaccgga gcctaccctg ccactggccc ctatggcgcc cctgctgggc cactgattgt gccttataac ctgcctttgc ctgggggagt ggtgcctcgc atgctgataa caattctggg cacggtgaag cccaatgcaa acagaattgc tttagatttc caaagaggga atgatgttgc cttccacttt aacccacgct tcaatgagaa caacaggaga gtcattgttt gcaatacaaa gctggataat aactggggaa gggaagaaag acagtcggtt ttcccatttg aaagtgggaa accattcaaa atacaagtac tggttgaacc tgaccacttc aaggttgcag tgaatgatgc tcacttgttg cagtacaatc atcgggttaa aaaactcaat gaaatcagca aactgggaat ttctggtgac atagacctca ccagtgcttc atataccatg atataa **806**

7.2 **Appendix B** Drug Dilutions used in *in vitro* Toxicity Assays

Table 7.2.1 Range of Chemotherapeutic Drug Dilutions used in *in vitro* Toxicity Assays for DLKP-Galectin-3 and Survivin transfectants

Adriamycin (ng/ml)	Taxol (ng/ml)	Carboplatin (μg/ml)
250	62.5	100
125	31.25	50
62.5	15.62	25
15.62	7.81	12.5
7.81	3.9	6.25
3.9	1.95	3.125
1.95	0.97	1.56
0.97	0.48	0.78
0.48	0.24	0.39

Table 7.2.2 Range of Chemotherapeutic Drug Dilutions used in *in vitro* Toxicity Assays for DLKP Drug-treated variants

Taxotere (ng/ml)	Taxol (ng/ml)	Vincristine (ng/ml)	5-FU (μg/ml)	Adriamycin (ng/ml)	Cisplatin (μg/ml)
500	62.5	5000	10	250	50
250	31.25	2500	5	125	25
125	15.62	1250	2.5	62.5	12.5
62.5	7.81	625	1.25	15.62	6.25
31.5	3.9	62.5	0.62	7.81	3.125
7.8	1.95	31.25	0.31	3.9	1.56
1.95	0.97	3.12	0.15	1.95	0.78
0.97	0.48	1.56	0.078	0.97	0.39
0.48	0.24	0.39	0.039	0.48	0.15

Table 7.2.3 Range of Chemotherapeutic Drug Dilutions used in *in vitro* Toxicity Assays for RPMI 2650-Drug-treated variants

Vincristine (ng/ml)	5-FU (μg/ml)	CCNU (μg/ml)	Adriamycin (ng/ml)	Carboplatin (μg/ml)
300	20	25	250	60
150	10	12.5	125	30
75	5	6.25	62.5	15
37.5	2.5	1.56	15.62	7.5
18.75	1.25	0.78	7.81	3.75
9.38	0.62	0.39	3.9	1.88
4.69	0.31	0.19	1.95	0.94
2.34	0.15	0.09	0.97	0.47
1.17	0.078	0.04	0.48	0.23

7.4 **Appendix C** Table of Figures