Clonal Variation in Multidrug Resistant Human Cell Populations

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a thesis submitted for the degree of Ph.D.

Dublin City University

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

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July 1994.

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

Signed : Mary Heenan Date : 5/7/94.

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Abstract :

Heterogeneity within an adriamycin-selected multidrug resistant (MDR) human squamous lung carcinoma, DLKP-A, was examined by establishing and characterising nine clones of the cell line. MDR variants of the same lung cell line were established by selecting with VP-16. The resulting cell lines, DLKP/VP-3 and DLKP/VP-8, were characterised and their resistance profiles and mechanisms of resistance were compared with the DLKP-A cell line.

All the subpopulations of DLKP-A were found to possess an MDR profile, but their resistance to adriamycin spanned a 9-fold concentration range. The VP-16 selected cells were also MDR. All the cell lines were resistant to vincristine, adriamycin and VP-16 but not to 5-fluorouracil and the VP-16-selected cells were hyper-sensitive to cisplatin. The rank order of resistance was similar in all the cell lines. Heterogeneity was observed in the DLKP-A cell line pertaining to the cells' drug- and radiation-sensitivity, cell doubling time and biochemical alterations associated with the acquisition of an MDR profile. MDR, induced by the exposure of DLKP cells to adriamycin, did not confer cross-resistance to radiation. Alternatively, irradiation of the DLKP cell line did not result in an MDR cell line. The DLKP-A clones could be mixed to generate a cell population with a toxicity profile similar to the original DLKP-A population.

All the MDR cell lines exhibited biochemical alterations; P-glycoprotein expression was increased and mdr1 mRNA levels were overexpressed in the MDR cell lines. Adriamycin accumulation by VP-16-selected cells was reflective of their P-glycoprotein levels and was completely reversible by the circumventing agents, verapamil and cyclosporin A. These circumventing agents also enhanced the toxicity of chemotherapeutic drugs to DLKP variants. In addition, topoisomerase II levels were altered in the drug resistant cell lines. A significant decrease in the enzyme level was found in DLKP/VP-8 and DLKP-A, with other cells exhibiting a slight decrease. The topoisomerase II alterations were due to a decrease in the α subunit and these modifications were confirmed by mRNA analysis.

Metabolic co-operation was observed in all the variants of the DLKP cell line. While functional gap junctions were observed within the cell lines, intercellular adriamycin transfer did not occur and in general, gap junction inhibitors did not enhance the cells' sensitivity to the drug. Sensitivity to chemotherapeutic drugs was found to be a cell density-dependent phenomenon. Conditioned medium from a highly resistant variant of DLKP-A enhanced the adriamycin-sensitivity of a low resistant variant. This suggests that a factor, secreted by the cells and whose secretion and/or production was enhanced by the presence of adriamycin, could modify the sensitivity of the cells to the drug.

Acknowledgements

I would like to thank my supervisor, Professor Martin Clynes, for his encouragement and guidance throughout the course of this project.

I am grateful to Dr. Michael Maher, St. Luke's Hospital for co-ordinating the clinical link required for the radiation work. I would also like to thank Ms. Pauline O'Neill for her expert technical assistance in the radiation work.

I would like to thank Drs. W.T. Beck and G. Astaldi-Ricotti for their generous gifts of anti-topoisomerase II antibodies.

I appreciate the help and encouragement I received from former and present members of the NCTCC.

I am grateful to Ms. Lorraine O'Driscoll for her help with the PCR aspects of this research work, to Dr. Kevin Kavanagh for his helpful advice in the planning and analysis of the experimental work, to Ms. Shirley McBride for her helpful guidence in the Western blot analysis and to Dr. Mohamad Saleh for his design of the programme for the analysis of toxicity assay data.

Special thanks are owed to Joanne Keenan, Dr. Geri Grant, Lorraine O'Driscoll, Dr. Carmel Daly, Dr. Kevin Kavanagh, Dr. Gerry Doherty and Dr. Keara Hall for their help during the writing of this thesis. Their help in the typing, proof-reading, correction and printing of this thesis was immense.

I am also grateful to Dr. Eilis Corrigan for her help with the proof-reading and printing of this thesis and to Bernie for her help with the typing of the bibliography.

A special thanks to my family for their continual support and encouragement.

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1. Introduction

1.1 General Introduction

Lung cancer is the leading cause of cancer death, with approximately 90% of affected patients dying within one year of diagnosis (Makela *et al.*, 1991). On the basis of both clinical behaviour and prognosis, lung cancers can be divided into small cell lung cancer and non-small cell lung cancers (DeVita *et al.*, 1985). Small cell lung cancer accounts for approximately 25% of all new cases of lung cancer. Long-term survival or complete remission in small cell lung cancer is directly related to its response to cytotoxic therapy; surgical treatment is not a useful alternative due to the aggressive growth and metastatic activity of the cancer. Non-small cell lung cancers include adenocarcinomas, which account for 30% of all new lung cancer cases, squamous cell carcinoma, accounting for 25% of new cases and large cell carcinomas which account for approximately 15% of new lung cancer cases. For all non-small cell lung cancers, resection of the primary tumour is the major treatment used.

Due to its poor prognosis, lung cancer accounts for 22% of all cancers in men and 9% in women and is responsible for 35% of cancer-related deaths in men and 17% in women, research is ongoing into improving treatment for lung cancer patients. Small cell lung cancer differs both biologically and clinically from other histologic forms of lung cancer. Its unique response to chemotherapy, compared with that of non-small cell lung cancer, is well established and has led to improved survival and a small, but definite, cure rate. Combination chemotherapy is standard therapy for both extensive and limited small cell lung cancers. However, second line chemotherapy, after relapse following the initial combination chemotherapy, is largely unsuccessful, with only modest response rates of brief duration.

Therefore, non-small cell lung cancer tumours exhibit intrinsic chemoresistance, whereas small cell lung cancer tumours manifest an acquired drug resistance. The mechanisms underlying the various forms of lung cancer chemotherapy are complex and not completely understood. To help determine the molecular mechanisms of drug resistance in human lung tumours, researchers have made resistant sublines from drug-sensitive lung cell lines, by exposing them to cytotoxic drugs *in vitro*. The sensitive parental cell line can act as a control for the mechanism of resistance being investigated. Drug-resistant cell sublines have been derived, not only from lung cell lines, but from cell lines of all tumour types. From analysis of these cell lines, and tumour samples, it is apparent that a range of mechanisms of resistance exist in cells and tumours. These resistance mechanisms may be occurring individually or in combinations. The two most widely studied, and most commonly found, are the overexpression

of the P-glycoprotein drug efflux pump and alterations in DNA topoisomerase II (topoisomerase II) (Clynes *et al.*, 1993; Volm *et al.*, 1991, 1992(a,b)). However, the altered expression of other proteins has been noted in resistant cells and/or tumours; such proteins include glutathione-S-transferase (GST) and proteins homologous to, but distinct from, P-glycoprotein.

1.2 P-glycoprotein

In general, multidrug resistant (MDR) cells, generated by exposure to chemotherapeutic drugs (either anthracyclines, colchicine, vinca alkaloids, actinomycin D or epipodophylltoxins), express cross-resistance to the range of the other structurally unrelated drugs but exhibit unaltered sensitivity to 5-fluorouracil and platinum compounds (Clynes *et al.*, 1993; Nielson and Skovsgaard, 1992; Beck and Danks, 1991; Hill, 1993). Although a number of biochemical changes have been associated with MDR cells, one of the most important early biochemical studies on MDR was the identification of the overexpression of a 170 kDa. membrane associated glycoprotein, P-glycoprotein, in resistant cells, by comparison with their sensitive parental counterparts (Juliano and Ling, 1976). It has been well documented that a major determinant of the MDR phenotype is the overproduction of this protein, which acts as an energy dependent drug efflux pump (Safa *et al.*, 1987). Therefore, the protein functions by removing drug from the cells and thereby facilitating their survival in the presence of concentrations of drugs which are toxic to the parental cells.

Gene-associated cytogenetic abnormalities were observed in the early established MDR cell lines (Biedler *et al.*, 1980; Baskin *et al.*, 1981). Microscopically detectable aberrant chromosomal structures usually appear as non-banded homogeneously staining regions, abnormally banding regions of metaphase chromosomes or small paired extrachromosomal chromatin bodies, termed double minutes. These cytogenetic abnormalities have been observed in cell lines selected for vincristine-resistance (Meyers *et al.*, 1985; Benard *et al.*, 1989; Fojo *et al.*, 1987) and adriamycin-resistance (Slovak *et al.*, 1987; Fairchild *et al.*, 1987; Redmond *et al.*, 1990; Clynes *et al.*, 1992). The finding of these genetic markers in MDR cells suggested that MDR was associated with gene amplification. Genetic analysis of resistant cell lines has revealed that a family of genes, the mdr gene family, is responsible for the acquisition of a drug resistance profile by cells. This family consists of two members in humans and three in rodents, as shown in Table 1.1.

Table 1.1 : The MDR gene family

Class	Human[1]	Hamster[2]	Mouse[3]	
			Scheme A	Scheme B
I	mdr1	pgp1	mdr3	mdr1a
II		pgp2	mdr1	mdr1b
III	mdr3/2	pgp3	mdr2	mdr2

[1] Van der Bliek *et al.* (1986(a, b)); Roninson *et al.* (1986); Chen *et al.* (1986; 1990a); Schinkel *et al.* (1991); Chin *et al.* (1989)
[2] Endicott *et al.* (1987); Ng *et al.* (1989).
[3] Gros *et al.* (1986(a, b); 1988); Hsu *et al.* (1989); Croop *et al.* (1989); Raymond *et al.* (1990)

Both the hamster and mouse mdr gene families include three members, designated pgp1, pgp2 and pgp3, in the case of hamster. The human mdr cDNA sequence which shares homology with the hamster pgp3 gene was designated mdr3 by Van der Bliek *et al.* (1988(b)). This corresponds to the human mdr2 gene described by Roninson *et al.* (1986). Among the two rodent homologues of the human mdr1 gene, the pgp1 in Chinese hamster and the mdr3 in mouse (also called mdr1a) showed the most sequence similarity to human mdr1.

Although mdr1 and mdr3 genes encode highly homologous proteins, suggesting that the mdr3 gene product also functions as an efflux pump, the mdr3 gene does not appear to be involved in MDR. Expression of the human mdr3 or mouse mdr2 cDNA, inserted into mammalian expression vectors, does not result in resistance to drugs associated with MDR (Gros *et al.,* 1988; Van der Bliek *et al.,* 1988(b)).

The two human mdr genes are located on chromosome 7; at position 7q21.1 and approximately 300bp apart. The human mdr locus covers about 230kb (Lincke *et al.*, 1991). The mdr3 gene is located downstream of the mdr1 gene and both genes are transcribed in the same direction (Lincke *et al.*, 1991). The nucleotide sequence of all MDR genes share extensive homology. Sequence conservation in the nucleotide-binding regions is maintained by gene conservation (Endicott *et al.*, 1987; Van der Bliek *et al.*, 1988; Hsu *et al.*, 1989). The mdr1 gene includes 28 introns, 26 of which interrupt the protein coding sequence. Although both halves of the protein-coding sequence are composed of approximately the same number of exons, only two intron pairs, both within the nucleotide-binding domain, are located at conserved positions in the two halves of the protein (Chen *et al.*, 1990a). This suggests that the mdr1 gene originated by the fusion of genes coding for two related but distinct proteins, rather by internal duplication. The mdr1 gene codes for a 4.5-5.0 kb mRNA that is overexpressed in MDR cell lines of human, mouse and hamster origin (Roninson *et al.*, 1986; Gros *et al.*, 1986(a,b)).

Analysis of nucleotide and deduced amino acid sequences of human, Chinese hamster and mouse P-glycoprotein genes (Gerlach *et al.*, 1986; Gros *et al.*, 1986a; Chen *et al.*, 1986) provide suggestive evidence for the structure and function of the protein. The gene products are of approximately 1,280 amino acids (Chen *et al.*, 1986) and contain two homologous halves, joined by a linker region. That P-glycoprotein requires the presence of both intact halves to confer drug-resistance has been confirmed by transfection studies (Currier *et al.*, 1989). The degree of homology varies throughout the sequence and is much stronger near the C terminus, than in the rest of the protein. Each half has a short hydrophilic amino terminal segment, six hydrophobic membrane spanning domains that form three transmembrane loops and a hydrophilic carboxy-terminal region containing consensus sequences for a nucleotide-binding site. There are several potential glycosylation sites on the first external loop, near the amino terminal of the molecule. It is proposed that the 12 trans-membrane domains associate to form a pore or channel-like structure, through which substrates, including the drugs involved in MDR, are transported from the cytoplasm to the outside of the cell. Other researchers (Higgins and Gottesman, 1992) suggest that the protein acts as a 'flippase'. In this model, following drug binding to the protein in the inner lipid bilayer of the cell membrane, the protein flips the drug to the outer lipid bilayer and into the extracellular medium.

P-glycoprotein shares notable homology with various bacterial transporters (Gros *et al.*, 1986a; Chen *et al.*, 1986; Gerlach *et al.*, 1986). There is strong homology between P-glycoprotein and the bacterial α-haemolysin transport protein, HlyB (Gerlach *et al.*, 1986; Holland *et al.*, 1991). Homology also exists between P-glycoprotein and the yeast STE6 gene product responsible for the export of the hydrophobic a-factor pheromone, the *pf*mdr1 gene implicated in chloroquine resistance in *Plasmodium falciparum* (Foote *et al.*, 1989) and the human cystic fibrosis gene product, CFTR (Hyde *et al.*, 1990).

That mdr1 codes for P-glycoprotein has been verified by the transfection of the gene into drug-sensitive cells, resulting in the generation of MDR cells and the detection of gene product expression. The transfection of genomic DNA from MDR cells into sensitive cells, has resulted in the generation of cells exhibiting an MDR phenotype and overexpressing mdr1 gene product (Roninson *et al.*, 1991; Gros *et al.*, 1991; Robertson *et al.*, 1984). Transfection with full length mdr1 cDNA, in a mammalian expression vector, has also resulted in MDR cell lines (Gros *et al.*, 1986a). In contrast, transfection of the mdr3 gene does not confer drug resistance (Schinkel *et al.*, 1991; van der Bliek *et al.*, 1988). The transfection of cells with genomic DNA or mdr1 cDNA, require the selection of transfected cells, by exposing them to drug. The selective agent is generally one of the drugs whose exposure to cells naturally results in the generation of an MDR profile. Therefore, the extent of the cells' resistance to a drug, due to the presence of the foreign DNA, is unclear. However, insertion of DNA which codes for a

stretch of the antisense strand of mdr1 into MDR cells, has led to a decrease in P-glycoprotein expression and drug-resistance in the cell lines (Rivoltini *et al.*, 1990; Efferth and Volm, 1993; Clynes *et al.*, 1992, 1993). These experiments were carried out without exposing the cells to drug, and so the decrease in P-glycoprotein and drug-resistance can be directly associated with expression of the mdr1 gene. Kobayashi *et al.* (1994) also reported that transfection of an mdr1 ribozyme, in a mammalian expression vector, into a P-glycoprotein overexpressing human acute leukaemia cell line, resulted in a reduction of the level of resistance and the amount of mdr1 RNA expressed by the cell.

The drug cross-resistance profile exhibited by cells is associated with the sequence of the P-glycoprotein which is expressed. Several point mutations have been shown to influence the cross resistance profile conferred by P-glycoprotein, presumably because they are at sites involved in recognition, binding or release of drug. Dhir *et al.* (1993) observed that changing the serine residues located at position 939 of the P-glycoprotein coded by the mouse mdr3 gene and at position 941 of the P-glycoprotein coded by the mouse mdr1 gene, limited the ability of the resulting altered P-glycoproteins to confer resistance to vinblastine. When alanine or cysteine were inserted at these positions no alteration in the adriamycin and colchicine cross-resistance profile was noted. However, when tyrosine tryptophan or aspartic acid were inserted, almost complete loss of adriamycin and colchicine resistance was observed. Devine *et al.* (1992) also observed that transfection of the mutated form of P-glycoprotein, which was expressed by DC-3F cells selected for resistance to actinomycin D, conferred a cross-resistance phenotype on the transfected cells. However, the resistance profile was altered significantly from that conferred by transfection of cDNA coding for the normal protein. Choi *et al.* (1988) have found that drug-resistant KB cells exhibit preferential resistance to colchicine, accompanied by a decrease in vinblastine resistance. This resistance profile occurred simultaneously with a cluster of point mutations in the mdr 1 gene, leading to a $Glycine_{185} \rightarrow$ Valine substitution in the P-glycoprotein sequence. Transfection studies (Safa *et al.*, 1990) utilizing both the normal and mutated forms of the mdr 1 cDNA, have resulted in the conferring of altered cross-resistance patterns on cell lines.

P-glycoprotein has been detected in a variety of normal tissues, most notably tissue which is inherently resistant to chemotherapy. In humans, high levels of mdr1 mRNA and P-glycoprotein have been detected in liver, kidney, pancreas, adrenal glands, small intestine colon and placenta, whereas low levels are detected in most other tissue (Fojo *et al.*, 1987; Sugawara *et al.*, 1988; Cordon-Cardo *et al.*, 1989, 1990; Favrot *et al.*, 1991). The localisation of P-glycoprotein to specialised cells in human tissue (Tatsuta *et al.*, 1992; Sugawara *et al.*, 1990; Cordon-Cardo *et al.*, 1989, 1990; Thiebaut *et al.*, 1989), together with its homology to a number of transport proteins, implies that it may have a function in the secretion of toxins.

Chao Yeh *et al.* (1992) have described MDR breast cancer MCF-7 cells, with increased P-glycoprotein expression, which are cross resistant to benzo[a]pyrene, and the rates of efflux of benzo[a]pyrene were higher in MDR cells than in sensitive parental cells, suggesting the P-glycoprotein function in the efflux of benzo[a]pyrene in these cells.

P-glycoprotein is a functional component of the blood-brain barrier (Tatsuta *et al.*, 1992) and it is confined to the luminal surface of epithelial cells which comprise the capillary vessels of the brain and spinal cord (Sugawara *et al.*, 1990). P-glycoprotein has also been localised to the luminal surface of the brush border of proximal renal tubule; the luminal surface of biliary hepatocytes and on the mucosal surface of the columnar epithelial cells of the small and large intestine (Thiebaut *et al.*, 1987).

1.3 Modifications, other than P-glycoprotein, observed in MDR cells

Reduced drug accumulation and its perturbed distribution may account for a form of resistance observed in some cells, both those overexpressing P-glycoprotein and those exhibiting no alteration in P-glycoprotein levels. For drugs to be effective chemotherapeutic agents, they must be capable of entering cells and reaching their intracellular target. Any alterations in these capabilities may result in the cells exhibiting resistance to the drugs.

Alterations in the intracellular distribution of drugs has been reported in the literature. Coley *et al.* (1993) reported observing this shift in adriamycin location, from the nucleus to a cytoplasmic- or Golgi-location, in both P-glycoprotein overexpressing MDR cells and resistant cells not exhibiting altered P-glycoprotein expression. Gervasoni *et al,* (1991) also reported altered drug distribution in MDR cells which are both positive and negative for P-glycoprotein overexpression. Perturbed intracellular adriamycin localisation was noted in a number of MDR variants of the human lung cell line, SW-1573 (Keizer *et al.*, 1989; Schuurhuis *et al.*, 1991; Mulder *et al.*, 1993). A decrease in the nuclear- to cytoplasmic-fluorescence ratio (due to drug location) was observed, by comparison with drug sensitive parental cells and although some MDR variants did not overexpress P-glycoprotein, they accumulated less drug. Versantvoort *et al.* (1992) observed a decrease in daunorubicin accumulation in the SW-1573/2R120 and GLC_4/ADR variants of the human lung cell lines. In GLC_4/ADR cells, enhanced drug efflux was noted and the intracellular pH was decreased by 0.3 pH units.

The enhanced drug efflux in the absence of P-glycoprotein overexpression implies that other proteins, that also function as drug efflux pumps, may be overexpressed in the MDR cells. Cole *et al.* (1992a) have reported the expression of a 36 kDa. membrane-associated phosphorylated protein in the MDR small cell lung carcinoma H69AR, a cell line that does not overexpress P-glycoprotein. DNA sequencing and restriction endonuclease mapping revealed this protein to be a member of the annexin II/lipocortin family of Ca^{2+} and phospholipid-binding proteins. Cole *et al.* (1992b; 1993) also reported the overexpression of a member of the adenosine triphosphate (ATP)-binding transporter family, distinct from P-glycoprotein, in these cells. This multidrug resistant related protein (MRP) was also overexpressed in the small cell lung carcinoma selected for resistance to adriamycin (GLC_4/ADR) but not in ten MDR sublines of SW-1573, a non-small cell lung carcinoma (Zaman *et al.*, 1993). Direct evidence that MRP can confer MDR on cells has been presented by Grant *et al.* (1994). Transfection of MRP cDNA into HeLa cells has resulted in cells overexpressing a 190 kDa. membrane protein recognised by anti-MRP antibodies and which were resistant to adriamycin, vincristine and VP-16, but exhibited unaltered sensitivity to

cisplatin. MRP was also expressed, and associated with MDR, in HL60/ADR cells (Krishnamachary and Centre, 1993).

Altered expression of cell surface membrane proteins (of 24.5 kDa. to 34.5 kDa.) and non-membrane associated proteins (36 kDa., 47 kDa. and 55 kDa.) have also been observed in the H69AR and the MDR human fibrosarcoma cells (Mirski and Cole, 1989). The adriamycin-selected MDR human large cell lung cancer cell line, COR L23/R, which lacked P-glycoprotein but exhibited reduced drug accumulation, expressed a 190 kDa. membrane protein, distinct from P-glycoprotein (Barrand *et al.*, 1993). Marquardt *et al.* (1990) also reported the overexpression of a 190 kDa. membrane protein in MDR variants of HL-60 cells, overexpressing P-glycoprotein. In addition, March and Center (1989) noted that the leukaemia HL-60/ADR cells contained a 150 kDa. membrane glycoprotein. Although this protein was also expressed in drug-sensitive HL-60 cells, the protein in the resistant cells was modified by phosphorylation. An 85 kDa. membrane protein was also overexpressed in another drug-resistant leukaemia cell line, K562/ADR, but transfection studies have shown that expression of the protein does not result in the acquisition of a resistance profile (Sugimoto *et al.*, 1993). An 85 kDa. membrane protein and a 42 kDa. membrane-associated protein, were also detected in a mitoxantrone-resistant human MCF-7 breast cancer subline, using a polyclonal antibody directed against a synthetic peptide corresponding to the putative ATP binding domain of P-glycoprotein (Nakagawa *et al.*, 1992). A 95 kDa. membrane protein was expressed in two small cell lung cancer cell lines which possessed inherent resistance to VP-16 and has been detected in small cell lung cancer cell lines obtained from patients following relapse after chemotherapy (Doyle *et al.*, 1993).

Alterations in epidermal growth factor (EGF) receptor levels have been associated with the acquisition of an MDR profile by cells. However a direct correlation cannot be made between levels of EGF receptors in cells and their resistance to chemotherapeutic drugs. While Reeve *et al.* (1990) observed reduced levels of EGF receptors in the drug-resistant lung cell line, COR L23/R, Meyers *et al.* (1993) and Shin *et al.* (1991) observed the simultaneous amplification in the expression of EGF receptors and mdr1, in lung cells.

The acquisition of drug-resistance by cells is often associated with alterations in the levels of glutathione and glutathione-related enzymes (Cole *et al.*, 1990; Chen *et al.*, 1990b; Chao Yeh *et al.*, 1992; Kramer *et al.*, 1988). However, the transfection of GST cDNA, in mammalian expression vectors, into human cell lines has generally resulted in little alteration in the cells' resistance to drugs (Fairchild *et al.*, 1990; Leyland-Jones *et al.*, 1991; Lavoie *et al.*, 1992). GST is a multifunctional phase II detoxification enzyme that catalyses the conjunction of electrophilic substances and endogeneous xenobiotics to glutathione, forming stable secretable metabolites. As the formation of free-radicals is one of the mechanisms by

which adriamycin exerts its toxicity on cells (Booser and Nortobagyi, 1994), the expression of a detoxification system by cells may enable them to survive in the presence of toxic levels of adriamycin.

1.4 Topoisomerase II

In vivo, DNA topology is regulated by two classes of highly conserved enzymes known as type I and type II DNA topoisomerases. These enzymes catalyse the interconversion of topological forms of single stranded and double-stranded DNA, respectively. Wang (1969) reported that activity in *E. coli* extracts was capable of relaxing supercoiled DNA due to a single enzyme, topoisomerase I. Gellert *et al.* (1976) reported the presence of a DNA gyrase-like enzyme (topoisomerase II) which reduced the linking number of a double-stranded DNA ring in an ATP-dependent fashion. Topoisomerase II (EC 5.99.1.3), is an enzyme that alters the linking number of DNA by a factor of two. Topoisomerase II was isolated from mammalian cells (Liu *et al.*, 1980; Miller *et al.*, 1981a) and has been characterised as a homodimeric protein, with a molecular weight of approximately 170 kDa./subunit.

Topoisomerase II is required for the relaxation of supercoiled DNA, the unlinking activity necessary for fork propagation during DNA replication and segregation of newly synthesised daughter molecules. It is also necessary for chromosome condensation and is a structural component of mitotic chromosome scaffolds (Osheroff *et al.*, 1983; Yang *et al.*, 1987; Adachi *et al.*, 1991; Earnshaw *et al.*, 1985).

Topoisomerase II was first isolated from human cells, HeLa, by Miller *et al.* (1981). cDNA encoding human topoisomerase II has been sequenced and localised to a single copy gene on chromosome 17q21-22 (Tsai-Pflugfelder *et al.*, 1988). The topoisomerase II cDNA encodes a 6.2kb mRNA which in turn codes for a 1,530-amino-acid protein of deduced molecular mass of 174 kDa. This topoisomerase II shares extensive homology with other eukaryotic topoisomerase II enzymes. Purification of topoisomerase II from 4'-(-9-Acridinylamino)methanesulfin-*m*-anaiside (amsacrine)-resistant P388 leukaemia cells revealed two forms of the protein possessing catalytic activity; a 170 kDa. and a 180 kDa. form (Drake *et al.*, 1987). These two forms of the enzyme are now referred to as topoisomerase II-α and topoisomerase II-ß, respectively. The two enzymes possessed similar requirements for ATP. However, the optimum KCl concentration for catalytic activity of the 180 kDa. form was 20-30mM higher than for the 170 kDa. form, while the 170 kDa. form was the more thermally stable. When Chung *et al.* (1989) characterised cDNA clones coding for the two forms of human topoisomerase II, the cDNA coded for the 170 kDa. protein was identical to that previously described by Tsai-Pflugfelder *et al.* (1988). Topoisomerase II-ß shares extensive nucleotide (75%) and predicted peptide (92%) sequence homology with the first two-thirds of topoisomerase II-α (Chung *et al.*, 1989). The coding region of topoisomerase II-ß is 4,863 and encodes a polypeptide of predicted molecular weight of 183.705 kDa. (Jenkins *et al.*,

1992). The extreme N terminus and the last 25% of the C terminal domain show the greatest divergence. Topoisomerase II-ß has been mapped to chromosome 3p24 (Tan *et al.*, 1992; Jenkins *et al.*, 1992). Austin *et al.* (1993) also published the complete coding sequence of the topoisomerase II-ß isoform in HeLa cells, which coded for a 1,621-amino-acid protein. Davies *et al.* (1993) revealed a second form of topoisomerase II-ß, which arose due to differential gene splicing. The topoisomerase II-ß1 described by Davies *et al.* (1993) is similar to the topoisomerase II-ß described by Jenkins *et al.* (1992), while the topoisomerase II-ß2 encodes a protein containing an extra 5 amino acids after $valine_{23}$ of the topoisomerase II-ß1 sequence.

Topoisomerase II alters the topological state of DNA by passing an intact helix of DNA through a transient double-stranded break, which it generates in a separate DNA helix (Osheroff, 1989). Topoisomerase II produces the double-stranded breaks in a non-random manner with a 4 base pair (bp) 5' overhang. The two enzyme subunits covalently bind to the 5' phosphoryl ends of the DNA break (Liu *et al.*, 1983; Sander and Hsien, 1983). The removal of supercoils in DNA is accompanied by the hydrolysis of ATP to inorganic phosphate and the corresponding nucleoside diphosphate (Osheroff *et al.*, 1983). Magnesium is also required as a cofactor, but it is not known when the cofactors interact with the enzyme and/or DNA (Sander and Hsien, 1983; Osheroff, 1986). Magnesium is not necessary for enzyme binding to the DNA but is required for its activity (Osheroff, 1987; Osheroff and Zechiedrich, 1987). The beta- and gamma-phosphate groups, the 2'-OH of the ribose sugar and the C_6-NH_2 of the adenine ring of the ATP molecule are important for its interaction with the enzyme. The binding of ATP to topoisomerase II is sufficient to induce DNA strand passage events, but ATP hydrolysis is required for enzyme turnover (Osheroff *et al.*, 1983).

Osheroff *et al.*, (1991) suggested that the steps involved in topoisomerase II catalytic activity can be outlined as follows and as shown in Figure 1.1.

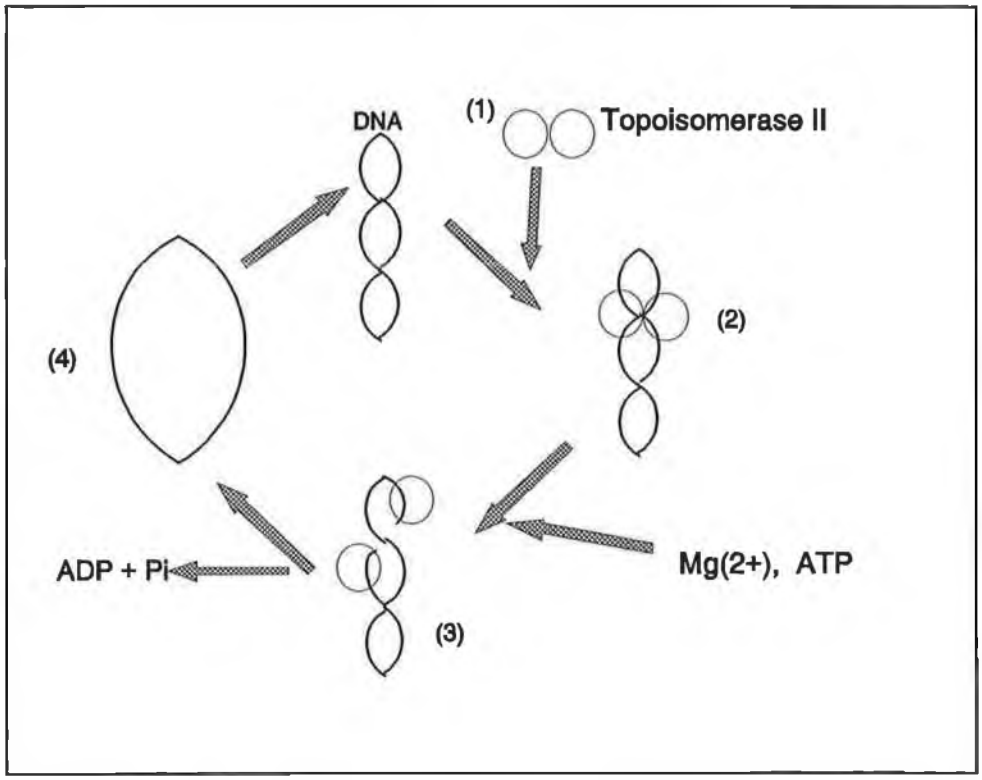
- Substrate recognition and binding to DNA
- DNA cleavage / ligation
- DNA strand passage
- DNA cleavage / ligation
- ATP hydrolysis
- Enzyme dissociation from DNA

Topoisomerase II binding to the double-stranded DNA initiates its catalytic reaction. The specificity of topoisomerase II binding to the DNA is determined by both the topological structure and the nucleotide sequence of the DNA. The enzyme preferentially interacts with negative supercoiled DNA rather than with relaxed molecules and also preferentially binds to DNA crossovers (Osheroff *et al.*, 1983; Zechiedrich and Osheroff, 1990). It therefore recognises DNA topology and can distinguish between its substrate and product.

Topoisomerase II-mediated cleavage of DNA is highly site-specific. However, different research groups have shown that topoisomerase II-cleavage sites vary in different cells. Kas and Laemmli (1992) found a major characteristic of a topoisomerase II binding site to be a prominent GC-rich core, flanked by an extensive array of oligo(dA).oligo(dT) tracts. Haung *et al.* (1992) found porcine spleen topoisomerase II cut preferentially in an energetically unstable region at the 3' side of an adenine, leaving the 5' protruding end with a 2 base pair stagger. Spitzner *et al.* (1990) have shown that topoisomerase II cleavage occurred predominantly at sites located within, or at the boundary of, alternating purine/pyrimidine tracts, whereas Fosse *et al.* (1991) demonstrated that thymidine is always present at the 3' end of at least one strand of the cleavage site and the dinucleotide AT or GC at the 3' end of the break plays a major role in complex stability. Drake *et al.* (1989) showed that topoisomerase II-α preferentially cut an AT-rich sequence and topoisomerase II-ß a GC-rich sequence, although topoisomerase II-α possessed the capacity to cut both types of sequences.

Following binding of topoisomerase II to DNA, the enzyme cleaves the DNA. DNA cleavage requires the presence of a divalent cation, and while Mg^{2+} is the cation most associated with the enzyme function, Ca^{2+} can also sustain DNA cleavage and re-ligation (Osheroff, 1987; Osheroff and Zechiedrich, 1987). Topoisomerase II breakage of DNA results in a 4 base pair 5' overhang (Sander and Hsieh, 1983; Liu *et al.*, 1983). Following cleavage, the subunits of topoisomerase II covalently attach to the 5' termini of the cleaved DNA *via* O^4-phosphotyrosine bonds (Rowe *et al.*, 1986a), resulting in the generation of a temporary structure known as the topoisomerase II-DNA cleavage complex. Succeeding the generation of a double-stranded DNA break, topoisomerase II passes a separate intact double-stranded DNA helix through the break, in a step requiring the presence of both the divalent cation and ATP. Upon completion of the DNA strand passage event, the cleaved DNA is re-ligated. This post-strand passage topoisomerase II-DNA cleavage complex is the target for many antineoplastic drugs (Robinson and Osheroff, 1990). As a prelude to enzyme turnover, bound ATP is hydrolysed to ADP and orthophosphate (Osheroff, 1989).

a.



b.

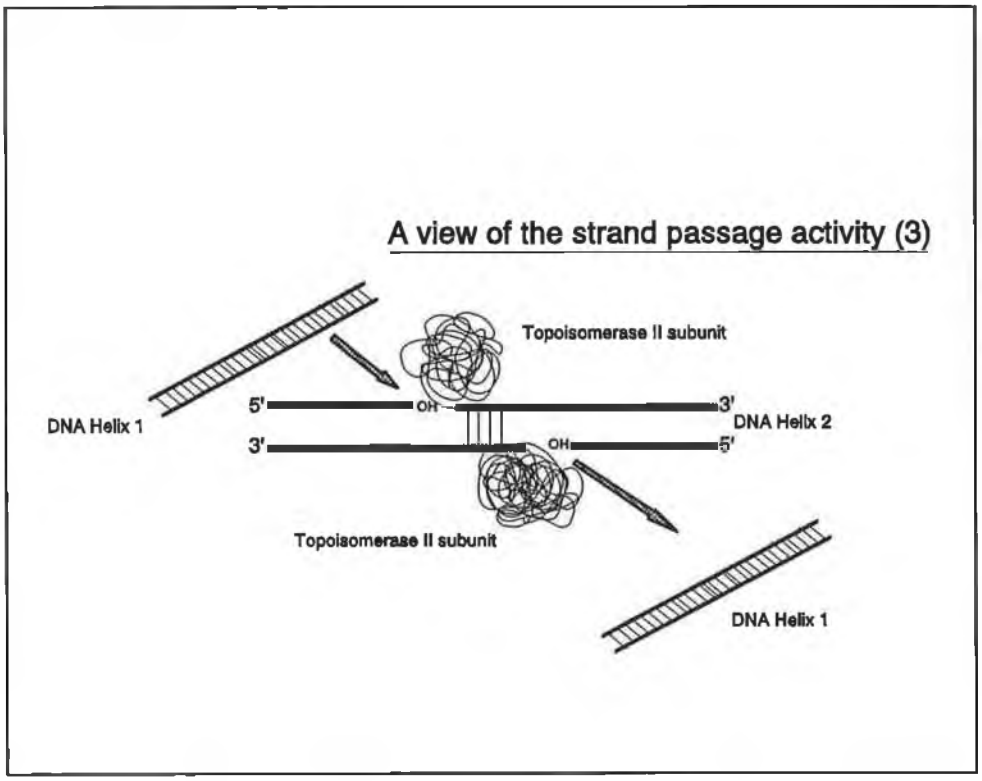


Figure 1.1 : Figure 1.1(a) depicts the catalytic cycle of topoisomerase II (as described by Osheroff *et al.,* 1991). The homodimeric enzyme is represented by paired circles (1). The reaction involves the recognition and binding of the enzyme to the supercoiled DNA (2), followed by the transient breaking of one double-stranded DNA helix (3). The enzyme is covalently bound to the 5' end of the broken DNA strand. A second DNA strand passes through the transient break. The enzyme then detaches from the DNA and the DNA is re-ligated (4). The reaction requires the presence of a divalent cation and the hydrolysis of ATP. A more detailed outline of the strand passaging event (3) is depicted in Figure 1.1(b).

Topoisomerase II is generally located in the nucleus but the protein is differentially localised during various stages of the cell cycle. Berrios *et al.* (1985) noted that topoisomerase II in *Drosophila* larvae was located in the nucleus. However, in interphase nuclei it was excluded from the nucleolus and equally distributed throughout the remainder of the nucleus. During mitosis the topoisomerase II was distributed diffusely throughout the cell. Monoclonal antibodies to the α and ß forms of topoisomerase II gave distinct staining patterns in HeLa and K652 human myeloid cells (Negri *et al.*, 1992; Zini *et al.*, 1992). Topoisomerase II-α antibodies stained mainly the nucleoplasm while anti-ß antibodies stained only the nucleolus (mainly the dense fibrillar component). Topoisomerase II-α was more abundant during logarithmic cell growth and the ß form was mainly present during the plateau phase. Wolverton *et al.* (1992) observed a 'patchy' topoisomerase II staining in nucleus of human rhabdomyosarcoma cells and exposure of the cells to a topoisomerase II inhibitor increased the intensity and homogeneity of nuclear staining in a subpopulation of the cells. Petrov *et al.* (1993) have localised topoisomerase II in Chinese hamster fibroblasts using antibodies described by Chung *et al.* (1989). Both the α and ß forms were present in the nucleolus and nucleoplasm. In the nucleoplasm, both forms localised at the periphery of heterochromatin regions. The nucleolus contained more detectable topoisomerase II-α and ß than the nucleoplasm and both forms localised in the fibrillar zone. During mitosis, both isoforms were detected in the cytoplasm.

Topoisomerase II is a sensitive and specific marker for proliferating cells (Heck and Earnshaw, 1986). The appearance of the enzyme parallels the onset of replication. Therefore, topoisomerase II levels are cell cycle dependent and the enzyme activity fluctuates with cell cycle progression (Chou and Ross, 1987). In synchronised HeLa cells topoisomerase II activity was 4-fold to 15-fold greater when the cells were in M phase than in S phase, while cells in G_1 and G_2 phases had an intermediate level of activity (Estay *et al.*, 1987). The activity was acquired abruptly in late G_2 and was lost abruptly in early G_1 phase. Kaufmann *et al.* (1991) also noted that the topoisomerase II levels in HL-60 human progranulocytic leukaemia cells increased 2-fold from G_1 to G_2/M and when the cells were induced to mature the levels of topoisomerase (both α and ß) diminished. Heck *et al.* (1988) also found that topoisomerase II underwent significant cell cycle dependent alterations in both amount and stability. During transition from M to G_1 much of the topoisomerase II was degraded. Topoisomerase II levels in primary human skin fibroblasts decreased to non-detectable levels due to serum starvation, while the re-addition of serum led to an increase in topoisomerase II (Hsaing *et al.*, 1988a). The topoisomerase II levels were decreased when the fibroblast cells were confluent. Chapuis *et al.* (1992) found a significant increase in VP-16 and VM-26 resistance in unfed plateau compared to exponential phase cells. This was reflected in the 5-fold to 15-fold increase in

quiescent cells present in the plateau phase.

The two forms of topoisomerase II are distinguishable by their association with different stages of the cell cycle. The 170 kDa. form decreases as the cells reach plateau and the 180 kDa. form, which is low during rapid cell proliferation, increases (Drake *et al.*, 1989). Prosperi *et al.* (1992) found levels of topoisomerase II-α to be 3-fold higher than those of the ß form in exponentially growing cells, whereas topoisomerase II-α, but not the ß form, was markedly reduced in plateau cells. Woessner *et al.* (1991) also found topoisomerase II-α and -ß to be differentially expressed in NIH-3T3 cells. Following serum starvation, topoisomerase II-α levels were undetectable until the cells reached the late S phase, peaked in G_2/M phase and decreased as cells completed mitosis. The ß form was constantly expressed when the cells entered the cell cycle. When the cells were induced to enter the G_0 phase (by serum starvation), levels of topoisomerase II-α decreased in parallel with the cells' exit from S and G_2/M phases, and were undetectable once all cells entered G_0. Topoisomerase II-ß was still present once all cells entered G_0 and was more tightly associated with chromatin than the α form. Differential expression of topoisomerase II-α and -ß was also noted during murine brain development (Tsutsui *et al.*, 1993). Topoisomerase II-α levels were found to be high in embryonic brain and 2-day-old newborn murine cerebellum, followed by rapid decrease to undetectable levels 4 weeks after birth. Topoisomerase II-ß was present throughout embryonic and postnatal stages. In developing cerebellum, topoisomerase II-α containing cells were confined to the external granular layer but topoisomerase II-ß was detected over the entire region. Therefore, topoisomerase II-α appeared to be associated with cell proliferation and the ß form associated with a process not directly related to proliferation.

The activity of topoisomerase II in cells is not only modified by altering the enzyme levels but also the level of phosphorylation. Topoisomerase II is phosphorylated *in vivo*, with phosphate incorporation being highest in the G_2 and M fraction of the cell cycle (Heck *et al.*, 1989). Ackerman *et al.* (1985) showed that the phosphorylation of *Drosophila melanogaster* topoisomerase II stimulated its DNA relaxation activity. Topoisomerase II activity in Chinese hamster ovary cells was dependent on the degree of phosphorylation of the protein, not just the protein level (Burden *et al.*, 1993) and both were cell-cycle-dependent. The protein levels were maximum in G_2 phase, intermediate in S and M phase and minimum in G_1. Phosphorylation was maximum in M phase, lower in S, and lowest in G_2, while topoisomerase II activity was maximum in M, lower in G_2 and minimum in S phase. DeVore *et al.* (1992) also noted that the sensitivity of topoisomerase II to induce DNA strand breaks in the presence of VP-16 and amsacrine could be modulated by altering the phosphorylation state of the enzyme. Phosphorylated enzyme had an increased rate of DNA relaxation. Phosphorylation had little effect on the cleavage/re-ligation equilibrium in the absence of drug, but in the

presence of drug, phosphorylated enzyme has a decreased ability, compared to the unphosphorylated enzyme, to cleave DNA and so alters the enzyme's ability to form DNA strand breaks.

A range of drugs currently in use as chemotherapeutic agents function *via* topoisomerase II-mediated cell kill. This is accomplished by stabilising a ternary complex that is formed between the cleaved DNA and the covalently bound enzyme (Liu, 1989). VP-16 and VM-26 stimulated site-specific DNA cleavage by purified calf thymus topoisomerase II (Ross *et al.*, 1984) and Chen *et al.* (1984) have shown that VP-16 and VM-26 interfere with the breakage/re-ligation reaction of topoisomerase II, by stabilising the cleavable complex. *In vitro*, topoisomerase II-mediated DNA damage by adriamycin has been demonstrated by Tewey *et al.* (1984). Nelson *et al.* (1984) noted that mammalian topoisomerase II was a primary target for amsacrine and that drug-induced complex formation between the DNA and enzyme was the cause of cytotoxicity. Investigations into drug induced DNA damage resulted in specific cleavage at different sites in the presence of different drugs (Pommier *et al.*, 1991(a, b); 1992; Capranico *et al.*, 1990) whereby a preference for A at the 3' end in the presence of doxorubicin and C at the 3' end in the presence of VM-26 was noted.

Quantitative and qualitative alterations in the α form of topoisomerase II have been associated with cellular resistance to chemotherapeutic drugs. These modifications include alterations in both enzyme level and activity, genetic mutations and post-translational modifications. Topoisomerase II-mediated drug-resistance may be the only identified mechanism of resistance in cells or it may co-exist with alternative resistance mechanisms. To gain a better understanding of the clinical implications of the modification of topoisomerase, especially in relation to chemotherapeutic drug resistance, topoisomerase II has been studied in MDR cell lines.

Decreased topoisomerase II levels, and therefore reductions in enzyme activity, are commonly noted characteristics of drug-resistant cells. Kamath *et al.* (1992) observed that both topoisomerase II levels and the enzyme's cleavage activity were decreased in MDR cells of P388, while Friche *et al.* (1991) also found decreased topoisomerase II levels and activity in MDR EHR2/DNR+ cells that did not overexpress P-glycoprotein. Topoisomerase II and I activity is decreased in a P388 cell line selected for resistance to amsacrine (Per *et al.*, 1987) and both the topoisomerase II protein and the α and ß mRNA levels was decreased in MDR K562 leukaemia cells selected for resistance to VP-16 (Ritke *et al.*, 1994). VP-16-induced damage and DNA-topoisomerase II-cleavable complexes also disappeared faster, following VP-16 removal, in the resistant cells. Hoban *et al.* (1992) showed that MDR Chinese hamster ovary cells, derived by exposing the cells to increasing concentrations of adriamycin, exhibited several mechanisms of resistance including P-glycoprotein and GST overexpression and a

reduction in transcripts coding for topoisomerase II. Cole *et al.* (1991) found reduced levels of topoisomerase II in adriamycin-selected MDR NCI-H69 cells, when compared to the parental cells. The cells did not overexpress P-glycoprotein but were cross-resistant to vinca alkaloids, implying other mechanisms of resistance must also be present.

Alterations in topoisomerase II levels in MDR cell lines generally do not occur in isolation. The most commonly observed other modifications in cells exhibiting altered topoisomerase II are either the overexpression of P-glycoprotein and/or an alteration in the level and activity of topoisomerase I. The topoisomerase I enzyme is also involved in the modification of the topological form of DNA. However, unlike topoisomerase II, it modifies the topological state of DNA by transiently breaking and rejoining one of the two strands of the DNA helix. Modifications in topoisomerase I are generally associated with resistance to a range of drugs (most notably, camptothecin and its analogues) not included in the MDR profile of resistance (reviewed by Pessina, 1993; Liu, 1989). Nevertheless, alterations in topoisomerase II levels and activity may by balanced by modifications in topoisomerase I, thereby balancing the physiological functions of the topoisomerases. Ferguson *et al.* (1988) showed that human nasopharyngeal carcinoma KB cells, selected for resistance to VP-16, had decreased topoisomerase II levels and activity, while the topoisomerase I levels were increased slightly in the resistant cells. Lefevre *et al.* (1991) found a decrease in topoisomerase II activity and an increase in topoisomerase I and GST activity in MDR human breast cancer cells selected by exposure to amsacrine. Sugimoto *et al.* (1990) showed that camptothecin-resistant human lung (A549) and colon (HT-29) cell lines have reduced levels of topoisomerase I activity but increased topoisomerase II activity over the parental cells. However, Matsuo *et al.* (1990) found a decrease in topoisomerase II protein, mRNA and activity in VM-26 resistant KB cells, but topoisomerase I activity was similar in resistant and sensitive cells. De Jong *et al.* (1990) also showed that topoisomerase II and the decatenation activity in the cell extract were reduced in adriamycin-selected human small cell lung cancer cells which did not overexpress P-glycoprotein. Again the cells' topoisomerase I levels were unaltered. Therefore, although modifications in topoisomerase I are associated with alterations in topoisomerase II, the association varies from cell line to cell line.

Alteration in the quantity of topoisomerase II present in cells, is not the only modification of topoisomerase II observed in MDR cells. Post-translational modifications, resulting in either altered cellular localisation of the protein or an alteration in enzyme activity have also been reported.

Deffie *et al.* (1992) noted that while topoisomerase II levels and the enzyme's cleavage and catalytic activities were reduced in adriamycin-resistant Chinese hamster ovary cells and mitoxantrone resistant HeLa cells by comparison with their parental cell lines, no

differences were noted by Northern or Southern analysis. Glisson *et al.* (1986a) also found that a Chinese hamster ovary cell line resistant to VP-16 had levels of topoisomerase II activity similar to the parental cells but the enzyme in the resistant cells was more resistant to drug-induced stimulation of its activity. Although equal levels of topoisomerase II activity were observed in CHOK1 cells and a DNA-break-repair-deficient subpopulation of the cell line, the mutant cells were more sensitive to drug-induced cytotoxicity (Warters *et al.*, 1991). Schneider *et al.* (1994) also found that the human breast cancer cell line, MCF7/VP, selected for resistance to VP-16, possessed similar topoisomerase II-α and ß levels to the parental cells but topoisomerase II from the resistant cells was more resistant to drug-induced cleavage complex formation than the parental cells. No mutation was detected in either the putative ATP binding or breakage/resealing regions of either the α or ß form of topoisomerase II, suggesting a possible post-translational modification. Although the topoisomerase II activity in two VP-16 resistant KB cell lines was similar to that in the parental cell line (Takano *et al.*, 1991), the protein and RNA levels were lower in the resistant lines. VP-16 induced DNA cleavage was reduced in the resistant cells and the relative specific phosphorylation was higher in the resistant cells. Phosphorylation occurred at a serine residue in both parental and resistant cells.

In cases where the topoisomerase II protein was purified and characterised, although altered enzyme activity was observed in drug-resistant cells, the activity of the purified protein was similar to that of the enzyme isolated from sensitive cells. Campain *et al.* (1993) reported that a VP-16-selected melanoma cell line, which possessed equal levels of topoisomerase II as the parental cell line, was less susceptible to drug-induced cleavable complex formation. This was only observed when live cells or cell lysates were tested and not isolated nuclei, suggesting some component of the cytoplasm, cell membrane or a factor removed during nuclei isolation is responsible for the resistance to VP-16 exhibited by these cells. Zwelling *et al.* (1990) noted that the difference between VP-16-induced DNA cleavage in HT1080 cells and in an adriamycin-resistant variant was much greater in cell extract than in isolated nuclei, suggesting some cytosolic factor influences the susceptibility of cells to the DNA cleavage activity of drugs. De Isabella *et al.* (1990) noted that in MDR P388 leukaemia cells, which exhibited a 1.7-fold reduction in their topoisomerase II activity in comparison to the parental cells, although the drug stimulated cleavage was 10-fold lower in the resistant cells, partially purified topoisomerase II from the resistant and sensitive cells exhibited similar drug sensitivities. This suggests that a modulating factor was present in the resistant cells, which was lost during purification.

Modifications occurring in topoisomerase II protein levels and/or activity due to genetic mutations usually occur in one of two domains; the putative ATP-binding domain (located at amino acid 416 to 513, in the protein sequence) or the putative breakage/resealing

domain (located around the active tyrosine$_{804}$, which is situated at amino acid 686 to 882) (based on sequence homology with gyrase A of *E. coli* (Wyckoff *et al.*, 1989; Worland and Wang, 1989)).

Lee *et al.* (1992) found that the topoisomerase II-α gene in two independently derived MDR leukaemia cell lines, both of which had been selected with amsacrine, had arginine$_{486}$ mutated to a lysine. Sequence comparison indicated that the position of the mutation of the topoisomerase II-α corresponded to that of a point mutation in the gyrase B gene of *Escherichia coli* which conferred nalidixic acid resistance. Hinds *et al.* (1991) also noted that topoisomerase II in a human leukaemia cell line selected for resistance to amsacrine, HL-60/AMSA, was more resistant to drug induced inhibition of its activity than the enzyme of the parental line. A single base change was observed in the topoisomerase II genomic DNA of the resistant cells, which changed base pair 1493 from G → A and so also changed an arginine to a lysine in the enzyme. Danks *et al.* (1988) found that nuclear extracts of CCRF-CEM leukaemic cell lines selected for resistance to VM-26, CEM/VM-1 and CEM/VM-1-5, possessed similar levels of immunodetectable topoisomerase II but had altered levels of catalytic activity, when compared to the parental cells. The extract required higher ATP levels to achieve levels of activity equal to that of the enzyme in the parental cells (Danks *et al.*, 1989). Bugg *et al.* (1991) have detected the expression of a mutant topoisomerase II in these cells. The cDNA was found to contain an altered sequence, having a G → C base change in the region corresponding to the consensus ATP-binding region. The base change resulted in the replacement of the arginine at position 449 with a glutamine. Both the normal and altered sequences were present in the drug- resistant cells, while only the normal sequence was present in the parental cells. An extra mutation, the substitution of T_{2404} → A, resulting in serine$_{802}$ → proline was noted by Danks *et al.* (1993). However, no mutation in the putative ATP or DNA binding regions of 15 blast cell samples from patients with relapsed acute lymphocytic leukaemia, previously treated with VP-16 or VM-26, was noted. Chan *et al.* (1993) found that, although topoisomerase II expression was similar in drug-resistant, Vpm^R-5, and sensitive Chinese hamster ovary cell lines, the DNA cleavage activity in the resistant line was not stimulated by drug. A mutation from G → A at nucleotide 1478 was observed in the cDNA of the resistant cells, changing amino acid 493 from arginine to glutamine. This alteration is also located adjacent to the putative ATP binding site of topoisomerase II.

Although point mutations have resulted in the modification of topoisomerase II in drug-resistant cell lines, most notably by affecting their ATP-binding potential, alternative splicing of topoisomerase II mRNA has also been detected in a number of resistant cell lines. This production of an alternative length mRNA may result in production of a non-functional

protein, or of a functional protein which is located at an alternative site within the cell. Northern blot analysis of topoisomerase II in an adriamycin-resistant P388 leukaemia cells (Deffie *et al.*, 1989a) revealed two mRNA splices; a 6.6 kb transcript in sensitive cells and reduced by 7-fold to 8-fold in resistant cells, and a shorter transcript only present in resistant cells (Deffie *et al.*, 1989b). Southern blot analysis revealed two different alleles in the resistant cells, one identical to that found in sensitive cells but with lower gene copy number and a second allele containing a mutation. This suggests that the decrease in the topoisomerase II noted in the resistant cells (Deffie *et al.*, 1989a) was due to decreased gene levels and that the mutated gene codes for the shortened transcript. Mc Pherson *et al.* (1993) investigated this gene rearrangement and determined that the shorter 4.5 kb transcript only contained 3.5 kb of the topoisomerase II sequence, from the 5' terminus onwards. This encodes topoisomerase II-α until nucleotide 3494 with the remaining 956 bases encoding the promoter, exon 1 and part of the first intron of the murine retinoic acid receptor α gene locus, in an antisense orientation. Retenoic acid α receptor expression has also been reported in tumour samples expressing topoisomerase II (Keith *et al.*, 1993). Tan *et al.* (1989) noted that a gene re-arrangement had occurred in one topoisomerase II allele and that this gene was hyper-methylated in the amsacrine-resistant P388 cell line previously described by Per *et al.* (1987). The resistant cells exhibited reduced topoisomerase II activity and mRNA levels in comparison to the parental cells. The VP-16 resistant variants of the small cell lung carcinoma, H209, which exhibited decreased levels of topoisomerase II-α also possessed an α form of the protein with an apparent molecular weight of 160 kDa. (Mirski *et al.*, 1993). The level of corresponding mRNA was reduced and a second topoisomerase II-α mRNA of 4.8 kb was also present in the resistant cells. When Binaschi *et al.* (1992) characterised a rearrangement at the 3' terminus of one allele of the topoisomerase II gene, in a drug-resistant small cell lung carcinoma cell line NCI-H69, in addition to the normal 6.2 kb mRNA, the resistant cells expressed a 7.4 kb transcript. Although longer than the normal mRNA, the 7.4kb transcript lacked a substantial portion of the 3' terminus. Feldhoff *et al.* (1994) demonstrated that human small cell lung cancer cell line H209 contained the 170 kDa. form of topoisomerase II-α, but VP-16-selected variants contained a 160 kDa., and no 170 kDa., form of the enzyme. The resistant cells had a 5-fold decrease in topoisomerase II-α and a 2- to 3-fold decrease in topoisomerase II activity, although both forms of purified topoisomerase II-α have similar catalytic activities. The 160 kDa. topoisomerase II-α was located in the cytoplasm but the 170 kDa. topoisomerase II-α and topoisomerase II-ß were located in the nucleus, suggesting the deleted sequence was not necessary for catalytic activity but was required for nuclear localisation of the enzyme.

When the alterations in topoisomerase II observed in cell lines are correlated to clinical settings, it is observed that alterations in topoisomerase II exist in a large range of

tumours and that enzyme levels are a good indication of the susceptibility of the tumour to chemotherapy. In general, topoisomerase II is not altered in isolation in cancers, but co-exists with other genetic alterations.

In the clinical setting, highest levels of topoisomerase II are associated with cells undergoing cell proliferation. When Holden *et al.* (1992) measured topoisomerase II activity in a variety of normal and neoplastic tissues, highest activity was noted in spleen and thymus normal tissue. Therefore, the highest levels were observed in tissues that have high proliferation rates and the highest level of enzyme activity in neoplastic tissue was in the same range as that found in normal non-proliferating tissue. Holden *et al.* (1990(a, b)) also noted that tissue in which a high proportion of the cells were proliferating (melanoma, hepatocellular carcinoma) contained predominantly topoisomerase II-α and small amounts of the ß subunit, which corresponds to what is found in cell lines. In terminally differentiated organs or neoplastic tissue with low cell cyclings, topoisomerase II levels were undetectable in whole cell extracts. In term placenta this was because the tissue contained predominantly the ß form in low concentrations, which could be detected after partial concentration. However, Nelson *et al.* (1987) found elevated levels and activity of topoisomerase II in rat prostatic adenocarcinomas, compared with levels detected in normal rat dorsal prostate. A greater fraction of the adenocarcinoma cell nuclei containing detectable levels of topoisomerase II, than the normal tissue cell nuclei.

Most of the literature on topoisomerase II levels in tumour samples concentrates on the correlation between enzyme levels and the responsiveness of the tumour to chemotherapy. Ramachandran *et al.* (1993) found that adriamycin-induced DNA breaks and topoisomerase II activity inversely correlated to the degree of adriamycin sensitivity in 5 human melanoma cells. Van der Zee *et al.* (1994) also noted topoisomerase II catalytic activity to be lower in ovarian tumours which had undergone platinum/cyclophosphamide chemotherapy, in comparison with untreated tumours, while no difference in topoisomerase I activity was noted. Topoisomerase II-α was detected in 4 of the 8 untreated and 3 of the 9 treated tumours, while all tumours possessed topoisomerase II-ß (detected as the 150 kDa. protein, using the antibodies described by Negri *et al.* (1992)). Giaccone *et al.* (1992) correlated topoisomerase II levels and sensitivity to topoisomerase II inhibitors in eight human lung cancer cell lines. Kim *et al.* (1992) investigated the relationship between mRNA expression of mdr1, GST-π and topoisomerase II and the responsiveness of the tumour to adriamycin, in two oesophageal, two gastric and two colon cancers. Topoisomerase II levels were detected in all tumours and higher levels corresponded to responsiveness to adriamycin. Kim *et al.* (1991) also noted that topoisomerase II mRNA expression significantly correlated with the clinical responsiveness of 6 breast cancers, 3 hepatocellular carcinomas, 5 liver metastatic breast cancers and 1 gastric

cancer. Brain tumours, which are inherently resistant to chemotherapy, have been associated with overexpression of P-glycoprotein (Becker *et al.*, 1991; Favrot *et al.*, 1991; Cardon-Carlo *et al.*, 1989; Sugawara *et al.*, 1990). However, Mousseau *et al.* (1993) also correlated the resistance in 67 human brain tumours with altered topoisomerase II. Topoisomerase II RNA was absent in all normal brain and 64% of the tumours, with GST-π overexpressed in only 38% of tumours (mdr1 and dihydrofolate reductase were only occasionally expressed). Volm *et al.* (1993) noted an inter-relationship between increased expression of P-glycoprotein and GST-π and down regulation of topoisomerase II in 38 human renal cell carcinomas, while in previous studies (Volm *et al.*, 1991; 1992(a, b)) a relationship was noted between the cells' resistance, measured *in vitro*, and overexpression of P-glycoprotein or GST-π and down-regulation of topoisomerase II in renal and non-small cell carcinomas. Efferth *et al.* (1992) also found a tendency for increased P-glycoprotein and a decrease in topoisomerase II in kidney carcinoma cell lines, while Keith *et al.* (1993) showed that, in a survey of 50 breast cancer biopsy samples, prior to treatment, where 6 samples had an amplified erbB2 region, the topoisomerase II-α locus was co-amplified in 3 cases. Amplification was accompanied by high levels of topoisomerase II-α expression. Amplification of the retinoic acid α receptor was also observed in the same three samples.

1.5 Correlation between chemoresistance and radioresistance

Most patients with locally advanced lung cancer are not curable by either chemotherapy or radiotherapy alone and therefore, the combination of the two has been used to improve the treatment outcome. Radiation therapy is effective for the reduction of tumour volume in localised recurrent lung cancer after chemotherapy. However, there is a growing field of research examining the cross-resistance between anti-cancer drugs and radiation.

It is the size and localised nature of the energy-deposition events caused by ionizing radiations which explains its efficacy in damaging biological systems. Radiation's toxicity to cells is mediated by causing DNA damage. Radiation damage also delays the progression of cells through the cell cycle by blocking the cells at the G_2 phase of the cell cycle.

Cells may manifest resistance to radiation by a variety of mechanisms. One of the most important is through disturbance of the cell cycle. Although different cell lines have different sensitivities to radiation (Grenman *et al.*, 1991) and proliferating cells are more sensitive to radiation-induced cell kill than quiescent cells (Swarts *et al.*, 1990), many cells have a resistant period in their cell cycle. The radiosensitivity of cells differs in different phases of the cell cycle. Cells in late S phase have the highest probability of survival after radiation, while cells in the G_2/M phase are the most sensitive to damage by radiation (Iliakis and Okayasu, 1990; Northdurft *et al.*, 1992; Quiet *et al.*, 1991).

Radiation also exerts its toxicity on cells by the generation of oxygen radicals. Cells irradiated in the presence of oxygen are more sensitive to radiation than cells irradiated under conditions of severe hypoxia. Therefore, tumours may also exert a form of resistance by their physical location. Cells located at the centre of a tumour mass are generally oxygen deprived and so may be more resistant to radiation.

Cells with altered levels of GST may also exhibit altered sensitivity, as those enzymes are known to be involved in protecting cells from the detrimental effects of free-radicals, formed following cell irradiation. When Louie *et al.* (1985) characterised the radioresistance of sublines of the human ovarian cancer cell line A2780 (generated by incubation with stepwise increasing concentrations of either adriamycin (to a final resistance level of 100-fold for adriamycin), malphalan (10-fold resistant) or cisplatin (10-fold resistant)) and two additional cell lines which were established from drug-refractory patients, the melphalan-resistant, cisplatin-resistant and two drug-refractory cell lines were found to exhibit cross-resistance to radiation. These cells also had elevated glutathione levels with respect to the parental cell line. The adriamycin-resistant cells, which remained sensitive to radiation, had glutathione levels which were only marginally higher than those of the parental cell line.

Buthionine sulfoximine (which depleted glutathione), significantly, but not completely, increased the radiation sensitivity of the malphalan- and cisplatin-selected cell lines, suggesting glutathione levels play an important role in the radiation response, but not an absolute one. Twentyman *et al.* (1991) compared the radiation resistance of three human lung cancer cell lines (a small cell, a large cell and an adenocarcinoma) adapted to be cisplatin-resistant (3-fold to 5-fold). The cell lines were all melphalan-resistant. The parent lines show a wide range of glutathione content per cell, with the cell line with the highest glutathione content showing the greatest inherent resistance to cisplatin, melphalan and radiation. Although there was a correlation between glutathione content and radiosensitivity of the parent cell lines, acquired resistance to cisplatin was not accompanied by increased glutathione content, yet two of the three cisplatin resistant lines (the small cell and large cell carcinomas) showed significantly increased radioresistance. However, when Miura and Sasaki (1991) investigated the mechanism of radioresistance in a subclone of a murine squamous cell carcinoma, the radioresistance of the cells appeared to be mediated by a mechanism other than glutathione. Saito *et al.* (1991) also noted that glutathione levels alone could not act as an indicator of radiosensitivity in rat glioma cells.

A correlation exists between the clinical responses of small cell lung cancer tumours and the *in vitro* chemosensitivity and radiosensitivity of the respective cell lines (Tanio *et al.*, 1990). Therefore mechanisms associated with conferring chemoresistance to cells may also function in altering their radiosensitivity.

Alterations in the expression of the two main markers for chemoresistance in lung carcinomas, P-glycoprotein and topoisomerase II, may also affect the sensitivity of the cells to radiation. Topoisomerase II is an enzyme involved in regulating DNA topology, and quantitative and qualitative changes in the presence of the enzyme in cells may result in cells exhibiting resistance to radiation. Cunningham *et al.* (1991) found good correlation between the reduction in topoisomerase II activity in transfected murine cells and their radioresistance. Lynch *et al.* (1991) observed increased DNA relaxation in radioresponsive bladder carcinomas while Giocanti *et al.* (1993) observed that cells arrested in the G_2 phase of the cell cycle, following cell irradiation, proved to be hypersensitive to the topoisomerase II poison, VP-16.

Conflicting correlations between the expression of P-glycoprotein and radiosensitivity have been reported in the literature. Cases have been cited where cells, overexpressing P-glycoprotein following exposure to chemotherapeutic drugs, exhibit resistance to radiation (Shimm *et al.*, 1988; Zijlstra *et al.*, 1987). However, P-glycoprotein-expressing cells are also found to have unaltered radioresistance, by comparison with non-P-glycoprotein expressing parental cells (Glisson and Alpeter, 1992). The literature has also revealed cell lines selected following drug exposure, which do not express P-glycoprotein and which exhibit altered

radiosensitivity (Miller *et al.*, 1992). Some radiation-exposed cell lines have been cited as exhibiting altered P-glycoprotein expression and chemosensitivity, with other radiation-exposed cells have been found to exhibit unaltered chemosensitivity (Mattern *et al.*, 1991; Hill, 1993). In cases where P-glycoprotein overexpression was induced by radiation treatment, the resulting drug resistance profile was generally different from the resistance profile induced by chemotherapy treatment.

Shimm *et al.* (1988) reported an MDR human lymphoblastic cell line, selected for resistance to vincristine and overexpressing P-glycoprotein, which exhibited resistance to radiation. However, a cell line derived from a mouse tumour, generated by inoculating with the MDR cell line, showed loss of gene amplification (which mediated P-glycoprotein overexpression) but did not lose all its radiation resistance, suggesting radiation resistance was not totally due to P-glycoprotein expression. An adriamycin-selected MDR small cell lung cell line which overexpressed P-glycoprotein and exhibited resistance to radiation has also been described by Zijlstra *et al.* (1987). In contrast, a VP-16-selected MDR variant of a small cell lung cell line described by Glisson and Alpeter, (1992) overexpressed P-glycoprotein but exhibited unaltered sensitivity to radiation. Miller *et al.* (1992) found that an adriamycin-resistant human fibrosarcoma subline (HT1080/DR4), which was 222-fold resistant to doxorubicin and expressed a multidrug resistant profile by a mechanism independent of P-glycoprotein, exhibited cross-resistance to radiation.

Mattern *et al.* (1991) have reported a human epidermoid lung carcinoma cell line which acquired a P-glycoprotein overexpressing, drug-resistant profile, following radiation treatment. Nonetheless, in general, cell lines which acquire a drug-resistant profile due to radiation treatment exhibit an altered profile of cross resistance, in that they are not resistant to adriamycin but exhibit resistance to vincristine and VP-16. Hill *et al.* (1990a) reported such a profile in a range of radiation-treated cell lines, which did not overexpress P-glycoprotein. However, the MDR variant of the Chinese hamster cell line isolated by McClean and Hill (1994) overexpressed P-glycoprotein, but exhibited a similar drug-resistance profile as the non-P-glycoprotein expressing cells described by Hill *et al.* (1990a).

1.6 MDR lung cell lines

An analysis of the mechanisms of resistance in drug-selected and inherently-resistant MDR human lung cell lines may enable a better understanding of resistance in lung tumours. The first striking conclusion obtained from the information pertaining to MDR lung cell lines is that a range of mechanisms exists in the cells and the combination of resistance mechanisms displayed by the cells varies between different cell lines selected by exposure to the same drug, between the same cell lines selected with different drugs and even between two independent selections of the same cell line with the same drug. However, in general, all MDR variants of lung cell lines exhibit cross-resistance to the same range of chemotherapeutic drugs.

Twentyman *et al.* (1986a) and Mirski *et al.* (1987) established adriamycin-resistant variants of the small cell lung carcinoma, NCI H69, which exhibited similar cross-resistance profiles. However, their underlying mechanisms of resistance varied. The cell line described by Twentyman *et al.* (1986a) overexpressed P-glycoprotein and exhibited decreased drug accumulation (Twentyman *et al.*, 1986a). The MDR variant selected by Mirski *et al.* (1987), H69AR, possessed decreased topoisomerase II levels, with no alteration in its P-glycoprotein or drug accumulation levels. H69AR cells also overexpressed a range of membrane-associated and non-membrane-associated proteins not detectable in the sensitive parental cells (Mirski and Cole, 1989). These include a calcium- and phospholipid-binding protein (Cole *et al.*, 1992a) and MRP (Cole *et al.*, 1992b). The implications of the expression of these proteins, or of the altered topoisomerase II levels, on the resistance profile of the cell variant described by Twentyman *et al.* (1986a) are unknown, due to lack of information pertaining to their expression in the cell line. A P-glycoprotein-negative MDR human small cell lung cancer cell line, GLC_4/ADR (Zijlstra *et al.*, 1987), possessed decreased topoisomerase II activity by comparison with drug sensitive parental cells. However, the cells additionally exhibited decreased drug accumulation and rapid drug efflux, obviously mediated by an as yet unidentified transport protein. Furthermore, these cells possessed reduced glutathione levels (which were restored when the cells were grown in the absence of drug) and were cross-resistant to radiation (Zijlstra *et al.*, 1987; Meijer *et al.*, 1987; de Jong *et al.*, 1990, 1993).

Nygren *et al.* (1991) and Twentyman *et al.* (1986a) have described MDR variants of small cell lung carcinomas (U1650/150, U1285/250) and a large cell lung cell line (COR L23/R), respectively. Although none of the cell lines overexpress P-glycoprotein, all possess reduced drug accumulation, which in the case of COR L23/R cells was accompanied by a reduction in the nuclear localisation of the drug (Coley *et al.*, 1993). The cytoplasmic free Ca^{2+} concentration was elevated in U1690/150 and U1285/250 cells (Nygren *et al.*, 1991),

while the COR L23/R cells exhibited reduced EGF receptor levels (Reeve *et al.*, 1990).

VP-16-selected MDR variants of the small cell lung cell line, NCI H69, have been established by Glisson and Alpeter, (1992) and Jensen *et al.* (1992). The HCI H69/VP cell line described by Jensen *et al.* (1992) exhibits an energy-dependent decrease in drug accumulation, implying the presence of an active efflux pump (possibly P-glycoprotein, but this has not been confirmed). The H69/VPR-2 cells established by Glisson and Alpeter, (1992) overexpressed mdr1 mRNA and, although no alteration in topoisomerase II activity was observed, the cells exhibited a decrease in the capacity of VP-16 to induce DNA strand breaks (which is a function associated with topoisomerase II activity). The VP-16-selected lung cells, SBC-3/ETP (a small cell lung carcinoma derived by Takigawa *et al.* (1992)), A549(VP)28 (a lung adenocarcinoma derived by Long *et al.* (1991)) and OC-NYH/VP (a small cell lung carcinoma derived by Jensen *et al.* (1991)) also exhibited individual mechanisms of resistance. The resistance profile of the SBC-3/ETP cells was mediated *via* overexpression of P-glycoprotein (resulting in decreased drug accumulation), reduction in topoisomerase II and topoisomerase I activity and an elevation in the cells' GST-π levels (Takigawa *et al.*, 1992). However, resistance in the other cell lines appeared to be as a result of P-glycoprotein-independent modifications. While direct evidence of unaltered mdr1 expression was presented for A569(VP)28 cells (Long *et al.*, 1991), the unaltered drug accumulation exhibited by OC-NYH/VM cells (Jensen *et al.*, 1991) also implies unaltered mdr1 expression.

When a cisplatin-resistant variant of NCI H69 cells was characterised, the cell line's MDR profile appeared to be supported, exclusively, by an increase in GST and metallothionine II levels (no changes in P-glycoprotein, drug accumulation or topoisomerase II were detected) (Hong *et al.*, 1988; Kasahara *et al.*, 1991). In contrast, the MDR profile of a small cell lung carcinoma, established from a patient following combination chemotherapy and radiotherapy, appeared to be supported not only by increased GST-π and glutathione levels, but by the overexpression of P-glycoprotein and reduced drug accumulation (Berendsen *et al.*, 1988; de Vries *et al.*, 1989). However, levels of drug accumulation by the cells did not correlate to their P-glycoprotein levels.

The multiple mechanisms of resistance exhibited by MDR lung cell lines are clearly demonstrated by MDR variants of a human squamous lung carcinoma, SW-1570, established by exposure to varying concentrations of adriamycin (Keizer *et al.*, 1989a). All variants exhibited an MDR profile to the same range of drugs, with the levels of cross-resistance a function of the concentration of adriamycin to which the cells were selected. However, the underlying mechanism of resistance varied. The cells resistant to lower levels of drug overexpressed neither mdr1 mRNA nor P-glycoprotein, whereas cells resistant to higher levels of drug overexpressed both (Baas *et al.*, 1990). Decreased drug accumulation, altered

intracellular localisation of the drug (the shift from nuclear to cytoplasmic localisation of the drug was dependent on the level of resistance exhibited by the cells) and increased cytosolic pH were also detailed mechanisms of resistance pertaining to these cells (Keizer *et al.*, 1989a; Schuurhuis *et al.*, 1991; Keizer and Joenje, 1989). The non-P-glycoprotein-expressing MDR variants also possessed lower levels of topoisomerase II than the parental cells (Eijdems *et al.*, 1992) and overexpressed a 110 kDa. vesicular protein (Scheper *et al.*, 1993). Varying cytogenetic aberrations were also observed and were dependent on whether the cells exhibited a P-glycoprotein- or a non-P-glycoprotein-mediated mechanism of resistance.

In conclusion, an analysis of the literature pertaining to drug-resistance in lung cell lines reveals that MDR can not be correlated to one specific mechanism. Different resistance mechanisms are equally important in various cell lines, either in isolation or in combination with other mechanisms. Although similar toxicity profiles are exhibited, in general, by MDR lung cell lines, the underlying mechanisms of resistance may not be inferred from this knowledge.

1.7 MDR in lung tumours

The correlation of the mechanisms of drug-resistance observed in lung cell lines to resistance observed in clinical samples is distorted by the lack of analysis of the full range of genetic and biochemical alterations in tumour samples. Predominantly, the genetic alterations examined in lung tumours are confined to P-glycoprotein, topoisomerase II and GST-π. The results of these investigations show variations in terms of the correlation between their level of expression and the sensitivity of the tumour to chemotherapy.

An investigation into mdr1 gene alterations, by DNA slot blot analysis, in 23 untreated non-small cell lung carcinomas by comparison with 14 adjacent normal lung samples showed low levels of DNA amplification in only one adenocarcinoma and two squamous cell carcinomas (Shin *et al.*, 1992). A concurrent investigation into mRNA overexpression (by RNA slot blot analysis) revealed only low mRNA expression in the six tumour samples tested. PCR analysis of mdr1 mRNA expression in 32 lung carcinomas, prior to and following chemotherapy, was detailed by Holzmayer *et al.* (1992). In the untreated tumours, 4 of 8 small cell lung carcinomas, 13 of 16 adenocarcinomas and 3 of 4 unclassified lung tumours had detectable levels of mdr1 mRNA. Analysis of lung tumours, following treatment with a range of MDR-associated drugs, revealed mdr1 mRNA expression in all cases studied (one small cell lung carcinoma, one adenocarcinoma and two unclassified lung tumours).

Volm *et al.* (1992a) investigated the association between the proliferation rate of 36 untreated non-small cell lung tumours, the expression of P-glycoprotein and GST-π and their sensitivity to adriamycin. No correlation between the proliferation rate of the samples and GST-π expression was observed. However, a link between GST-π and P-glycoprotein expression and a correlation between the resistance of the tumours to adriamycin and P-glycoprotein or GST-π expression was noted. Volm *et al.* (1992(a, b)) also correlated cells' resistance to chemotherapeutic drugs (measured *in vitro*), overexpression of P-glycoprotein or GST-π and down-regulation of topoisomerase II, in 94 non-small cell lung carcinomas and Volm *et al.* (1991) established a correlation between the expression of P-glycoprotein, GST-π, smoking history and adriamycin sensitivity for non-small cell lung cancers. However, no correlation with topoisomerase II expression was observed. In addition, seven small cell lung cell lines, established from biopsy samples, did not overexpress P-glycoprotein, even though three of the cell lines were established from patients following chemotherapy with MDR associated drugs (Milroy *et al.*, 1992).

An analysis of 86 untreated non-small cell lung carcinomas by Scagliotti *et al.*

(1991) also revealed that the lung tumour samples expressed P-glycoprotein. Fifteen of the samples had more than one in four cells stain positive for P-glycoprotein. However, heterogeneity in the level of staining was observed. While some tumour samples possessed a few cells that stained positive for P-glycoprotein, others were composed of nearly all P-glycoprotein-positive cells, revealing heterogeneity within tumours in relation to the expression of MDR-associated proteins.

1.8 Clonal variation in tumour cell lines.

It is generally assumed that most, if not all, tumours begin from a single mutant cell. However, the constantly expanding supply of literature on inter- and intra-tumoural heterogeneity has led to the observation that cancers are composed of complex subpopulations of cells. Tumours are heterogeneous in several ways. There is the heterogeneity among cancers in different individuals who nominally have the same type of disease. A second type is that seen within the same patient over the course of time, as the tumour progresses. Heterogeneity is also seen within a single tumour at any given time. Therefore, it must be assumed that mechanisms of the origin of tumour heterogeneity also include the generation of diverse subpopulations from a single clone, a process which may be due to random genetic mutation and/or production of cellular variants, as in normal tissue differentiation.

At any given time inter-tumour and intra-tumour heterogeneity may be occurring concurrently. When Wilson *et al.* (1993) investigated heterogeneity in the potential doubling time of 30 colorectal carcinomas, by assaying multiple biopsies from each tumour sample, both inter- and intra-tumour variations were observed. Von Hoff *et al.* (1986) analyzed the drug sensitivity profiles of 99 pairs of tumour samples. Though the sensitivity profiles generated by the two different samples of the same primary tumour were quite similar, tumour heterogeneity was observed when primary tumours and their metastases were tested. The agreement between the drug sensitivity profiles of different metastases was also poor. Nyugen *et al.* (1993) noted that tumour heterogeneity appeared to be a common characteristic of early cervical carcinomas, when the carcinomas were analyzed in terms of their DNA index. Tumour heterogeneity was observed in primary and metastatic tumours and, using DNA index to determine tumour origin, 60% of the metastases could not be traced to their primary origin.

Histological examination of tumour samples often reveals considerable differences in the morphology of cancer cells. The co-existence of multiple subpopulations of cells within a single tumour has been repeatedly demonstrated in diverse types of tumours. These include melanoma (Tsuruo and Fidler, 1981; Jelinek *et al.*, 1993), lymphoma-leukaemia (Okamura *et al.*, 1981), sarcoma (Okabe *et al.*, 1983) and carcinomas (Vindelov *et al.*, 1980; Worsham *et al.*, 1993; Nyugen *et al.*, 1992). Tumour heterogeneity is manifested by a variety of phenotypic differences, including differences in cellular morphology and tumour histopathology (Okabe *et al.*, 1983; Maekawa *et al.*, 1993; Bronson *et al.*, 1980), growth properties (Fried *et al.*, 1993), antigenic properties (Fried *et al.*, 1993; Shapiro *et al.*, 1981), the ability to metastasise (Maekawa *et al.*, 1993), karyotypic differences (Worsham *et al.*, 1993; Berens *et al.*, 1993) and sensitivity to chemotherapeutic drugs (Dolfini *et al.*, 1993; Tsuruo and Fidler, 1981; Allalunis-Turner *et al.*, 1993) and radiation (Powell and McMillan, 1991;

Allalunis-Turner *et al.*, 1993; Allam *et al*, 1993).

Formal proof of the existence of tumour subpopulations requires their isolation and characterisation. This has now been accomplished with a growing list of cancers from a broad range of organs, using cell cultures which have been maintained and passaged *in vitro* prior to cloning. Maekawa *et al.* (1993) established two highly metastatic tumour clones, D-1 and F-3, from a single murine skin squamous cell carcinoma, both of which had distinct morphology and features undergoing pulmonary metastases. Fried *et al.* (1993) characterised five osteogenic cell subpopulations of the bone marrow stroma cell line, MBA-15. The clones differed in their morphology, proliferation rate, quantities and distribution of extracellular matrix protein, levels of alkaline phosphatase activity and activation of adenylate cyclase by parathyroid hormone and/or prostaglandin E. Berens *et al.* (1993) isolated three clonal cell lines from the canine astrocytoma cell line DL3580, which exhibited varying chromosome modal number. Bronson *et al.* (1980) characterised a cell line, that had been established from a metastasis of a human testicular germ cell tumour, that consisted of four histological types of tumour cells. Okabe *et al.* (1983) isolated 25 clonal strains from a single human sarcoma of the stomach and two different types of clones have been identified by their morphology and behaviour *in vitro*. One set of clones produced tumours in mice while the other did not.

Only rarely have the subpopulations been obtained directly from the patient. This raised the possibility that the production of heterogeneous variants was a consequence of the *in vitro* environment and occurred sometime after removal of the tumour from the patient. However, a number of published results have shown that heterogeneity is a characteristic of tumours *in vivo*. Shapiro *et al.* (1981) karyotyped tumour cells from fresh samples of human gliomas within 6-72h. after surgery. An array of unique karyotypes was found in each tumour. Simultaneously, dissociated tumour cells were cloned by limiting dilution plating of the cells and the clones were karyotyped. By matching karyotypes of the clones with those in the fresh samples, it was possible to show that the clones were present in the tumour at the time of surgery. In this way, eight gliomas were each shown to contain between 3 and 21 subpopulations. The different clones from the same tumour also differed in morphology and growth kinetics; in addition, antigenic heterogeneity has also been reported in clones derived from one of the glioma tumours. Therefore, heterogeneity is a phenomenon detectable in tumour samples and is not purely a characteristic of cells in culture. Mehle *et al.* (1993) investigated tumour heterogeneity in renal carcinomas in ten patients, by DNA fingerprint analysis. Two tumour samples were taken from four patients and three samples from six patients. DNA fingerprinting of the samples revealed variation in the fingerprint patterns between samples from different parts of the same tumour, where up to three different cell clones were detected in some tumours.

It is often assumed that long term culture of cell lines results in a decrease in heterogeneity, due to the selection of one population, by the culturing conditions. The presence of distinct subpopulations in established cell lines argues against this hypothesis. Worsham *et al.* (1993) observed heterogeneity in a cell culture established from skin; Fried *et al.* (1993) demonstrated heterogeneity in a bone marrow stroma cell line; Vindelov *et al.* (1980) have shown that clonal variation existed in lung cultures; Bronson *et al* (1980) investigated tumour heterogeneity in testicular cancer; Fey *et al.* (1993) observed variation in gastric cell lines; Zangwill *et al.* (1993) have shown that heterogeneity is a characteristic of an ovarian carcinoma in culture; Dolfini *et al.* (1993) have isolated clones from a colon carcinoma cell line which exhibited variations in their sensitivity to chemotherapeutic drugs.

Additional evidence that human cancers contain tumour cell subpopulations comes from comparison between primary tumours and metastases. Tanigawa *et al.* (1984) observed variability in the chemosensitivity of different parts of the same tumour and between primary and metastases, although a strong correlation was observed between the chemosensitivity of metastases from different sites. Schlag and Schremi (1982) also observed considerable differences in primary tumours and their metastases, taken simultaneously from the same patient, with respect to their sensitivity to a range of chemotherapeutic agents. Evans *et al.* (1986) examined the adriamycin-sensitivity of two tumour cell subpopulations derived from a primary 13762 rat mammary adenocarcinoma, clone MTC, and a lung metastasis in the same animal, clone MTLn3. The MTC cells were significantly more resistant to adriamycin than the MTLn3 cells.

Heterogeneity in the metastatic phenotype may result from the metastatic tumours being composed of cell populations which only consist of a small percentage of the primary tumour. Subpopulations never existing in the primary tumour may exist in the metastasis, due to spontaneous genetic variation or induction by the primary treatment. Therefore, different characteristics, such as sensitivity to chemotherapeutic drugs or radiation, may exist in different metastases arising from a single primary tumour. As the metastases may bear little resemblance to the primary tumour in terms of chemosensitivity, treatments used in the eradication of the primary tumour may be ineffective in the treatment of the metastases.

The observation that tumours are made up of subpopulations of varying characteristics poses a problem for the development of effective therapy. Tumour subpopulations differing in susceptibility to both chemotherapy and radiotherapy have been characterised. The existence of resistant variants within primary cell lines, and presumably tumours, may have serious implications for therapy, since the existence of even one cancer cell at the end of treatment could result in a regeneration of the tumour. If this cancer cell possesses an altered, more aggressive, phenotype, it would result in a poor prognosis for future therapy.

Allalunis-Turner *et al.* (1993) concurrently established two aneuploid cell lines which differ in their inherent sensitivity to ionising radiation and chemotherapeutic agents, from a single specimen obtained from a patient with glioblastoma. M059J cells were approximately 30-fold more sensitive to radiation than were M059K cells and M059J cells were also more sensitive to bleomycin, nitrogen mustard and N,N-bis(2-chloroethyl)-N-nitrosourea. Tsuruo and Fidler (1981) demonstrated that differences in drug response exist among cells populating parental tumours; the tumours investigated were of three murine tumours, the B16 melanoma, the K-1735 melanoma and the UV-2237 fibrosarcoma and the A-375 human melanoma. Allam *et al.* (1993) observed significant intra-tumoural heterogeneity of radiation sensitivity in some malignant gliomas, by comparing the intrinsic radiation sensitivity of different glioma sublines derived from the same tumour. Powell and McMillan (1991) also observed clonal heterogeneity in response to ionising radiation in a human glioma cell line, IN859. Significant differences in DNA repair fidelity occurred between the most radioresistant and sensitive clones, and between the sensitive clone and the parental cell line. Dolfini *et al.* (1993) isolated two cellular clones of LoVo colon adenocarcinoma cells. LoVo C1.7 was intrinsically resistant to adriamycin while LoVo C1.5 showed the same resistance index as the mixed parental population. Therefore, the phenotype of the cell line is not dictated by the most aggressive subpopulation, but is modulated by the presence of other cells with differing characteristics. The behaviour of a cancer is the result of all the subpopulations within it. Knowledge of the characteristics of the individual subpopulations is insufficient to predict the behaviour of the whole cancer. The fact that one can characterise individual subpopulations from cancers is meaningless unless knowledge of how these characteristics may be modified by neighbouring cells and/or how these cells may in turn modify the characteristics of the surrounding cells.

The problem of selecting effective therapy for heterogeneous tumours may be further complicated by the observation that tumour heterogeneity is a dynamic process. Evidence shows that individual subpopulations from tumours are heterogeneous in their stability. Chambers *et al.* (1981) observed variations in the stability of the metastatic phenotype in clones of the murine KHT sarcoma cells. Therefore, heterogeneity in the stability of the phenotype of a cell line or tumour sample may give rise to new variants with altered properties. This may explain, at least partially, the variation observed between primary tumours and their metastases in such properties as chemosensitivity (Tanigawa *et al.*, 1984; Schlag and Schremi, 1982). Tumour cell heterogeneity provides a mechanistic basis for the concept of neoplastic progression, that is, the acquisition by tumours of new characteristics during tumour development. Changes in cancer behaviour could be ascribed to progressive selections of different clones resulting in shifts in the predominant cell subpopulations and, therefore, altering any cell interactions that may be occurring. Poste *et al.* (1981) also showed that instability in

the metastatic phenotype of B16 melanoma cells became evident after cloning, whereas individual clones growing together as mixed cell populations retain their characteristic degree of metastatic ability. Therefore, tumour cell interactions can influence subpopulation behaviour and the characteristics of the tumour may be defined by more than the individual properties of its subpopulations. The demonstration of tumour cell heterogeneity focuses on differences among multiple cell populations and these differences are shown most convincingly when the subpopulations are grown and compared in isolation. Tumour cells and cell subpopulations do not, however, exist independently of each other, but rather as part of a mixed cell population. The existence of interactions between the tumour cells is, therefore, not unexpected and the interactions may either enhance or diminish a characteristic of the surrounding cells.

There are many ways that tumour subpopulations can influence each other's growth and behaviour. Host and/or tumour cell factors may be involved. Some interactions require cell contact while others act systemically. Miller *et al.* (1989) observed that when subpopulations of a murine mammary tumour, with varying intrinsic sensitivity to methotrexate were mixed the resulting tumours exhibited altered sensitivity to methotrexate. The cell line consisting of a mixture of cell lines 66 and 4TO7 tended to be as responsive to methotrexate toxicity as 4TO7 alone, the most sensitive clone, whereas line 168 plus 4TO7 tended to be less responsive than line 4TO7 alone. The tumour arising after exposure to methotrexate tended to contain more cell line 66 or 168 than untreated mixed tumours. In another experiment (Miller *et al.*, 1981b), the sensitivity of cells to methotrexate *in vitro* was altered when cells of differing sensitivity were grown in contact. In the presence of the methotrexate-sensitive subpopulation of the murine mammary tumour, 410.4, the sensitivity of lines 168 and 67 was increased, but the sensitivity of the relatively resistant cell line, T68H clone 8, was not affected. Miller *et al.* (1991) also found that when the melphalan-sensitive cell line 4TO7 was grown mixed with the less sensitive cell line 66, in tumours in mice, line 66 was more sensitive than when grown alone. The melphalan-sensitivity of tumours derived from cell line 66, grown on the opposite side of mice bearing 4TO7 tumours, was not altered by comparison with the sensitivity of line 66 tumours when grown alone. Furthermore, line 66 was not more sensitive when grown mixed with 168TFAR, a melphalan-sensitive subpopulation, than when grown alone. Miller *et al.* (1981b) also observed that individual tumour subpopulations which differ in sensitivity to chemotherapeutic drugs can influence each other's drug sensitivity, even when they are not in physical contact. When mice were injected with cyclophosphamide sensitive cells, 168, and resistant cells, 410, on opposite flanks, the sensitivity of line 168 was not affected by 410 cells but the sensitivity of 410 was increased by the presence of 168 cells. Injections of line 168 or 410 alone on opposite flanks, did not alter the cells sensitivity. Therefore, interactions can occur between cell subpopulations which are not in physical contact and the consequence of the

interaction may be uni-directional. Miller *et al.* (1988) also noted that line 4TO7 suppressed the growth of line 168 when grown in contact. Line 168 in the absence of cell line 4TO7 had a doubling time one half that of 4TO7 cells, yet when a ratio of even 100:1, in favour of the cell line 168, was injected into nude mice, the resulting tumour consisted primarily of 4TO7 cells. The suppression did not occur when the two cell lines were injected simultaneously at different subcutaneous sites, nor did it occur when line 168 was injected in mixtures with lethally irradiated cell line 4TO7, implying cell contact was necessary for interactions resulting in the modification of growth in the cell lines. Intercellular interactions among two subpopulations of an Ehrlich ascites carcinoma were noted during *in vivo* cell growth, as tumours, in nude mice (Aabo *et al.*, 1987). A growth inhibitory interaction by a slow growing subpopulation (E1.15) on a fast growing subpopulation (E1.95) was observed, but only when the cells were growing in contact, as tumours. Ascites from the slow growing E1.15 cells exhibited no inhibitory effect on E1.95 cells.

Leith *et al.* (1987) noted that growth kinetics of various artificial heterogeneous tumours, composed of mixtures of the two subpopulations of the human colon adenocarcinoma DLD-1, clone A and D, could not be predicted from knowledge of the growth parameters of the pure tumour cell lines, grown as xenograft tumours. It was found that mixed tumours became zonal in composition, as a function of time, and that mixtures of extreme composition, 9% A and 91% D or 91% A and 9% D, remained stable while those of intermediate composition, 50% A and 50% D, did not. Given the clonal nature of cellular propagation and the limited mobility of cells in solid tissue or in culture on a solid support, subpopulations which may be in competition for limited space or resources would be expected to be arranged in zones rather than completely mixed together. Michelson *et al.* (1987) also observed that when neoplasms were grown *in vivo* as xenografts made from varying proportions of the two clonal subpopulations of DLD-1, regions composed primarily of single subpopulations emerged in all cases.

Although tumours are mixed populations of cells, knowledge of the characteristics of individual components is not sufficient to predict the behaviour of the whole. Individual cancer subpopulations can interact to affect each others' growth, immunogenicity, ability to metastasise, sensitivity to drugs and clonal stability. However, cancer cell subpopulations can exist within a tumour and/or cell line without altering each other's phenotype or modifying their response to external stimuli. Barranco *et al.* (1988) observed that when two clones of a human astrocytoma cell line were mixed 1:1, one sensitive and one resistant to 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-1-nitrosourea, where both cell lines had similar doubling times, cell cycle distribution and colony forming efficiency, exposure of the cell mix to three weekly treatments of the drug resulted in the generation of a population consisting of the

resistant subclone. Leith *et al.* (1988) constructed artificial heterogeneous tumours using two clonal cell lines, A and D, originally isolated from the heterogeneous DLD-1 human colon adenocarcinoma, mixed at the ratio 1:9 and 9:1, respectively. Mitomycin C killed A cells more efficiently than D cells, by a factor of about 2.3. After treatment of mixes with mitomycin C, the tumours reached stable, albeit altered (based on the individual survival of A and D cells from pure cultures) percentage mixtures of subpopulations.

However, exposure of mixed cell populations to external stimuli may also alter the tumour composition. Leith *et al.* (1988) observed that the artificial heterogeneous xenograft tumours produced by injecting mixtures of the two clonal cell lines A and D isolated from the DLD-1 human colon adenocarcinoma, at a ratio of 9:1 and 1:9, showed a significant change in the percentage composition of the tumour following radiation treatment. Both mixtures became significantly enriched in the subpopulation that was originally predominant, whereas the composition of untreated mixtures remained constant.

In itself, tumour heterogeneity provides a rationale for combination therapy since subpopulations resistant to one treatment might be sensitive to another. However, the major problem of combination chemotherapy is the ability of cells to be either inherently resistant, or to acquire resistance, to the chemotherapeutic drugs. As tumour cells are heterogeneous in relation to the susceptibility to chemotherapeutic drugs, it follows that drug-resistant tumour cells may also exhibit variations in their sensitivity. Heterogeneity within drug-resistant cells may occur by selection of the more resistant variants of the primary cells, in cases where resistance is observed to occur following primary therapy, or by inducing new drug-resistant variants in the cells by mutagenistic effects of the chemotherapeutic drugs. Heterogeneity within MDR cell lines is implied in cases where exposure of cell lines by different research groups to the same chemotherapeutic drug has resulted in the generation of MDR cell lines of differing resistance profiles and mechanisms.

Numerous MDR variants of the small cell lung carcinoma, NCI-H69, have been established. Twentyman *et al.* (1986), Mirski *et al.* (1987) and Jensen *et al.* (1989) have all described MDR cell lines selected by exposure of the HCI-H69 cells to adriamycin. Twentyman *et al.* (1986) adapted the NCI-H69 cells to grow in 0.4μg/ml adriamycin, over a 7 month period. Mirski *et al.* (1987) cultured the cells in gradually increasing concentrations of adriamycin up to 0.8μM, over 14 months. Jensen *et al.* (1989) passaged the NCI-H69 cell line three times in 0.1μg/ml adriamycin and then maintained the cells in drug-free medium for 8 weeks prior to characterisation. All three cell lines exhibited the MDR phenotype of cross resistance to a range of structurally unrelated drugs, but while the cell lines established by Twentyman *et al.* (1986) and Jensen *et al.* (1989) overexpressed P-glycoprotein, the MDR variants isolated by Mirski *et al.* (1987) did not. When Yang and Trujillo (1990) established

two independently-derived MDR sublines of the human colon carcinoma LoVo, the phenotype of the resistant cells varied. AdR1.2 was developed by exposing the cells to stepwise increasing concentrations of adriamycin, from 0.01μg/ml to 1.2μg/ml, over 16 months, and was then maintained in the absence of drug. SRA1.2 cells were selected by exposing the LoVo cells to 9 one-hour drug exposures, with 1.2μg/ml of adriamycin, allowing the cells time to recover between each treatment. The AdR1.2 cells were approximately 40-fold more resistant to adriamycin than SRA1.2 cells, yet both exhibited cross-resistance and collateral sensitivity to the same chemotherapeutic drugs. Both drug-resistant cell lines exhibited overexpression of a 130 kDa. membrane protein, which does not cross-react with an anti-P-glycoprotein monoclonal antibody, but only AdR1.2 cells showed over-production of a 150 kDa. membrane bound protein which cross-reacted with C219 antibody. AdR1.2 cells also possessed an activated drug efflux pump, overexpression of GST-π and decreased nuclear-bound drug, whereas SRA1.2 cells only exhibited the decrease in the nuclear location of the drug. However, it cannot be confirmed whether the altered phenotypes are a result of selecting out different clonal cell lines from a heterogeneous parental population (Dolfini *et al.* (1993) have isolated clones of the LoVo cell line which exhibited altered adriamycin sensitivity) or whether the MDR cell lines are themselves heterogeneous.

In order to confirm clonal variation within an MDR cell line, the individual variants must be isolated and characterised. This has been carried out by Yang *et al.* (1993). A drug-resistant variant of the human colon carcinoma cell line, LoVo, was established by exposing the cells to stepwise increasing concentrations of cisplatin, from 0.005 to 2.0μg/ml, over 20 months. Morphologically distinct subpopulations were observed in the resistant cells and 22 clones were selected by limiting dilution. All clones had one of two distinct morphologies, designated CP2.0 and RT, and these two cisplatin-resistant clones also exhibited differing drug-resistant phenotypes. CP2.0 cells were spindle-like in shape and were resistant to cisplatin, mustargen, VP-16, 5-fluorouracil, adriamycin and vincristine to a fold resistance of 6.4, 2.3, 1.1, 2.6, 2.6 and 7.8, respectively, relative to LoVo cells. The RT cells appeared rounded, with vacuoles, and possessed a level of fold resistance to the above mentioned drugs, of 2.1, 1.9, 1.0, 1.6, 0.6 and 0.3, respectively. CP2.0 cells also overexpressed P-glycoprotein and accumulated less adriamycin, relative to LoVo cells. RT cells, on the other hand, did not overexpress P-glycoprotein and accumulated higher levels of adriamycin, by comparison with LoVo cells. Therefore, intercellular heterogeneity in the expression of MDR-associated mechanisms of resistance and any cell-cell interactions which may be occurring between subpopulations of the cell line, may result in a modification of the cells' resistance and may be of importance in analysing the overall resistance of the MDR cells. However, the literature details only a few comprehensive studies on heterogeneity in cells' sensitivity to chemotherapeutic drugs and interactions which may modify that sensitivity.

1.9 Aims of this thesis

Following the observations in the literature that heterogeneity is a property of many cancers and cell lines and that few studies pertaining to heterogeneity within MDR cell lines have been documented, an analysis of heterogeneity within an MDR variant of a lung cell line was undertaken. Therefore, the aims of this thesis were to determine if heterogeneous subpopulations exist within an adriamycin-selected MDR variant of the human squamous lung carcinoma, DLKP-A, and to characterise these subpopulations, in terms of their MDR profile, their sensitivity to radiation and the biochemical and genetic alterations pertaining to drug resistance, exhibited by these variants. The mechanisms by which the subpopulations survive and interact, within the heterogeneous population was to be elucidated. Finally, the characteristics exhibited by the adriamycin-selected MDR cell line were to be compared with a VP-16-selected MDR variant of the same cell line. This was to determine if MDR variants of the same cell, derived by exposure to different chemotherapeutic drugs, resulted in similar MDR cell lines and if similar biochemical and genetic mechanisms supported the MDR profiles.

2. Materials and Methods

2.1 Water

A Millipore Milli-RO 10 Plus system, fed to an Elga Elgastat UHP was used to produce ultra-high quality water for use in the production of all media and solutions. This consisted of a pre-treatment which utilised activated carbon, pre-filtration and anti-scaling, prior to a reverse osmosis step. This was followed by organic adsorption, ion exchange, ultra-microfiltration, photo-oxidation and ultrafiltration. The quality of the water was monitored on-line, with a specification of 18 megaohms/cm resistivity at 25°C.

2.2 Glassware

All glassware and bottle-caps which came in contact with medium or reagents were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific; RBS-25) in warm water for 1 - 2h. The bottles were then scrubbed, rinsed in warm water, and washed by machine using Neodiser detergent. The glassware and bottle-caps were then rinsed twice with distilled water and once with ultrapure water.

2.3 Sterilisation

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

2.4 Medium preparation

Growth medium was prepared as outlined in Table 2.1, the pH adjusted to pH 7.45-7.55 by the addition of sterile 1.5M NaOH, and the volume adjusted to 5L. The medium was then filtered through a sterile 0.22μm bell filter (Gelman; G.1423S) into sterile 500ml bottles and stored at 4°C. Sterility checks were carried out on the medium, which included performing turbidity checks and setting up Columbia (Oxoid; CM331) blood agar plates and Sabauraud (Oxoid; CM 217) dextrose and Thioglycollate (Oxoid; CM173) broths. ATCC medium was made by mixing Dulbecco's Modified Eagles medium (DMEM) and Hams F12 in a ratio of 1:1. All medium was supplemented with 2mM L-glutamine (Gibco; 043-05030) and 5% foetal calf serum (FCS) (Seralab; 101024), prior to use. Minimum essential medium Eagle (MEM) was also supplemented with 1% MEM non-essential amino acid solution (Sigma; M7154)

Table 2.1 : Preparation of growth medium

	DMEM	**MEM**	**HamsF12**
10X Medium	500ml 10X DMEM Gibco 042-02501M	500ml 10X MEM Gibco 042-01430M	HamsF12 powder Gibco 074-01700N
Ultrapure H_2O	4300ml	4300ml	4700ml
1M Hepes (pH 7.5)* Sigma H9136	100ml	100ml	100ml
7.5% $NaHCO_3$ BDH 30151	45ml	45ml	45ml

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

2.5.1 Cell lines

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Holton or Gelman Cytoguard) and strict aseptic technique was adhered to at all times. All cell lines which were routinely used throught this research work are outlined in Table 2.2. and are anchorage dependent. Cells were grown in 25cm^2 flasks (Costar; 3050) or 75cm^2 flasks (Costar; 3075). Cells were grown at 37°C in the presence of 5% CO_2 and fed every two to three days or when a medium pH change was observed (as determined by a change in the colour of the medium due to the presence of a phenol red indicator).

Table 2.2 : Cell lines used in the course of this research work.

Cell Line	Growth Medium	Detail of cell type	Source
DLKP	ATCC	Human Lung Squamous Cell Carcinoma	G. Grant NCTCC
DLKPA	ATCC	DLKP adapted to multidrug resistance by increasing stepwise exposure to Adriamycin (Farmatalia)	A. Redmond NCTCC
T24	MEM	Human Bladder Carcinoma	ATCC
SKMES1	ATCC	Human Squamous Lung Carcinoma	ATCC
HT29	MEM	Human Colon Carcinoma	ATCC
OAW-42	ATCC	Human Ovarian Carcinoma	ATCC
Hep-2	ATCC	Human Epidermoid Carcinoma of the Larynx (HeLa markers)	ATCC
Hep-2A	ATCC	Hep-2 adapted to multidrug resistance by increasing stepwise exposure to Adriamycin (Farmitalia)	A. Redmond NCTCC

2.5.2 Sub-culture of cell lines

On reaching confluency, or when required for use in assays, the cells were sub-cultured by enzymatic detachment. Waste medium was poured from the cells and the cells were rinsed with 2ml of trypsin/EDTA (0.25% trypsin (Gibco; 043-05090), 0.01% EDTA (Sigma; EDS) solution in PBS (Oxoid; BR14a)), which had been preincubated at 37°C for 15 min. The cells were then incubated with a further 2ml of the trypsin/EDTA solution for 10-15 min. or until a single-cell suspension was obtained. An equal volume of medium, containing foetal calf serum, was added. The cell suspension was transferred to a 30ml sterile universal (Sterilin; 128a) and centrifuged at 120 g. for 5 min. The medium was poured from the universal and the cell pellet was resuspended in 5ml of growth medium. A cell count was performed, as outlined in Section 2.5.3. Approximately $2x10^4$ or 10^5 cells were added to 10ml of medium in a $25cm^2$ flask or 20ml of medium in a $75cm^2$ flask, respectively. These were then incubated at 37°C in 5% CO_2.

2.5.3 Cell counting

A sample of a single cell suspension was mixed in a ratio of 4:1 with trypan blue dye (Gibco; 525). Following a 2 min. incubation at room temperature, a sample of the cell mixture was applied to a haemocytometer, such that it was held in the areas between the haemocytometer and coverslip. Cells in the 16 squares of the four outer corner grids were counted, and the average of the four squares was multiplied by 10^4, to determine the cell number per ml. Cells which stained blue were non-viable and those which remained unstained were viable.

2.5.4 Large-scale cell culture

Cells required in large numbers were cultivated in roller bottles. Approximately 100ml of growth medium was allowed to equilibrate in the roller bottle at 37°C for 30 min., prior to the addition of a single cell suspension (approximately $2x10^7$ cells in total). The roller bottle was incubated at 0.25 r.p.m. overnight, then the rotor speed increased to 0.50 r.p.m. The cells in the roller bottle were fed every 3-4 days until the reached 80% confluency, at which stage they could be used where large cell numbers were required.

2.6 *Mycoplasma* detection

To establish that *Mycoplasma* infection of cell lines was not occurring, the cells were examined using the Hoechst 33258 indirect staining method and the mycoplasma culture method of *Mycoplasma* detection. The cell lines which were being tested were grown, for a minimum of three passages following thawing, in an antibiotic- and drug-free medium. Medium was exposed to a flask of nearly-confluent cells for three days. The medium was then asceptically collected and used for the detection of *Mycoplasma* contamination of the cell line.

2.6.1 Hoechst 33258 indirect stain

NRK cells (2 x 10^3) were grown overnight at 37°C in 5% CO_2 on sterile coverslips in 1ml of DMEM supplemented with 5% FCS and 2mM L-glutamine, in individual sterile dishes. One ml of the collected sample medium was added to duplicate coverslips of NRK cells and incubated for 5 days (the cells should have reached approximately 50% confluency at this stage). The medium was removed from the coverslips. The coverslips were then washed twice with PBS, washed once with a 1:1 solution of ice-cold PBS : Carnoy's fixative (this was a freshly prepared 1:3 solution of glacial acetic acid (Sigma; A0808) : methanol (BDH; 101584W) which had been stored at -20°C for 30 min.), and fixed for 10 min. in Cornoy's fixative. The coverslips were then allowed to air dry and 2ml of Hoechst 33258 stain (Sigma; B2883) at 50*n*g/ml in PBS was added to each coverslip and incubated in darkness for 10 min. The coverslip was then washed with water and mounted onto a glass slide, using 50% glycerol (BDH; 101184K) in 0.1M citric acid, 0.2M disodium phosphate, pH 5.5 as the mounting solution. The slides were then examined for *Mycoplasma* contamination under oil immersion using a mercury fluorescent lamp. Nucleic acid is stained by the Hoechst 33258 stain. Therefore, the NRK cell nucleus stains brightly and any extra-nuclear stain is an indication of the presence of *Mycoplasma*. Appropriate controls (medium not exposed to cells as the negative control and a sample of known *Mycoplasma* contaminated medium as the positive control) were included in the procedure.

2.6.2 *Mycoplasma* culture method

Ninety mls. of *Mycoplasma* agar (Oxoid; CM401) and *Mycoplasma* broth (Oxoid; CM403) bases were supplemented with 16.33% FCS, 0.002% DNA (BDH; 42026), 2μg/ml fungizone (Gibco; 05290), 2x10^3 units penicillin (Sigma; Pen-3) and 10ml of a 25% (w/v) yeast extract solution (which had been boiled for 5 min. and filtered through a 0.2μm filter). Sample medium (0.5ml) from the cell line being tested was incubated with 3ml of the broth, for 48h. at 37°C. A streak from the broth was then innoculated onto 10ml agar plates and incubated for up to 3 weeks at 37°C in a CO_2 environment, with constant microscopic analysis for colony formation. The presence of 'fried egg' type colonies indicated the existence of *Mycoplasma* contamination of the cell line.

2.7.1 Long Term Storage of Animal Cells

For cryo-preservation, a single cell suspension was prepared (Section 2.5.2) from a sub-confluent large-scale culture of cells (Section 2.5.4). The cell pellet was resuspended in FCS. An equal volume of 10% (v/v) DMSO (Sigma; D5879) in FCS was added dropwise, with constant mixing, to the cell suspension, to give a final cell concentration of 10^7 cells/ml The cell suspension was then added, in 1.5ml volumes, to cryovials (Greiner; 122 278) which were placed in the vapour phase of liquid nitrogen for 2.5h. They were then stored in the liquid phase until required.

2.7.2 Recovery of Frozen Stocks

A frozen vial of cells was removed from liquid nitrogen storage and thawed rapidly in a 37°C water bath. The thawed cell suspension was transferred to a sterile universal with 5ml of medium containing 5% FCS and 2mM L-glutamine and was centrifuged for 5 min. at 120 g. The medium was gently removed and the cell pellet resuspended in 2ml of the medium. The cells were then transferred to a 25cm^2 flask with 10ml of medium and the cells were incubated for 24h. to allow cell attachment. Following cell attachment, the cells were re-fed with fresh medium.

2.8 Safe Handling of Cytotoxic Drugs.

Due to the serious potential risks incurred from the handling of chemotherapeutic agents, extreme care was exercised in their handling and disposal. All work with cytotoxic drugs was carried out in Gelman 'Cytoguard' laminar air flow cabinets (CG Series). Face masks and double gloves were worn when dealing with concentrate solution and all drugs were stored and disposed as outlined in Table 2.2.

Table 2.2 : Chemotherapeutic drugs used in the research work

CYTOTOXIC DRUG	SUPPLIER	DISPOSAL	STORAGE
DOXORUBICIN 2mg/ml	Farmitalia	Inactivate with 1% hyperclorite solution and dispose with excess water.	Stock @ 4°C, in darkness
VP-16 (etoposide) 20mg/ml	Bristol Myers Pharmaceuticals	Incineration	Store @ room temp. Working stock @ -20°C, in darkness
VINCRISTINE 1mg/ml	David Bull Laboratories Ltd.	Inactivate by autoclaving and dispose with excess water.	Store @ 4°C in darkness
5-FLUOROURACIL 25mg/ml	Farmitalia	Inactivate by addition of equal volume of 5M NaOH and dispose with excess water.	Store @ room temp. in darkness
CIS-PLATIN 1mg/ml	Lederle	Incineration	Store @ room temp. in darkness

2.9 Conditioned Medium

DLKP-A cells were grown, as outlined in Section 2.5.4, for large-scale cell culture. When cells were approximately 80% confluent, the medium was removed and replaced with 100ml of fresh medium, which was again incubated overnight. This medium was collected, spun at 120 g. for 5 min. to pellet any cells and filtered through a 0.22μm gelman filter. The conditioned medium was stored at 4°C.

2.10 Isolation of variants of DLKP

2.10.1 Adaption of MDR variants

The cell lines being adapted to drug resistance were grown in the selective drug, VP-16, at a concentration which gave 10% survival as determined by miniaturised toxicity assays (Section 2.11.1). Cells were grown at this concentration until they appeared healthy by microscopic examination and had attained high numbers. The drug concentration to which the cells were exposed was then doubled. This process was repeated until good cell growth was obtained in the desired concentration of VP-16. Overall, the DLKP cell line was selected for drug resistance by exposing the cells to increasing concentrations of VP-16, from an initial concentration of 0.3μg/ml VP-16 to final concentrations of 3.0μg/ml (in the case of the cell line **DLKP/VP3**) and 8.0μg/ml (in the case of the cell line **DLKP/VP8**).

2.10.2 Selection of MDR Clones

The DLKP-A cell line was sub-cultured, a cell count was performed and the cells were plated at 50 cells per 35mm tissue culture petri-dish (Greiner; 627160), in 2ml of medium (Section 2.10) supplemented with 10% FCS and 1% L-glutamine. The cells were grown for 24h. in 5% CO_2 at 37°C to allow cell attachment. The plates were microscopically examined to ensure cells adhered singularly and in a dispersed manner. The cells were grown, with constant monitoring and feeding with a 1:1 mixture of medium to conditioned medium, until individual cells had formed single colonies of approximately 50 cells. The colonies were individually sub-cultured. Cloning rings (stainless steel rings, with an inner diameter of 8mm) were used to form individual trypsination chambers. This involved removing the medium from each petri-dish and washing the cells with sterile PBS. The PBS was removed from the plate and a sterile cloning ring, with its end covered in siliconised grease, was placed around an individual colony, isolating it from other colonies on the plate and forming a separate sub-culturing chamber. Trypsin/EDTA (Section 2.5.2)(100μl) was added to the colony and the cells incubated at room temperature for 5 min. The trypsin/EDTA was then repeatedly pipetted up and down, using a Gilson micropipette, to ensure all cells had detached. The cells were transferred to a single well of a 96-well plate (Costar; 3599). Medium (100μl) was added to the well, and the cells were allowed to attach overnight at 37°C in 5% CO_2. Following

attachment, the medium was removed from the well and 200μl of fresh medium added. When the cells had reached approximately 90% confluency, they were sub-cultured into a single well of a 48-well plate (Costar; 3548). This procedure was followed to repeatedly transfer the cells into a 24-well plate (Costar; 3424), 6-well plate (Costar; 3406), 25 cm^2 flask (Costar; 3050) and 75 cm^2 flask (Costar; 3075). Stocks of the randomly selected clones were maintained frozen (Section 2.7.1) and all clones were routinely maintained in the absence of adriamycin, when in culture. All assays were carried out within a ten passage span following revival of the cells from cryo-preservation.

2.10.3 Irradiation of DLKP cells

Cells to be exposed to radiation were subcultured into a 75 cm^2 tissue culture flask at a density of 5 x 10^6 cells/flask and allowed to attach overnight. Following a 24h. incubation the growth medium was replaced with fresh medium and the flask of cells was irradiated, at room temperature, with a single defined radiation dose from a linear accelerator at a dose rate of 2.5-2.6 Gray (Gy.)/min. One cm of tissue equivalent bolus was placed on top of the plate during irradiation, to ensure electronic equilibrium. Following irradiation, the cells were maintained at 37°C in 5% CO_2 overnight. The next morning the medium in the flask was replaced with fresh medium. The cells were allowed to grow until they appeared healthy and had attained high numbers, at which stage they were again exposed to another dose of radiation. The radiation doses to which the cells were exposed is outlined in Table 2.10.3 and the cell line which resulted from the radiation treatment was designated DLKP/RAD.

Day	Radiation Dose	Passage Number
1	150 cGy	17
15	600 cGy	18
21	900 cGy	19
28	900 cGy	19
48	1200 cGy	19
77	1200 cGy	20
83	1200 cGy	20

Table 2.10.3 : The radiation treatment profile used in the generation of the cell line DLKP/RAD, from the parental cell line, DLKP.

2.11 Miniaturised assays

In all assays, cells were pre-treated in a similar manner to ensure that the cells were healthy and in a logarithmic phase of growth prior to analysis. Two days prior to the assay, the cells were subcultured into 75cm^2 flasks at a density of 2 x 10^5 cells/flask. The cells were allowed to attach overnight and the next day the cells were fed with fresh medium. On the day the assay was set up, the cells were sub-cultured and a single cell suspension obtained as described in Section 2.5.2.

2.11.1 Toxicity Assays : 96 Well Plate

Cells were plated, from a single cell suspension, into a 96 well plate (Costar; 3599) at a cell density of 10^3 cells/well in 100μl of medium. The first column of the plate was used as the plate control and contained only medium. Cells were allowed to attach for 24h. at 37°C in 5% CO_2, then 100μl of the required drug dilution (which was diluted to 2X the final concentration required in the assay) was added to each well, in replicas of eight. Drug concentrations used in each assay ranged from 0.0M to a concentration which achieved approximately 100% cell kill. The plates were then incubated for a further 6 days at 5% CO_2 and 37°C, or until control wells (no drug added) reached 80% confluency. Drug toxicity was then determined by acid phosphatase analysis (Connolly *et al.*, 1986; Martin and Clynes, 1991). Medium was removed and the assay plates rinsed twice with PBS. Then 100μl of assay substrate, 10mM *p*-nitrophenyl phosphate (Sigma; 104-0) in 0.1M sodium acetate, 0.1% triton X-100 (Sigma; X100), pH 5.5 was then added to each well and the plate incubated at 37°C for 2h. in darkness. The enzyme reaction was then stopped by the addition of 50μl of 1.0M NaOH and the plate read in a dual beam plate reader at 405nm (reference wavelength 620nm.).

2.11.2 Radiation Assay : 24 Well Plate

Cells were plated from a single cell suspension into 24 well plates (Costar; 3424), in replicas of six, at a cell density of 400 cells/well, in 500μl of complete medium. As the complete assay plate was exposed to one dose of radiation, a separate plate was set up for each radiation dose. The plates were incubated overnight at 37°C in 5% CO_2 to allow cell

attachment. The plates were then individually irradiated, at room temperature, with a single defined radiation dose from a linear accelerator, at a dose rate of 2.5 - 2.6 Gy./min. One cm. of tissue-equivalent bolus was placed on top of the plate during irradiation, to ensure electronic equilibrium. Following radiation exposure, the cells were maintained at 37°C in 5% CO_2 overnight. The medium was then replaced with fresh medium, and the cells maintained, with constant medium replacement every 4-5 days, until the control plate (which was not irradiated) had reached approximately 80% confluency. The medium was then removed, the plate washed twice with PBS and the cells fixed with 10% formalin (Sigma; F1635) for 10 min. The formalin was removed and the plate allowed to dry. The cells were stained with 0.025% crystal violet (Sigma; C3886) for 10 min. The plate was then washed to remove excess stain and again allowed to air dry. The stain was eluted with 300μl of 33.3% glacial acetic acid and 200μl of this was then transferred to a 96 well plate. The plate was read in a dual beam plate reader at 570nm (reference wavelength 620nm.).

2.11.3 Plating density assay : 24 Well Plate

Cells were plated from the single cell suspension into 24 well plates at a cell density of 200 cells/well, in 500μl of complete medium. The plates were incubated overnight at 37°C in 5% CO_2 to allow cell attachment. Control plates, containing 2000 cells/well were set up in parallel. Then, 500μl of a 2X drug dilution was added to each well, in replicas of three. Plates were incubated at 37°C in 5% CO_2 until the control wells (no drug added) reached 80% confluency. The medium was then removed, the plate washed twice with PBS and the cells fixed with 10% formalin for 10 min. The formalin was removed and the plate allowed to dry. The cells were then stained with 0.025% crystal violet for 10 min. The plates were washed to remove excess stain and allowed to air dry. The stain was then eluted with 300μl of 33.3% glacial acetic acid and 200μl of this was then transferred to a 96 well plate. The plate was read in a dual beam plate reader at 570nm (reference wavelength 620nm).

2.11.4 Drug Replacement Assay : 24 well plate

Triplicate plates of cells were plated from a single cell suspension into 24 well plates at a cell density of 200 cells/well, in 500μl of medium. The plates were incubated

overnight at 37°C in 5% CO_2 to allow cell attachment. Triplicate control plates, containing 2000 cells/well were set up in parallel. Then, 500μl of a 2X drug dilution was added to each well, in replicas of three. Plates were incubated at 37°C in 5% CO_2. Fresh concentrate drug was added to one of the triplicates every 48h., to replace the drug which was inactivated (adriamycin has a half-life of 29h. at 37°C in growth medium (Hilderbrand-Zanki and Kern, 1986)). A complete drug and medium change was given to a second of the triplicate plates every 48h. The drug in the third of the triplicate plates was neither supplemented with additional drug nor replaced with fresh drug and medium. Plates were maintained at 37°C in 5% CO_2 until the control wells (no drug added) reached 80% confluency. The medium was then removed, the plate washed twice with PBS and the cells fixed with 10% formalin for 10 min. The formalin was removed and the plate allowed to dry. The cells were then stained with 0.025% crystal violet for 10 min. The plate was washed to remove excess stain and allowed to air dry. The stain was eluted with 300μl of 33.3% glacial acetic acid and 200μl of this was then transferred to a 96 well plate. The plate was then read in a dual beam plate reader at 570nm (reference wavelength 620nm).

2.11.5 Circumvention Assay : 96 Well Plate

Cells were plated, from single cell suspension, into a 96 well plate at a cell density of 10^3 cells/well in 100μl of complete medium. The first column acted as a plate control and contained only medium. Cells were allowed to attach for 24h. at 37°C in 5% CO_2. Then 50μl of a 4X concentration of the required drug and/or circumventing agents (verapamil (Sigma; V4629) and cyclosporin A (Sandoz; Sandimmune)) was added to the appropriate wells, in replicas of eight. Medium was added to wells which required a volume adjustment in order to bring the final volume to 200μl/well. The plates were incubated for a further 6 days at 5% CO_2 and 37°C, or until the control wells (no drug or circumvention agent added) reached 80% confluency. Drug-induced cell kill was then determined by acid phosphatase analysis. Medium was removed and the assay plates rinsed twice with PBS. Then 100μl of 10mM *p*-nitrophenyl phosphate in 0.1M sodium acetate, 0.1% triton-X-100, pH 5.5 was added to each well and the plate incubated at 37°C for 2h. in darkness. The enzyme reaction was stopped by the addition of 50μl of 1.0M NaOH and the plate read in a dual beam plate reader at 405nm (reference wavelength 620nm).

2.12 Protein Analysis

2.12.1 Topoisomerase II isolation

All cells used in the determination of the cellular content of topoisomerase II were in the exponential phase of cell growth when the protein was extracted. To prevent proteolytic degradation of the topoisomerase II during the isolation procedure, all solutions used to prepare the samples for electrophoresis contained the following freshly prepared cocktail and unless otherwise stated, all procedures were carried out on ice or at 4°C : Phenylmethylsulfonyl fluoride (PMSF) (Sigma; P7626) (1mM); benzamidine (Sigma; B6506) (1mM); Soybean trypsin inhibitor (Sigma; T9003) (1mg/ml); leupeptin (Sigma; L2884) (50μg/ml); pepstatin A (Sigma; P6425) (1μg/ml) and aprotinin (Sigma; A1153) (20μg/ml).

2.12.1.1 Nuclear extraction

This protocol has been adapted from the protocol by Danks *et al.* (1988). Exponentially growing cells (10^8) were trypsinised from a large cell culture (as outlined in Section 2.5.2) and permeablised by resuspending the PBS-washed cell pellet in 1.75ml of hypotonic buffer (5mM KH_2PO_4, 2mM $MgCl_2$, 4mM DTT, 0.1mM Na_2EDTA, pH 7.0) and incubating for approximately 20 min. When approximately 90% of the cells stained with trypan blue, they were centrifuged at 400g for 5 min. The cells were resuspended in hypotonic buffer containing 0.25M Sucrose at a cell density of $5x10^8$cells/8ml. Each 8ml aliquot was layered over 3ml of hypotonic buffer containing 0.3M Sucrose and centrifuged at 2000g for 20 min. The supernatant was removed and the pellet volume determined. The pellet was resuspended in a half volume of lysis buffer (5mM KH_2PO_4, 4mM DTT, 1mM Na_2EDTA, pH 7.0) and incubated for 15 min. Nuclear topoisomerase II was then extracted by adding half the pellet volume of a 1.0M salt extraction buffer (40mM Tris, 2.0M NaCl, 4mM DTT, 20% glycerol, pH 7.5). The solutions were vortexed and incubated for 30 min, followed by a 100,000g centrifugation for 1h. The clear supernatant was then removed and the pellet re-centrifuged. The second supernatant was pooled with the first and either used immediately or stored at -170°C until required for the determination of topoisomerase II content in the cells. If stored, the extract was used within three days.

2.12.1.2 Total cell extraction

Cells used for the determination of the topoisomerase II content in total cell extract were grown until approximately 10^7 cells were obtained. These cells were trypsinised and centrifuged at 120 g. for 5 min. The cell pellet was washed with PBS and recentrifuged. The resulting cell pellet was resuspended in 0.2ml of DNase 1 buffer (10mM Tris, 1mM $MgCl_2$, 0.1mM CaCl and 1mM DTT, pH 7.8). The cells were incubated on ice for 15-20 min. and were then checked for cell lysis (by trypan blue staining). If the cells did not lyse within 20 min., the cell density was too high and more DNase 1 buffer was added. When cell lysis was achieved, 120 units of DNase 1 enzyme (Promega; RQ1 RNase-free DNase) was added and the cells were incubated for 20 min. in a 37°C waterbath. The sample was used immediately or stored at -170°C until required for the determination of topoisomerase II content in the cells. If stored, the extract was used within three days.

2.12.2 Cell membrane preparations

Membrane preparations for Western blot analysis were prepared from 2 x 10^7 cells which were in the exponential growth phase. Following trypsinisation the cells were washed three times in PBS. All the following steps in the procedure were performed while the cells were kept on ice. The cell pellet was resuspended in 5ml of ice-cold lysis buffer (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris, pH 7.4 and 2mM PMSF was added immediately prior to use). The cells were sonicated until lysis was observed by microscopical analysis. The lysed cells were then centrifuged at 100,000 g. for 1h at 4°C. The pellet obtained was resuspended in 500μl of lysis buffer. An aliquot was removed for protein quantification and the remainder was divided into 100μl aliquots and lyophilised overnight. The lyophilised samples were stored at -80°C until required for Western blot analysis.

2.12.3 Quantification of protein

The protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad; 500-0006) and by using several concentrations of bovine serum albumin (BSA) (Sigma; A9543) as standards. The dye reagent was provided as a 5-fold concentrate. The appropriate standards (0.1ml) and test samples (0.1ml) were placed in clean, dry test tubes. The diluted dye reagent (5ml) was added and the mixture vortexed. After a period of 5 min.

to 1h., the OD_{595} was measured, against a reagent blank. From the plot of the OD_{595} of the standards versus their concentrations, the concentration of protein in the test samples was determined.

2.12.4 Gel electrophoresis

The protein present in the cell preparations were separated on a size basis using a 7.5% SDS polyacrylamide gel. The resolving gel was made by mixing 3.8ml of an acrylamide stock (29.1g acrylamide (Sigma; A8887) and 0.9g NN-methylene bis-acrylamide (Sigma; N7256) made up to 100ml with water), 8.0ml of water and 3.0ml of 1.875M Tris-HCl, pH 8.8. To this solution, 150μl of 10% SDS (Sigma; L4509), 50μl of 10% ammonium persulphate (Sigma; A1433) and 7.5μl of TEMED (Sigma; T8133) were added. The gel was immediately poured into two clean 10cm x 8cm gel cassettes. Each gel cassette comprised of a glass and aluminium plate separated by two 0.75cm plastic spacers, one on either outer edge. The gel was overlayed by saturated butanol and allowed to set. Once set, the saturated butanol was removed, the gel tops washed with water and the stacking gels poured. The stacking gels comprised of 0.8ml of the acrylamide stock mixed with 3.6ml of water and 0.5ml of 1.25M Tris-HCl, pH 6.8. To this solution 50μl of 10% SDS, 17μl of 10% ammonium persulphate and 5μl of TEMED were added, the solution mixed and poured onto the resolving gels. A comb of the appropriate thickness and well size was immediately inserted and the gel allowed to set. When the wells had formed, the gel combs were removed and the gels transferred to a mini-electrophoresis apparatus. The gels were flooded with running buffer (1.9M glycine (Sigma; G6761), 0.25M Tris, 0.1% SDS. The pH of the buffer should be 8.3 without adjustment).

Protein samples were loaded into the wells, based on equal protein loading. The samples to be loaded were diluted 1:1 with loading buffer (50mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol (Sigma; M6250), 5% glycerol and 0.1% bromophenol blue) and boiled for 2 min. They were then loaded onto the gel, as were appropriate molecular weight markers. The gels were run for approximately 1.5h. with voltage set at 250V and current set at 45mA.

2.12.5 Western blotting procedure

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

2.12.5.1 P-glycoprotein

Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked for 2h. at room temperature in TBS (500mM NaCl, 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed twice with TBS and was exposed to the primary antibody (0.25μg/ml C219 antibody (Centicor Diagnostics) in 10ml TBS, 0.1% Tween-20) at 4°C overnight. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary antibody (a 1/9,500 dilution of rabbit anti-mouse IgG (Sigma; A-1902) in TBS, 0.1% Tween-20) for 1.5h. at room temperature. The nitrocellulose was again washed three times in TBS and was developed as outlined in Section 2.12.6.

2.12.5.2 Topoisomerase II

Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked at 4°C overnight in blocking solution (10mM Tris-HCl, pH 7.4, 140mM NaCl, 5%

non-fat dried milk, 3% BSA, 0.2% Tween-20). The primary antibodies (a polyclonal rabbit serum that recognises topoisomerase II and pre-immune rabbit serum (as a negative control), which were gifts from Dr. W.T. Beck and decribed by Friche *et al.* (1991)) were diluted 1/700 with blocking solution. The nitrocellulose was incubated with the primary antibody for 48h. at 4°C. The nitrocellulose was then washed three times with 10mM Tris, 140mM NaCl, pH 7.4 and incubated with the secondary antibody (a 1/13,000 dilution of goat anti-rabbit IgG (Sigma; A-8025) in blocking solution) at room temperature for 2h. The nitrocellulose was again washed three times with 10mM Tris, 140mM NaCl, pH 7.4 and was developed as outlined in Section 2.12.6.

2.12.5.3 Topoisomerase II-α and Topoisomerase II-ß

Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked at 4°C overnight in blocking solution (PBS containing 3% BSA). The primary antibodies (monoclonal antibodies 6G2 and 8F8 which recognise topoisomerase II-α and topoisomerase II-ß, respectively, were gifts from Dr. G. Astaldi-Ricotti and decribed by Negri *et al.* (1992)) were diluted 1/50 (6G2) and 1/300 (8F8) with PBS containing 0.1% BSA. The nitrocellulose was incubated with the primary antibody overnight at 4°C. The nitrocellulose was then washed three times with PBS containing 0.1% Tween-20 and incubated with the secondary antibody (a 1/9,500 dilution of rabbit anti-mouse IgG (Sigma; A-1902) in PBS, containing 0.1% BSA, 0.1% Tween-20) at room temperature for 2h. The nitrocellulose was again washed three times with PBS, containing 0.1% Tween-20 and was developed as outlined in Section 3.12.6.

2.12.6 Developing blots

The blots were developed by incubating the blots in a developing substrate until bands and/or background colour appeared. The developing substrate consisted of a mixture of 2.5ml of solution A and 1ml of solution B in 22.5ml of a solution consisting of 0.5M Tris-HCl, pH 8.9 containing 0.1ml of 1.0M $MgCl_2$. Solution A consisted of 0.1% Nitroblue tetrazolium (Sigma; H-5514) in 10ml Tris-HCl, pH 8.9. Solution B consisted of 5mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma; B-0274) in Dimethyl formamide (Sigma; D-8654). Once the blots had developed, they were washed in water at room temperature and allowed to dry. They were then stored in the dark.

2.13 RNA Analysis

2.13.1 Total RNA extraction

All steps in the RNA analysis protocol were carried out in sterile conditions, where possible. The cell were grown on 135mm-diameter petri-dishes until they reached 80% confluency. The medium was then removed and the cells rinsed twice with PBS. The cells on five replica plates were lysed by the addition of 5ml of a 4M guanidinium thiocyanate (GnSCn) solution. The 4M GnSCn consisted of 50g of guanidinium thiocyanate (Sigma; G6639), 0.5g of N lauroyl sarcosine (Sigma; L5125) and 5ml of 1M Na citrate (RDH; 32320), pH 7.0. The solution was brought to 100ml with water and filtered through a 0.45μm filter. The solution was stored at room temperature in the dark, until required. Immediately before use, 700μl/100ml of ß-mercaptoethanol and 330μl/100ml of antifoam A (Sigma; A5758) were added.

The cell lysate solution was then centrifuged at 120 g. for 5 min. and the solution layered over 5.5ml of a 5.7M cesium chloride cushion in ultracentrifuge tubes. The 5.7M cesium chloride solution consisted of 95.8g of CsCl (Sigma; C3032) and 2.5ml of 1M sodium citrate, pH 7.0 per 100ml of water. The solution was filter sterilised and 100μl of diethyl procarbonate (DEPC) (Sigma; D5758) was added, the mixture left at room temperature for 2h. and the solution was then autoclaved.

The cell extract was then centrifuged at 100,000 g. for 21-24h. at 15°C. in a swinging bucket centrifuge. The guanidinium thiocyanate and the "jelly-like" layer below the GnSCn/CsCl interface were removed by aspiration. The cesium chloride was then removed to the last 1ml The tubes were inverted and the bottom, containing the RNA pellet, cut from the rest of the tube using a heated scalpel blade. The pellet was washed with 95% ethanol at room temperature and resuspended in 200μl of DEPC-treated water, by pipetting up and down, on ice. The resuspended pellet was transferred into eppendorf tubes and the pellet remains were rinsed into the eppendorf tube with a further 200μl The RNA was precipitated by the addition of 3M sodium acetate (to a final concentration of 0.3M) and two volumes of absolute ethanol and incubation at -20°C or -80°C overnight. The RNA was pelleted by spinning at 4°C in a microfuge at maximum speed. The pellet was washed with 70% ethanol and resuspended in 100μl of DEPC-treated water. The RNA was stored at -80°C.

2.13.2 Quantification of RNA

The RNA was quantified spectrophotometrically at 260nm and 280nm. An optical density of 1 at 260nm is equivalent to 40mg/ml RNA. A ratio of A_{260}/A_{280} of 2 is indicative of pure RNA.

2.13.3 Reverse transcriptase reaction

The cDNA was formed on the RNA template by the following reactions, which were incubated on ice unless otherwise stated. One μl of Oligo(dT) 12-18 primers (1μg/μl), 1μl of total RNA (1μg/μl) and 3μl of water were mixed, heated to 70°C for 10 min. and then chilled on ice. To this solution the following components were added: 4μl of a 250mM Tris-HCl pH 8.3, 375mM KCl and 15mM $MgCl_2$ buffer, 2μl of DTT (100mM), 1μl of RNasin (40U./μl), 1μl of dNTPs (10mM each), 6μl of water and 1μl of Moloney murine leukemia virus-reverse transcriptase (200U./μl). The reaction mixture was incubated at 37°C for 1h. followed by 2 min. at 95°C.

2.13.4. Polymerase chain reaction (PCR)

Five μl of cDNA was incubated with relevant primers in the PCR for 30 cycles. The resulting product was analysed by gel electrophoresis and densitometry. Water (24.5μl) was mixed with 5μl of a 100mM Tris-HCl pH 9.0 at 25°C, 50mM KCl, 1% Triton X-100, 3μl of 25mM $MgCl_2$, 8μl of 1.25mM dNTP, 1μl of 250μg/ml first strand target primer, 1μl of 250μg/ml second strand target primer, 1μl of 250μg/ml ß-actin first strand primer, 1μl of 250μg/ml ß-actin second strand primer, 0.5μl of 5U./μl *Taq* DNA polymerase enzyme and 5μl of cDNA from the reverse transcriptase reaction (which had been heated to 95°C. for 3 min., followed by cooling on ice). A drop of mineral oil was added to each reaction tube and the cDNA amplified by the following procedure: 95°C for 1.5 min., 30 cycles of 95°C for 1.5 min., 55°C for 1 min. and 72°C for 3 min., 72°C for 7 min. and then held at 4°C. Ten μl of gel loading buffer was added to each tube and 10μl of the cDNA solution was then separated by electrophoresis for 4h. at 100V on a 4% agarose (Nusieve agarose) gel containing ethidium bromide with TBE (22.5mM Tris, 22.5M boric acid (Sigma; B7901), 0.5mM EDTA, pH 8.0) as the running buffer.

2.14 Gap junctional intercellular communication

2.14.1 Detection of functional gap junctions in cells

The presence of functional gap junctions in variants of the DLKP cell line was determined using the lucifer yellow scrape loading method of analysis (El-Fouly *et al.*,1987). The cells were plated onto a sterile coverslip in individual sterile dishes, in 1 ml of medium, at a cell density of approximately 5 x 10^4 cells. The coverslip was incubated at 37°C in 5% CO_2 until the cells reached confluency. The medium was then removed from the cells and the cells rinsed twice, for 2 min. each time, in assay buffer (130mM NaCl, 2.8mM KCl, 2mM $MgCl_2$, 10mM Hepes, pH 7.2). Lucifer yellow (Sigma; L0144) (0.5mg/ml) in assay buffer was added to the cells and a blade was used to score the cells, at a central location on the coverslip. The cells were incubated in this solution for 2 min. at 37°C in 5% CO_2 in darkness. The lucifer yellow solution was then removed and the cells washed twice with 2 ml of the assay buffer containing 1mM $CaCl_2$. The cells were then viewed under a fluorescent microscope.

In cases where gap junctional-intercellular communication was being inhibited with heptanol (Sigma; H6129), the procedure was carried out as outlined above, with the following modifications. The cells were pre-incubated in assay buffer containing 1.5mM heptanol for 1.5 h. prior to scrape loading the lucifer yellow into the cells. The scrape loading section of the assay was also carried out in heptanol containing buffer. However, heptanol was omitted from the final wash buffer.

The ability of adriamycin to be transported intercellularly *via* gap junctions was also investigated. The procedure used for this experiment was the same as that used in the investigation of the intercellular transfer of lucifer yellow, except that the lucifer yellow was replaced by 100μM adriamycin and the cells were exposed to the adriamycin-containing solution for 5 min. following the scoring of the cells. The effect of cyclosporin A on adriamycin transfer by gap junctions was investigated by adding 30μg/ml cyclosporin A to all solutions containing adriamycin.

2.14.2 Investigation of gap junctional-mediated adriamycin toxicity

The effect of the blocking of gap junctional-communication on adriamycin toxicity to variants of DLKP was investigated in a miniaturised assay. The cells were set up in four 24 well plates; one plate at 200 cells/well, one at 2000 cells/well, one at 400 cells/well and the final plate at 4000 cells/well, in a volume of 500μl/well. The cells were allowed to attach overnight at 37°C in 5% CO_2. Following cell attachment, 250μl of 6mM heptanol (the heptanol was originally dissolved in DMSO and then diluted with medium) was added to the plates containing 400 cells/well and 4000 cells/well. An equal volume of medium, containing the same concentration of DMSO as was used to dissolve the heptanol, was added to the two remaining plates. A concentration of heptanol of 1.5mM had resulted in 50% cell kill, by comparison with a DMSO control, when cells were exposed to the chemical in a preliminary assay (carried out as in Section 2.11.1). The cells were incubated for a further 24h. at 37°C in 5% CO_2. Adriamycin (250μl of a 4X final concentration) was then added to the plate in replicas of three and the plates incubated until the control wells reached approximately 80% confluency. Cell growth was then measured by analysing the intensity of crystal dye staining, as performed in the plating density assay (Section 2.11.3).

2.15 Adriamycin accumulation

Cells were plated, from a single cell suspension, into a 6 well plate at a cell density of 5 x 10^5 cells/well in 5ml of growth medium. The cells were incubated at 37°C in 5% CO_2 for 48h. The medium was then removed from the cells and 4ml of fresh medium containing 10μM adriamycin (and an appropriate concentration of circumventing agent, if required in the assay) was added to duplicate wells. Control wells were set up containing adriamycin-free medium. The cells were incubated at 37°C in 5% CO_2. At specific time intervals, the medium was removed from the cells and the cells washed twice with ice-cold PBS. Ice cold water (2ml) was added to each well and left for approximately 5 min. or until cell lysis was observed, as determined by microscopical analysis. Two mls of an ice-cold 0.6M HCl solution in methanol was added to each well and the plates further incubated for 3 min. The solution was then centrifuged at 4000g for 10 min. The fluorescence of the supernatant was measured, against the control blanks, at an excitation range of 470nm and an emission of 585nm, using a Perkin Elmer LC50 luminescence spectrometer. The concentration of adriamycin in the supernatant was determined from a linear standard curve, prepared from the fluorescence of known concentrations of adriamycin.

2.16 Conditioned medium assay

The effect that conditioned medium from one cell line, generated in the presence and absence of adriamycin, exerted on the drug sensitivity of another cell line was investigated. Four 75cm^2 flasks of DLKPA2B and DLKPA5F cells were set up at 10^6 cells per flask. The cells were allowed to attach overnight at 37°C in 5% CO_2. The medium was removed from the cells and two flasks of each cell line were re-fed with 30ml of fresh medium. The remaining two flasks of each cell line were re-fed with 30ml of fresh medium containing 2.5μg/ml adriamycin. The flasks were incubated for a further 24h. at which time the conditioned medium was collected from each flask and the media from replica flasks were pooled. The conditioned media was centrifuged at 120 g. and the supernatant filtered through a 2μm low protein-binding filter. The adriamycin content of the conditioned media was determined from their level of fluorescence (as determined using a Perkin Elmer LC50 luminescence spectrometer, with an excitation of 470nm and an emission of 585nm) as compared to freshly diluted adriamycin standards. In each case the conditioned medium from a cell line was analysed using the appropriate adriamycin-free conditioned medium produced by the same cell line as a blank. Fresh adriamycin was added to a sample of the conditioned medium, generated by the four individual procedures, re-adjusting the adriamycin concentration to 2.5μg/ml. Adriamycin was also added to fresh medium, to a final concentration of 2.5μg/ml (Table 2.16).

Simultaneously, DLKPA2B cells were set up as for a plating density assay (Section 2.11.3), such that by this stage of the production of adriamycin- containing conditioned media, the cells had been allowed to attach overnight. The adriamycin-containing conditioned media were now diluted, using the appropriate adriamycin-free medium, and 500μl of these adriamycin dilutions were added to triplicate wells of the 24 well plate. The control in each case was the adriamycin-free medium, produced by that cell line. The plates were incubated in 5% CO_2 at 37°C until the control wells were approximately 80% confluent, at which stage the level of cell growth was ascertained using crystal violet staining, as outlined in Section 2.11.3.

Exposure of cells to adriamycin.	Determination of adriamycin concentration.	Adjustment of adriamycin concentration.	Dilution of adriamycin-containing medium.	Use of the conditioned medium.
Adriamycin was added to fresh ATCC medium, to a final concentration of 2.5μg/ml.			Serial dilutions of the drug-containing medium were performed, using fresh ATCC medium.	The medium was added to the DLKPA2B cells in a 24 well assay.
ATCC medium, containing 2.5μg/ml adriamycin, was exposed to DLKPA2B for 24h. at 37°C. The conditioned medium was collected, centrifuged and filtered through a low protein-binding 0.22μm filter.	The concentration of adriamycin present in the conditioned medium was determined by its fluorescence.	Fresh adriamycin was then added to an aliquot of the conditioned medium, to a final concentration of 2.5μg/ml.	This drug-containing conditioned medium was diluted with drug-free conditioned medium.	The conditioned medium was added to the DLKPA2B cells in a 24 well assay.
ATCC medium, containing 2.5μg/ml adriamycin, was exposed to DLKPA5F for 24h. at 37°C. The conditioned medium was collected, centrifuged and filtered through a low protein-binding 0.22μm filter.	The concentration of adriamycin present in the conditioned medium was determined by its fluorescence.	Fresh adriamycin was then added to an aliquot of the conditioned medium, to a final concentration of 2.5μg/ml.	This drug-containing conditioned medium was diluted with drug-free conditioned medium.	The conditioned medium was added to the DLKPA2B cells in a 24 well assay.
Fresh ATCC medium was exposed to DLKPA2B cells for 24h. at 37°C. The conditioned medium was then collected, centrifuged and filtered through a low protein-binding 0.22μm filter.		Adriamycin was then added to an aliquot of the conditioned medium, to a final concentration of 2.5μg/ml.	Serial dilutions were performed on the drug-containing conditioned medium, using the drug-free conditioned medium.	The conditioned medium was added to the DLKPA2B cells in a 24 well assay.
Fresh ATCC medium was exposed to DLKPA5F cells for 24h. at 37°C. The conditioned medium was then collected, centrifuged and filtered through a low protein-binding 0.22μm filter.		Adriamycin was then added to an aliquot of the conditioned medium, to a final concentration of 2.5μg/ml.	Serial dilutions were performed on the drug-containing conditioned medium, using the drug-free conditioned medium.	The conditioned medium was added to the DLKPA2B cells in a 24 well assay.

Table 2.16 : Protocol followed in the generation of adriamycin-free and adriamycin-containing conditioned media.

3. Results

3.1 Establishment of novel MDR cell lines

3.1.1 Establishment of DLKP/VP-3 and DLKP/VP-8

Two new multidrug resistant variants of the human squamous cell carcinoma cell line DLKP (Law *et al.*, 1992) were established, as outlined in Section 2.9. DLKP adapted readily to continuous growth in VP-16. The initial selective pressure to which the cell line was exposed was 0.3μg/ml VP-16. The cell line adjusted to growth in 3.0μg/ml VP-16 within six months and to a final concentration of 8.0μg/ml VP-16 within an additional three months. The cell lines selected to and maintained in a concentration of 3.0μg/ml VP-16 and 8.0μg/ml VP-16 were designated DLKP/VP-3 and DLKP/VP-8, respectively.

3.1.2 Cross-resistance patterns of DLKP/VP-3 and DLKP/VP-8

To ensure that the VP-16 selected cells possessed the multidrug resistance characteristic of exhibiting cross-resistance to a range of structurally unrelated chemotherapeutic drugs, their sensitivities to five such drugs were determined. These drugs included: the selective agent, VP-16; the anthracycline, adriamycin; the alkylating agent, cisplatin; the vinca alkaloid, vincristine and the anti-metabolite, 5-fluorouracil. The sensitivity of DLKP/VP-3 and VP-8 cells to the chemotherapeutic agents were tested a minimum of three times with eight replicas performed in each assay. Table 3.1.2.1 presents the average IC_{50} values for the chemotherapeutic agents acting on the parental cell line, DLKP and the two VP-16 selected variants obtained from their toxicity profiles, as described in Section 2.12.1, with the IC_{50} values determined as outlined in Appendix A. Table 3.1.2.2 represents the fold increase in the IC_{50} values of the variants with respect to the parental cells.

DLKP/VP-3 and DLKP/VP-8 both possessed multidrug resistance phenotypes and exhibited cross-resistance and hyper-sensitivity to a similar range of drugs. They were resistant to their selective agent VP-16 and cross-resistant to adriamycin and vincristine. They exhibited collateral sensitivity to cisplatin and no significant alteration in their sensitivity to 5-fluorouracil (Table 3.1.2.1).

In terms of fold resistance, both selected cell lines were most resistant, not to their selective agent, but to vincristine (approximately 1700- and 1000-fold resistant, respectively), next to adriamycin (270- and 90-fold, respectively), with the least fold increase in relation to VP-16 (approximately 100- and 60-fold, respectively). The increase in their

resistance to vincristine was approximately one order of magnitude greater than their resistance to adriamycin or VP-16 (Table 3.1.2.2). The fold resistance ratio displayed by both DLKP/VP-3 and DLKP/VP-8 with respect to vincristine and VP-16 was approximately constant, *i.e.* when the resistance to one drug was doubled, both doubled. However, DLKP/VP-8 was three times more resistant to adriamycin than DLKP/VP-3. Cell lines DLKP/VP-3 and DLKP/VP-8 were approximately 3- and 4-fold, respectively, more sensitive to cisplatin than the parental cell line, DLKP.

IC_{50} (nM)	DLKP	DLKP/VP-3	DLKP/VP-8
Adriamycin	24.83 ± 14.48	2237.07 ± 328.79*	6764.31 ± 945.35*
Cisplatin	873.00 ± 387.33	281.67 ± 149.00*	191.67 ± 50.33*
Vincristine	1.21 ± 0.30	1317.66 ± 194.91*	2102.28 ± 420.59*
VP-16	146.45 ± 68.81	8527.35 ± 338.60*	14826.71 ± 958.21*
5-Fluorouracil	9551.11 ± 6255.96	4448.12 ± 3631.82	8621.06 ± 4067.64

Table 3.1.2.1 : IC_{50} values for a range of chemotherapeutic drugs acting on the cell lines DLKP, DLKP/VP-3 and DLKP/VP-8. The values are given as the average IC_{50} value ± the standard deviation, on a minimum of three repeats. The IC_{50} values were determined as outlined in Appendix A.
* The values are significantly different from the corresponding DLKP values.

FOLD RESISTANCE	DLKP/VP-3	DLKP/VP-8
Adriamycin	90.10 ± 13.24	272.42 ± 38.07
Cisplatin	0.32 ± 0.17	0.22 ± 0.06
Vincristine	1088.98 ± 161.08	1737.42 ± 347.60
VP-16	58.23 ± 2.31	101.24 ± 6.54
5-Fluorouracil	0.47 ± 0.38	0.90 ± 0.43

Table 3.1.2.2 : The fold increase in resistance to chemotherapeutic drugs in DLKP/VP-3 and DLKP/VP-8 with respect to DLKP, as determined by comparing their IC_{50} values.

3.2 Establishment of clones of an MDR cell line

3.2.1 Establishment of Clones

To study the heterogenous nature of the DLKP-A cell line and to determine if different sub-populations within the cell line were responsible for various characteristics of the overall population, nine clones of the MDR cell line were established as outlined in section 2.10. During the initial stages of isolating clones of DLKP-A, poor growth was observed when the DLKP-A cells were plated at the low densities necessary to ensure that all colonies arose from single, well-dispersed cells. When conditioned medium (Section 2.10) from the DLKP-A cell line was used to partially feed the cells at this stage of the isolation procedure (at a 1:1 dilution with fresh medium), it appeared to stimulate the cells to grow, in comparison with the growth observed when only fresh medium was used. The clones of DLKP-A were maintained as individual cell lines without selective pressure and randomly designated DLKPA2B, DLKPA6B, DLKPA9B, DLKPA11B, DLKPA3C, DLKPA10C, DLKPA2D, DLKPA5D and DLKPA5F.

3.2.2 Cross-resistance profiles of DLKP-A Clones

To determine if the clonal populations present in the DLKP-A cell line were multidrug resistant, or whether specific clones were resistant to certain drugs and the combination of clones was responsible for the overall MDR profile, the sensitivities of the clones to a variety of chemotherapeutic compounds were investigated. These agents included adriamycin, VP-16, vincristine, cisplatin and 5-fluorouracil. Table 3.2.2.1 presents the IC_{50} values obtained for the parental cell line DLKP, its MDR adapted variant DLKP-A and the nine clones established from the MDR variant. The sensitivity of all cell lines to the different chemotherapeutic agents was tested a minimum of three times, with eight replicas performed in each assay. The IC_{50} values were evaluated as outlined in Appendix A. Table 3.2.2.2 presents the fold increase in the IC_{50} values of the MDR variant, DLKP-A, and its clones, with respect to the drug sensitive parental cell line, DLKP.

DLKP-A, as previously described by Clynes *et al.*, (1992), exhibited an MDR profile. Under the experimental conditions used in this study, DLKP-A was found to be resistant, not only to its selective agent, adriamycin (250-fold resistant), but also highly cross-resistant to vincristine (1500-fold resistant) and resistant to a lesser extent to VP-16 (60-fold resistant). It showed neither significant cross-resistance nor collateral sensitivity to cisplatin (1.46-fold resistant) or 5-fluorouracil (1.75-fold resistant). The cell line exhibited a high level of deviation in its toxicity profile with respect to all drugs examined.

IC_{50} (nM)	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKP	24.83 ± 14.48*	873.00 ± 387.33	1.21 ± 0.30*	146.45 ± 68.81*	9551.11 ± 6255.96
DLKPA	6310.34 ± 2965.52#	1277.33 ± 364.00	1820.15 ± 855.90#	8885.49 ± 1172.27#	16679.48 ± 7071.48
DLKPA2B	913.79 ± 396.55*,#	500.00 ± 63.33*	276.27 ± 85.59*,#	2660.55 ± 1219.84*,#	12259.80 ± 7355.88
DLKPA6B	2348.28 ± 810.34*,#	616.67 ± 66.67*	444.20 ± 198.27*,#	4359.50 ± 1862.05*,#	7363.57 ± 3120.68
DLKPA11B	2082.76 ± 310.34*,#	543.33 ± 233.33*	397.62 ± 196.10#	3688.41 ± 287.12*,#	5149.88 ± 953.11*
DLKPA5F	8208.62 ± 1701.73#	1213.33 ± 23.33	1542.80 ± 756.23#	7410.81 ± 1182.47#	9362.03 ± 4481.17
DLKPA9B	6087.24 ± 1194.83#	796.67 ± 100.00	2171.72 ± 200.00#	8961.94 ± 1979.27#	10591.85 ± 53.80
DLKPA3C	8136.21 ± 1218.97#	953.33 ± 123.33	2401.95 ± 410.62#	5176.69 ± 62.86*,#	8554.96 ± 3382.01
DLKPA10C	2206.90 ± 1908.62#	825.00 ± 73.00	1178.76 ± 98.59#	3380.90 ± 1868.84*,#	9362.03 ± 1691.01
DLKPA2D	4109.48 ± 305.17#	1461.67 ± 73.00#	2154.93 ± 49.84#	6223.24 ± 2891.61#	13912.38 ± 1929.29
DLKPA5D	5294.83 ± 879.31#	1701.67 ± 73.00*,#	907.91 ± 191.77#	14085.97 ± 506.29#	9684.86 ± 3697.16

Table 3.2.2.1 : IC_{50} values obtained for a range of chemotherapeutic drugs towards DLKP, DLKP-A and its clones. The values given are the mean IC_{50} value ± the standard deviation. The average IC_{50} values was calculated as outlined in Appendix A and the standard deviation was determined using a minimum of triplicate repeat assays, in all cases.

* The value is significantly different from the corresponding DLKP-A value.

the value is significantly different from the corresponding DLKP value.

FOLD RESISTANCE	Adriamycin	Cisplatin	Vincristine	VP-16	5-Fluorouracil
DLKPA	254.14 ± 119.43	1.46 ± 0.42	1504.26 ± 707.36	60.67 ± 8.00	1.75 ± 0.74
DLKPA2B	36.80 ± 15.97	0.57 ± 0.07	228.32 ± 70.74	18.17 ± 8.33	1.28 ± 0.77
DLKPA6B	94.59 ± 32.64	0.71 ± 0.08	367.11 ± 163.86	29.77 ± 12.71	0.77 ± 0.33
DLKPA11B	83.88 ± 12.50	0.62 ± 0.27	328.61 ± 162.07	25.19 ± 1.96	0.54 ± 0.10
DLKPA5F	330.59 ± 68.53	1.39 ± 0.03	1275.04 ± 624.98	50.60 ± 8.07	0.98 ± 0.47
DLKPA9B	245.16 ± 48.12	0.91 ± 0.11	1794.81 ± 165.29	61.19 ± 13.51	1.11 ± 0.06
DLKPA3C	327.68 ± 49.09	1.09 ± 0.14	1985.08 ± 339.36	35.35 ± 0.43	0.90 ± 0.35
DLKPA10C	88.88 ± 76.87	0.95 ± 0.08	974.18 ± 81.48	23.09 ± 12.76	0.98 ± 0.18
DLKPA2D	165.50 ± 12.29	1.62 ± 0.08	1780.93 ± 41.19	42.49 ± 19.74	1.46 ± 0.20
DLKPA5D	213.21 ± 35.41	1.95 ± 0.08	750.34 ± 158.49	96.18 ± 3.46	1.01 ± 0.39

Table 3.2.2.2 : Fold increase in resistance towards chemotherapeutic drugs of DLKP-A and its clones, with respect to DLKP. The fold resistance was determined by dividing the average IC_{50} value for the cell line of interest by the corresponding average IC_{50} value for DLKP.

All clones exhibited similar cross-resistance profiles to that of DLKP-A, in that they were highly resistant to the MDR-associated drugs, adriamycin, vincristine and VP-16. As was observed with the DLKP-A cells, high levels of deviation existed within the IC_{50} values determined, especially in respect to vincristine resistance where up to 50% standard deviation was noted *e.g.* the standard deviation on the average IC_{50} value of vincristine toxicity to DLKPA5F was 49%. The high inter-assay variability means that an exceedingly large difference must exist between two cell populations, with respect to their sensitivity to a chemotherapeutic drug, before a significant difference is noted.

As was found for DLKP-A, all the clones exhibited the same rank order of resistance, *i.e.* significantly more resistant to vincristine than to adriamycin and less resistant to VP-16. Very low levels of resistance or sensitivity to cisplatin or 5-fluorouracil were exhibited by some clones. Within the clonal sub-populations, there was a 9-fold variation in resistance levels to adriamycin. This variation ranged from a 37-fold resistance in DLKPA2B to 331-fold resistance in DLKPA5F. There was an 8-fold variation in resistance levels to vincristine (228-fold resistance level in DLKPA2B and 1985-fold resistance in DLKPA3C). With respect to VP-16, there was a 5-fold variation within the clonal population; DLKPA2B was 18-fold resistant whereas DLKPA5D was 96-fold resistant. The cloned cell line most resistant to one specific MDR associated drug was not most resistant to all MDR drugs, but DLKPA2B was the least resistant clone with regard to resistance to the typical MDR drugs. With respect to DLKP-A, the clonal cell lines DLKPA2B, DLKPA6B and DLKPA11B exhibited collateral sensitivity to cisplatin (with a fold resistance of 0.57 $\pm$ 0.07, 0.71 $\pm$ 0.08 and 0.62 $\pm$ 0.27 respectively), but not by comparison with the cisplatin sensitivity of the parental DLKP cell line (an IC_{50} of 873.00 $\pm$ 387.33 nM was detected for DLKP while the IC_{50} values for DLKPA2B, DLKPA6B and DLKPAIIB were 500.00 $\pm$ 63.33 nM, 616.67 $\pm$ 66.67 nM and 543.33 $\pm$ 233.33 nM, respectively). DLKPA11B also exhibited sensitivity to 5-fluorouracil (fold resistance of 0.54 $\pm$ 0.10), in comparison to the level of sensitivity noted in DLKP-A (fold resistance of 1.75 $\pm$ 0.74). DLKPA5D showed a slight increase in resistance with respect to DLKP-A in terms of cisplatin toxicity (fold resistance of 1.95 $\pm$ 0.08).

The relative resistance levels exhibited to the five chemotherapeutic drugs varied for each clone. Table 3.2.2.3 presents the relative fold resistance ratio of adriamycin, vincristine and VP-16, for DLKP-A and the nine clonal sub-populations. The fold resistance to adriamycin, for each cell line, was taken as the unit fold resistance and the cells resistance to vincristine and VP-16 normalised to this. DLKP-A possessed a cross-resistance profile of 1:5.92:0.64 in relation to adriamycin, vincristine and VP-16, respectively. Although all clonal cell lines of DLKP-A retained the property of most resistance to vincristine, less to adriamycin and least resistant to VP-16, no clone had a similar ratio of fold resistance to the three drugs

as that observed in DLKP-A. DLKPA9B exhibited a fold resistance ratio most like that of DLKP-A. It possessed a fold resistance ratio of 1:7.32:0.25 with respect to resistance to adriamycin, vincristine and VP-16. The relative ratio of fold resistance to adriamycin and VP-16 was similar for both the DLKP-A and DLKPA9B cells, but DLKPA9B exhibited a higher fold resistance to vincristine, when normalised to adriamycin resistance, than that observed for DLKP-A. DLKP-A and DLKPA3C also showed similar fold resistance ratios to adriamycin and vincristine (1:5.92 for DLKP-A and 1: 6.06 for DLKPA3C) but the ratios of adriamycin to VP-16 fold resistance for both cell lines were significantly different (1:0.24 for DLKP-A and 1:0.11 for DLKPA3C).

The cell clones DLKPA10C and DLKPA2D exhibited similar fold resistance ratios. DLKPA10C possessed a ratio of 1:10.96:0.26 while DLKPA2D possessed a ratio of 1:10.61:0.26, with respect to adriamycin, vincristine and VP-16 fold resistance. However, the DLKPA2D cells were twice as resistant to the drugs as the DLKPA10C cells (165.50 $\pm$ 12.29 and 88.88 $\pm$ 76.87 fold resistance, respectively). Although both cell lines exhibited a ratio of adriamycin to VP-16 resistance similar to DLKP-A cells, the relative ratio of adriamycin to vincristine resistance was twice that noted for DLKP-A. The cell lines DLKPA6B and DLKPA11B also possessed similar fold resistance ratios DLKPA6B exhibited a ratio of adriamycin, vincristine and VP-16 resistance of 1:3.88:0.31, while that of DLKPA11B was 1:3.92:0.30. Both cell lines were also resistant to adriamycin to a similar level (the IC_{50} of adriamycin toxicity to DLKPA6B was 2348.20 $\pm$ 810.34 nM, while that of DLKPA11B was 2082.76 $\pm$ 310.34 nM). Although the relative fold resistance ratio of adriamycin resistance to vincristine resistance was similar for DLKPA5F (1:3.86) as that detected for DLKPA6B (1:3.88) and DLKPA11B (1:3.92), the fold resistance ratio of adriamycin to VP-16 in DLKPA5F (1:0.15) was half that observed for DLKPA6B (1:0.31) and DLKPA11B (1:0.30). DLKPA5F cells were also 4-fold more resistant to adriamycin and vincristine than DLKPA6B and DLKPA11B (Table 3.2.2.2).

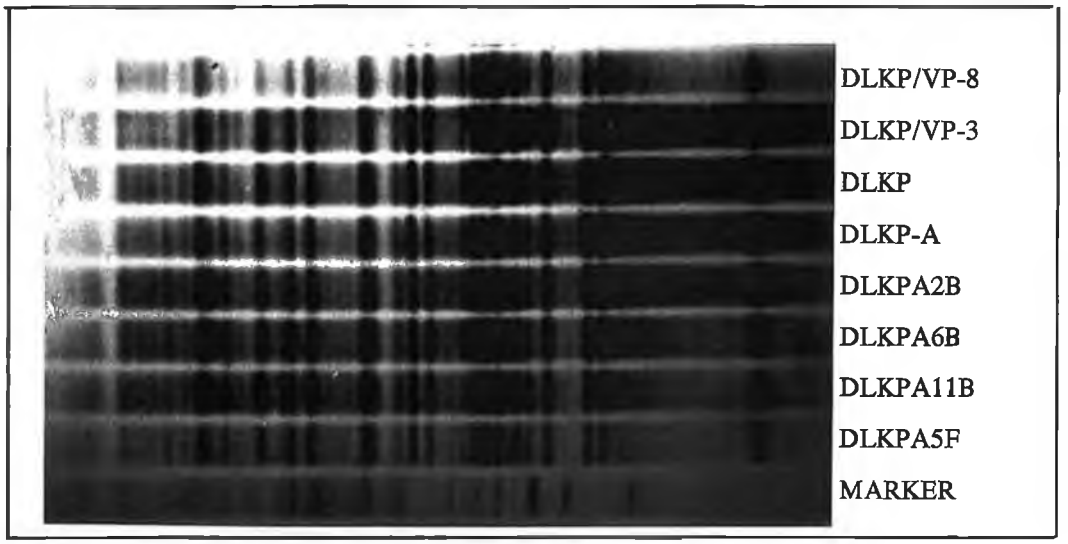
CELL LINE/CLONE	Adriamycin	Vincristine	VP-16
DLKP-A	1	5.92	0.24
DLKPA2B	1	6.20	0.49
DLKPA6B	1	3.88	0.31
DLKPA11B	1	3.92	0.30
DLKPA5F	1	3.86	0.15
DLKPA9B	1	7.32	0.25
DLKPA3C	1	6.06	0.11
DLKPA10C	1	10.96	0.26
DLKPA2D	1	10.61	0.26
DLKPA5D	1	3.52	0.45

Table 3.2.2.3 : The ratio of the relative fold resistance of the MDR cell lines to adriamycin, vincristine and VP-16. The fold resistance level to adriamycin in each cell line is given as a unit resistance and the fold resistance to vincristine and VP-16 was normalised to the adriamycin resistance.

3.3 DNA fingerprint analysis of the drug resistant variants

DNA fingerprint analysis was carried out to ensure that the VP-16-selected MDR cell lines were variants of DLKP and that the clones DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F were variants of DLKP-A. DNA was extracted from the cell lines by Cellmark Diagnostics and DNA fingerprints were produced with the multi locus probes, 33.15 and 33.6 (Case Number R932939). As may be observed from Figures 3.3, the DNA fingerprints of all DLKP variants had 56 identical bands and so share approximately 99% homology. Hence, all cell lines must have originated from a common source. DLKP, DLKP-A and DLKPA6B had one additional band each, at different positions, within their fingerprint. DLKP/VP-3 and DLKPA11B had two additional bands each within their fingerprints, one of which was present in both cell lines.

a.



b.

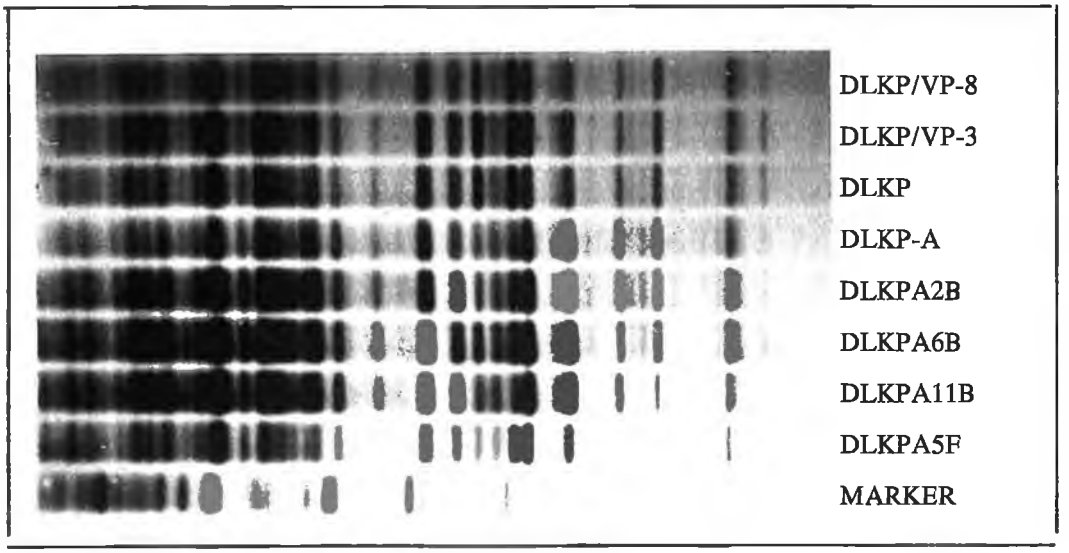


Figure 3.3 : DNA fingerprint analysis of DLKP variants, produced with the multi locus probes 33.15 (a) and 33.6 (b).

3.4 Stability of the toxicity profiles of drug resistant variants of DLKP

3.4.1 Stability of VP-16 toxicity profiles of VP-16-selected MDR variants of DLKP

The stability of the drug resistant phenotype was studied by maintaining the MDR cells in the absence of VP-16 for up to three months. As can be determined from Figures 3.4.1.1 and 3.4.1.2, the toxicity profile of the cell lines, DLKP/VP-3 and DLKP/VP-8, with respect to VP-16, were maintained for up to three months with little deviation. These lines were therefore characterised as being stable cell lines, with respect to their level of resistance to VP-16.

3.4.2 Stability of adriamycin toxicity profiles of DLKP, DLKP-A and DLKPA5F

To ensure that the level of resistance to adriamycin observed in the clones was a real and stably expressed characteristic, the stability of their adriamycin toxicity profiles was investigated.

DLKP cells were sensitive to adriamycin and the level of adriamycin toxicity remained stable when thawed and passaged (Figure 3.4.2.1(a)). The adriamycin resistance of DLKP-A cells remained stable when the cells were cultured in the absence of selective pressure for three months (Clynes *et al.*, 1992). The stability of the adriamycin toxicity profile when the cells were repeatedly frozen and thawed and/or subcultured was confirmed (Figure 3.4.2.1(b)). The effect of cryopreservation and subculturing on the adriamycin toxicity profile of DLKPA5F, as a representive of a clone of DLKP-A, was also examined. Though the clone was maintained in the absence of any selective pressure, the adriamycin toxicity profile was stable following continuous freeze/thawing and subculturing (Figure 3.4.2.2). Any deviation in the toxicity profile appeared to be due to inter-assay variability.

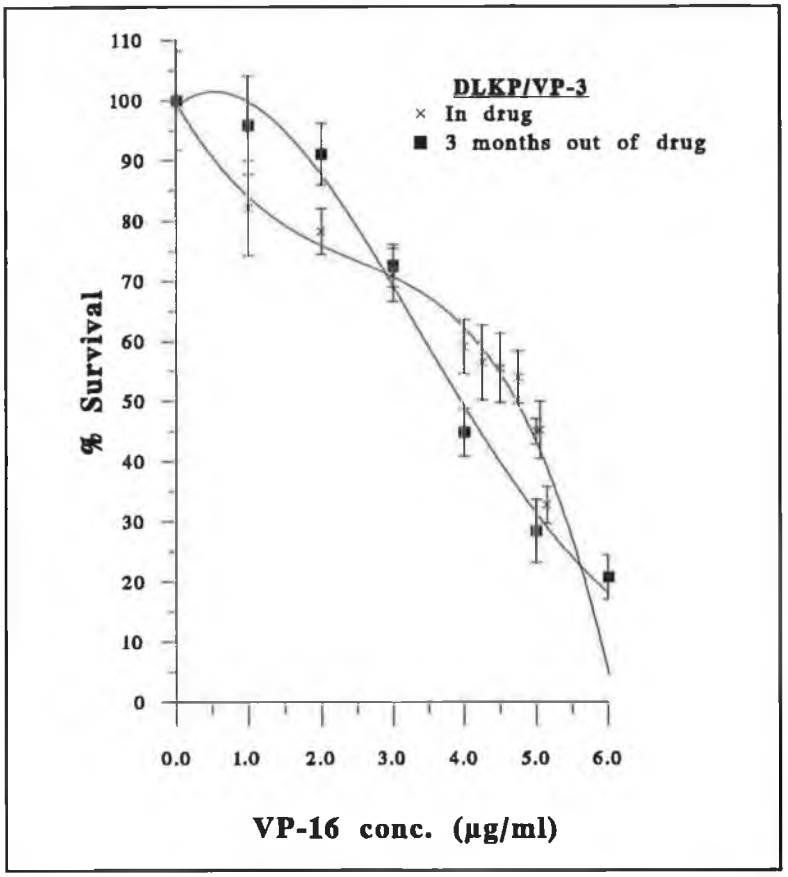


Figure 3.4.1.1 : Stability of the VP-16 toxicity profile of DLKP/VP-3 when maintained in drug-free medium, for up to three months. The error bars represent the standard deviation on the mean of eight replicas.

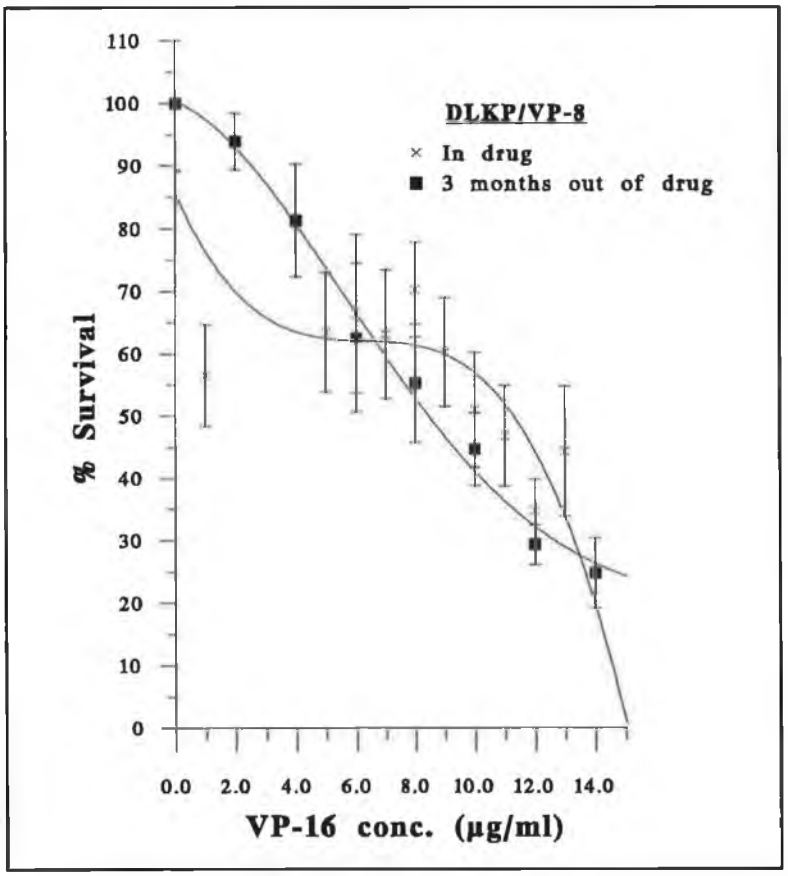
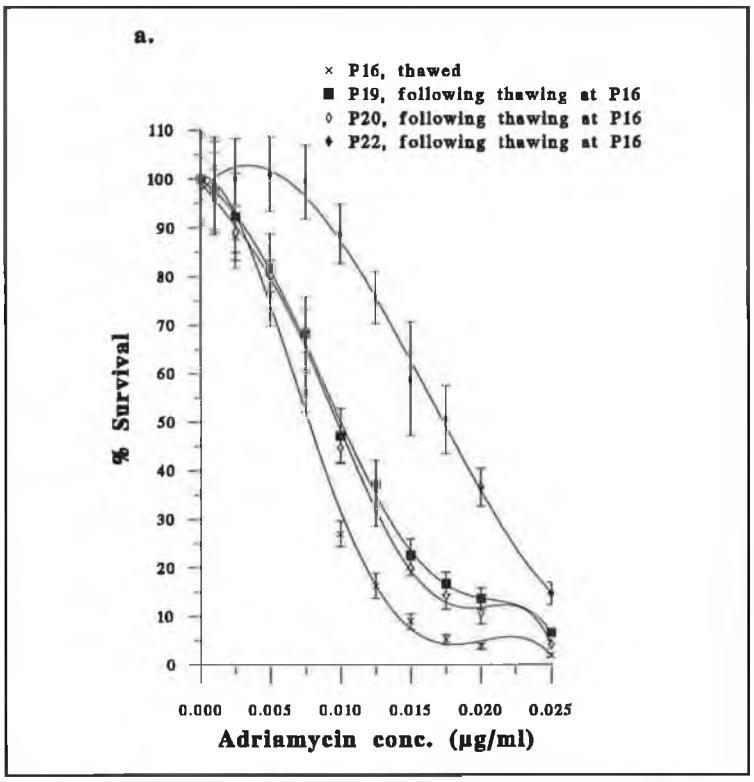


Figure 3.4.1.2 : Stability of the VP-16 toxicity profile of DLKP/VP-8 when maintained in a drug-free medium, for up to three months. The error bars represent the standard deviation on the mean of eight replicas.



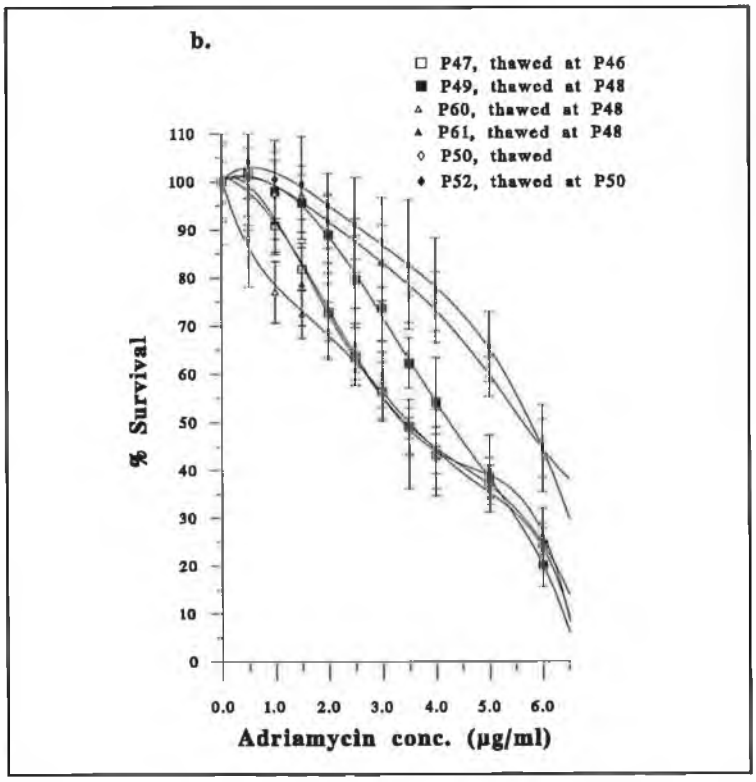
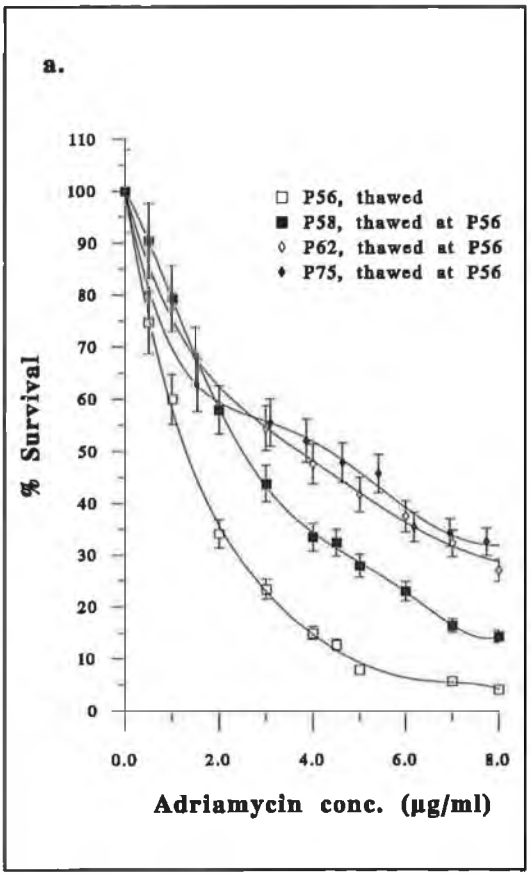
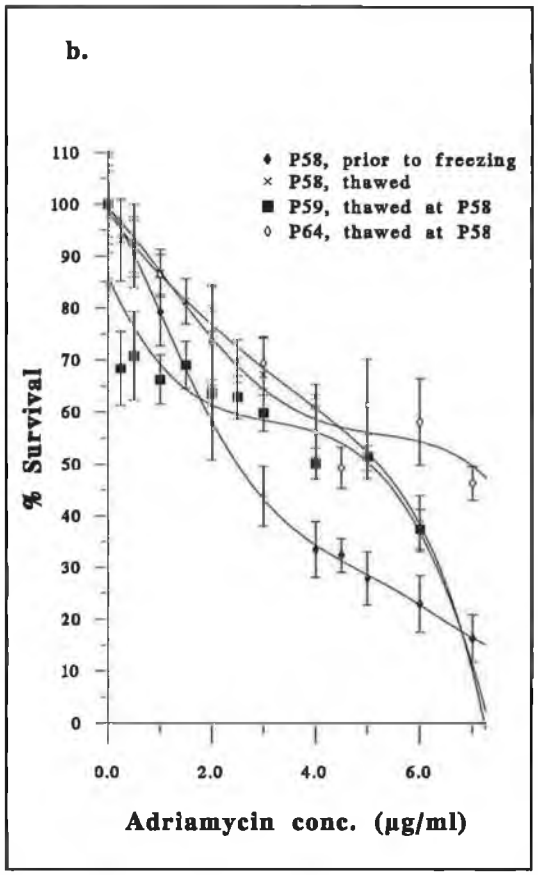


Figure 3.4.2.1 : Stability of the adriamycin toxicity profiles of DLKP (a) and DLKP-A (b), following thawing and passaging in the absence of adriamycin. The error bars represent the standard deviation on the mean of eight replicas.





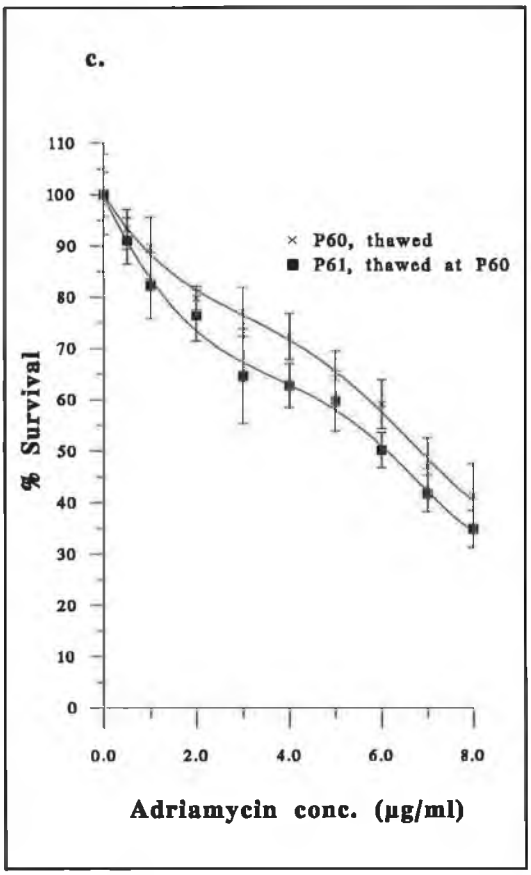


Figure 3.4.2.2 : Stability of the adriamycin toxicity profile of DLKPA5F, following thawing and passaging in the absence of adriamycin. Figure 3.4.2.2(a) presents the adriamycin toxicity profiles of DLKPA5F when thawed at P56 and its profile at subsequent passage numbers. Figure 3.4.2.2(b) presents the adriamycin toxicity profiles of DLKPA5F at P58, immediately prior to and after thawing and its profile at subsequent passage numbers. The cells at P58 prior to freezing are the same cells as DLKPA5F thawed at P56 and passaged twice, as in Figure 3.4.2.2(a). Figure 3.4.2.2(c) presents the adriamycin toxicity profile of DLKPA5F at P60, immediately following cell thawing and after passaging the cell line. The error bars represent the standard deviation on the mean of eight replicas.

3.5 Radiation studies

Exposure of cells to chemotherapeutic drugs has, in some cases, been associated with conferring cross-resistance to radiation on these cells, with the converse also found (Mattern *et al.*, 1991; Hill *et al.*, 1990b; Tanio *et al.*, 1990). In order to investigate if this were true for the MDR variant of the squamous cell carcinoma cell line, DLKP-A and its clonal cell lines, their toxicity profiles with respect to radiation were examined and compared to that found in DLKP. To determine if resistance to radiation leads to drug cross-resistance for this cell line, DLKP was exposed to varying increasing doses of radiation and studied to detect any alterations in its toxicity profile with respect to a range of clinically used drugs.

3.5.1 Toxicity of chemotherapeutic drugs to radiation treated cells

Following exposure of DLKP to increasing doses of radiation, allowing time for the cells to recover between each exposure (as outlined in Table 3.5.1), DLKP did not exhibit an MDR profile, *i.e.* it showed no significant alteration in its level of sensitivity to any of the five chemotherapeutic agents tested (Table 3.5.2). The toxicity profile, as deduced by IC_{50} values, was unaltered with respect to vincristine and VP-16 and is decreased, but not by a significant amount, in terms of adriamycin, cisplatin and 5-fluorouracil.

Day	Radiation Dose	Passage Number
1	150 cGy	17
15	600 cGy	18
21	900 cGy	19
28	900 cGy	19
48	1200 cGy	19
77	1200 cGy	20
83	1200 cGy	20

Table 3.5.1 : The radiation treatment profile used in the generation of the cell line DLKP/RAD, from the parental cell line, DLKP.

IC_{50} (nM)	DLKP	DLKP/RAD
Adriamycin	24.80 ± 14.48	13.02 ± 0.36
Cisplatin	873.00 ± 387.33	500.00 ± 13.00
Vincristine	1.21 ± 0.30	1.20 ± 0.60
VP-16	146.45 ± 68.81	158.00 ± 15.80
5-Fluorouracil	9551.11 ± 6255.96	3239.82 ± 1191.39

Table 3.5.2 : IC_{50} values of a range of chemotherapeutic drugs to DLKP and DLKP/RAD. The IC_{50} value presented is the mean value calculated from triplicate repeat assays. It is presented as the average IC_{50} ± standard deviation and is calculated as outlined in Appendix A.

3.5.2 Toxicity of radiation to MDR Cells

Exposure of a squamous cell carcinoma to a chemotherapeutic drug, such that it acquired an MDR phenotype of cross-resistance to a range of structurally unrelated drugs, as in the case of DLKP-A, resulted in no notable alteration in the cells' susceptibility to cell kill by radiation. The radiation toxicity was determined by the cells' IC_{50} value, the quasi-threshold value, D_q (which represents the size of the shoulder on the curve and reflects the cells ability to accumulate and repair irradiation damage) and D_o (which represents the slope of the straight line part of the curve) (see Appendix B). The MDR cell line DLKP-A, exhibited a slight increase in its IC_{50} value, extrapolation number and D_o, indicating a slight increase in the cell line's resistance to radiation, with respect to DLKP (Table 3.5.2). The level of radiation sensitivity observed in the DLKP-A clones ranged from more sensitive than the parental cell line (DLKP), to more resistant than the DLKP-A cell line.

The clonal cell lines of DLKP-A exhibited variation with respect to their radiation profile. DLKPA2B, DLKPA6B, DLKPA3C, DLKPA10C and DLKPA2D all possessed IC_{50} values within the range of DLKP-A (242 ± 32 cGy). However, DLKPA6B was the only one of these clones which also possessed an extrapolation number similar to DLKP-A. DLKPA2B and DLKPA3C had lower D_q values and slightly higher D_o values, with respect to DLKP-A. DLKPA10C and DLKPA2D had both slightly lower D_q and D_o values. DLKPA5F was notably more resistant to radiation than DLKP-A, as determined from its higher IC_{50} and D_q values. Both cell lines had similar D_o values, but DLKPA5F had an IC_{50} and D_q value of 303 ± 35 cGy and 177.05 cGy, respectively, as compared to the values obtained for DLKP-A of 242 ± 32 cGy and 130.43 cGy, respectively. In comparison with DLKP-A, DLKPA11B and DLKPA5D were more sensitive to cell kill by radiation.

Cell line	Radiation Data		
	D_o (cGy)	D_q (cGy)	IC_{50} (cGy)
DLKP	115.17	88.13	193 ± 83
DLKPA	167.39	130.43	242 ± 32
DLKPA2B	214.41	68.58	215 ± 83
DLKPA6B	120.63	125.40	215 ± 15
DLKPA11B	188.48	62.83	134 ± 30
DLKPA5F	164.77	177.05	303 ± 35
DLKPA9B	163.35	109.95	165 ± 65
DLKPA3C	191.75	72.68	208 ± 47
DLKPA10C	128.76	98.15	191 ± 52
DLKPA2D	122.11	90.53	194 ± 60
DLKPA5D	230.77	27.69	124 ± 21

Table 3.5.2 : Toxicity of ionising radiation to DLKP variants, as determinable from the IC_{50} of radiation to the cells, the quasi-threshold dose, D_q, and D_o. The IC_{50} value shown is the mean of the values obtained from duplicate assays. The IC_{50} value was calculated as outlined in Appendix A and the D_o and D_q values were determined as shown in Appendix B.

3.6 Doubling time of cell lines

The doubling time, calculated during the exponential phase of cell growth, was determined for variants of DLKP, while growing in ATCC medium supplemented with 1% L-glutamine and 5% foetal calf serum (Sera Lab : Batch number 101024) and the results are presented in Table 3.6.1. The doubling time was determined from a single experiment which contained eight internal replicas.

DLKP had a doubling time of 36.19h., while the doubling time observed in its MDR variant, DLKP-A, was reduced to 31.72h. The VP-16-selected MDR cell lines also had reduced doubling times with respect to DLKP. DLKP/VP-8 had a doubling time of 33.38h., while DLKP/VP-3 had a greatly reduced doubling time of 19.77h. All DLKP-A clones isolated had their own unique doubling times, ranging from 15.60h. for DLKPA2D to 32.86h. for DLKPA5F.

Cell line	Doubling time (hours)
DLKP	36.19
DLKPA	31.72
DLKPA2B	31.74
DLKPA6B	32.91
DLKPA11B	24.01
DLKPA5F	32.86
DLKPA9B	19.78
DLKPA3C	20.63
DLKPA10C	16.80
DLKPA2D	15.60
DLKPA5D	20.59
DLKP/VP-3	19.77
DLKP/VP-8	33.38

Table 3.6.1 : Doubling times of variants of the DLKP cell line, determined during the exponential phase of cell growth.

3.7 Regeneration of DLKP-A-like population

All clones isolated from DLKP-A possessed the MDR phenotype of cross-resistance to a range of structurally unrelated chemotherapeutic drugs (Section 3.2.2). However, no clone had an identical toxicity profile to that observed in DLKP-A. To determine if a DLKP-A-like cell population could be regenerated, the four clones DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F were mixed at varying ratios, to establish a population similar to DLKP-A, as determined by its toxicity to adriamycin. As can be seen from Figure 3.7.1, a mixture of DLKPA5F, DLKPA2B, DLKPA6B and DLKPA11B in the ratio 2:1:1:1, respectively, generated a cell line with a toxicity profile most like that of DLKP-A. Most deviation between DLKP-A and the mixed populations occurred at low drug concentrations. While 1μg/ml adriamycin resulted in negligible cell kill in DLKP-A, 10% kill was observed in the regenerated cell line most like DLKP-A and 20-35% kill was noted in all other regenerated cell lines at this concentration of adriamycin. Therefore, a factor(s) present in the DLKP-A cell line, which contributed to its adriamycin-resistance, was missing from the mixed clones, or if present, its effect was masked.

The regenerated cell line most like DLKP-A with respect to adriamycin toxicity, was examined to determine if it also had similar VP-16 and vincristine toxicity profiles. The resulting toxicity profiles, generated from performing concurrent toxicity assays on the mixed population of clones and the DLKP-A cell line (in duplicate), showed that the mixed cell line also exhibited similar levels of resistance to vincristine and VP-16 to that observed in DLKP-A (Figure 3.7.2).

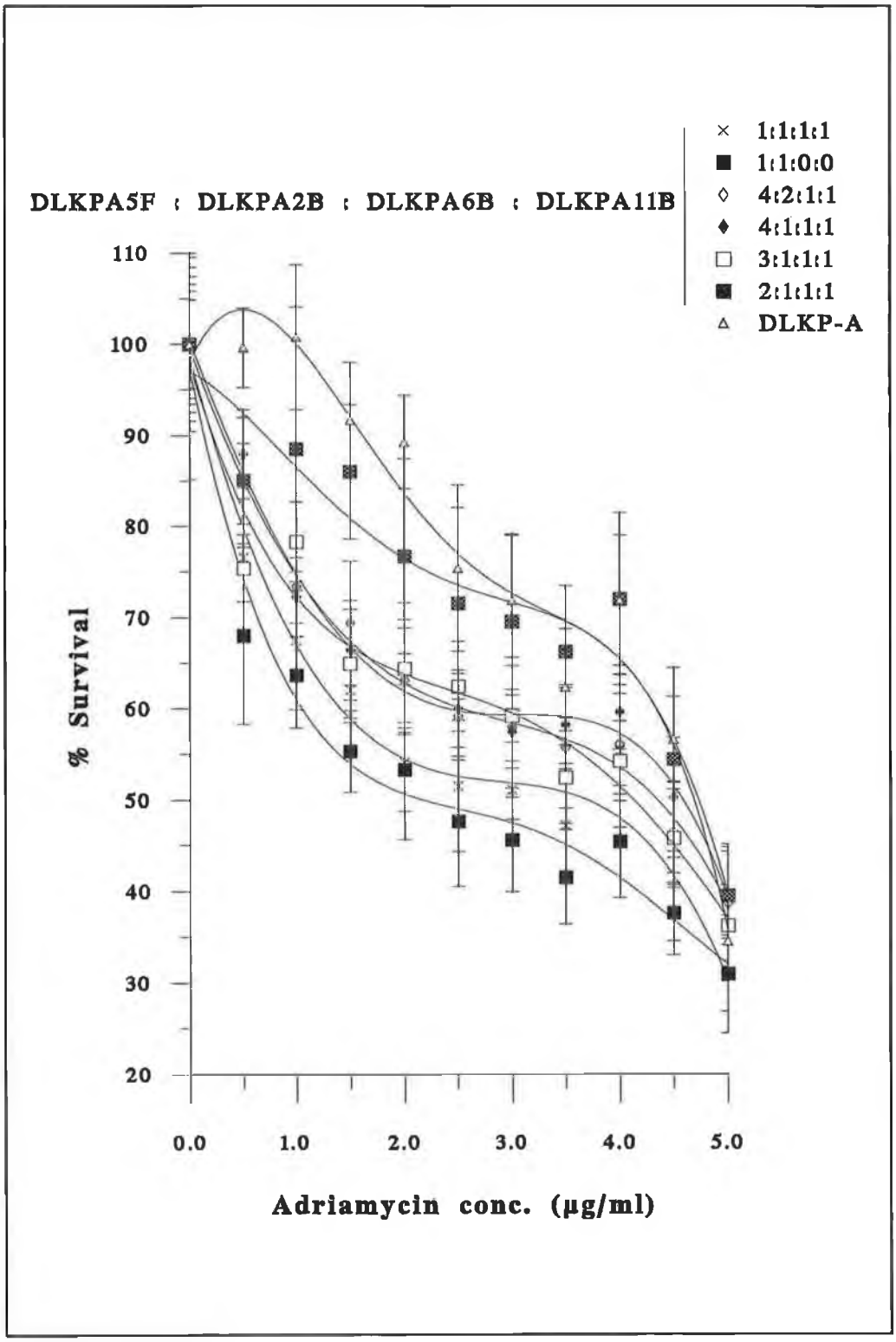
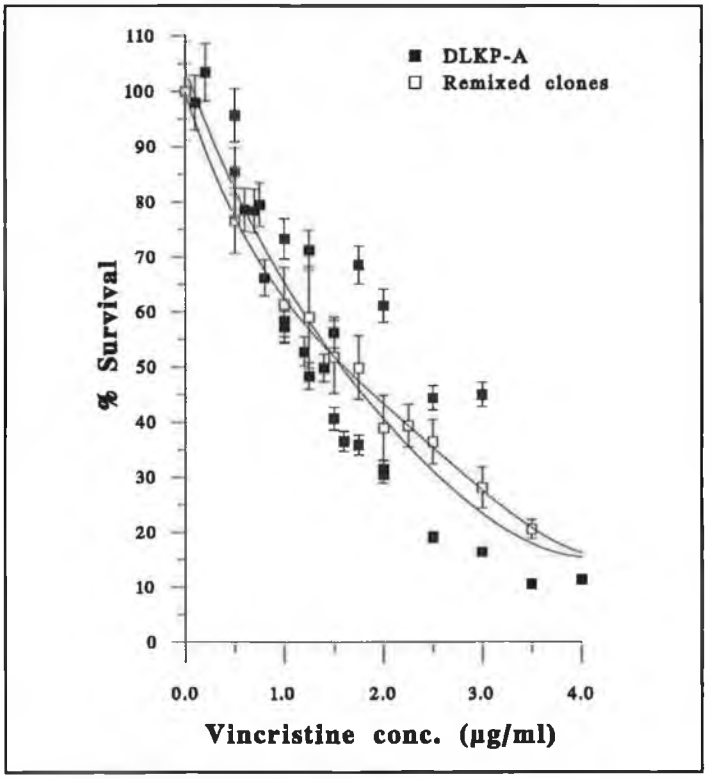


Figure 3.7.1 : Toxicity profile of DLKP-A and mixed cell lines, consisting of various ratios of DLKPA5F, DLKPA2B, DLKPA6B and DLKPA11B, to adriamycin. The error bars represent the standard deviation on eight replicas.

a.



b.

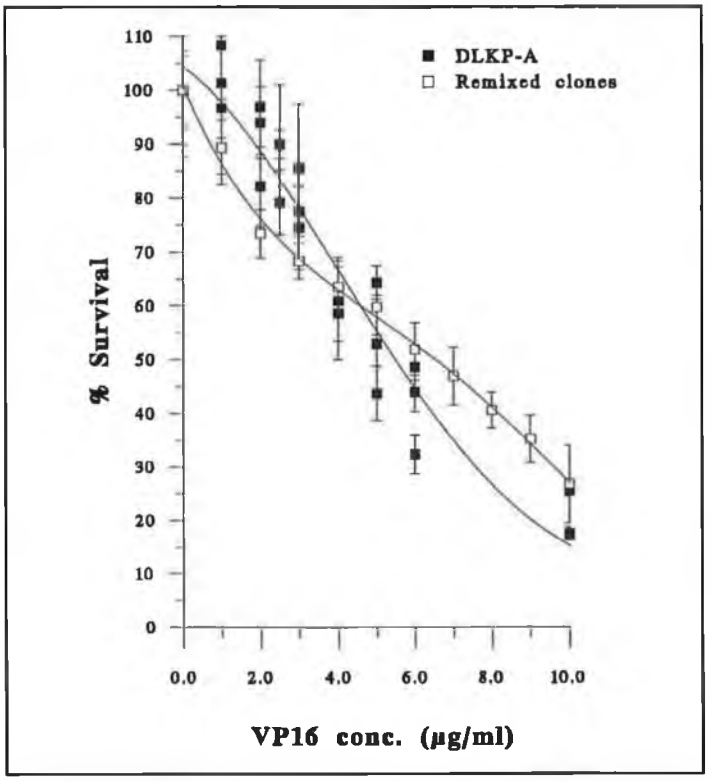


Figure 3.7.2 : Toxicity profile of DLKP-A and the mixed clones, in the ratio 2:1:1:1 of DLKPA5F, DLKPA2B, DLKPA6B and DLKPA11B, respectively, to vincristine (a) and VP-16 (b). The error bars represent

3.8 mRNA analysis

Alterations in the expression of proteins involved in conferring an MDR profile on cells, were investigated at the mRNA level in DLKP, its VP-16 and adriamycin selected MDR variants and four clones of DLKP-A, DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F. The level of gene transcripts, corresponding to mdr1, topoisomerase II, topoisomerase II-α and topoisomerase II-ß, were examined by RT-PCR analysis. The analysis was performed on one RNA extraction from each cell line. Duplicate batches of cDNA were generated, using the Moloney murine leukaemia virus reverse transcriptase enzymes. The duplicate cDNA aliquots were used to individually assess the mRNA levels of interest in the original sample, by PCR amplification of sequences specific to each RNA. The level of transcript of interest in each cell line was normalised to the level of the ubiquitously expressed ß-actin mRNA and so corrected for inter-reaction variations in the amplification efficiency. The primers used to specifically amplify ß-actin mRNA are detailed by O'Driscoll *et al.* (1993) and amplify a 383bp stretch of ß-actin cDNA. In each case, 30 cycles of amplification of the mRNA of interest were performed. The PCR product concentration is proportional to the starting target DNA only as long as the product accumulation remains exponential. As the point at which the exponential accumulation plateaus was not assessed prior to analysis, the determination of the mRNA levels in these experiments was only semi-quantitative.

3.8.1 Analysis of mdr1 transcripts.

The mdr1-specific primers used in this study have been previously detailed by Noonan *et al.* (1990). They amplify a 157bp region of the mdr1 cDNA, from position 2596 to 2752 on the mdr1 cDNA sequence. The primers flank a sequence that crosses an intron, so contaminating DNA can be detected by the production of a 1257bp band. When these primers were used to detect mdr1 mRNA levels in variants of DLKP, the level of the mdr1 transcripts was increased in all the MDR cell lines by comparison with DLKP, which expressed no detectable levels of mdr1 mRNA (Figure 3.8.1). The only significant difference in mdr1 mRNA levels detected were those noted when any of the MDR cell lines were compared with DLKP. When compared to each other, the relative differences in the intensity of the bands resulting when mdr1 mRNA levels were examined in all the MDR cell lines was minimal. In the VP-16-selected cell lines, DLKP/VP-3 appeared to possess slightly higher levels of mdr1 mRNA than the more resistant cell line, DLKP/VP-8 (Table 3.8.1), but the differences in their mRNA levels was not significant. DLKP/VP-8 exhibited an equal level of the mdr1 transcript

to that noted in DLKP-A. All clones of DLKP-A appeared to express less mdr1 mRNA than that detected in DLKP-A. Levels detected in DLKPA5F were slightly lower than those found in DLKP-A. The cell lines, DLKPA2B and DLKPA11B had equal, but lower, levels of mdr1 mRNA than DLKPA5F. The mdr1 mRNA level detected in DLKPA6B was significantly different from that noted in DLKP-A. DLKPA6B possessed approximately half the amount of mdr1 mRNA observed in DLKP-A.

3.8.2 Analysis of topoisomerase II mRNA transcripts

The primers used to determine the levels of topoisomerase II mRNA in the cell lines, allowed the detection of topoisomerase II in general, by amplifying a region that is common to both the α and ß isoforms of the enzyme. The primers, detailed by O'Driscoll *et al.* (1993), amplify a 216bp region homologous to the region spanning base pairs 1395 to 1610 of topoisomerase II, as described by Tsai-Pflugfelder *et al.* (1988). Analysis of topoisomerase II mRNA levels in the cell lines revealed only a slight reduction in the levels of the mRNA in the DLKP/VP-3 and DLKP/VP-8 cell lines by comparison with the levels noted in DLKP (Figure 3.8.1). Although no significant alterations in mRNA levels were observed in DLKP-A compared to DLKP, a reduction in transcript levels was noted in the DLKP-A clones. However, the level of topoisomerase II mRNA expression was similar in all clones.

3.8.3 Analysis of topoisomerase II-α mRNA transcripts

Analysis of mRNA levels which code for the α subunit of the topoisomerase II enzyme revealed no significant alteration in the MDR cell lines examined (Figure 3.8.1). All cell lines expressed detectable levels of topoisomerase II-α. A 139bp region, from base 4052 to 4190, of the topoisomerase II-α sequence published by Tsai-Pflugfelder *et al.* (1988) was detected in this case. Although the region amplified was slightly homologous to topoisomerase II-ß, the primers were specific for topoisomerase II-α (O'Driscoll *et al.*, 1993).

3.8.4 Analysis of topoisomerase II-ß mRNA transcripts

RT-PCR analysis of mRNA levels coding for topoisomerase II-ß was carried out using primers to bases 4335-4364 and 4425-4452 of the topoisomerase II-ß cDNA described by Jenkins *et al.* (1992). These primers amplified a 118bp region unique to topoisomerase II-ß (O'Driscoll *et al.*, 1993). RT-PCR analysis revealed that all the cell lines expressed detectable, but low levels of the mRNA (Figure 3.8.1). The intensity of the topoisomerase II-ß homologous band, relative to the intensity of the ß-actin band, was approximately one fifth of that observed for the other transcripts. Once again, any alterations observed in the topoisomerase II-ß mRNA levels in the cell lines were not significant by comparison with the levels detected in DLKP.

CELL LINE/ CLONE	mdr1	Topo II	Topo II-α	Topo II-ß
DLKP/VP-8	0.583, 0.688	0.636, 0.545	0.455, 0.500	0.048, 0.050
DLKP/VP-3	0.737, 0.750	0.520, 0.625	0.555, 0.500	0.039, 0.050
DLKP	0.000*, 0.000*	0.625, 0.647	0.555, 0.555	0.120, 0.091
DLKPA	0.647, 0.688	0.667, 0.533	0.500, 0.211	0.100, 0.100
DLKPA2B	0.500, 0.500	0.400, 0.368	0.571, 0.555	0.130, 0.050
DLKPA6B	0.333, 0.100	0.364, 0.458	0.500, 0.600	0.053, 0.063
DLKPA11B	0.500, 0.500	0.263, 0.478	0.444, 0.429	0.091, 0.0*
DLKPA5F	0.563, 0.579	0.316, 0.389	0.500, 0.500	0.091, 0.053

Table 3.8.1 : Densitometrical analysis of mRNA levels of mdr1, topoisomerase II, topoisomerase II-α and topoisomerase II-ß, as determined by RT-PCR. The densitometer readings were normalised to the level of the ß-actin transcript present in each cell line, which was co-amplified with the transcript of interest, by PCR.
* The transcript of interest was not detectable by densitometrical analysis in this case, but a band corresponding to ß-actin was observed.

3.8.5 Conclusions

The mRNA levels of genes associated with MDR were altered in all drug resistant cell lines examined, as determined by RT-PCR analysis. All the resistant cell lines over-expressed mdr1 mRNA, by comparison with the drug-sensitive parental cells. However, any alterations in the mRNA levels of topoisomerase II, or its α and ß isoforms, in DLKP-A, DLKP/VP-3 and DLKP/VP-8 was not significant. A small reduction in the topoisomerase II mRNA levels in the four clones of DLKP-A, was observed.

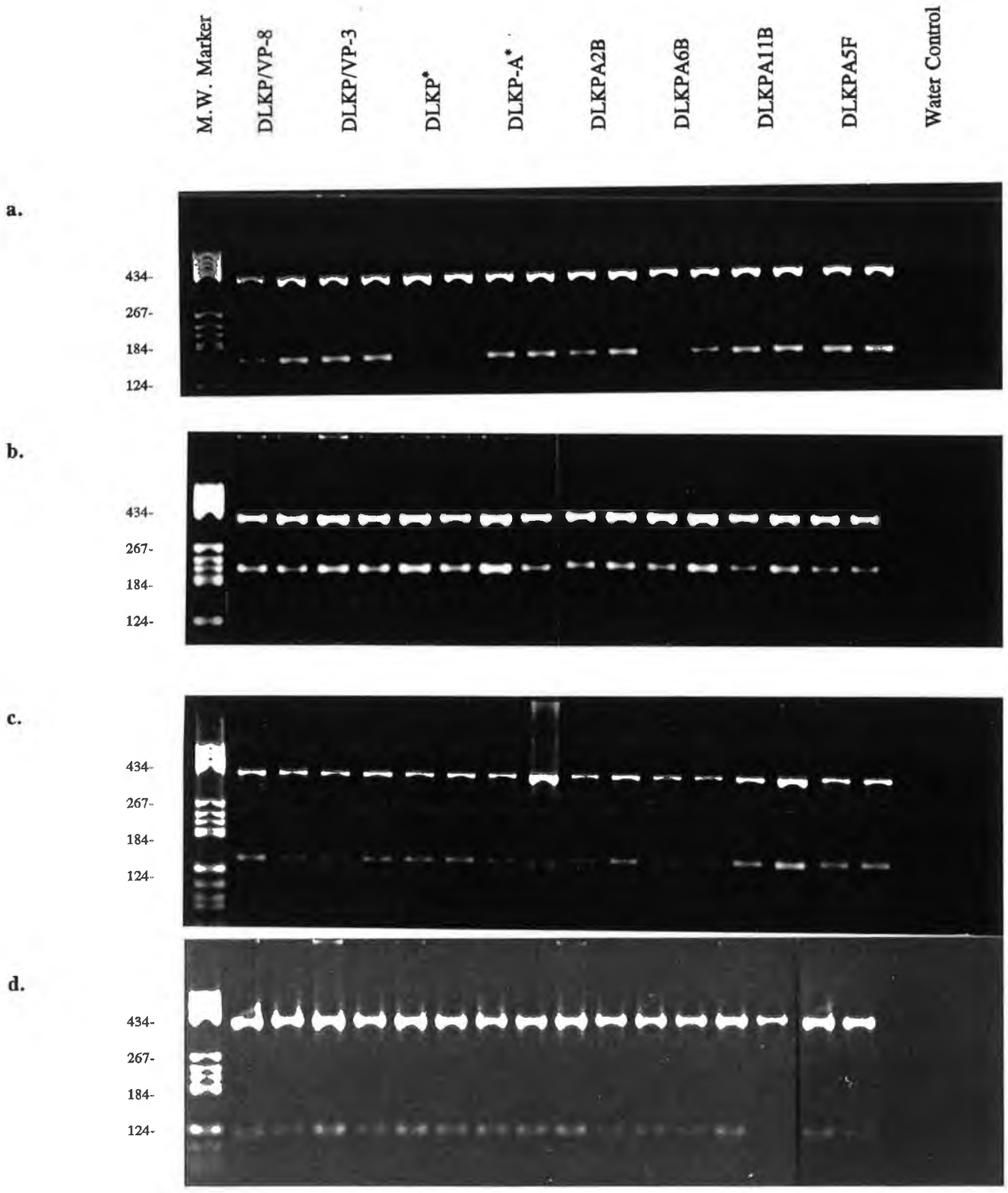


Figure 3.8 : Gel electrophoresis of the products of RT-PCR analysis of the mRNA levels of mdr1 (a), topoisomerase II (b), topoisomerase II-α (c) and topoisomerase II-ß (d) in DLKP variants. The left-hand lane contains the DNA molecular weight marker,which contains 22 fragments with the following number of base pairs; 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 8. The right hand, last two lanes contain water controls, where cDNA was not added to the PCR reaction, but was replaced by an equal volume of ultra-pure water. This acted as the negative control for the PCR reaction. The central lanes contain the products of the RT-PCR analysis on the cell lines of interest, in duplicate. Two bands were produced in these cases, the upper band by the ß-actin primers and the lower band by the primers to either mdr1 (a), topoisomerase II (b), topoisomerase II-α (c) or topoisomerase II-ß (d). The ß-actin band acted as an internal positive control and as a means to normalise the intensity of the bands of interest.
* RT-PCR analysis on these cell lines was originally performed by Ms. Lorraine O'Driscoll, M.Sc. and was repeated here to enable comparisons between their mRNA levels and those of other DLKP variants.

3.9 Protein analysis

Alterations in the expression of proteins associated with MDR was investigated by Western blot analysis. Over-expression of P-glycoprotein and alterations in the levels of topoisomerase II α and ß subunits were examined in the VP-16-selected MDR cells and the clones isolated from DLKP-A. Comparisons were made with the levels observed in the DLKP and DLKP-A cell lines.

3.9.1 Western blot analysis of topoisomerase II levels.

3.9.1.1 Topoisomerase II levels in total cell extract.

Topoisomerase II levels, as determined by Western blot analysis of total cell extract, with a polyclonal antibody to topoisomerase II (Fernandes *et al.*, 1990) showed that all cell lines possessed immunologically reactive topoisomerase II (Figure 3.9.1.1.1). The antibody detected a 170 kDa. (topoisomerase II-α) and 180 kDa. (topoisomerase II-ß) isoform of the enzyme, in all cell extracts. Western blot analysis with pre-immune rabbit serum revealed no detectable immunologically reactive compounds (Figure 3.9.1.1.1). The band intensity in all cases was analyzed by densitometrical means and the results detailed in Table 3.9.1.1. Topoisomerase II levels were determined in two separate cell extractions and the results were found to correlate.

Topoisomerase II levels varied for each cell line studied. DLKP, the parental cell line, possessed the highest levels of immunologically detectable topoisomerase II while a stronger band was noted for the 180 kDa. form than the 170 kDa. form. Topoisomerase II levels were reduced in DLKP-A, in comparison with DLKP and again the 180 kDa. band appeared to be the stronger of the two bands detected. Detectable levels of topoisomerase II in the VP-16-selected cell lines varied. The level of topoisomerase II in DLKP/VP-3 was increased slightly over that observed in DLKP. This increase was reflected by an increase in band intensity of both the 170 kDa. and 180 kDa. form, with the 180 kDa. band staining strongest. The overall topoisomerase II level in DLKP/VP-8 was reduced by comparison with DLKP and the band corresponding to the 180 kDa. form was the stronger of the two bands. The relative intensity of the 180 kDa. to the 170 kDa. bands for each cell line described above, remained constant.

Topoisomerase II levels in total cell extracts from four clones of DLKP-A

were examined. Both the 170 kDa. and 180 kDa. form were detected with the polyclonal antibody. DLKPA2B, the most adriamycin-sensitive clone, had a topoisomerase II level similar to the drug sensitive parental cells, DLKP, and the relative intensity of the 170 kDa. to 180 kDa. band was constant, in comparison with the levels observed in DLKP. DLKPA6B, which was 95-fold resistant to adriamycin relative to DLKP, had decreased levels of detectable topoisomerase II, when compared to DLKP and possessed approximately 1.5 times the level detected in DLKP-A. The relative level of the two isoforms was altered in this cell extract, by comparison with the levels detected in DLKP. The detected 170 kDa. band was more intense than the 180 kDa. band. DLKPA11B, which was 85-fold resistant to adriamycin, also possessed more immunologically detectable topoisomerase II than DLKP and in this case the intensity of both enzyme isoforms were equal. DLKPA5F, the most resistant clone, had an overall topoisomerase II level similar to DLKP and to the most drug sensitive clone, DLKPA2B. In contrast to both these cell lines, total cell extract from DLKPA5F contained equal levels of detectable 170 kDa. and 180 kDa. forms of the protein.

The levels of the α and ß subunits of topoisomerase II were individually examined by Western blot analysis of the total cell extract with monoclonal antibodies which individually identify the α and ß subunits (Figure 3.9.1.1.2). The topoisomerase II-α isoform detected was a 170 kDa. protein. The topoisomerase II-ß form banded at 150 kDa. and 180 kDa. The 150 kDa. product has been characterised as a possible breakdown product of the 180 kDa. topoisomerase II-ß subunit (Negri *et al.*, 1992).

All cell lines tested contained detectable levels of both α and ß topoisomerase II subunits (Figure 3.9.1.1.2). DLKP had the highest levels of topoisomerase II-α detected in total cell extract. The VP-16-selected MDR cells exhibited reduced levels of the α subunit. DLKP/VP-3 showed only a slight reduction in the level of the protein, while the level of the immunoreactive protein was decreased 3-fold in DLKP/VP-8. DLKP-A possessed a similar level of topoisomerase II-α to that observed in DLKP/VP-8. All DLKP-A clones had reduced levels of detected topoisomerase II-α, by comparison with DLKP, but had slightly higher levels than those noted in DLKP-A. Of the four clones, the levels of topoisomerase II-α were greatest in DLKPA11B, DLKPA2B had slightly lower amounts and DLKPA5F and DLKPA6B had the lowest, but relatively equal amounts of topoisomerase II-α.

DLKP and its MDR variants DLKP-A, DLKP/VP-3 and DLKP/VP-8 all possessed a form of topoisomerase II-ß that banded at 150 kDa., with very low levels of the 180 kDa. form observed. DLKP again possessed highest levels of the immunologically reactive enzyme. By comparison with DLKP, DLKP-A and DLKP/VP-3 had equal and

slightly lower levels of the enzyme, with DLKP/VP-8 having lowest levels of the protein. The relative difference in topoisomerase II-ß levels between the cell lines was small in comparison with the relative differences observed for topoisomerase II or its α subunit.

Western blotting analysis on DLKP-A clones resulted in the detection of only a 150 kDa. form of topoisomerase II-ß. DLKPA5F and DLKPA11B had protein levels similar to that noted in DLKP. DLKPA2B and DLKPA6B had equal but greatly reduced levels, when compared to the amount detected in DLKP.

3.9.1.2 Topoisomerase II levels in nuclear extract.

Topoisomerase II levels in cell nuclear extracts from variants of the DLKP cell line were examined with duplicate nuclear extractions from each cell line. The level of the enzyme detected differed for each variant. The polyclonal antibody to topoisomerase II detected a nuclear protein of 170-180 kDa. (Figure 3.9.1.2.1), with highest levels of the immunologically reactive protein present in DLKP. The VP-16-selected MDR cell lines exhibited decreased levels of the protein, with the level of protein inversely proportional to their level of selection to VP-16. The level of detectable protein was greatly decreased in nuclear extracts from DLKP-A, which had less immuno-detectable protein than DLKP/VP-8, though both cell lines exhibited similar resistance profiles. Examination of topoisomerase II levels in nuclear extracts from DLKP-A clones revealed that both DLKPA5F and DLKPA2B, the most and least adriamycin-resistant clones respectively, had approximately equal levels of the protein, which was comparable with the level noted in DLKP-A. DLKPA11B and DLKPA6B had comparable levels of topoisomerase II and an approximately 3-fold increase in their enzyme levels, when compared with DLKP-A.

Analysis of topoisomerase II-α levels in nuclear extracts from the cell lines, revealed the presence of an immunoreactive protein that banded at 170 kDa. (Figure 3.9.1.2.2). VP-16-selected MDR cells had decreased levels of the α subunit in comparison with DLKP, with the greatest decrease noted in the cell line with highest resistance. Topoisomerase II-α levels were also decreased in DLKP-A. The protein level in clones of DLKP-A was substantially higher than that observed in DLKP-A, but remained less than that noted in DLKP. DLKPA5F had topoisomerase II-α levels slightly lower than that observed in DLKP, while the topoisomerase II-α levels in DLKPA6B cells were marginally less than that noted in DLKPA5F. DLKPA11B and DLKPA2B had approximately a 4-fold and 8-fold decrease, respectively, in the level of their α subunit when compared to DLKP.

Analysis of immunologically detectable topoisomerase II-ß levels in cell

nuclear extracts revealed the presence of only the 180 kDa. form of the topoisomerase II-ß enzyme in all cell lines (Figure 3.9.1.2.2). No 150 kDa. form of the enzyme, which was observed when the total cell lysate was examined, was detected. The level of the protein was approximately equal in all nuclear extracts, with only slight decreases observed in DLKP and DLKPA2B.

CELL LINE/ CLONE	Topo II	Topo IIα	Topo IIß	Topo II	Topo IIα	Topo IIß
	Total cell lysate			Nuclear extract		
DLKP/VP-8	0.61	0.18	0.08	1.22	0.31	1.93
DLKP/VP-3	1.27	0.51	0.11	2.09	0.49	2.35
DLKP	1.14	0.54	0.17	3.47	0.93	1.44
DLKPA	0.44	0.19	0.11	0.72	0.05	1.68
DLKPA2B	1.12	0.27	0.02	0.80	0.11	1.44
DLKPA6B	0.67	0.20	0.02	2.40	0.60	2.10
DLKPA11B	1.41	0.33	0.15	2.24	0.22	2.65
DLKPA5F	1.14	0.21	0.16	0.96	0.84	2.17

Table 3.9.1.1 : Densitometrical analysis of topoisomerase II detection by Western blotting.

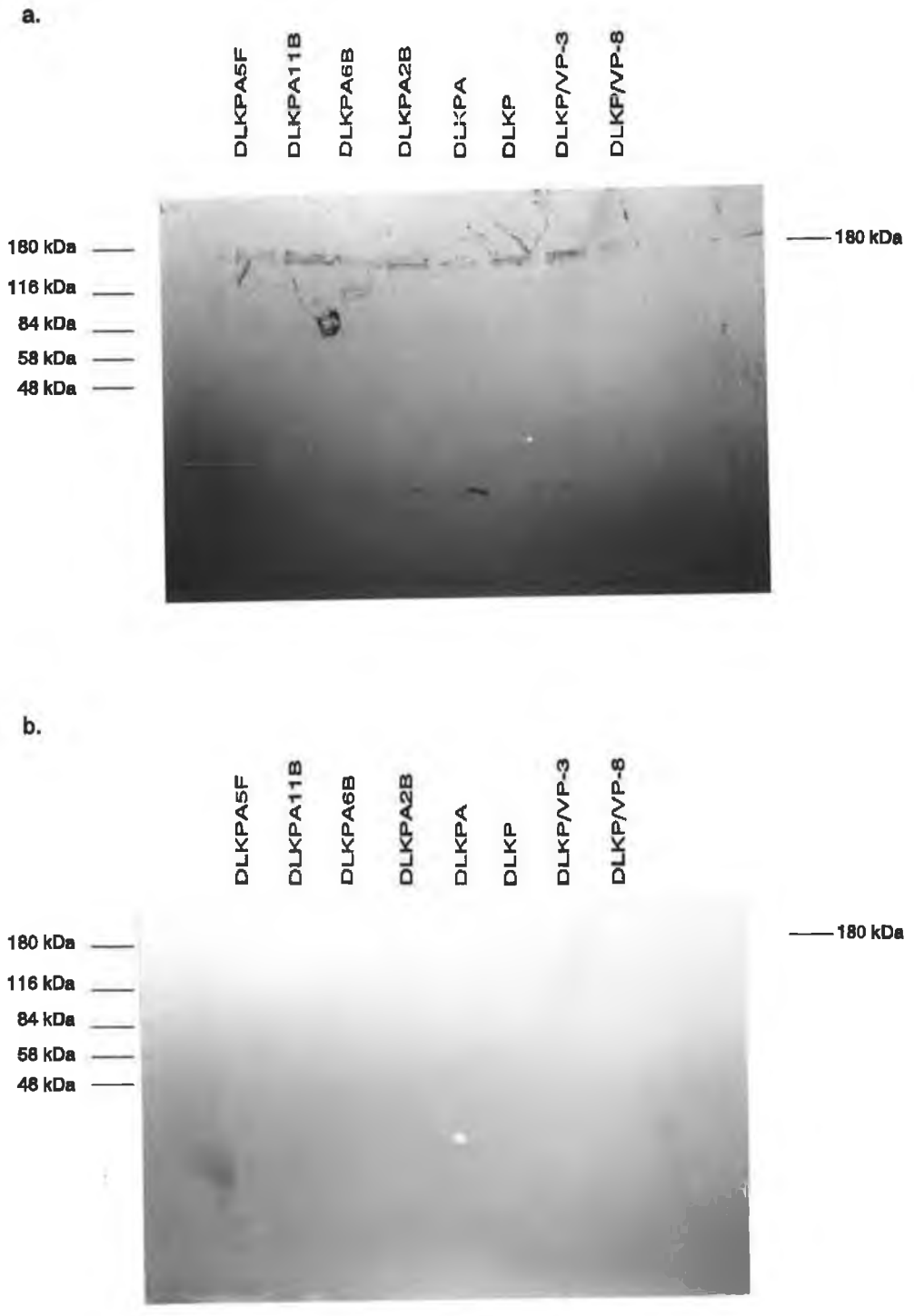


Figure 3.9.1.1.1 : Western blot detection of topoisomerase II protein in total cell extracts, isolated from DLKP variants. The protein was detected using a polyclonal antibody directed against topoisomerase II (a), with the pre-immune serum acting as the negative control (b). In all cases, 45μg of protein was loaded per lane. The left and right lanes contained molecular weight markers.

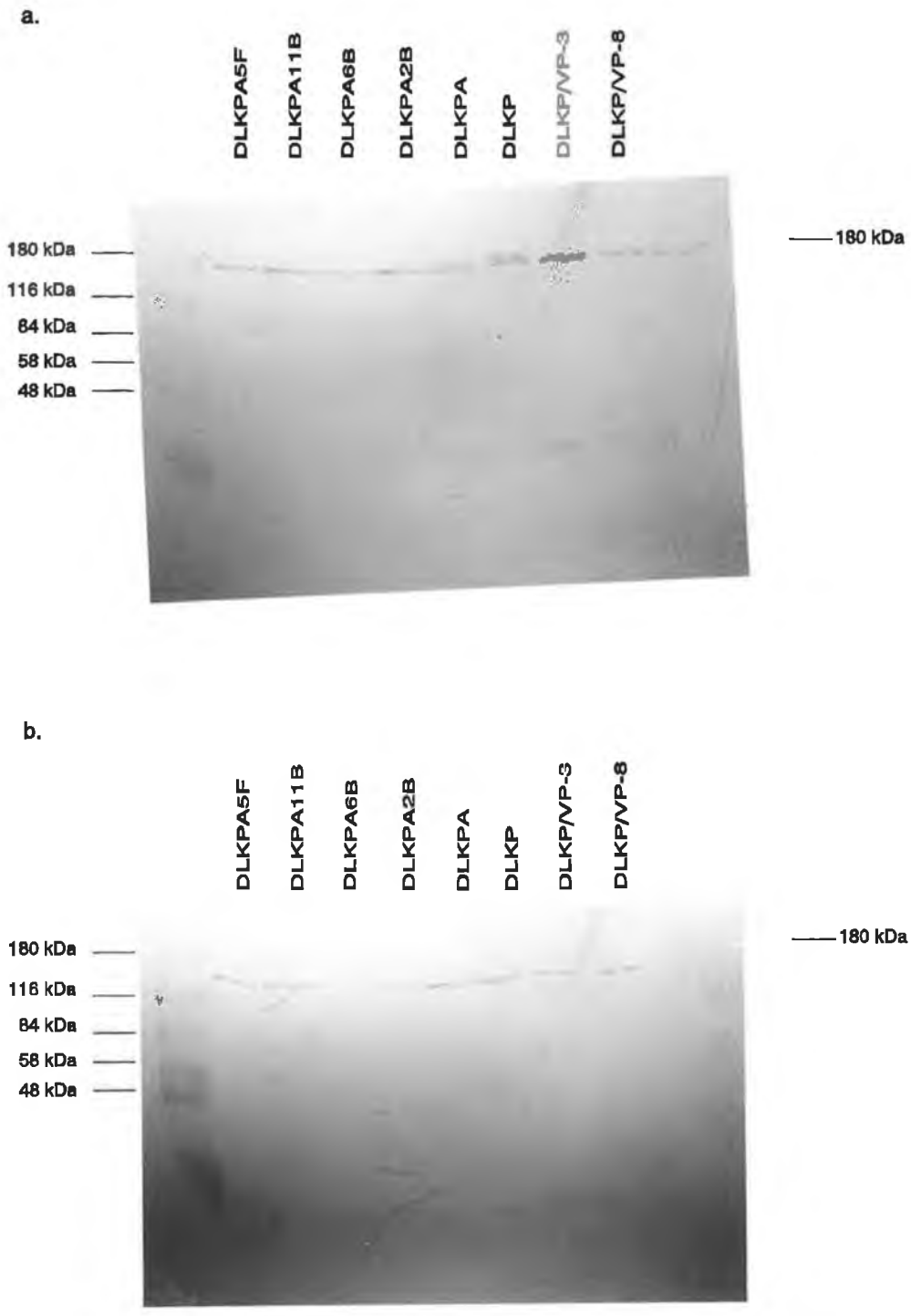


Figure 3.9.1.1.2 : Western blot detection of topoisomerase II-α (a) and topoisomerase II-ß (b) in total cell extract from DLKP variants. The proteins were detected by monoclonal antibodies directed against the specific forms of the enzyme. A total of 45μg of protein was loaded per lane. The left and right lanes contained molecular weight markers.

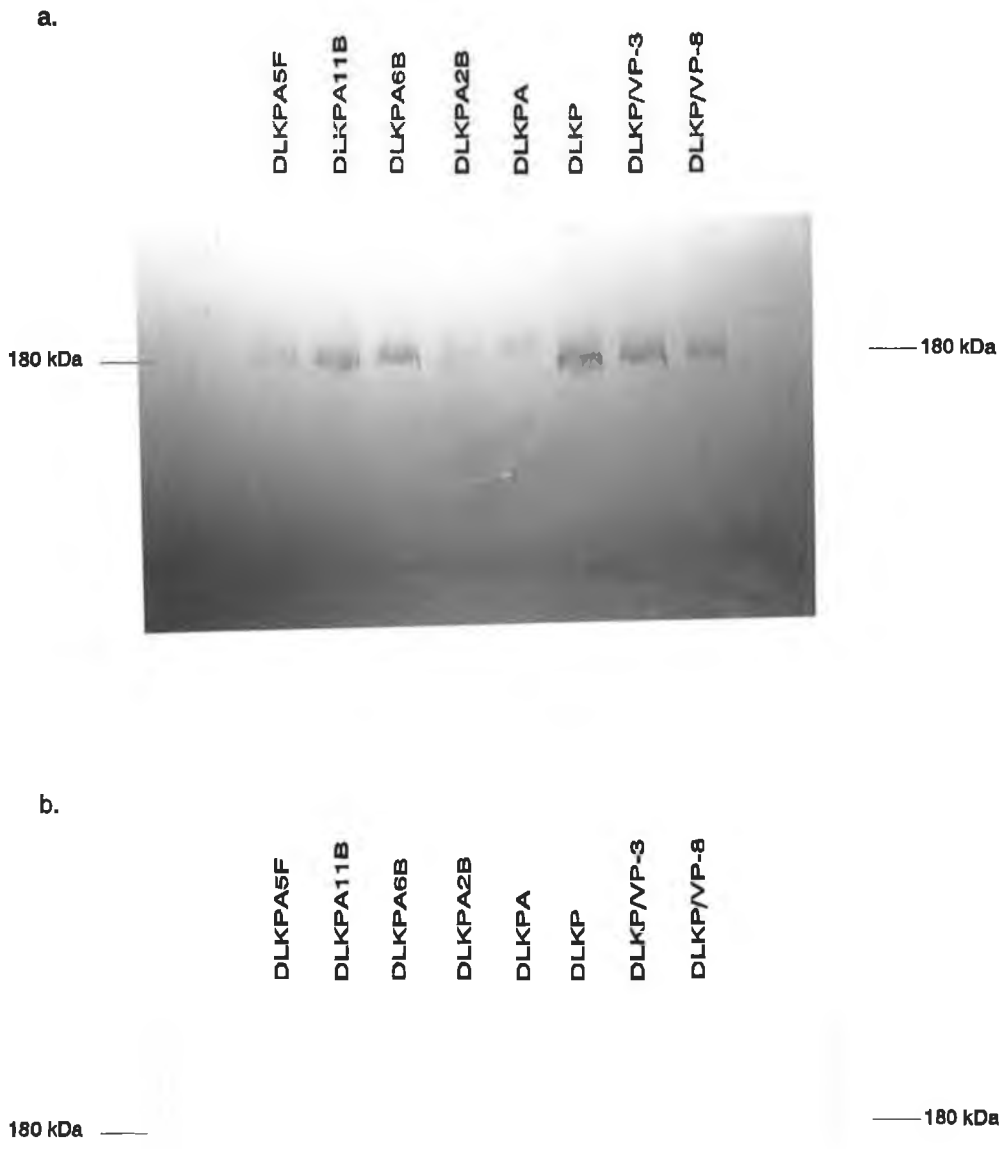


Figure 3.9.1.2.1 : Western blot detection of topoisomerase II in nuclear extract, isolated from DLKP variants. The protein was detected with a polyclonal antibody directed against topoisomerase II (a) and with the pre-immune serum, as the negative control (b). In all cases 35μg of protein was loaded per lane. The left and right lanes contained molecular weight markers.

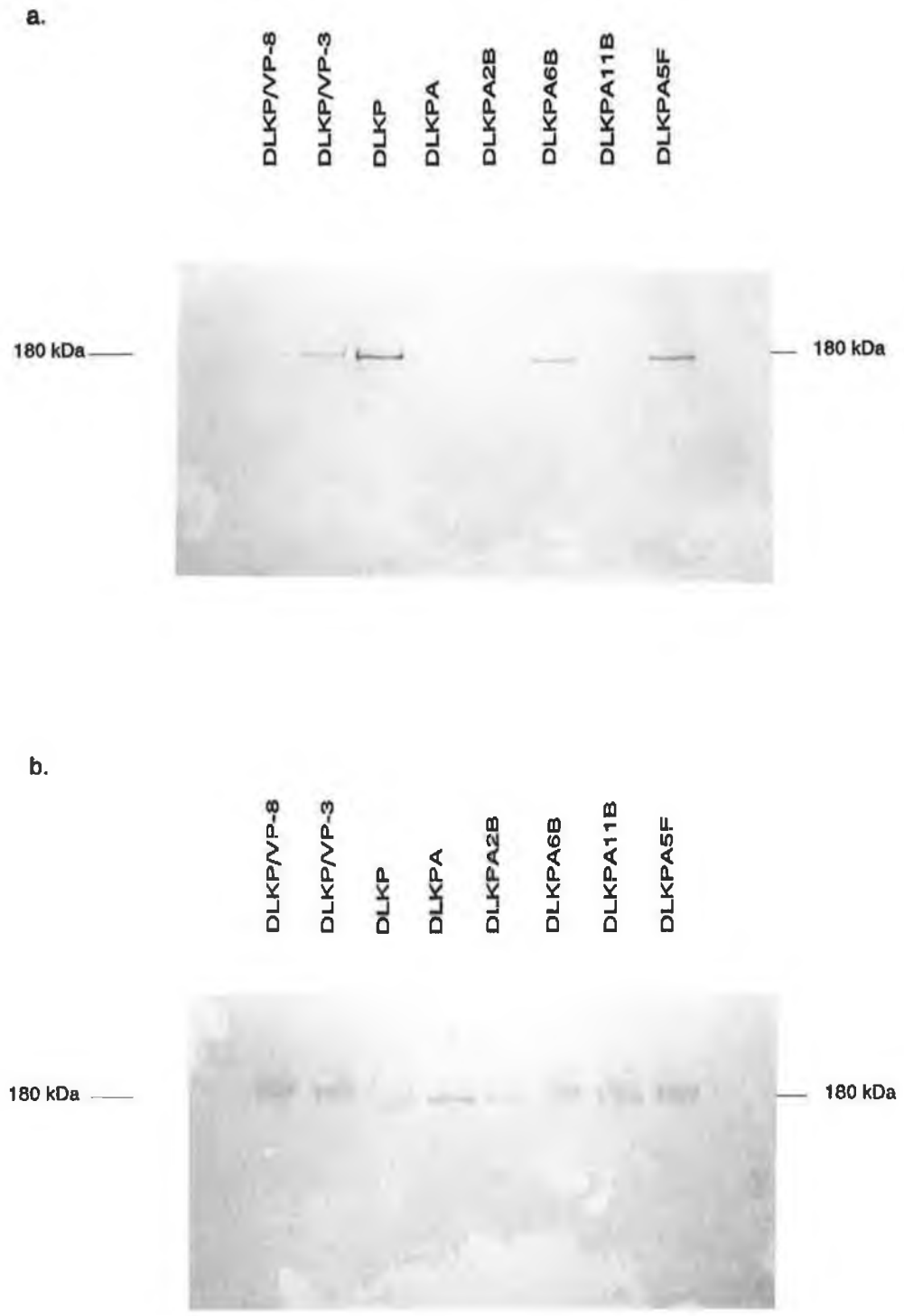


Figure 3.9.1.2.2 : Western blot detection of topoisomerase II-α (a) and topoisomerase II-ß (b) in nuclear extract from DLKP variants. The proteins were detected with monoclonal antibodies directed against the specific form of the enzyme. A total of 35μg of protein was loaded per lane. The left and right lanes contained molecular weight markers.

3.9.2 Western blot analysis of p-glycoprotein levels.

P-glycoprotein, as determined by Western blot analysis on membrane preparations, was over-expressed in all MDR cell lines (Figure 3.9.2.1). The analysis was performed in duplicate from a single cell preparation, and the results obtained were correlated. 10μg of protein was separated by gel electrophoresis and checked for cross-reactivity with the anti-P-glycoprotein antibody, C219, a 170 kDa. protein was seen to be elevated in the VP-16-selected MDR cells. DLKP/VP-3, the less resistant variant, expressed higher levels of the protein than the more resistant DLKP/VP-8 cell line, as was confirmed by densitometrical analysis (Table 3.9.2.1). All clones of DLKP-A over-expressed P-glycoprotein to greater levels than DLKP-A, which has been previously characterised (Clynes *et al.*, 1992). When the clones were compared, it was noted that the most resistant clone, DLKPA5F, expressed the lowest levels of immunodetectable P-glycoprotein while the remaining clones expressed approximately equal levels of the protein. However, the variation in P-glycoprotein expression between the clones was slight.

CELL LINE/ CLONE	P-Glycoprotein
DLKP/VP-8	3.97
DLKP/VP-3	4.17
DLKP	0.00
DLKPA	0.03
DLKPA2B	0.68
DLKPA6B	0.75
DLKPA11B	0.76
DLKPA5F	0.59

Table 3.9.2.1. : Densitometrical analysis of P-glycoprotein levels detected by Western blotting.

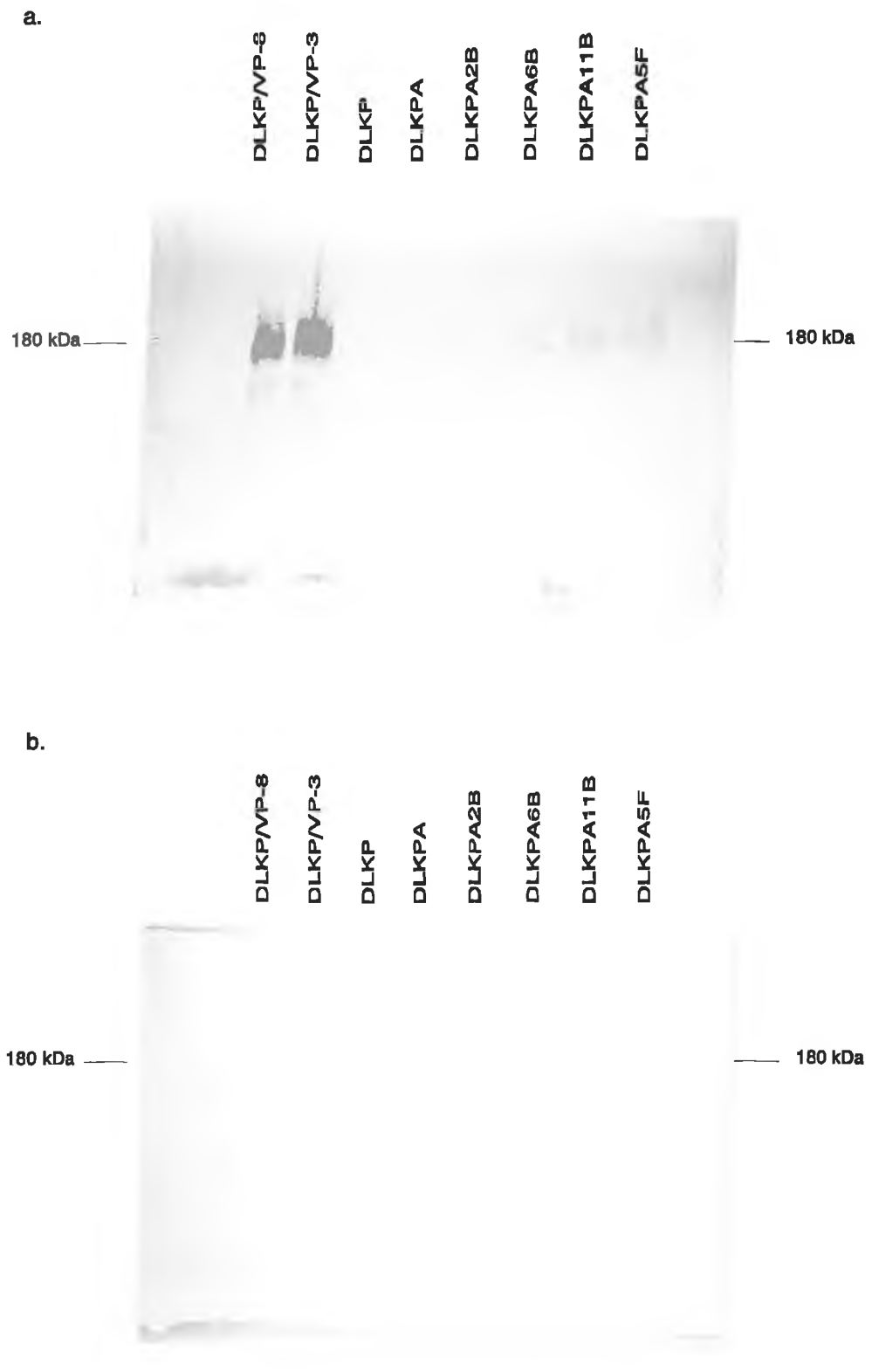


Figure 3.9.2.1 : Western blot detection of cell membrane preparations from DLKP variants with the monoclonal antibody, C219, directed against p-glycoprotein (a). The negative control is shown in figure b, where the primary antibody, C219, was replaced by an equal volume of antibody diluent, with the remainder of the blotting procedure carried out as for the positive blot. In all cases 10μg of protein was loaded per lane. The left and right lanes contained molecular weight markers.

3.9.3 Conclusions

Protein analysis of the levels of topoisomerase II, its α and ß subunits and P-glycoprotein present in the cell lines studied in this research work revealed that they were altered in the MDR cell lines by comparison with the parental cell line, DLKP. P-glycoprotein was over-expressed in all the drug-resistant cells and to highest levels in the VP-16-selected variants of DLKP. In general, the levels of topoisomerase II and its α subunit were reduced in the drug-resistant cells, by comparison with DLKP cells. However enzyme levels varied in the cell lines, depending on whether the levels of enzyme present in the isolated nucleus or total cell extract were determined. The levels of topoisomerase II-ß did not vary as significantly, between drug resistant and sensitive cell lines. The anti-topoisomerase II-ß monoclonal antibody detected two homologous proteins, with respect to their immunological reactivity, a 180 kDa. protein and a smaller 150 kDa. protein.

3.10 Adriamycin accumulation by VP-16-selected MDR cell lines

The level of adriamycin accumulated by VP-16-selected MDR cells was measured and compared to that accumulated by the drug-sensitive parental cell line. The effect of a calcium channel antagonist, verapamil and an immunosuppressive agent, cyclosporin A, on the level of adriamycin accumulation by the cells, was examined.

Adriamycin accumulation was reduced in the MDR cells selected by exposure to increasing concentrations of VP-16, in comparison to the level of drug accumulated by the parental cell line (Figure 3.10.1(a), 3.10.3(a)). DLKP accumulated approximately 1 nanomole of adriamycin per 10^6 cells within 4 hours. DLKP/VP-3 accumulated 100 - 200 picomoles of adriamycin per 10^6 cells in the same time period. DLKP/VP-8 accumulated slightly more adriamycin than DLKP/VP-3, within the 4 hours. These levels were the maximum accumulated by each cell line under the experimental conditions.

When the level of adriamycin accumulated by DLKP in the presence of cyclosporin A (10μg/ml and 30μg/ml) was studied, only slight changes in either the accumulation rate or the maximum level of adriamycin accumulated by the cells was observed (Figure 3.10.1(b)). The addition of cyclosporin A altered both the accumulation rate and the final level of adriamycin accumulated by DLKP/VP-3 and DLKP/VP-8 (Figure 3.10.2). In the presence of both 10μg/ml and 30μg/ml cyclosporin A, the level of adriamycin uptake by both cell lines reverted to that observed in the DLKP cell line under similar experimental conditions.

Verapamil also enhanced the accumulation of adriamycin by the drug resistant cells (Figure 3.10.4). Verapamil, at 10μg/ml and 30μg/ml, altered neither the accumulation rate of adriamycin by DLKP nor the maximum level accumulated (Figure 3.10.3). However, the presence of verapamil at both concentrations, resulted in an increase in adriamycin accumulation in DLKP/VP-3 cells, to levels comparable with the parental cell line (Figure 3.10.4(a)). With respect to DLKP/VP-8, exposing the cells to verapamil at 10μg/ml, resulted in intercellular adriamycin levels comparable with DLKP. However, when the cells were exposed to 30μg/ml verapamil, the adriamycin was accumulated to a level greater than that observed in the parental cell line (approximately 1.4 nanomoles of adriamycin accumulated in DLKP/VP-8, in comparison to 1.2 nanomoles in DLKP, under similar conditions) (Figure 3.10.4(b)).

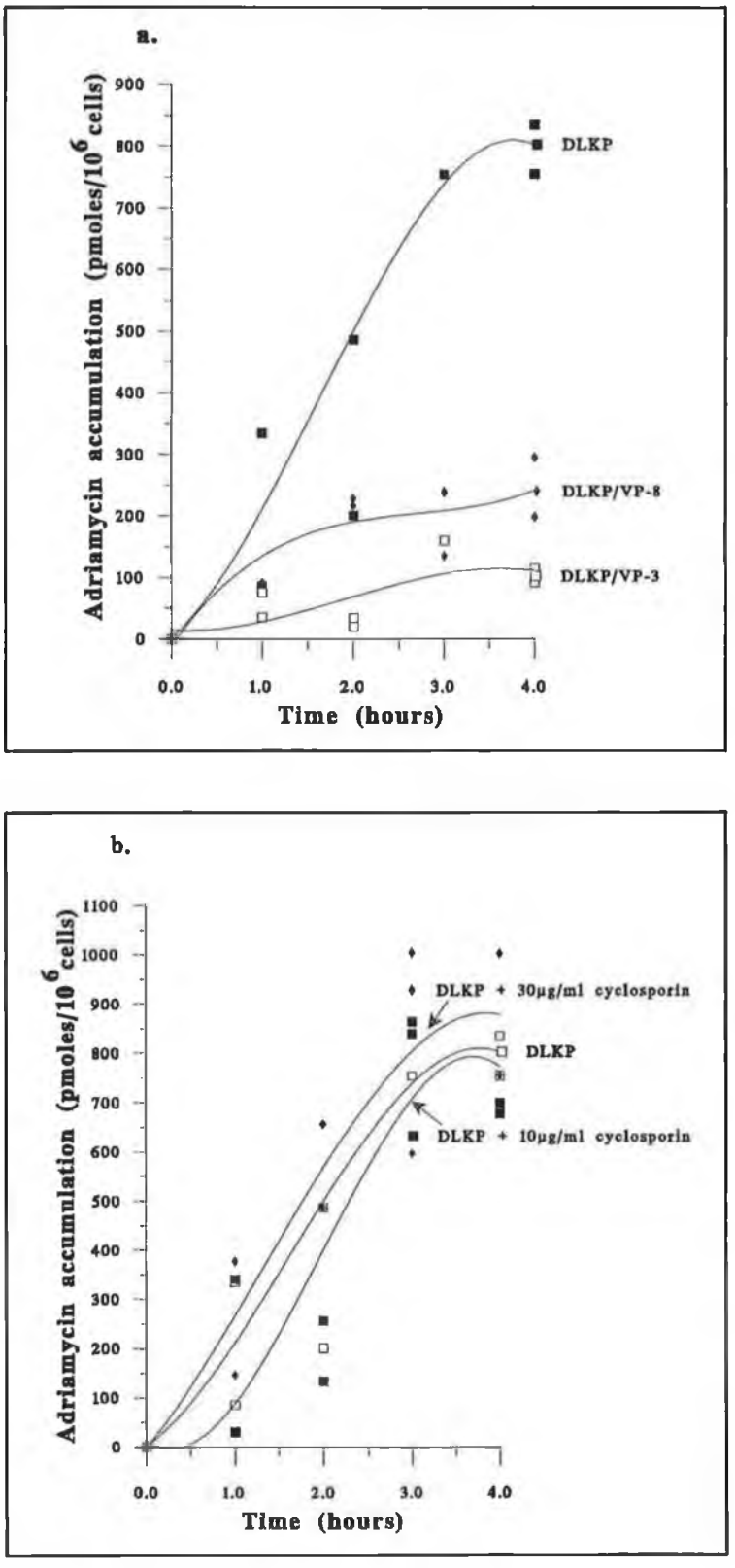
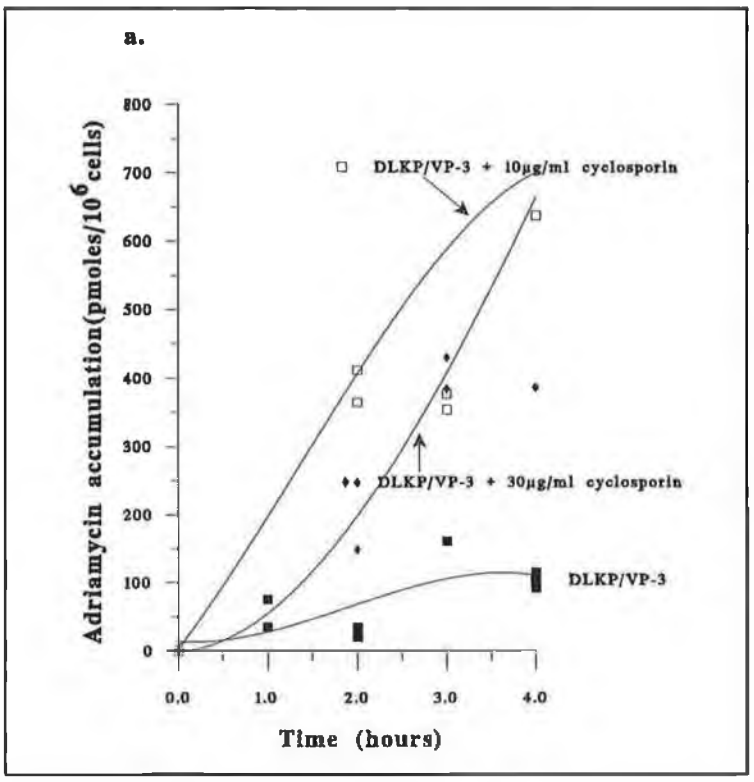


Figure 3.10.1 : Adriamycin accumulation in DLKP, DLKP/VP-3 and DLKP/VP-8 over a 4h. time period (a) and the effect of cyclosporin A, at 10μg/ml and 30μg/ml, on adriamycin accumulation by DLKP over the same time period (b).



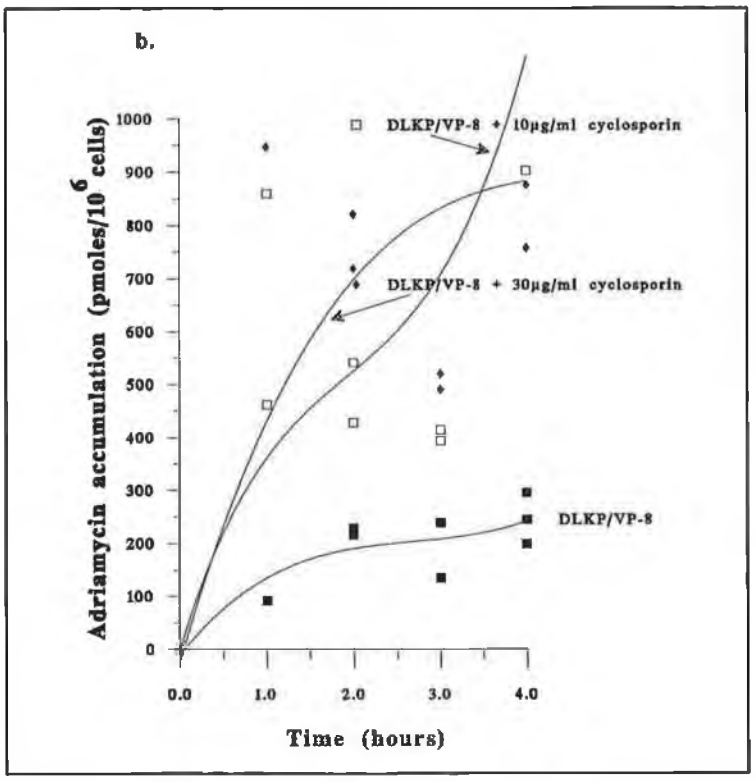
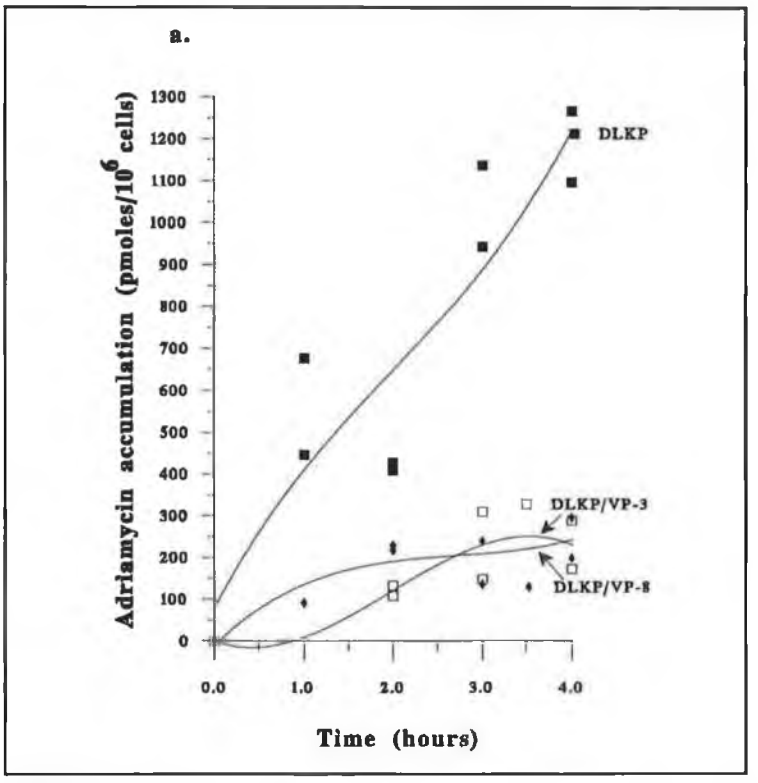


Figure 3.10.2 : The effect of cyclosporin A, at 10μg/ml and 30μg/ml, on adriamycin accumulation by DLKP/VP-3 (a) and DLKP/VP-8 (b) over a 4h. time period.



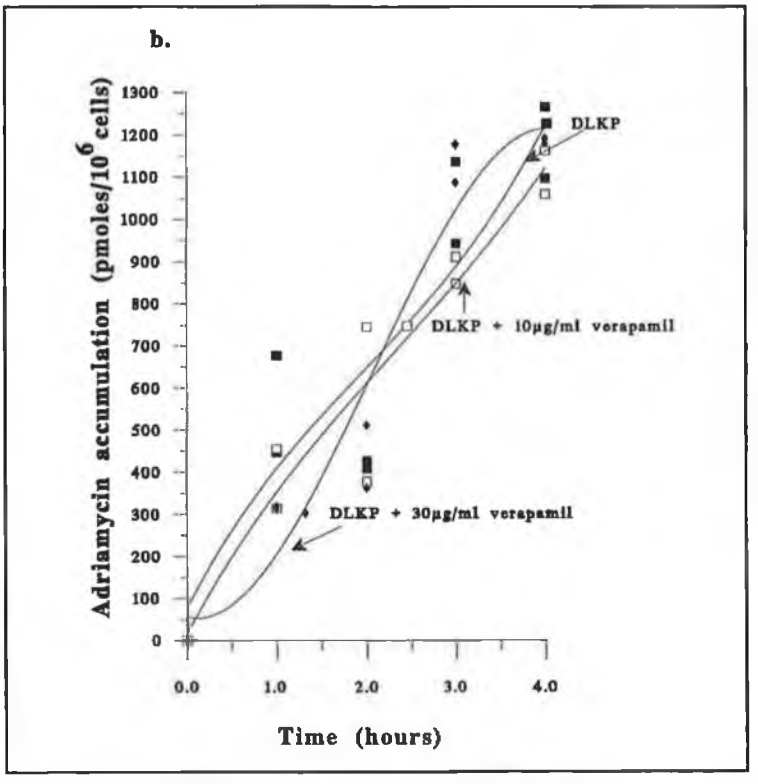
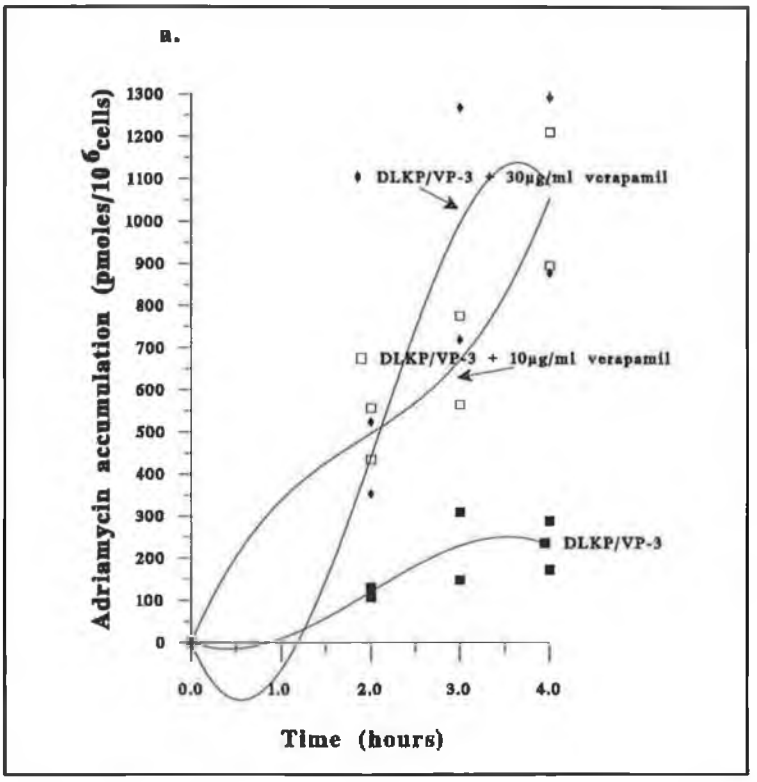


Figure 3.10.3 : Adriamycin accumulation in DLKP, DLKP/VP-3 and DLKP/VP-8 over a 4h. time period (a) and the effect of verapamil, at 10μg/ml and 30μg/ml, on adriamycin accumulation by DLKP over the same time period (b).



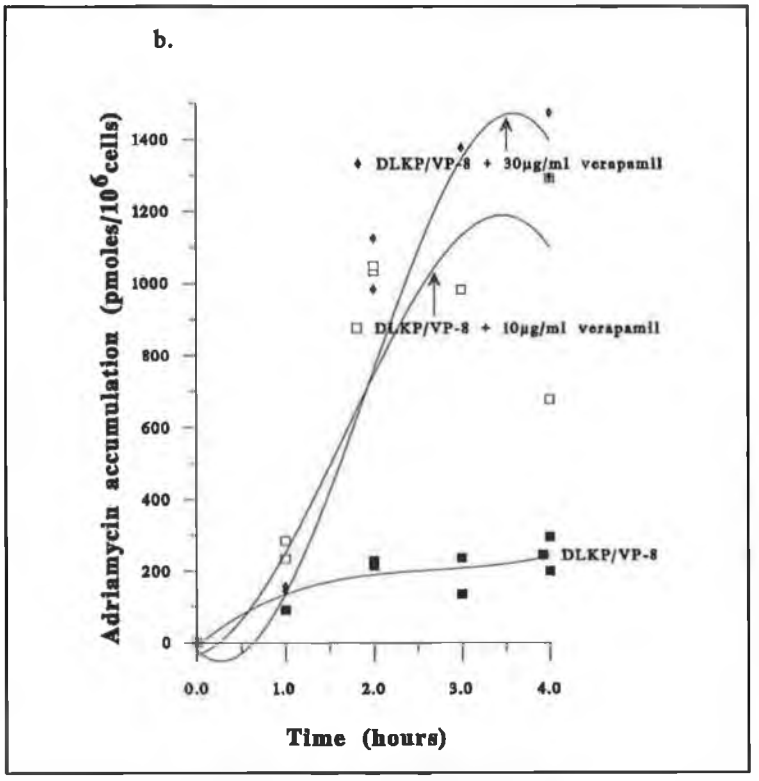


Figure 3.10.4 : The effect of verapamil, at 10μg/ml and 30μg/ml, on adriamycin accumulation by DLKP/VP-3 (a) and DLKP/VP-8 (b) over a 4h. time period.

3.11 Circumvention of DLKP variants

The ability of the calcium channel antagonist, verapamil and the immunosuppressive agent cyclosporin A, to overcome drug-resistance, either partially or completely, when used in combination with chemotherapeutic drugs, was investigated in relation to variants of the cell line DLKP. The toxic effects of both cyclosporin A and verapamil to the cell lines, at the concentrations used in these assays, are summarised in Table 3.11.1. Summary tables are also given of the ability of the circumventing agents to enhance the toxicity of adriamycin (Table 3.11.6.2), vincristine (Table 3.11.6.3) and VP-16 (Table 3.11.6.4).

3.11.1 Toxicity of the circumventing agents to variants of DLKP

The circumventing agents, cyclosporin A and verapamil, proved to be least toxic to DLKP in comparison to its MDR variants. When DLKP was exposed to up to 3.0μg/ml verapamil, under the assay conditions outlined in Section 2.12.5, it proved non-toxic to DLKP cells (102.69 $\pm$ 3.23% survival). Cyclosporin A, at concentrations of up to a maximum of 2.0μg/ml, also exhibited little toxicity on DLKP (92.61 $\pm$ 8.01% survival). However, both circumventing agents proved to be toxic to all the MDR variants of DLKP. This was most notable in the case of the VP-16-selected MDR cells, DLKP/VP-3 and DLKP/VP-8. When the cells were incubated with 3.0μg/ml verapamil, under assay conditions, only 48.11 $\pm$ 7.98% and 30.20 $\pm$ 7.48% cell growth was noted, respectively.

CELL LINES	Cyclosporin A			Verapamil		
	0.2μg/ml	1.0μg/ml	2.0μg/ml	0.2μg/ml	1.0μg/ml	3.0μg/ml
DLKP	95.23 (4.42)	93.38 (8.05)	92.61 (8.01)	101.34 (3.66)	101.23 (5.71)	102.69 (3.23)
DLKP/VP-3	95.34 (6.82)	91.58 (9.03)	84.02 (15.14)	102.71 (15.24)	67.64 (7.27)	48.11 (7.98)
DLKP/VP-8	96.96 (16.03)	92.84 (14.74)	90.71 (10.32)	93.75 (7.47)	51.71 (10.41)	30.20 (7.48)
DLKP-A	103.18 (7.27)	98.45 (8.95)	70.15 (13.81)	92.58 (13.31)	73.37 (16.35)	50.34 (19.11)
DLKPA5F	97.14 (5.85)	85.75 (13.12)	68.60 (5.02)	97.98 (15.36)	81.41 (20.15)	65.98 (19.44)
DLKPA11B	88.23 (6.50)	76.37 (8.45)	77.83 (12.60)	99.94 (5.99)	97.87 (8.46)	88.56 (3.45)
DLKPA6B	93.63 (3.73)	81.82 (5.04)	61.40 (9.23)	99.20 (11.38)	91.89 (11.13)	86.30 (11.24)
DLKPA2B	93.93 (3.34)	83.88 (7.95)	66.41 (8.02)	89.22 (10.16)	79.36 (12.96)	63.90 (16.09)

Table 3.11.1 : The toxicity of cyclosporin A and verapamil, at varying concentrations, to the DLKP variants. The data presented in this table is a summary of the data in Tables 3.11.1.1 - 3.11.4.20. The percentage growth in the absence of cyclosporin A and verapamil is taken as 100% survival. Growth in the presence of varying concentrations of the circumvention agents is presented as the percentage growth relative to that observed in the 100% controls. The values in brackets are the standard deviations on the percentage survival values.

3.11.2 Circumvention of DLKP

Cyclosporin A was relatively non-toxic to the cell line DLKP, with greater than 90% growth observed, at a concentration of up to 2.0μg/ml (Table 3.11.2.1). At all concentrations up to 2.0μg/ml, cyclosporin A enhanced the toxicity of adriamycin, vincristine and VP-16. Although 45.06 ± 3.34% cell growth occurred in the presence of 0.02μg/ml adriamycin, only 27.52 ± 7.21% growth occurred when 0.2μg/ml cyclosporin A was also present. Growth was further reduced, to 13.03 ± 1.41%, when the concentration of the circumventing agent was increased to 2.0μg/ml. Cyclosporin A was a more potent enhancer of vincristine toxicity than adriamycin toxicity. The presence of 0.001μg/ml vincristine resulted in 98.44 ± 5.66% cell growth with respect to the drug-free control, but with the combined presence of 0.001μg/ml vincristine and 2.0μg/ml cyclosporin A (which was non-toxic and resulted in 99.77 ± 3.79% cell growth) only 3.63 ± 0.47% cell survival was observed. The toxicity of VP-16 to DLKP was also enhanced by cyclosporin A. Cell growth in 0.075μg/ml VP-16 resulted in 91.69 ± 2.91% cell survival, while its combination with 2.0μg/ml cyclosporin A resulted in only 50.04 ± 3.43% cell survival (cell survival in 2.0μg/ml cyclosporin A alone was 97.09%).

Verapamil was also an effective circumventing agent, when used in combination with adriamycin, vincristine or VP-16 to enhance their toxicity to the DLKP cell line (Table 3.11.2.2), but overall it appeared to be less potent than cyclosporin A. Verapamil was non-toxic to DLKP up to a concentration of 3.0μg/ml (108.14 ± 4.03% growth). The presence of 3.0μg/ml verapamil in combination with 0.02μg/ml adriamycin enhanced its toxicity, decreasing the percentage survival from 41.98 ± 7.12% to 24.94 ± 2.49%. As was observed with cyclosporin A, the combination of the circumventing agent, verapamil, with vincristine, appeared to be the most potent combination for DLKP toxicity. The addition of 3.0μg/ml verapamil decreased the percentage survival from 94.12 ± 5.72% cell survival in the presence of 0.001μg/ml vincristine alone to only 51.18 ± 4.97% in the presence of the combination. The toxicity of VP-16 to DLKP was less notably amplified by the addition of verapamil. The addition of 3.0μg/ml verapamil reduced the survival in the presence of drug from 98.61 ± 3.02% in the presence of 0.075μg/ml VP-16 alone to 90.62 ± 4.73% survival for the combination.

% Survival	Cyclosporin A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (4.83)	97.41 (5.80)	97.56 (3.52)	98.22 (10.09)	97.45 (5.26)
0.01 μg/ml	97.76 (3.90)	96.56 (5.88)	94.28 (4.10)	91.65 (6.61)	85.81 (4.72)
0.0 μg/ml	100 (7.60)	95.30 (6.15)	87.35 (3.00)	87.65 (6.44)	87.94 (3.92)
0.02 μg/ml	45.06 (3.34)	27.52 (7.21)	14.53 (1.41)	6.93 (1.84)	13.03 (1.41)
Vincristine					
0.0 μg/ml	100 (5.86)	99.45 (4.84)	99.41 (7.54)	98.32 (5.98)	99.77 (3.79)
0.001 μg/ml	98.44 (5.66)	86.52 (7.03)	21.52 (2.89)	6.68 (1.33)	3.63 (0.47)
0.0 μg/ml	100 (7.41)	87.83 (4.85)	82.33 (4.00)	83.33 (5.26)	80.78 (12.96)
0.002 μg/ml	12.01 (1.40)	5.76 (0.95)	1.65 (0.35)	1.50 (0.30)	1.45 (0.30)
VP-16					
0.0 μg/ml	100 (2.44)	96.14 (3.39)	100.24 (4.96)	97.40 (3.39)	97.09 (4.76)
0.075 μg/ml	91.69 (2.91)	86.10 (4.69)	65.35 (5.39)	58.43 (3.66)	50.04 (3.43)

Table 3.11.2.1 : Effect of cyclosporin A on adriamycin, vincristine and VP-16 toxicity to DLKP. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% Survival	Verapamil				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (11.33)	106.48 (4.92)	108.56 (6.14)	111.48 (9.87)	108.14 (4.03)
0.01 μg/ml	101.19 (8.09)	100.34 (7.25)	103.18 (6.36)	109.87 (14.70)	106.82 (7.37)
0.0 μg/ml	100 (3.46)	96.65 (7.30)	99.14 (3.04)	103.90 (3.81)	102.67 (2.72)
0.02 μg/ml	41.98 (7.12)	37.40 (6.66)	35.54 (2.86)	29.42 (3.40)	24.94 (2.49)
Vincristine					
0.0 μg/ml	100 (2.51)	99.41 (4.10)	101.82 (5.84)	100.28 (4.10)	102.05 (4.14)
0.001 μg/ml	94.12 (5.72)	94.79 (5.72)	81.53 (6.47)	68.03 (5.29)	51.18 (4.97)
0.0 μg/ml	100 (4.59)	101.71 (6.55)	93.08 (4.66)	100.69 (6.69)	100.60 (6.41)
0.002 μg/ml	14.90 (1.38)	11.99 (1.48)	5.86 (0.92)	4.01 (0.46)	3.27 (0.42)
VP-16					
0.0 μg/ml	100 (3.54)	102.43 (3.82)	103.54 (6.28)	102.54 (4.06)	100.00 (3.38)
0.075 μg/ml	98.61 (3.02)	98.81 (3.54)	96.26 (3.42)	94.87 (3.94)	90.62 (4.73)

Table 3.11.2.2 : Effect of verapamil on adriamycin, vincristine and VP-16 toxicity to DLKP. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

3.11.3 Circumvention of drug resistance in VP-16-selected cell lines

Cyclosporin A and verapamil enhanced the toxicity of chemotherapeutic drugs to the VP-16- selected cell lines, DLKP/VP-3 and DLKP/VP-8 (Tables 3.11.3.1 - 3.11.3.4 and summarised in Tables 3.11.6.1 - 3.11.6.4). Cyclosporin A was relatively non-toxic to DLKP/VP-3 and DLKP/VP-8, up to a concentration of 2.0μg/ml (Tables 3.11.3.1 and 3.11.3.2, respectively), but verapamil was toxic at a concentration of 2.0μg/ml to DLKP/VP-3 (Tables 3.11.3.3 and 3.11.1) and 1.0μg/ml to DLKP/VP-8 (Tables 3.11.3.4 and 3.11.1).

In relation to DLKP/VP-3, cyclosporin A enhanced the toxicity of adriamycin, vincristine and VP-16 (Table 3.11.3.1). The presence of 1.5μg/ml adriamycin resulted in 81.01 $\pm$ 19.09% cell survival relative to a drug-free control. However, cell survival was decreased to 10.44 $\pm$ 3.06% in the presence of 2.0μg/ml cyclosporin A. Likewise, the addition of cyclosporin A decreased the percentage cell survival in the presence of 1.2μg/ml vincristine from 75.91 $\pm$ 7.51% to 21.10 $\pm$ 2.43%. Cyclosporin A also enhanced the toxicity of VP-16. The presence of 2.0μg/ml cyclosporin A decreased the percentage cell survival from 51.60 $\pm$ 6.64% in the presence of 5.0μg/ml VP-16 alone, to 8.05 $\pm$ 2.30% in the presence of the combination. A similar effect was noted for DLKP/VP-8 in relation to the ability of cyclosporin A to enhance the toxicity of cytotoxic drugs (Table 3.11.3.2). The presence of 2.0μg/ml cyclosporin A reduced the percentage cell survival from 74.49 $\pm$ 12.93% to 12.88 $\pm$ 2.30% in the presence of 4.0μg/ml adriamycin, from 48.54 $\pm$ 8.44% to 9.27 $\pm$ 1.67% in the presence of 2.0μg/ml vincristine and from 69.09 $\pm$ 3.04% to 26.36 $\pm$ 4.55% in the presence of 8.0μg/ml VP-16. For both DLKP/VP-3 and DLKP/VP-8, cyclosporin A appeared equally effective at enhancing the toxicity of all three chemotherapeutic drugs.

Although verapamil was relative toxic to both DLKP/VP-3 and DLKP/VP-8, it still remained a potent enhancer of the toxicity of adriamycin and vincristine for both cell lines (Table 3.11.3.3 and 3.11.3.4 respectively). At a concentration of 3.0μg/ml, verapamil enhanced the toxicity of 2.0μg/ml adriamycin in DLKP/VP-3 cells. This can be seen from the decrease in cell survival from 71.16 $\pm$ 6.93% in the absence of the circumventing agent to 12.09 $\pm$ 1.45% in its presence, while the toxicity due to verapamil alone was 54.97 $\pm$ 5.60% (Table 3.11.3.3). The same concentration of verapamil also increased the toxicity of vincristine when used in combination. Cell survival dropped from 61.16 $\pm$ 5.43% in the presence of 2.0μg/ml vincristine alone to 11.97 $\pm$ 1.58% in the presence of the combination of verapamil and vincristine. In respect to DLKP/VP-8, 3.0μg/ml verapamil enhanced the toxicity of 4.0μg/ml adriamycin. The percentage cell survival dropped from 82.74 $\pm$ 13.31% for adriamycin alone to 6.83 $\pm$ 0.53% for the combination, while the percentage cell survival with respect to verapamil alone accounting for 27.46 $\pm$ 4.30% survival (Table 3.11.3.4). The

toxicity of vincristine was also intensified by verapamil, with 45.80 ± 7.07% cell survival observed in the presence of 2.0μg/ml vincristine, while this was decreased to 5.22 ± 1.45% by the addition of 3.0μg/ml verapamil.

When verapamil was added to VP-16-selected cells in the presence of VP-16, the combination partially masked their individual toxic effects (Tables 3.11.3.3 and 3.11.3.4). Following the addition of verapamil to cells in the presence of VP-16, an increase in the percentage cell kill was obtained with regard to that observed for either verapamil or VP-16 alone. However, the percentage cell kill did not represent the product of the individual cell kill for both compounds. The addition of 3.0μg/ml verapamil to DLKP/VP-3 cells in the presence of 5.0μg/ml VP-16 decreased the percentage cell survival from 50.65 ± 5.44% to only 32.51 ± 3.91% (Table 3.11.3.3), while the percentage cell survival in the presence of 3.0μg/ml verapamil alone was 47.81 ± 11.83%. Likewise, the addition of 3.0μg/ml verapamil to DLKP/VP-8 cells in the presence of 12.0μg/ml VP-16 decreased the percentage cell survival from 37.05 ± 2.88% to 21.57 ± 5.09%, but cell survival in the presence of 3.0μg/ml verapamil alone was only 26.83 ± 4.13% (Table 3.11.3.4). When cell kill by VP-16 and verapamil in combination was examined by the Chou and Talalay (1984) method of analysis, an antagonistic effect was observed.

% Survival	Cyclosporin A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (13.17)	102.11 (13.71)	96.10 (15.93)	97.57 (15.08)	98.95 (12.87)
1.5 μg/ml	81.01 (19.09)	77.95 (17.30)	49.58 (15.72)	25.00 (5.80)	10.44 (3.06)
0.0 μg/ml	100 (6.35)	99.89 (4.50)	97.70 (7.45)	98.98 (8.57)	97.43 (8.52)
2.0 μg/ml	71.40 (9.59)	68.77 (6.37)	38.30 (5.62)	18.00 (4.28)	10.93 (0.91)
Vincristine					
0.0 μg/ml	100 (17.08)	98.23 (9.06)	98.67 (9.94)	93.48 (11.27)	87.40 (12.49)
1.2 μg/ml	75.91 (7.51)	68.95 (6.52)	45.52 (5.75)	32.37 (4.09)	21.10 (2.43)
0.0 μg/ml	100 (15.70)	90.52 (6.61)	77.20 (17.86)	73.87 (10.68)	67.89 (13.10)
2.0 μg/ml	53.59 (4.19)	48.13 (4.83)	28.14 (1.55)	15.28 (3.39)	11.89 (2.76)
VP-16					
0.0 μg/ml	100 (22.62)	85.95 (15.45)	88.25 (16.48)	69.60 (9.45)	68.45 (7.54)
5.0 μg/ml	51.60 (6.64)	48.15 (4.73)	24.39 (4.47)	9.83 (2.04)	8.05 (2.30)

Table 3.11.3.1 : Effect of cyclosporin A on adriamycin, vincristine and VP-16 toxicity to DLKP/VP-3. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% Survival	Cyclosporin A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (7.17)	98.75 (9.44)	94.26 (7.24)	92.21 (10.58)	89.42 (7.24)
4.0 μg/ml	74.49 (12.93)	69.95 (9.79)	47.53 (7.99)	26.41 (5.79)	12.88 (2.30)
0.0 μg/ml	100 (10.01)	70.02 (7.91)	66.48 (6.89)	64.71 (7.45)	76.26 (16.20)
5.0 μg/ml	44.60 (6.70)	35.10 (4.56)	22.53 (3.63)	15.46 (1.86)	13.59 (2.05)
Vincristine					
0.0 μg/ml	100 (6.85)	106.40 (6.54)	104.36 (5.84)	108.81 (7.74)	105.42 (9.64)
2.0 μg/ml	48.54 (8.44)	48.03 (7.97)	24.11 (5.38)	14.09 (3.01)	9.27 (1.67)
0.0 μg/ml	100 (4.37)	91.28 (5.76)	85.93 (8.72)	93.74 (4.94)	91.19 (5.68)
1.5 μg/ml	42.47 (3.62)	40.33 (3.70)	27.41 (2.80)	21.65 (2.39)	16.38 (0.91)
VP-16					
0.0 μg/ml	100 (7.10)	97.51 (8.89)	101.91 (8.65)	101.66 (13.83)	98.24 (4.84)
12.0 μg/ml	29.16 (3.91)	26.58 (3.13)	15.93 (2.69)	10.31 (1.86)	7.23 (1.17)
0.0 μg/ml	100 (4.16)	117.79 (8.03)	104.12 (4.66)	97.51 (5.53)	83.73 (6.51)
8.0 μg/ml	69.09 (3.04)	69.67 (3.90)	50.65 (6.07)	36.77 (2.49)	26.36 (4.55)

Table 3.11.3.2 : Effect of cyclosporin A on adriamycin, vincristine and VP-16 toxicity to DLKP/VP-8. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% Survival	Verapamil				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (23.86)	89.86 (10.80)	54.66 (9.07)	44.52 (9.40)	36.52 (7.67)
1.5 μg/ml	80.79 (19.21)	73.87 (11.95)	36.93 (6.84)	18.96 (3.71)	12.45 (2.56)
0.0 μg/ml	100 (4.07)	91.12 (7.49)	70.03 (6.11)	55.92 (4.97)	54.97 (5.60)
2.0 μg/ml	71.16 (6.93)	63.35 (5.73)	31.68 (3.27)	16.25 (2.02)	12.09 (1.45)
Vincristine					
0.0 μg/ml	100 (10.74)	113.51 (10.00)	71.17 (10.85)	58.94 (8.19)	45.11 (7.77)
1.2 μg/ml	85.00 (12.45)	86.49 (9.29)	41.49 (5.64)	24.57 (3.40)	17.55 (2.98)
0.0 μg/ml	100 (8.08)	95.04 (5.84)	71.20 (6.25)	55.49 (5.32)	56.13 (94.56)
2.0 μg/ml	61.16 (5.43)	53.27 (5.55)	31.72 (3.27)	17.06 (2.34)	11.97 (1.58)
VP-16					
0.0 μg/ml	100 (11.38)	124.02 (15.27)	71.12 (10.53)	56.80 (10.06)	47.81 (11.83)
5.0 μg/ml	50.65 (5.44)	48.88 (3.91)	42.49 (6.75)	35.38 (6.27)	32.51 (3.91)

Table 3.11.3.3 : Effect of verapamil on adriamycin, vincristine and VP-16 toxicity to DLKP/VP-3. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% Survival	Verapamil				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
Adriamycin					
0.0 μg/ml	100 (11.22)	102.80 (6.52)	50.22 (6.43)	27.64 (2.80)	27.46 (4.30)
4.0 μg/ml	82.74 (13.31)	84.03 (18.10)	28.17 (5.90)	11.80 (1.15)	6.83 (0.53)
0.0 μg/ml	100 (7.73)	86.48 (4.15)	60.14 (4.76)	44.09 (4.76)	41.78 (2.61)
5.0 μg/ml	54.22 (9.83)	46.16 (3.15)	28.19 (3.53)	19.05 (1.15)	22.50 (8.53)
Vincristine					
0.0 μg/ml	100 (14.60)	95.94 (11.42)	31.88 (8.12)	27.19 (7.42)	24.52 (5.10)
2.0 μg/ml	45.80 (7.07)	48.17 (11.07)	11.65 (2.38)	4.00 (0.52)	5.22 (1.45)
0.0 μg/ml	100 (5.88)	83.39 (5.97)	57.18 (1.21)	39.60 (2.26)	37.18 (3.06)
1.5 μg/ml	40.73 (2.34)	36.45 (2.50)	19.92 (1.53)	12.50 (1.05)	10.00 (1.29)
VP-16					
0.0 μg/ml	100 (5.53)	99.33 (5.21)	52.44 (11.89)	34.71 (5.92)	26.83 (4.13)
12.0 μg/ml	37.05 (2.88)	43.05 (9.39)	30.52 (8.30)	24.11 (6.13)	21.57 (5.09)
0.0 μg/ml	100 (9.21)	94.54 (14.05)	57.41 (12.26)	39.02 (12.26)	23.41 (14.72)
8.0 μg/ml	61.32 (5.91)	59.98 (6.69)	42.25 (7.92)	20.18 (5.13)	16.28 (4.46)

Table 3.11.3.4 : Effect of verapamil on adriamycin, vincristine and VP-16 toxicity to DLKP/VP-8. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

3.11.4 Circumvention of drug resistance in DLKP-A

The toxicity of adriamycin, vincristine and VP-16 to DLKP-A was enhanced by the presence of cyclosporin A and verapamil (Tables 3.11.4.1 - 3.11.4.5 and summarised in Tables 3.11.6.1 - 3.11.6.4). As the concentration of circumventing agent was increased the synergistic effect was generally enhanced.

Cyclosporin A was relatively non-toxic to DLKP-A at concentrations below 1.0μg/ml. At 2.0μg/ml approximately 70% cell growth was noted (Table 3.11.4.1 - 3.11.4.3). Verapamil was more toxic to DLKP-A. In the presence of 1.0μg/ml verapamil, approximately 60 - 70% cell growth was obtained, while at 3.0μg/ml only approximately 40% growth was noted (Table 3.11.4.3 to 3.11.4.5).

Cyclosporin A was a potent enhancer of adriamycin toxicity towards DLKP-A. Adriamycin at 1.0μg/ml, allowed 100.74 $\pm$ 12.08% growth, but when combined with 2.0μg/ml cyclosporin A, only 50.56 $\pm$ 4.46% growth relative to DLKP-A, occurred (Table 3.11.4.1). When the adriamycin concentration was increased to 3.0μg/ml, resulting in 48.36 $\pm$ 12.09% growth, the presence of 1.0μg/ml cyclosporin A reduced the percentage survival to only 13.73 $\pm$ 5.43%.

Vincristine's toxicity to DLKP-A was also enhanced by cyclosporin A. The combined effect of 0.5μg/ml vincristine and 1.0μg/ml cyclosporin A resulted in a decrease in percentage cell survival, from 102.79 $\pm$ 5.43% (for vincristine alone) to 77.98 $\pm$ 4.96%. However, when the concentration of cyclosporin A was increased to 2.0μg/ml, only 34.89 $\pm$ 4.34% cell survival was noted. In the presence of 1.0μg/ml vincristine alone, 85.94 $\pm$ 9.50% cell survival was observed, but when combined with 1.0μg/ml cyclosporin A, only 41.65 $\pm$ 10.40% cell survival was noted, although cyclosporin A did not elicit a toxic response when used alone (100.72 $\pm$ 11.12% cell survival). When the vincristine concentration was further increased to 3.0μg/ml, resulting in 31.37 $\pm$ 7.21% cell survival, only 4.33 $\pm$ 1.51% survival was noted when 1.0μg/ml cyclosporin A was also present.

Cyclosporin A also enhanced the toxicity of VP-16 with respect to the cell line, DLKP-A (Table 3.11.4.3). The addition of 2.0μg/ml cyclosporin A to a toxicity assay in the presence of 2.0μg/ml VP-16 decreased the percentage cell survival from 69.01 $\pm$ 1.87% to 13.45 $\pm$ 5.31%, while the percentage survival in the presence of 2.0μg/ml cyclosporin A alone was 66.85 $\pm$ 20.58%

Verapamil increased the potency of VP-16, adriamycin and vincristine (Table 3.4.4.3 to 3.11.4.5). With respect to DLKP-A, 75.51 $\pm$ 4.07% cell growth occurred in the presence of 2.0μg/ml VP-16. This was reduced in the combined presence of 2.0μg/ml VP-16 and increasing concentrations of verapamil, to a minimum cell survival of 29.75 $\pm$ 1.05% in

the presence of VP-16 and 3.0μg/ml verapamil. In isolation, this concentration of verapamil resulted in 72.13 ± 4.62% cell growth (Table 3.11.4.3).

Table 3.11.4.4 shows the percentage survival for DLKP-A with various concentrations of verapamil and adriamycin. Again, the circumventing effect was more potent at increased concentrations of verapamil. While the presence of 1.0μg/ml adriamycin resulted in 93.54 ± 21.04% cell survival, the addition of 1.0μg/ml verapamil reduced this to 45.63 ± 13.96%. An increase in the concentration of verapamil to 3.0μg/ml resulted in a further decrease to only 27.71 ± 9.38% cell survival. For verapamil, a concentration of 1.0μg/ml and 3.0μg/ml resulted in 73.54 ± 20.42% and 36.46 ± 18.13% cell survival, respectively. The intensity of the circumvention effect was also dependent on the initial cell kill produced by the cytotoxic agent. While 1.0μg/ml verapamil resulted in 68.04 ± 12.55% cell growth of DLKP-A and 0.05μg/ml adriamycin allowed 109.74 ± 11.32% growth, the combination only resulted in 59.24 ± 12.48% cell growth. When the concentration of adriamycin was increased to 1.0μg/ml (resulting in 96.03 ± 8.32% cell growth), the presence of the same concentration of verapamil reduced cell survival to only 47.47 ± 6.28%. Cell growth in the presence of 3.0μg/ml adriamycin was 62.48 ± 12.48% but this was decreased to 14.43 ± 2.81% by the addition of 1.0μg/ml verapamil.

Verapamil also proved an efficient enhancer of vincristine toxicity towards DLKP-A (Table 3.11.4.5). Again, the circumventing ability was dependent on the concentration of both the cytotoxic drug and the circumvention agent. Vincristine, 0.5μg/ml, resulted in 96.37 ± 4.89% cell growth with no significant circumvention noted until the cells were exposed to a concentration of 3.0μg/ml verapamil. The combination of vincristine and verapamil resulted in only 26.18 ± 2.52% growth. No notable circumvention was observed when a combination of 1.0μg/ml vincristine and 1.0μg/ml verapamil was used, but when the level of vincristine was increased to 3.0μg/ml, the percentage survival fell from 18.91 ± 3.15% to 3.30 ± 1.07% in the absence and presence of verapamil, respectively.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
ADRIAMYCIN						
0.0 μg/ml	A	100 (7.16)	113.01 (12.45)	104.28 (9.11)	97.56 (9.11)	85.32 (9.29)
	B	100 (38.32)	101.13 (24.94)	108.56 (33.50)		
0.05 μg/ml	B	104.28 (16.62)	102.52 (28.84)	90.43 (22.80)		
1.0 μg/ml	A	100.74 (12.08)	102.60 (10.78)	90.52 (10.04)	75.28 (10.04)	50.56 (4.46)
3.0 μg/ml	B	48.36 (12.09)		13.73 (5.43)		

Table 3.11.4.1 : Effect of cyclosporin A on adriamycin toxicity to DLKP-A. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (7.33)	103.72 (5.89)	91.94 (9.30)	71.32 (6.20)	58.29 (6.82)
	B	100 (11.48)	105.17 (11.48)	100.72 (11.12)		
0.5 μg/ml	A	102.79 (5.43)	91.63 (5.27)	77.98 (4.96)	62.79 (5.43)	34.89 (4.34)
1.0μg/ml	B	85.94 (9.50)	87.80 (21.69)	41.65 (10.40)		
3.0 μg/ml	B	31.37 (7.21)		4.33 (1.51)		

Table 3.11.4.2 : Effect of cyclosporin A on vincristine toxicity to DLKP-A. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL	CYCLOSPORIN A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VP-16					
0.0 μg/ml	100 (3.98)	92.87 (5.12)	86.77 (6.38)	57.02 (4.51)	66.85 (20.58)
2.0 μg/ml	69.01 (1.87)	67.76 (1.39)	45.66 (2.02)	18.95 (3.90)	13.45 (5.31)
% SURVIVAL	**VERAPAMIL**				
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VP-16					
0.0 μg/ml	100 (5.29)	105.40 (3.34)	100.46 (5.40)	82.65 (5.90)	72.13 (4.62)
2.0 μg/ml	75.51 (4.07)	77.03 (4.44)	70.43 (4.94)	35.79 (3.48)	29.75 (1.05)

Table 3.11.4.3 : Effect of cyclosporin A and verapamil on VP-16 toxicity to DLKP-A. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% SURVIVAL		VERAPAMIL				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
ADRIAMYCIN						
0.0 μg/ml	A	100 (18.39)	71.46 (12.50)	73.54 (20.42)	36.25 (10.00)	36.46 (18.13)
	B	100 (8.06)	94.95 (15.58)	68.04 (12.55)		
0.05 μg/ml	B	109.74 (11.32)	104.55 (15.58)	59.24 (12.48)		
1.0 μg/ml	A	93.54 (21.04)	69.79 (21.67)	45.63 (13.96)	28.54 (8.33)	27.71 (9.38)
	B	96.03 (8.32)	82.03 (7.58)	47.47 (6.28)		
3.0 μg/ml	B	62.48 (12.34)		14.43 (2.81)		

Table 3.11.4.4 : Effect of verapamil on adriamycin toxicity to DLKP-A. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		VERAPAMIL				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (8.80)	89.43 (11.20)	68.14 (4.89)	48.74 (6.47)	42.43 (3.79)
	B	100 (24.90)	101.65 (35.53)	56.66 (15.47)		
0.5 μg/ml	A	96.37 (4.89)	85.96 (6.78)	64.04 (6.78)	47.48 (4.26)	26.18 (2.52)
1.0μg/ml	B	65.26 (7.16)	57.74 (13.25)	43.62 (14.33)		
3.0 μg/ml	B	18.91 (3.15)		3.30 (1.07)		

Table 3.11.4.5 : Effect of verapamil on vincristine toxicity to DLKP-A. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

3.11.5 Circumvention of drug resistance in DLKP-A clones

The ability of both cyclosporin A and verapamil, at relatively non-toxic levels, to enhance the toxicity of adriamycin, vincristine and VP-16 towards the four clonal cell lines DLKPA5F, DLKPA11B, DLKPA6B and DLKPA2B were investigated. The results outlined in Tables 3.11.5.1 to 3.11.5.20 and summarised in Tables 3.11.6.1 to 3.11.6.4.

With respect to the toxicity of the circumventing agents, cyclosporin A, at a concentration of 1.0μg/ml, resulted in approximately 90% cell growth of DLKPA5F. At the highest concentration used (2.0μg/ml), the percentage cell survival dropped to approximately 70% (Tables 3.11.5.1 - 3.11.5.3). Approximately 70% cell survival was also observed with the highest concentration (3.0μg/ml) of verapamil (Tables 3.11.5.3 to 3.11.5.5).

Cyclosporin A enhanced the toxicity of adriamycin towards DLKPA5F. The addition of 2.0μg/ml cyclosporin A, to a toxicity assay, decreased the percentage survival in the presence of 1.0μg/ml adriamycin, from 49.47 $\pm$ 10.59% to 8.17 $\pm$ 1.06% (Table 3.11.5.1). The toxicity of vincristine was also increased by cyclosporin A; the cell survival observed in the presence of 0.5μg/ml vincristine was reduced from 40.51 $\pm$ 2.52% to 6.83 $\pm$ 0.87%, by the addition of 2.0μg/ml cyclosporin A (Table 3.11.5.2). Cyclosporin A also enhanced the toxicity of VP-16. The cell survival resulting from growth in 4.0μg/ml VP-16 was decreased from 18.36 $\pm$ 4.49% to 9.72 $\pm$ 0.75% by the presence of 2.0μg/ml cyclosporin A (Table 3.11.5.3).

The addition of verapamil to DLKPA5F toxicity assays with VP-16 caused a slight increase in cell kill (Table 3.11.5.3). More notable effects were observed when combinations of verapamil and adriamycin were employed. The presence of 3.0μg/ml verapamil reduced the percentage cell survival in the presence of 1.0μg/ml adriamycin from 52.59 $\pm$ 3.96% to 12.13 $\pm$ 1.46% (Table 3.11.5.4), while the same concentration of verapamil reduced the survival in the presence of 0.5μg/ml vincristine from 29.92 $\pm$ 3.62% to 2.20 $\pm$ 1.56% (Table 3.11.5.5).

% SURVIVAL		CYCLOSPORIN A					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (6.31)		89.64 (8.37)	64.21 (14.94)	67.93 (4.78)	64.21 (10.62)
	B	100 (5.50)		95.28 (6.80)	95.32 (5.39)		
	C	100 (5.90)	95.14 (4.48)	93.12 (7.10)			
0.05 μg/ml	B	92.27 (4.81)		87.64 (3.22)	68.61 (6.49)		
	C	92.00 (4.86)	87.00 (2.84)	85.43 (4.63)			
1.0 μg/ml	A	49.47 (10.56)		40.04 (3.85)	20.05 (3.39)	13.88 (1.79)	8.17 (1.06)
	B	69.58 (5.12)		64.15 (2.96)	38.85 (1.50)		
	C	80.94 (5.23)	74.07 (3.74)	70.03 (4.26)			
3.0 μg/ml	B	53.11 (6.05)			21.72 (1.77)		
	C	71.15 (2.32)	67.56 (2.84)				

Table 3.11.5.1 : Effect of cyclosporin A on adriamycin toxicity to DLKPA5F. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (7.28)	99.95 (6.45)	84.14 (6.78)	81.68 (6.51)	74.08 (5.19)
	B	100 (8.47)	98.46 (6.28)	96.88 (5.63)		
0.05 μg/ml	B	85.16 (4.90)	82.93 (5.23)	61.45 (3.12)		
0.5 μg/ml	A	40.51 (2.52)	33.73 (3.94)	17.93 (1.48)	14.16 (2.13)	6.83 (0.87)
1.0μg/ml	B	56.67 (4.42)	47.87 (3.28)	26.71 (0.97)		
3.0 μg/ml	B	20.55 (2.19)		8.55 (0.57)		

Table 3.11.5.2 : Effect of cyclosporin A on vincristine toxicity to DLKPA5F. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL	CYCLOSPORIN A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VP-16					
0.0 μg/ml	100 (12.71)	106.40 (3.74)	88.21 (3.16)	79.65 (6.56)	67.52 (4.40)
4.0 μg/ml	18.36 (4.49)	19.19 (2.66)	12.96 (1.33)	11.54 (0.83)	9.72 (0.75)
% SURVIVAL	**VERAPAMIL**				
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VP-16					
0.0 μg/ml	100 (18.30)	122.73 (15.38)	100.80 (19.06)	89.67 (13.43)	176.58 (32.61)
4.0 μg/ml	16.65 (2.87)	17.34 (1.38)	17.57 (2.99)	13.89 (0.80)	14.24 (2.99)

Table 3.11.5.3 : Effect of cyclosporin A and verapamil on VP-16 toxicity to DLKPA5F. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% SURVIVAL		VERAPAMIL					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (8.13)		101.06 (3.67)	91.14 (4.43)	80.27 (7.95)	79.72 (5.64)
	B	100 (27.59)		104.41 (18.12)	83.06 (17.57)		
	C	100 (5.97)	90.33 (7.04)	89.95 (4.15)			
0.05 μg/ml	B	105.04 (21.12)		84.00 (26.48)	54.77 (12.14)		
	C	76.32 (5.84)	85.87 (4.33)	84.48 (4.33)			
1.0 μg/ml	A	52.59 (3.96)		45.70 (2.57)	29.84 (1.46)	16.66 (2.06)	12.13 (1.46)
	B	35.46 (3.86)		26.95 (4.33)	17.10 (4.26)		
	C	61.62 (2.20)	61.37 (4.27)	52.64 (4.02)			
3.0 μg/ml	B	38.06 (15.37)			6.70 (1.73)		
	C	48.93 (6.16)	46.55 (2.51)				

Table 3.11.5.4 : Effect of verapamil on adriamycin toxicity to DLKPA5F. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		VERAPAMIL				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (18.26)	77.54 (28.78)	47.62 (14.85)	85.15 (31.06)	52.23 (19.82)
	B	100 (11.05)	92.21 (5.26)	84.43 (10.07)		
0.05 μg/ml	B	71.34 (6.86)	76.93 (5.99)	39.71 (2.14)		
0.5 μg/ml	A	29.92 (3.62)	22.74 (6.97)	12.51 (7.17)	4.76 (1.71)	2.20 (1.56)
1.0μg/ml	B	29.78 (9.78)	18.49 (2.38)	9.05 (1.17)		
3.0 μg/ml	B	5.74 (1.70)		1.95 (0.39)		

Table 3.11.5.5 : Effect of verapamil on vincristine toxicity to DLKPA5F. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

With respect to the circumvention of drug resistance by cyclosporin A and verapamil, similar trends to those observed for DLKPA5F were also noted for DLKPA11B. Cyclosporin A enhanced the cell kill achieved by adriamycin (Tables 3.11.5.6 and 3.11.6.2), and vincristine (Tables 3.11.5.7 and 3.11.6.3) and resulted in a slight increase in the potency of VP-16 (Tables 3.11.5.8 and 3.11.6.4). This circumvention ability was also noted with verapamil, and to approximately the same level (Tables 3.11.5.8 - 3.11.5.10 and 3.11.6.1 - 3.11.6.4).

% SURVIVAL		CYCLOSPORIN A					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (24.18)		77.39 (16.24)	80.78 (29.19)	80.06 (29.29)	90.85 (30.32)
	B	100 (3.09)		93.97 (4.05)	76.18 (4.63)		
	C	100 (10.11)	95.38 (8.50)	91.96 (8.60)			
0.05 μg/ml	B	77.43 (3.14)		65.02 (2.89)	35.43 (1.78)		
	C	78.74 (3.23)	80.50 (4.25)	73.38 (4.25)			
1.0 μg/ml	A	71.74 (10.89)		68.76 (18.91)	41.42 (13.67)	46.25 (18.40)	34.94 (15.62)
	B	31.09 (1.15)		22.87 (1.89)	7.87 (1.05)		
	C	46.67 (2.59)	42.98 (1.76)	42.98 (2.22)			
3.0 μg/ml	B	14.50 (1.64)			3.54 (0.55)		
	C	34.01 (2.13)	30.78 (2.31)				

Table 3.11.5.6 : Effect of cyclosporin A on adriamycin toxicity to DLKPA11B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (6.09)	87.48 (10.60)	62.94 (11.44)	62.02 (11.60)	65.69 (10.10)
	B	100 (7.95)	84.50 (7.31)	76.31 (5.35)		
0.05 μg/ml	B	52.78 (1.64)	39.14 (2.78)	13.05 (2.40)		
0.5 μg/ml	A	46.41 (8.76)	41.24 (6.76)	35.39 (4.42)	27.80 (4.67)	19.12 (4.17)
1.0μg/ml	B	9.93 (1.20)	6.60 (0.87)	2.29 (0.33)		
3.0 μg/ml	B	3.71 (0.71)		0.60 (0.27)		

Table 3.11.5.7 : Effect of cyclosporin A on vincristine toxicity to DLKPA11B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL	CYCLOSPORIN A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VP-16					
0.0 μg/ml	100 (3.76)	94.06 (3.50)	85.62 (5.33)	83.41 (3.27)	76.94 (3.81)
2.0 μg/ml	24.73 (2.21)	23.44 (2.74)	19.56 (1.52)	15.83 (1.67)	14.92 (1.67)
% SURVIVAL	**VERAPAMIL**				
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VP-16					
0.0 μg/ml	100 (9.99)	105.42 (5.33)	98.16 (11.49)	83.82 (15.90)	91.54 (18.75)
2.0 μg/ml	24.54 (4.41)	25.18 (1.38)	26.10 (1.65)	20.13 (1.84)	18.84 (3.95)

Table 3.11.5.8 : Effect of cyclosporin A and verapamil on VP-16 toxicity to DLKPA11B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% SURVIVAL		VERAPAMIL					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (6.87)		96.54 (6.73)	91.01 (6.92)	89.62 (4.78)	89.37 (8.24)
	B	100 (10.53)		106.92 (10.78)	109.69 (10.37)		
	C	100 (9.19)	98.28 (8.33)	92.93 (5.07)			
0.05 μg/ml	B	67.93 (19.19)		71.46 (10.92)	58.24 (10.92)		
	C	76.65 (5.84)	75.31 (6.89)	77.80 (6.60)			
1.0 μg/ml	A	69.50 (4.21)		65.97 (4.91)	56.60 (4.72)	45.66 (3.58)	41.32 (3.08)
	B	20.41 (6.58)		17.15 (6.24)	8.27 (3.59)		
	C	41.91 (3.92)	38.76 (2.78)	35.22 (3.06)			
3.0 μg/ml	B	12.88 (2.92)			5.22 (1.63)		
	C	28.80 (2.87)	28.61 (2.68)				

Table 3.11.5.9 : Effect of verapamil on adriamycin toxicity to DLKPA11B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		VERAPAMIL (μg/ml)				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (5.82)	97.89 (7.47)	92.60 (4.21)	91.78 (4.76)	84.78 (5.91)
	B	100 (16.46)	27.25 (14.63)	56.12 (23.83)		
0.05 μg/ml	B	36.67 (12.55)	21.08 (9.80)	10.02 (5.35)		
0.5 μg/ml	A	53.74 (5.71)	54.15 (3.67)	44.16 (2.17)	34.99 (2.17)	26.90 (2.17)
1.0μg/ml	C	55.61 (5.13)	51.74 (5.03)			
3.0 μg/ml	B	2.69 (0.82)		0.37 (0.45)		

Table 3.11.5.10 : Effect of verapamil on vincristine toxicity to DLKPA11B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

In relation to the cell line DLKPA6B (Tables 3.11.5.11 - 3.11.5.15 and summarised in Tables 3.11.6.1 - 3.11.6.4), both cyclosporin A and verapamil enhanced the toxicities of adriamycin and vincristine. However, with respect to VP-16 toxicity, neither verapamil nor cyclosporin A caused a notable enhancement of toxicity. Cyclosporin A appeared to cause neither a synergistic nor an antagonistic effect on cell kill by VP-16, as determined using the Chou and Talalay (1984) method of analysis of two compounds in combination, while verapamil caused a slight decrease in the toxicity of VP-16 to the cells (Tables 3.11.5.13 and 3.11.6.4).

% SURVIVAL		CYCLOSPORIN A					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (23.43)		88.89 (17.30)	77.53 (23.36)	64.77 (17.42)	51.01 (19.57)
	B	100 (6.45)		91.91 (6.57)	82.93 (4.42)		
	C	100 (4.00)	91.73 (7.38)	100.18 (5.07)			
0.05 μg/ml	B	77.13 (4.35)		69.25 (3.73)	39.88 (3.11)		
	C	89.42 (3.56)	85.33 (3.64)	88.00 (6.40)			
1.0 μg/ml	A	56.06 (11.11)		38.13 (15.03)	15.53 (3.03)	14.14 (5.18)	9.22 (1.06)
	B	34.62 (3.11)		25.78 (3.32)	6.63 (0.69)		
	C	58.40 (3.82)	54.22 (2.49)	48.53 (2.48)			
3.0 μg/ml	B	18.38 (2.14)			2.14 (0.62)		
	C	42.13 (3.64)	35.20 (2.04)				

Table 3.11.5.11 : Effect of cyclosporin A on adriamycin toxicity to DLKPA6B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (4.19)	93.82 (6.12)	78.26 (4.59)	74.93 (4.26)	64.56 (6.25)
	B	100 (9.13)	92.77 (6.86)	90.01 (9.55)		
0.05 μg/ml	B	72.93 (11.04)	54.29 (5.29)	18.29 (2.68)		
0.5 μg/ml	A	28.32 (1.26)	20.35 (1.00)	5.45 (0.80)	2.59 (0.53)	1.26 (0.20)
1.0μg/ml	B	16.63 (2.39)	13.27 (1.34)	4.10 (0.30)		
3.0 μg/ml	B	5.96 (0.75)		2.83 (0.52)		

Table 3.11.5.12 : Effect of cyclosporin A on vincristine toxicity to DLKPA6B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL	CYCLOSPORIN A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VP-16					
0.0 μg/ml	100 (3.56)	94.19 (3.80)	80.38 (4.19)	79.21 (1.73)	68.64 (1.57)
2.0 μg/ml	21.24 (1.29)	21.69 (1.62)	15.20 (0.84)	11.96 (1.17)	14.42 (2.68)

% SURVIVAL	VERAPAMIL				
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VP-16					
0.0 μg/ml	100 (6.54)	111.51 (3.86)	108.84 (3.68)	93.77 (12.34)	88.66 (12.88)
2.0 μg/ml	21.90 (2.02)	23.74 (1.72)	27.42 (1.13)	23.86 (3.56)	25.04 (3.92)

Table 3.11.5.13 : Effect of cyclosporin A and verapamil on VP-16 toxicity to DLKPA6B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% SURVIVAL		VERAPAMIL					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (9.83)		100.94 (5.61)	94.60 (6.98)	96.22 (6.87)	96.17 (6.30)
	B	100 (11.74)		112.88 (15.47)	91.68 (10.11)		
	C	100 (6.77)	93.80 (8.48)	88.94 (4.10)			
0.05 μg/ml	B	65.83 (9.57)		90.34 (11.27)	64.40 (9.48)		
	C	80.65 (6.77)	83.13 (5.43)	74.26 (13.92)			
1.0 μg/ml	A	46.80 (3.83)		44.86 (3.62)	29.28 (2.62)	21.77 (1.52)	19.78 (1.42)
	B	32.29 (6.71)		19.50 (4.38)	8.68 (2.77)		
	C	51.86 (7.34)	47.66 (3.05)	44.23 (2.96)			
3.0 μg/ml	B	11.63 (3.58)			4.11 (2.42)		
	C	31.94 (4.29)	32.22 (3.62)				

Table 3.11.5.14 : Effect of verapamil on adriamycin toxicity to DLKPA6B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		VERAPAMIL				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (4.05)	95.31 (9.56)	84.60 (10.53)	71.63 (18.23)	74.07 (15.60)
	B	100 (20.72)	85.59 (26.24)	79.74 (23.49)		
0.05 μg/ml	B	47.60 (12.93)	42.62 (18.17)	33.89 (8.73)		
0.5 μg/ml	A	25.10 (1.41)	21.95 (2.25)	10.98 (2.12)	6.48 (1.48)	4.88 (0.83)
1.0μg/ml	B	9.87 (3.84)	8.30 (1.57)	4.80 (1.66)		
3.0 μg/ml	B	2.88 (1.05)		1.57 (0.44)		

Table 3.11.5.15 : Effect of verapamil on vincristine toxicity to DLKPA6B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

With respect to DLKPA2B, both cyclosporin A and verapamil caused a significant enhancement of the toxicities of adriamycin (Tables 3.11.4.16 - 3.11.4.19 and summarised in Table 3.11.6.2) and vincristine (Tables 3.11.4.17 - 3.11.4.20 and summarised in Table 3.11.6.3). Cyclosporin A, at a concentration of 2.0μg/ml, decreased cell survival in the presence of 1.0μg/ml adriamycin, from 35.30 ± 2.81% when the cells were exposed to adriamycin alone, to 6.94 ± 2.58% in the presence of the combination, while the cell survival in the presence of cyclosporin A alone was 72.08%. The presence of cyclosporin A, at 1.0μg/ml, reduced cell survival in the presence of 0.05μg/ml vincristine from 41.11 ± 4.65%, in the presence of vincristine alone, to 5.90 ± 0.71% in the presence of the combination, while the cyclosporin A alone supported 72.61 ± 11.64% cell growth. In terms of VP-16 toxicity, while cyclosporin A enhanced its toxicity, the combination of verapamil and VP-16 resulted in an antagonistic effect with respect to cell kill, as determined by the Chou and Talalay (1984) method of analysis (Tables 3.11.5.18 and 3.11.6.4).

% SURVIVAL		CYCLOSPORIN A					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (12.00)		96.97 (7.02)	88.63 (7.09)	84.05 (6.72)	72.08 (7.90)
	B	100 (12.36)		92.36 (5.18)	90.19 (4.22)		
	C	100 (3.14)	98.09 (3.78)	96.77 (4.04)			
0.05 μg/ml	B	65.64 (3.92)		59.37 (1.54)	36.49 (2.30)		
	C	59.41 (5.69)	51.55 (4.16)	46.66 (3.95)			
1.0 μg/ml	A	35.30 (2.81)		27.33 (1.92)	10.27 (2.07)	8.64 (0.74)	6.94 (2.58)
	B	24.55 (1.42)		16.54 (0.88)	5.09 (0.29)		
	C	20.10 (2.93)	14.62 (2.12)	9.31 (0.98)			
3.0 μg/ml	B	9.69 (0.75)			0.63 (0.17)		
	C	3.82 (0.64)	3.31 (0.51)				

Table 3.11.5.16 : Effect of cyclosporin A on adriamycin toxicity to DLKPA2B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		CYCLOSPORIN A				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (5.47)	94.03 (8.61)	72.61 (11.64)	73.86 (20.56)	60.74 (13.11)
	B	100 (8.73)	95.35 (4.42)	84.09 (6.97)		
0.05 μg/ml	B	41.11 (4.65)	29.18 (2.28)	5.90 (0.71)		
0.5 μg/ml	A	17.38 (1.71)	12.72 (2.56)	4.42 (0.47)	3.41 (0.47)	2.64 (0.39)
1.0μg/ml	B	8.09 (0.54)	6.08 (0.89)	5.05 (0.89)		
3.0 μg/ml	B	4.78 (0.40)		3.57 (0.36)		

Table 3.11.5.17 : Effect of cyclosporin A on vincristine toxicity to DLKPA2B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL	CYCLOSPORIN A				
	0 μg/ml	0.2 μg/ml	1.0 μg/ml	1.5 μg/ml	2.0 μg/ml
VP-16					
0.0 μg/ml	100 (5.31)	88.09 (9.08)	34.18 (5.88)	17.27 (2.61)	11.91 (1.86)
1.0 μg/ml	31.76 (1.34)	12.29 (1.27)	4.02 (0.60)	2.83 (0.67)	2.23 (0.67)

% SURVIVAL	VERAPAMIL				
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VP-16					
0.0 μg/ml	100 (7.35)	89.01 (7.94)	79.78 (12.28)	60.94 (7.74)	45.98 (9.60)
1.0 μg/ml	17.27 (2.31)	20.50 (3.60)	17.08 (3.42)	15.24 (2.12)	10.90 (1.94)

Table 3.11.5.18 : Effect of cyclosporin A and verapamil on VP-16 toxicity to DLKPA2B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data.

% SURVIVAL		VERAPAMIL					
		0.0 μg/ml	0.1 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
ADRIAMYCIN							
0.0 μg/ml	A	100 (6.56)		98.01 (14.21)	88.70 (10.66)	71.86 (23.31)	77.11 (20.82)
	B	100 (12.42)		80.85 (7.73)	80.16 (8.32)		
	C	100 (16.56)	100.28 (4.05)	104.00 (6.44)			
0.05 μg/ml	B	38.29 (11.57)		57.01 (9.92)	55.31 (5.12)		
	C	39.04 (13.12)	60.25 (4.09)	60.91 (6.63)			
1.0 μg/ml	A	27.86 (3.27)		23.81 (3.69)	16.92 (1.78)	12.86 (2.20)	10.09 (2.77)
	B	15.73 (4.11)		14.99 (2.99)	7.04 (1.81)		
	C	12.42 (2.92)	12.94 (3.15)	8.04 (1.46)			
3.0 μg/ml	B	4.57 (1.65)			2.45 (0.80)		
	C	1.65 (0.66)	2.07 (0.33)				

Table 3.11.5.19 : Effect of verapamil on adriamycin toxicity to DLKPA2B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A, B and C refer to repeat experiments and only values in a particular series are comparable.

% SURVIVAL		VERAPAMIL				
		0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml
VINCRISTINE						
0.0 μg/ml	A	100 (22.95)	77.35 (21.75)	57.83 (15.32)	50.42 (19.28)	68.62 (33.44)
	B	100 (9.41)	86.11 (16.30)	90.34 (10.45)		
0.05 μg/ml	B	22.98 (4.52)	15.68 (3.07)	12.32 (2.41)		
0.5 μg/ml	A	13.76 (2.97)	11.78 (2.31)	6.10 (2.06)	4.45 (1.73)	3.20 (0.99)
1.0μg/ml	B	3.90 (0.91)	2.90 (0.46)	2.70 (0.71)		
3.0 μg/ml	B	2.41 (0.46)		1.87 (0.29)		

Table 3.11.5.20 : Effect of verapamil on vincristine toxicity to DLKPA2B. Survival was calculated as percentage growth relative to that measured in a drug-free control and is the mean of eight replicas. The values in brackets are the standard deviations on the data. Series A and B refer to repeat experiments and only values in a particular series are comparable.

3.11.6 Conclusions

This study showed that both cyclosporin A and verapamil possessed the ability to modify the toxicity of adriamycin, vincristine and VP-16 of the DLKP cell line and its MDR variants. Both circumventing agents were less toxic to the drug-sensitive DLKP cells than to any of the MDR cell lines (Table 3.11.1). Cyclosporin A and verapamil were also more effective at enhancing the toxicity of adriamycin, vincristine and VP-16 to DLKP, in comparison with the drug-resistant cell lines (Tables 3.11.6.2 - 3.11.6.4). In all cases studied, the level of alteration in the drug's toxicity depended on both the concentration of the chemotherapeutic drug and the circumvention agent.

Cyclosporin A enhanced the toxicity of adriamycin, vincristine and VP-16, in all cell lines examined, as outlined in Table 3.11.6.1. Verapamil also enhanced adriamycin and vincristine toxicity to all the cell lines. However, verapamil exhibited an antagonistic effect on VP-16 toxicity to DLKP/VP-3, DLKP/VP-8, DLKPA2B and DLKPA6B.

Effect of circumventing agent on the toxicity of drugs	Verapamil			Cyclosporin A		
	Adriamycin	**Vincristine**	**VP-16**	**Adriamycin**	**Vincristine**	**VP-16**
DLKP	Synergistic[1]	Synergistic[1]	Synergistic[1]	Synergistic[1]	Synergistic[1]	Synergistic[1]
DLKP-A	Synergistic[1]	Synergistic[2,3]	Synergistic[1]	Synergistic[1]	Synergistic[1]	Synergistic[1]
DLKPA2B	Synergistic[1]	Synergistic[1]	**Antagonistic[2,3]**	Synergistic[1]	Synergistic[1]	Synergistic[1]
DLKPA6B	Synergistic[1]	Synergistic[1]	**Antagonistic[2,3]**	Synergistic[1]	Synergistic[1]	Synergistic[2,3]
DLKPA11B	Synergistic[1]	Synergistic[1]	Synergistic[2,3]	Synergistic[1]	Synergistic[1]	Synergistic[1]
DLKPA5F	Synergistic[1]	Synergistic[1]	Synergistic[2,3]	Synergistic[1]	Synergistic[1]	Synergistic[1,3]
DLKP/VP-3	Synergistic[1]	Synergistic[1]	**Antagonistic[2]**	Synergistic[1]	Synergistic[1]	Synergistic[1]
DLKP/VP-8	Synergistic[1]	Synergistic[1]	**Antagonistic[2]**	Synergistic[1]	Synergistic[1]	Synergistic[1]

[1] Determined by the multiple product method of analysis.
[2] As determined using the Chou and Talalay (1984) method of dose-effects analysis.
[3] A slight effect was noted at higher concentrations of the circumventing agent.

Table 3.11.6.1 : Summary table of circumvention of adriamycin, vincristine and VP-16 resistance in DLKP variants.

Adriamycin conc.	Cyclosporin A				Verapamil			
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	3.0 μg/ml
DLKP 0.02μg/ml	45.06	27.52	14.53	13.03	41.98	37.40	35.54	24.94
DLKP/VP-3 2.0μg/ml	71.40	68.77	38.30	10.93	71.16	63.35	31.68	12.09
DLKP/VP-8 5.0μg/ml	74.49	69.95	47.53	12.88	82.74	84.03	28.17	6.83
DLKP-A 1.0μg/ml	100.74	102.60	90.52	50.56	93.54	69.79	45.63	27.71
DLKPA5F 1.0μg/ml	49.47	40.04	20.05	8.17	52.59	45.70	29.84	12.13
DLKPA11B 1.0μg/ml	71.74	68.76	41.42	34.94	69.50	65.97	56.60	41.32
DLKPA6B 1.0μg/ml	56.06	38.13	15.53	9.22	46.80	44.86	29.28	19.78
DLKPA2B 1.0μg/ml	35.30	27.33	10.27	6.94	27.86	23.81	16.92	10.09

Table 3.11.6.2 : The enhancement of the toxicity of adriamycin to variants of DLKP, by various concentrations of cyclosporin A and verapamil. The data presented in this table is a summary of the data in Tables 3.11.2.1 - 3.11.5.20. The toxicity of adriamycin to the cells, at the concentration stated in the table, below the respective cell line, is presented and the toxicity of the circumvention agents to the cells may be determined from the summary table, Table 3.11.1. A more detailed analysis of the ability of the circumventing agents to enhance the toxicity of adriamycin to the cells, including the standard deviation on the data, may be found in the relevant tables mentioned above.

Vincristine conc.	Cyclosporin A				Verapamil			
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	3.0 μg/ml
DLKP 0.01μg/ml	98.44	86.52	21.52	3.63	94.12	94.79	81.53	51.48
DLKP/VP-3 2.0μg/ml	53.59	48.13	28.14	11.89	61.16	53.27	31.72	11.97
DLKP/VP-8 2.0μg/ml	48.54	48.03	24.11	9.27	45.80	48.17	11.65	5.22
DLKP-A 0.5μg/ml	102.79	91.63	77.98	34.89	96.37	85.96	64.04	26.18
DLKPA5F 0.5μg/ml	40.51	33.73	17.93	6.83	29.92	22.74	12.51	2.20
DLKPA11B 0.5μg/ml	46.41	41.24	35.39	19.12	53.74	54.15	44.16	26.90
DLKPA6B 0.5μg/ml	28.32	20.35	5.45	1.26	25.10	21.95	10.98	4.88
DLKPA2B 0.5μg/ml	17.38	12.72	4.42	2.64	13.76	11.78	6.10	3.20

Table 3.11.6.3 : The enhancement of the toxicity of vincristine to variants of DLKP, by various concentrations of cyclosporin A and verapamil. The data presented in this table is a summary of the data in Tables 3.11.2.1 - 3.11.5.20. The toxicity of vincristine to the cells, at the concentration stated in the table, below the respective cell line, is presented and the toxicity of the circumvention agents to the cells may be determined from the summary table, Table 3.11.1. A more detailed analysis of the ability of the circumventing agents to enhance the toxicity of vincristine to the cells, including the standard deviation on the data, may be found in the relevant tables mentioned above.

VP-16 conc.	Cyclosporin A				Verapamil			
	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	2.0 μg/ml	0.0 μg/ml	0.2 μg/ml	1.0 μg/ml	3.0 μg/ml
DLKP 0.075μg/ml	91.69	86.10	65.35	50.04	98.61	98.81	96.26	90.62
DLKP/VP-3 5.0μg/ml	51.60	48.15	24.39	8.05	50.65	48.88	42.49	32.51
DLKP/VP-8 8.0μg/ml	69.09	69.67	50.65	26.36	61.32	59.98	42.25	16.28
DLKP-A 2.0μg/ml	69.01	67.76	45.66	13.45	75.51	77.03	70.43	29.75
DLKPA5F 4.0μg/ml	18.36	19.19	12.96	9.72	16.65	17.34	17.57	14.24
DLKPA11B 2.0μg/ml	24.73	23.44	19.56	14.92	24.54	25.18	26.10	18.84
DLKPA6B 2.0μg/ml	21.24	21.69	15.20	14.42	21.90	23.74	27.42	25.04
DLKPA2B 1.0μg/ml	31.76	12.29	4.02	2.23	17.29	20.50	17.08	10.90

Table 3.11.6.4 : The enhancement of the toxicity of VP-16 to variants of DLKP by cyclosporin A and verapamil, at varying concentrations. The data presented in this table is a summary of the data in Tables 3.11.2.1 - 3.11.5.20. The toxicity of VP-16 to the cells, at the concentration stated in the table, below the respective cell line, in the absence of cyclosporin A and verapamil is presented and the toxicity of the circumvention agents to the cells may be determined from the summary table, Table 3.11.1. A more detailed analysis of the ability of circumventing agents to enhance VP-16 toxicity to the cells, including the standard deviation on the data, may be found in the relevant tables mentioned above.

3.12 Camptothecin toxicity to DLKP variants

The toxicity of camptothecin, a topoisomerase I inhibitor, to DLKP variants, was examined to determine if cell lines which were resistant to topoisomerase II inhibitors exhibited alterations in their sensitivity to topoisomerase I poisons. The experiment was performed twice with eight intra-assay replicas per assay.

Camptothecin proved to be least toxic to the parental cell line DLKP (which was also most sensitive to the topoisomerase II inhibitors) and the MDR clone of DLKP-A, DLKPA5F (which was highly resistant to both adriamycin and VP-16) (Figure 3.12.1). It exhibited slightly higher toxicity to DLKP-A, was even more toxic to DLKP/VP-3 and proved to be highly toxic to DLKP/VP-8. However, the range increase in toxicity, from the most sensitive to the most resistant cell line with respect to camptothecin, was small; a maximum of a 2-fold increase in the IC_{50} values was noted.

When the toxicity of camptothecin in combination with adriamycin, to the cells was examined, no synergism or antagonism between the two compounds was observed (Figure 3.12.2 - 3.12.6). The cell kill observed in DLKP, DLKP-A, DLKPA5F, DLKP/VP-3 and DLKP/VP-8, following exposure to the two drugs in combination, may be explained by the individual toxicity of the two compounds acting in an non-synergistic manner, when analyzed by the Chou and Talalay method of analysis of two compounds in combination at a fixed ratio (Chou and Talalay, 1984).

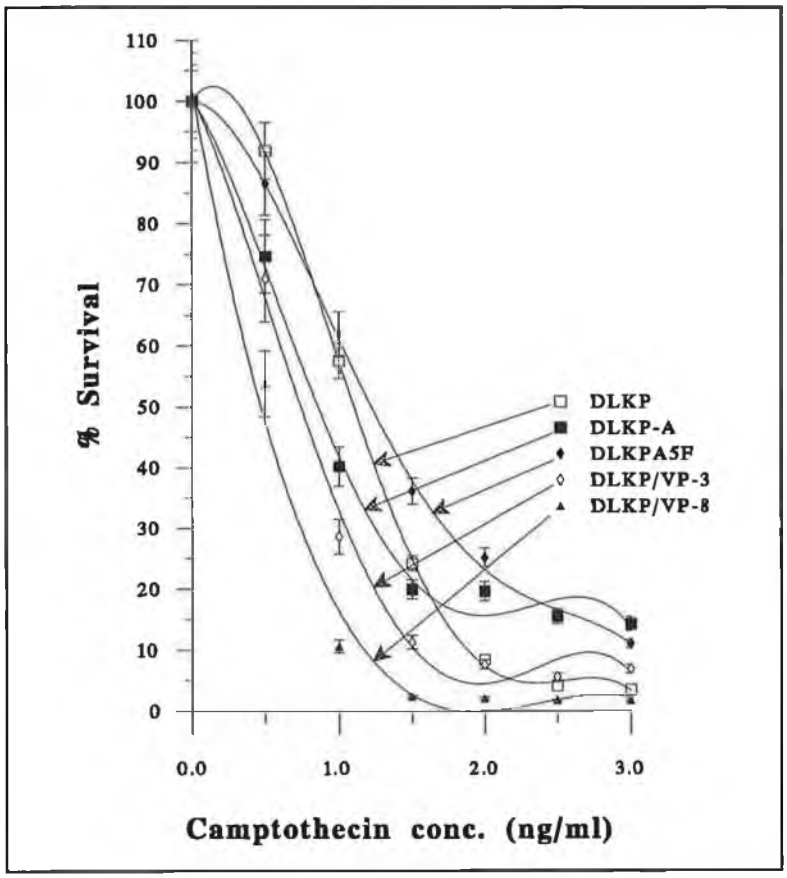


Figure 3.12.1 : Toxicity of camptothecin to DLKP, DLKP-A, DLKPA5F, DLKP/VP-3 and DLKP/VP-8. The error bars represent the standard deviation on the mean of eight replicas.

	Adriamycin (ng/ml)				Camptothecin (ng/ml)				Combination*		
	Conc.	% Survival	Std. Dev.		Conc.	% Survival	Std. Dev.		% Survival	Std. Dev.
A	1.875	99.72	4.04	A	0.5	91.92	4.96	A	91.46	3.90
B	3.75	96.51	7.99	B	1.0	57.56	1.88	B	52.41	3.08
C	5.625	99.49	5.01	C	1.5	24.21	1.56	C	21.50	1.52
D	7.5	93.59	5.61	D	2.0	8.41	0.67	D	6.77	0.31
E	9.375	85.53	5.30	E	2.5	4.10	0.71	E	3.43	0.53
F	11.25	68.17	11.53	F	3.0	3.47	0.62	F	3.43	0.49

Figure 3.12.2 : Effect of adriamycin and camptothecin, in combination, on DLKP. Analysis by the Chou and Talalay (1984) method of analysis of two compounds in combination, shows no synergistic or antagonistic effect over their individual toxicities.

* Adriamycin and camptothecin were combined at a fixed ratio, such that the data presented for combination A pertains to the percentage cell survival in the presence of the concentration of adriamycin and camptothecin corresponding to that letter.

	Adriamycin (μg/ml)				Camptothecin (ng/ml)				Combination*		
	Conc.	% Survival	Std. Dev.		Conc.	% Survival	Std. Dev.		% Survival	Std. Dev.
A	0.75	83.62	7.06	A	0.5	74.66	8.45	A	64.24	9.21
B	1.5	68.20	9.66	B	1.0	40.18	7.06	B	27.64	5.89
C	2.25	57.97	12.41	C	1.5	19.96	5.95	C	15.87	2.88
D	3.0	80.20	6.60	D	2.0	19.61	0.95	D	16.24	1.40
E	3.75	83.44	5.33	E	2.5	15.48	0.63	E	15.61	0.70
F	4.5	75.25	6.60	F	3.0	14.28	0.95	F	15.99	1.46

Figure 3.12.3 : Effect of adriamycin and camptothecin, in combination, on DLKPA. Analysis by the Chou and Talalay (1984) method of analysis of two compounds in combination, shows no significant synergistic or antagonistic effect over their individual toxicities.
* Adriamycin and camptothecin were combined at a fixed ratio, such that the data presented for combination A pertains to the percentage cell survival in the presence of the concentration of adriamycin and camptothecin corresponding to that letter.

	Adriamycin (μg/ml)				Camptothecin (ng/ml)				Combination*		
	Conc.	% Survival	Std. Dev.		Conc.	% Survival	Std. Dev.		% Survival	Std. Dev.
A	1.5	54.73	9.74	A	0.5	86.51	5.84	A	43.14	5.44
B	3.0	38.25	3.47	B	1.0	61.91	6.55	B	19.40	2.37
C	4.5	26.58	4.42	C	1.5	36.12	6.15	C	9.31	1.66
D	6.0	21.96	4.62	D	2.0	25.16	1.42	D	7.47	0.62
E	7.5	21.96	3.56	E	2.5	15.47	1.69	E	6.67	0.53
F	9.0	17.79	2.84	F	3.0	11.02	1.16	F	7.02	0.44

Figure 3.12.4 : Effect of adriamycin and camptothecin, in combination, on DLKPA5F. Analysis by the Chou and Talalay (1984) method of analysis of two compounds in combination, shows no significant synergistic or antagonistic effect over their individual toxicities.
* Adriamycin and camptothecin were combined at a fixed ratio, such that the data presented for combination A pertains to the percentage cell survival in the presence of the concentration of adriamycin and camptothecin corresponding to that letter.

	Adriamycin (μg/ml)				Camptothecin (ng/ml)				Combination*		
	Conc.	% Survival	Std. Dev.		Conc.	% Survival	Std. Dev.		% Survival	Std. Dev.
A	0.375	92.43	11.86	A	0.5	71.00	10.86	A	59.97	10.14
B	0.75	80.43	8.00	B	1.0	28.57	6.00	B	18.14	2.57
C	1.125	69.43	12.86	C	1.5	11.29	2.43	C	9.00	1.14
D	1.5	75.06	7.23	D	2.0	7.61	0.37	D	6.36	1.00
E	1.875	70.32	14.46	E	2.5	5.61	0.25	E	6.23	0.50
F	2.25	64.96	11.35	F	3.0	6.86	0.75	F	6.75	0.75

Figure 3.12.5 : Effect of adriamycin and camptothecin, in combination, on DLKP/VP-3. Analysis by the Chou and Talalay (1984) method of analysis of two compounds in combination, shows no significant synergistic or antagonistic effect over their individual toxicities.
* Adriamycin and camptothecin were combined at a fixed ratio, such that the data presented for combination A pertains to the percentage cell survival in the presence of the concentration of adriamycin and camptothecin corresponding to that letter.

	Adriamycin (μg/ml)				Camptothecin (ng/ml)				Combination*		
	Conc.	% Survival	Std. Dev.		Conc.	% Survival	Std. Dev.		% Survival	Std. Dev.
A	1.0	76.04	15.43	A	0.5	53.75	9.95	A	24.58	5.40
B	2.0	55.80	12.65	B	1.0	10.59	2.74	B	3.59	0.91
C	3.0	31.77	7.90	C	1.5	2.37	0.47	C	0.95	0.32
D	4.0	27.71	8.60	D	2.0	2.07	0.32	D	1.75	0.32
E	5.0	23.09	9.71	E	2.5	1.75	0.16	E	2.07	0.48
F	6.0	14.01	4.62	F	3.0	1.75	0.16	F	1.91	0.48

Figure 3.12.6 : Effect of adriamycin and camptothecin, in combination, on DLKP/VP-8. Analysis by the Chou and Talalay (1984) method of analysis of two compounds in combination, shows no significant synergistic or antagonistic effect over their individual toxicities.
* Adriamycin and camptothecin were combined at a fixed ratio, such that the data presented for combination A pertains to the percentage cell survival in the presence of the concentration of adriamycin and camptothecin corresponding to that letter.

3.13 Plating density effects on toxicity profiles of the cells

To determine if the sensitivity of the cell line, DLKP and its drug-resistant variants, to various chemotherapeutic drugs, was a cell density-dependent phenomenon, the toxicity of the drugs to the cell lines, when plated at 200 and 2000 cells/well, was investigated. All experiments were carried out in duplicate, with triplicate repeats in each assay and the results of both experiments were found to correlate.

3.13.1 Adriamycin toxicity of DLKP, DLKP-A and its clones

The effect of the initial cell plating density on the cells' ability to grow in the presence of adriamycin, was examined. When DLKP was plated at high (2000 cells/well) and low (200 cells/well) cell density in an adriamycin toxicity assay, slightly greater kill was observed in the toxicity assay set up at low plating density (Figure 3.13.1.1(a)). While 0.01μg/ml adriamycin resulted in 50% kill in DLKP at a plating density of 2000 cells/well, the IC_{50} value for the cells at a plating density of 200 cells/well was reduced to approximately 0.0075μg/ml. A greater difference in cell kill at high and low cell plating densities was observed for DLKP-A (Figure 3.13.1.1(b)). 4μg/ml resulted in 50% cell kill when cells were plated at 2000 cells/well, one fifth of this concentration (approximately 0.8μg/ml) resulted in the same level of kill at a plating density of 200 cells/well. The shape of the adriamycin toxicity curves generated by DLKP at high and low cell plating densities were similar. However, the shapes of the toxicity curves generated by DLKP-A at high and low plating densities differed (Figure 3.13.1.1(b)). High cell kill occurred at low adriamycin concentration when DLKP-A cells were plated at 200 cells/well, whereas low cell kill was noted at low drug concentrations at a plating density of 2000 cells/well.

The adriamycin toxicity profiles of the DLKP-A clones, DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F, were also cell density-dependent (Figure 3.13.1.2, 3.13.1.3). Although the cell density-dependent effect was generally greater than that observed for DLKP, it was substantially less than that noted for DLKP-A. The greatest cell density-dependent effect noted in the clones was observed in DLKPA5F and DLKPA2B. For DLKPA5F (unlike DLKP-A), at low adriamycin concentrations the shape of the toxicity profiles generated by high and low cell plating densities were similar and it was only at higher adriamycin concentrations that the profiles deviated (Figure 3.13.1.3(b)). The shape of the toxicity profiles generated when DLKPA2B was plated at high and low cell density were similar (Figure 3.13.1.2(a)).

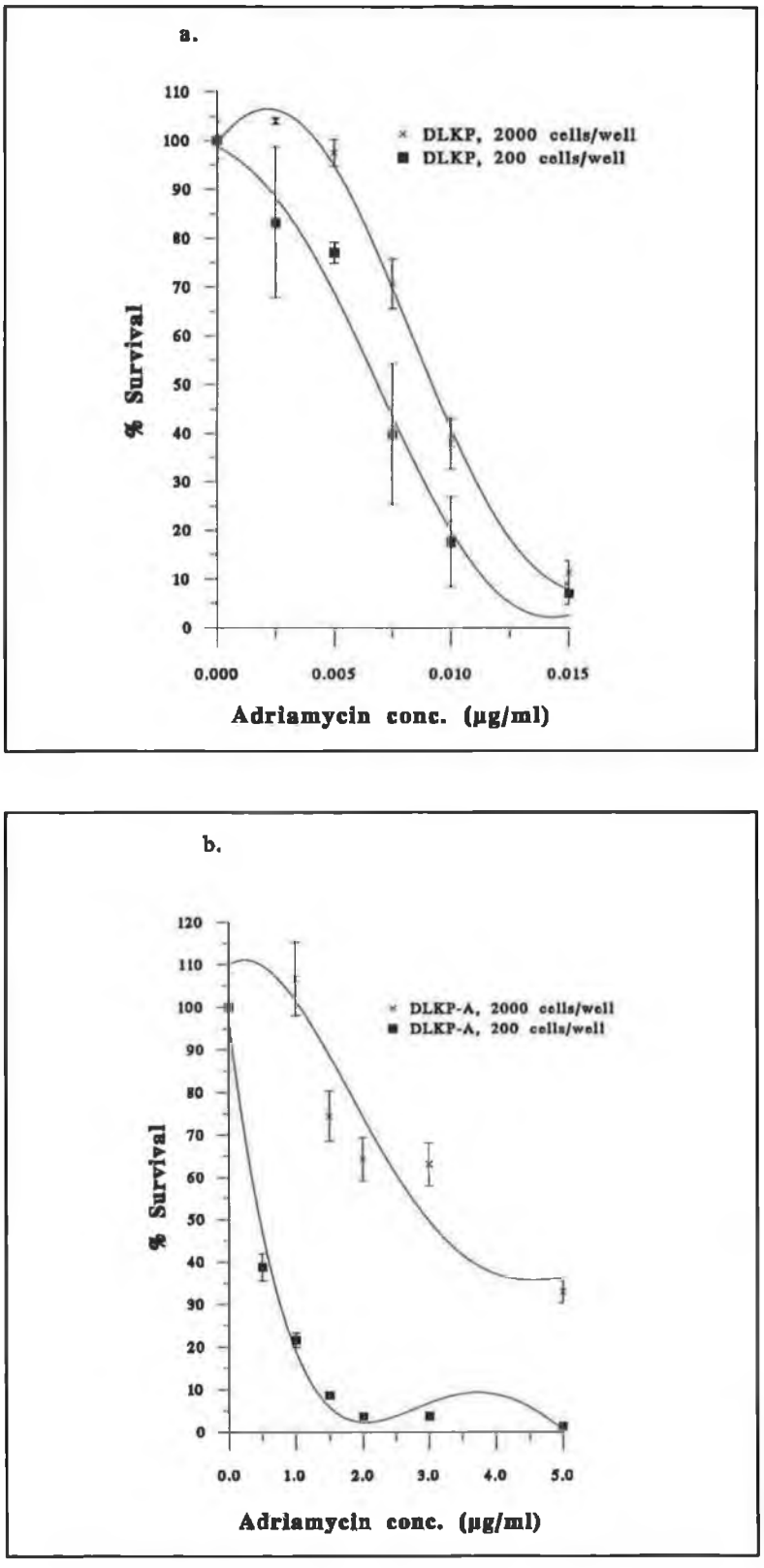


Figure 3.13.1.1 : Adriamycin toxicity to DLKP (a) and DLKP-A (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.

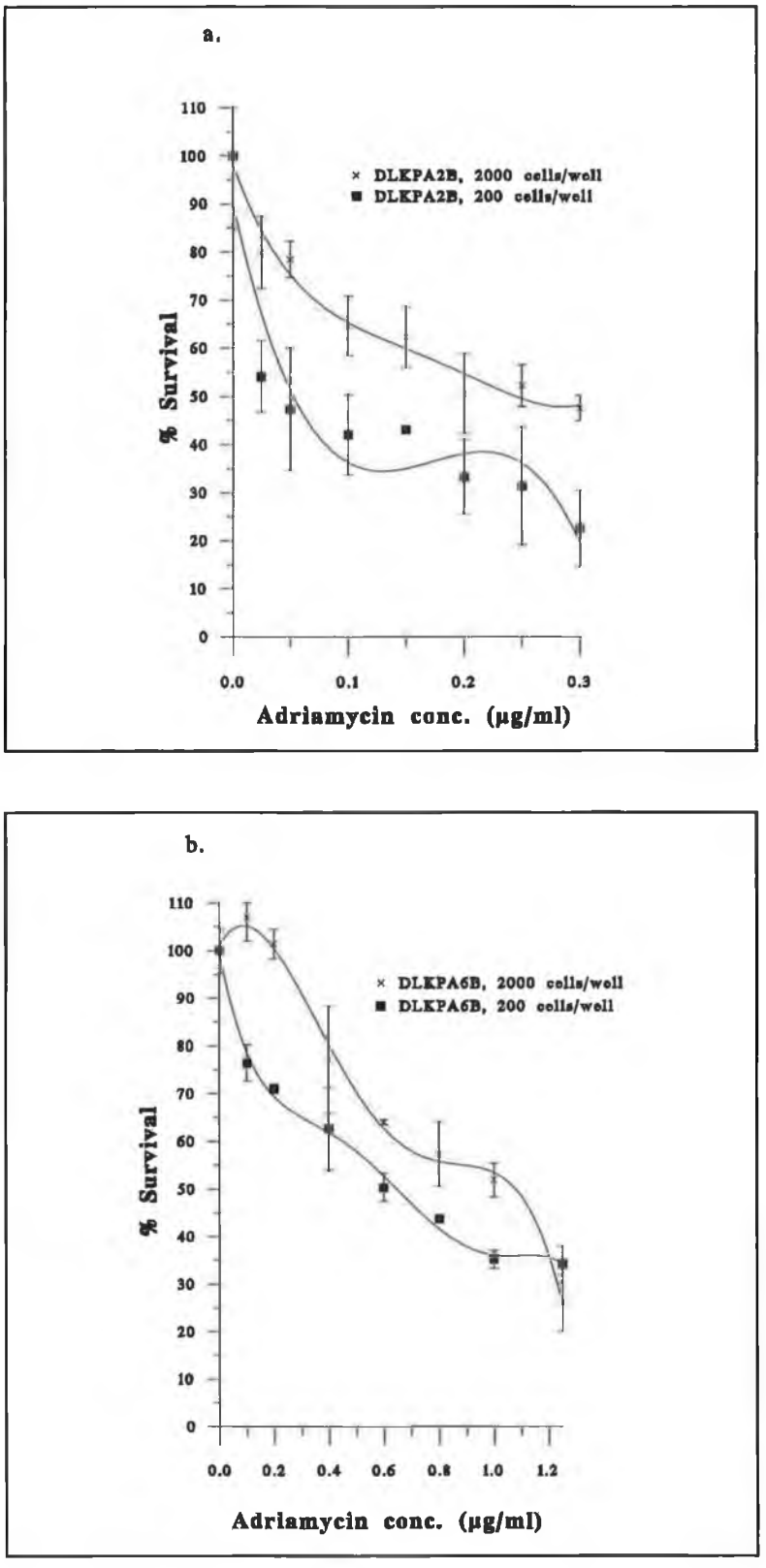
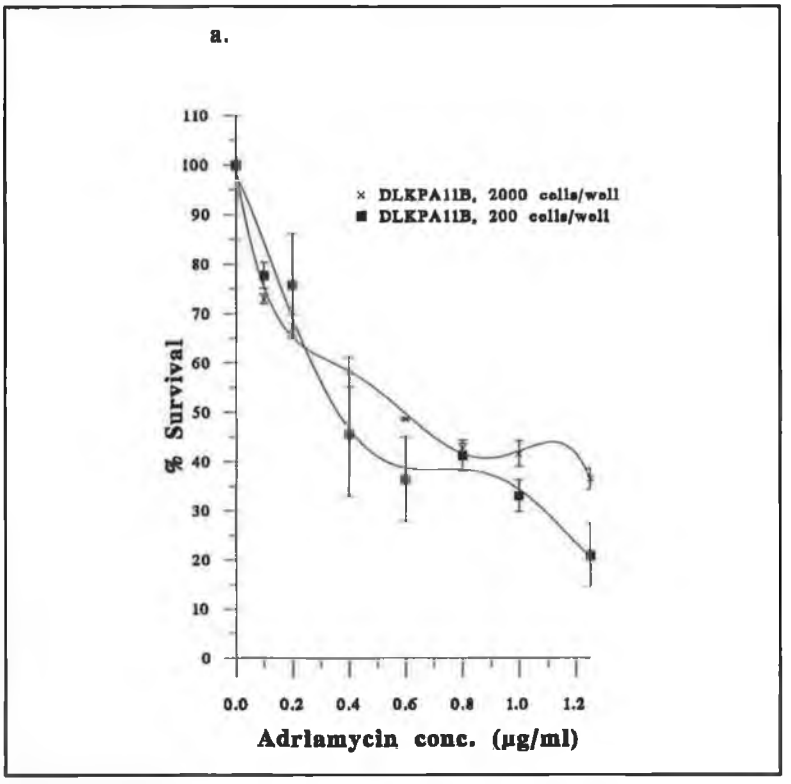


Figure 3.13.1.2 : Adriamycin toxicity to DLKPA2B (a) and DLKPA6B (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.



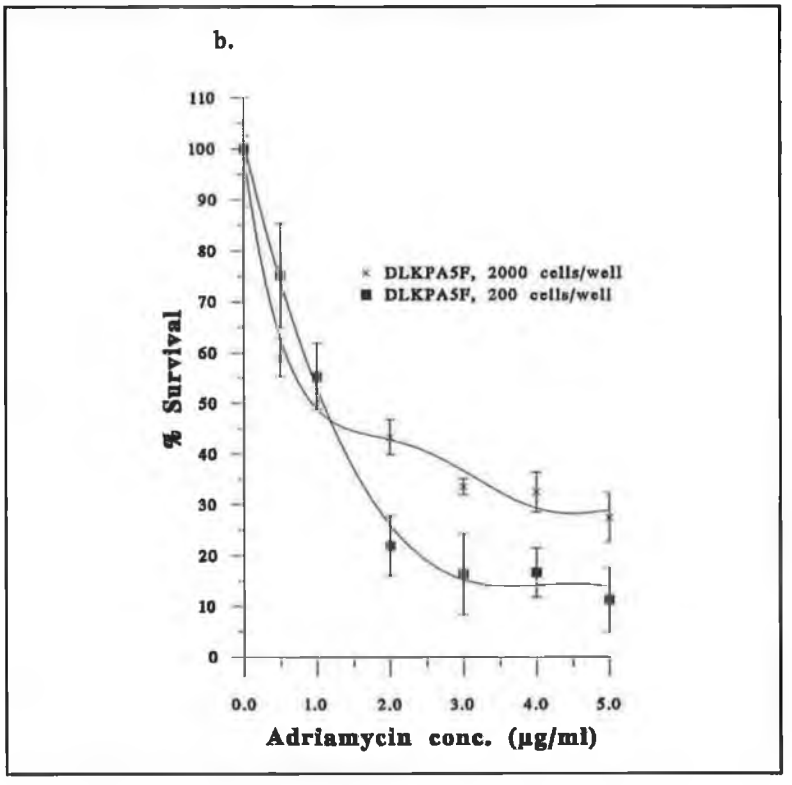


Figure 3.13.1.3 : Adriamycin toxicity to DLKPA11B (a) and DLKPA5F (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.

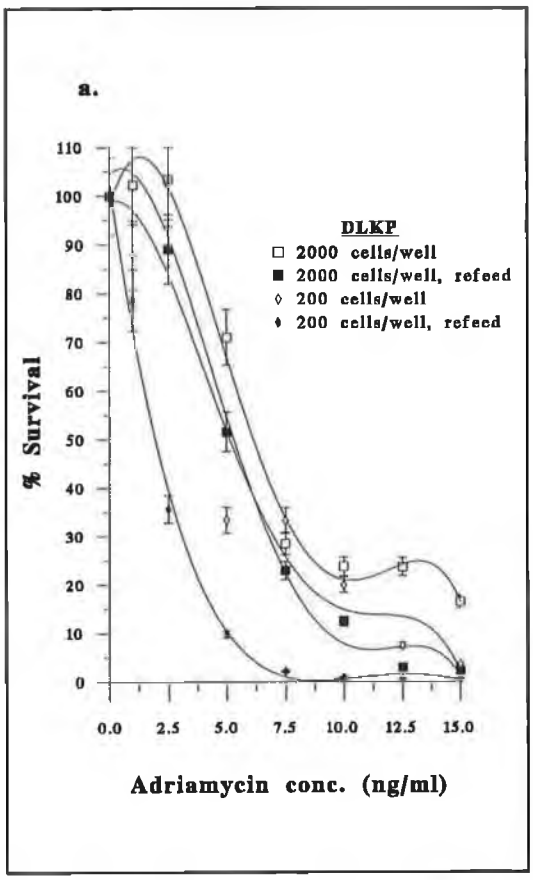
3.13.2 Adriamycin toxicity profiles with continuous drug replacement

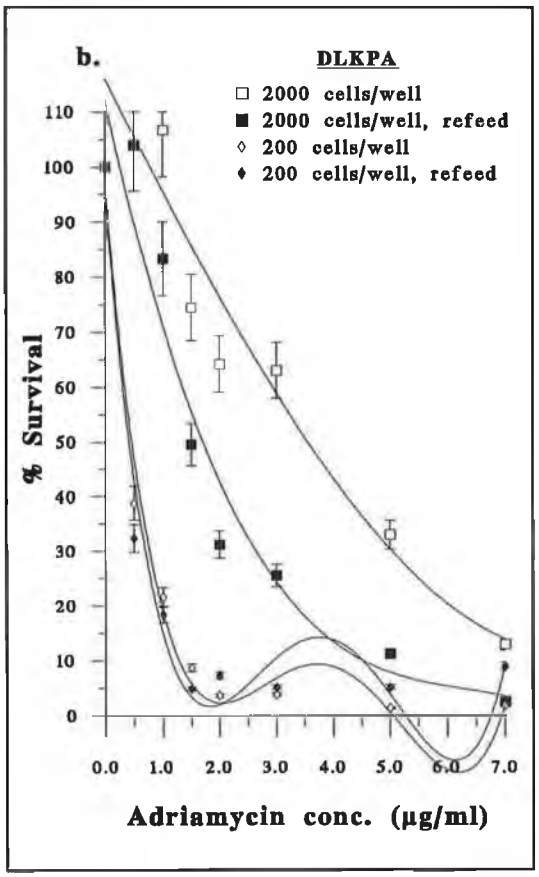
To ensure that the alterations in the toxicity profiles observed at different cell plating densities were not due to cell cycle variations, adriamycin toxicity assays were set up in which fresh drug was added every 48h. and the toxicity profiles analyzed. This ensured that the cells were continuously exposed to the initial drug concentration and therefore, cells could not have survived by being at a more resistant stage of the cell cycle, with respect to adriamycin toxicity, during the initial addition of adriamycin.

Refeeding with fresh adriamycin every 48h. did not alter the toxicity profile of DLKP at high cell plating density (Figure 3.13.2(a)). However, a slight decrease in the cells' resistance to adriamycin was observed when the cells were fed with fresh drug, at a low cell plating density. In both cases, the shape of the toxicity curves were similar to that noted when the toxicity profile was generated with only one drug addition.

Refeeding the DLKP-A toxicity assay with fresh adriamycin every 48h. resulted in a reduction of the adriamycin toxicity profile generated when the cells were plated at high density, in comparison with the toxicity profile generated by a single addition of adriamycin (Figure 3.13.2(b)). The toxicity profile obtained by refeeding with fresh drug maintained the shape of the curve generated by one addition of adriamycin. The DLKP-A cells still appeared significantly more resistant to adriamycin, when plated at high cell density and refed with fresh adriamycin every 48h., than when the cells were exposed to the same concentration of drug, at low cell plating density. Refeeding the low cell density assay with fresh drug resulted in negligible deviation from the toxicity profile generated by a single exposure to adriamycin.

The toxicity profiles generated by DLKPA5F at high cell plating density were similar whether they were obtained from one addition of adriamycin or refeeding with fresh drug every 48h. (Figure 3.13.2(c)). A slight decrease in the toxicity profile was observed at low cell plating density when fresh adriamycin was added, in comparison to the toxicity profile generated by one drug addition. Again, although the shape of the adriamycin toxicity profile was cell density-dependent, the addition of fresh drug did not alter the shape of the toxicity profile from that generated by a single addition of adriamycin.





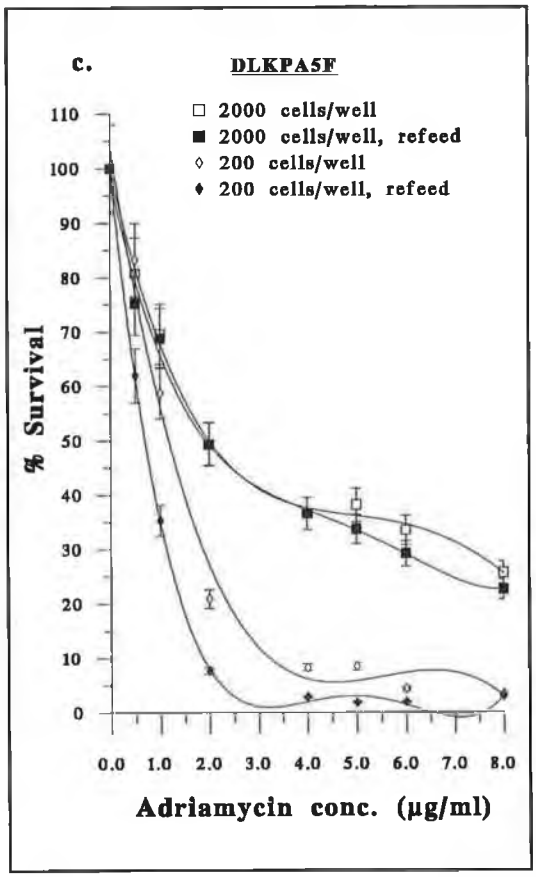
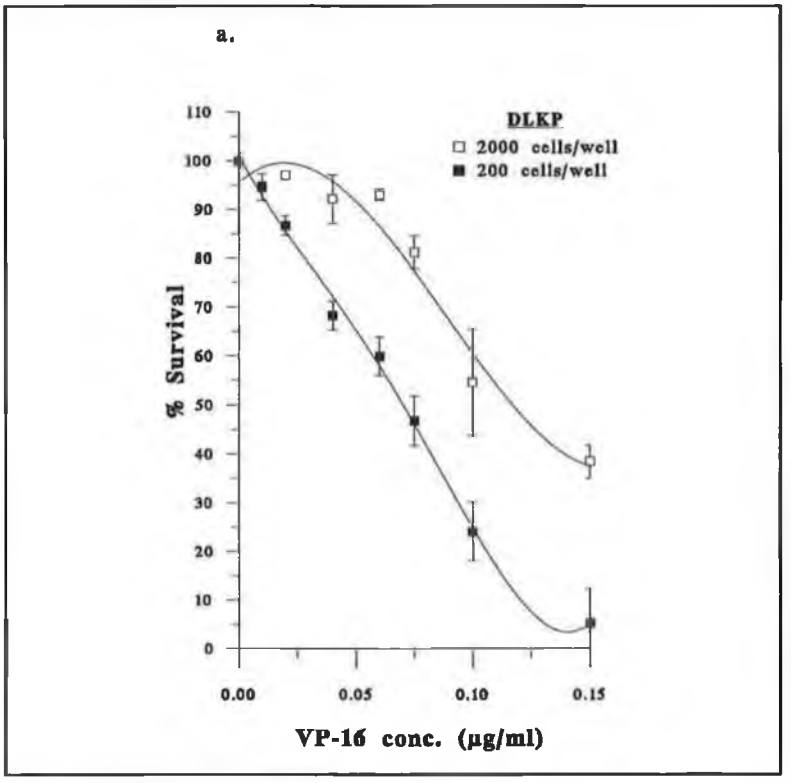


Figure 3.13.2 : The effect of the readdition, every 48h, of fresh adriamycin to a toxicity assay of DLKP (a), DLKP-A (b) and DLKPA5F (c), set up at plating densities of 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.

3.13.3 Effect of cell density on VP-16 toxicity to DLKP and DLKP-A

The cell lines, DLKP and DLKP-A were analyzed to determine if the cell plating density affected the cells' sensitivity to VP-16. As found for adriamycin, VP-16 was more toxic to both DLKP and DLKP-A at low cell plating density, than at high cell plating density (Figure 3.13.3(a)). An IC_{50} of approximately 0.075μg/ml VP-16 was noted when DLKP cells were plated at 200 cells/well, whereas the IC_{50} value was approximately 0.15μg/ml when the cells were plated at 2000 cells/well. The sensitivity of DLKP-A cells to VP-16 was also dependent on the plating density used in the assay, and to approximately the same extent as observed in DLKP (Figure 3.13.3(b)). An IC_{50} value of approximately 1.5μg/ml VP-16 was observed at a plating density of 200 cells/well and this was increased to 3.0μg/ml when the cell plating density was increased to 2000 cells/well. The shape of the toxicity curves generated by exposure of either DLKP or DLKP-A to increasing concentrations of VP-16 at high and low plating density were similar.



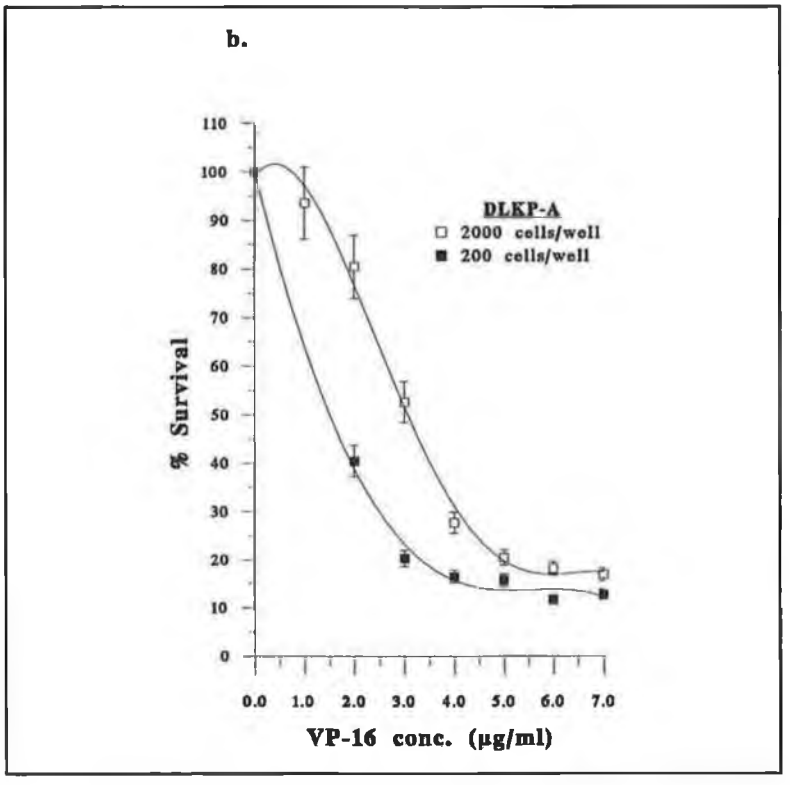


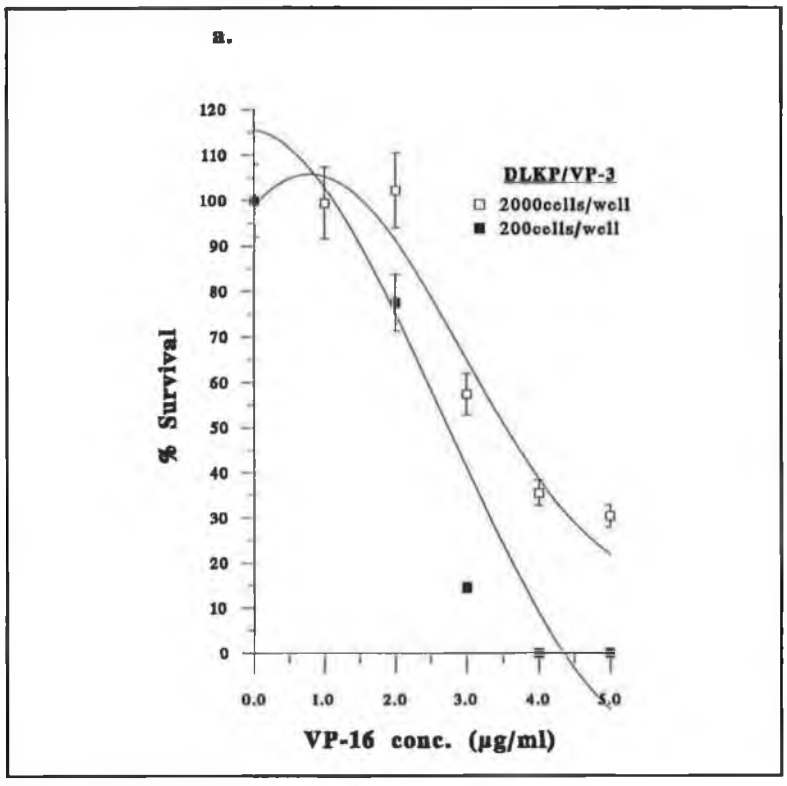
Figure 3.13.3 : VP-16 toxicity to DLKP (a) and DLKP-A (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.

3.13.4 Effect of cell density on VP-16 toxicity to VP-16-selected MDR cell lines

The VP-16-selected MDR cell lines, DLKP/VP-3 and DLKP/VP-8, were analyzed to determine if the cell density effects noted in DLKP and DLKP-A also occurred in the VP-16-selected cell lines. Cell toxicity profiles to VP-16, generated at low plating densities, in DLKP/VP-3, resulted in only slight increases in toxicity in comparison to that observed at high plating density (Figure 3.13.4(a)). The increase in the VP-16 sensitivity of DLKP/VP-3 cells resulted in a decrease in the IC_{50} from approximately 3.5μg/ml to 2.75μg/ml when the cells were plated at densities of 2000 cells/well and 200 cells/well, respectively. No significant alteration in VP-16 toxicity was observed in DLKP/VP-8 (Figure 3.13.4(b)).

3.13.5 Effect of cell density on adriamycin toxicity in VP-16 selected MDR cells

DLKP/VP-3 and DLKP/VP-8 were analyzed to determine if cell plating densities affected their sensitivity to adriamycin. Both cell lines' sensitivities to adriamycin were found to be cell density-dependent (Figure 3.13.5). In both cell lines, the IC_{50} values were decreased from approximately 3μg/ml adriamycin to 1μg/ml adriamycin when toxicity was analyzed at plating densities of 2000 cells/well and 200 cells/well, respectively. Adriamycin toxicity profiles generated by DLKP/VP-3 appeared to be more cell density-dependent than those generated by DLKP/VP-8. The shape of the DLKP/VP-8 adriamycin toxicity profile was similar at high and low plating densities. When DLKP/VP-8 were set up at 2000 cells/well, less than 10% kill occurred up to 1μg/ml adriamycin, with a rapid increase in cell kill noted at drug concentrations above this level. When the same cell line was exposed to adriamycin at 200 cells/well, high cell kill was observed even at very low drug concentrations. In relation to DLKP/VP-3, at high cell plating density, little cell kill occurred at adriamycin concentrations below 2.25μg/ml, after which an increase in drug caused a large increase in cell kill. The initial rate of cell kill noted for DLKP/VP-3 and DLKP/VP-8 were similar, at low plating densities, but the percentage cell survival fell to lower levels at a relatively low drug concentration in DLKP/VP-3 in comparison to DLKP/VP-8.



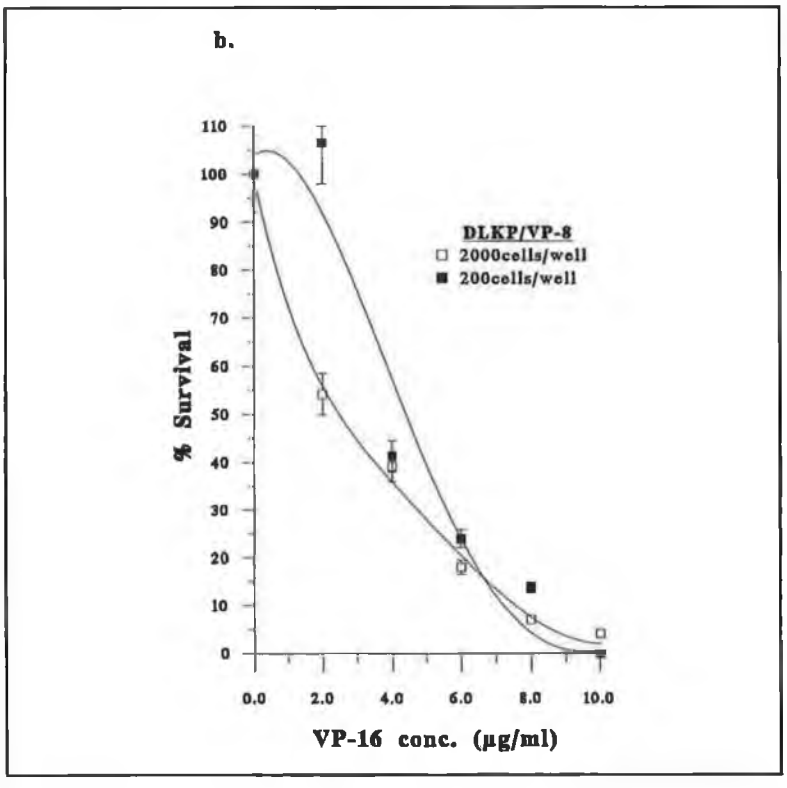
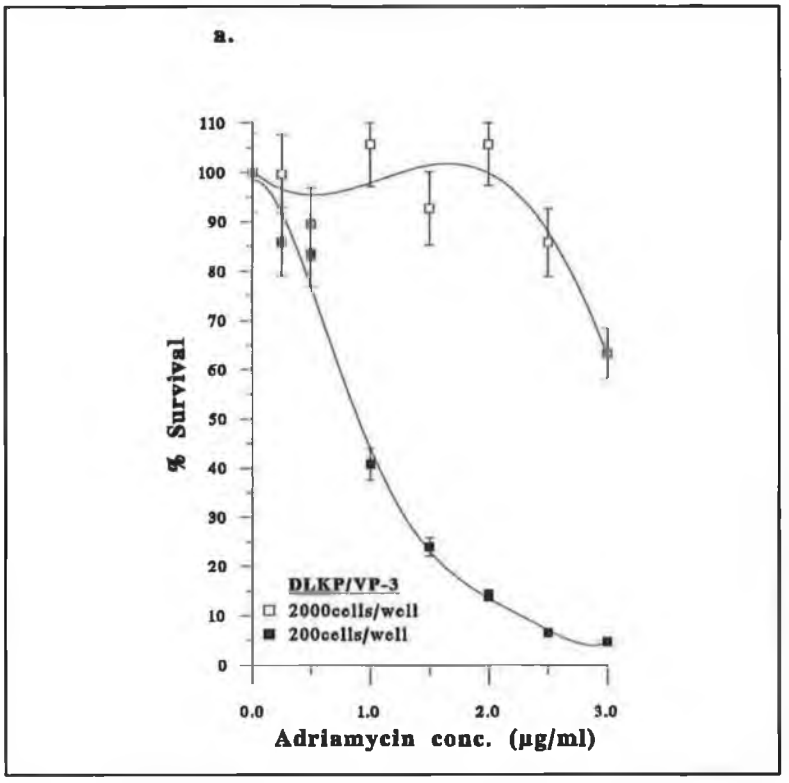


Figure 3.13.4 : VP-16 toxicity to DLKP/VP-3 (a) and DLKP/VP-8 (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.



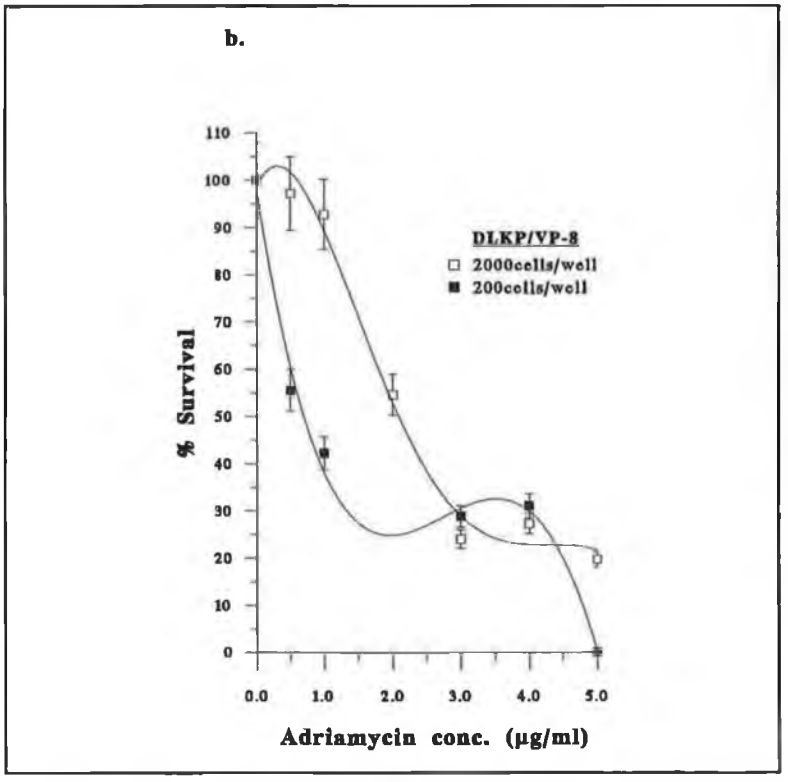


Figure 3.13.5 : Adriamycin toxicity to DLKP/VP-3 (a) and DLKP/VP-8 (b), when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.

3.13.6 Conclusions

The sensitivity of DLKP and its drug-resistant variants to chemotherapeutic agents was cell density-dependent. The toxicity of adriamycin to adriamycin-selected MDR cell lines, was dependent on the cells' density. Adriamycin was more toxic to cells which were plated at low density, by comparison with cells plated at high density. Adriamycin toxicity to DLKP cells was independent of the plating density of the cells. Cell density-dependent toxicity of adriamycin was also observed in the VP-16-selected cell lines. The differential adriamycin toxicity was not due to cell cycle effects. Changes in cell density caused only slight alterations to the toxicity of VP-16 to VP-16-selected, MDR variants of DLKP. However, VP-16 induced toxicity in DLKP and DLKP-A was cell density-dependent. The increased toxicity observed in DLKP-A at low cell plating density, by comparison with the level of VP-16 induced cell kill noted at high plating density, was less than that seen in the comparable study on cell density-dependent toxicity of adriamycin. Conversely, in the DLKP cells, the toxicity of VP-16 was found to be more strongly associated with the cell plating density, than the association observed for adriamycin toxicity.

3.14 Gap Junctional Communication

To determine if metabolic co-operation was occurring between cells, in variants of the cell line DLKP, the existence of functional gap junctions within the population was examined by means of lucifer yellow transfer. The literature has cited many examples of altered gap junctional intercellular communication in cancer cells. Therefore the existence of active gap junctions was investigated in a range of other cell lines established from solid tumours. Intercellular adriamycin transfer was examined in DLKP variants to determine if adriamycin was transported within the cell lines *via* active gap junctions.

3.14.1 Microscopic analysis of functional gap junctional communication

When lucifer yellow, in a calcium-free solution, was added to DLKP cells, for 2 minutes at 37°C, the dye was observed to be transported from scrape-loaded cells, in a confluent monolayer, to surrounding cells (Figure 3.14.1.1). This is a classical test for functional gap junctional communications in cells (El-Fouly *et al.*, 1987) and the ability of the fluorescent dye to be transferred from one cell to surrounding cells in which it is in physical contact, suggested the presence of active gap junctions in these cells. The adriamycin-selected MDR variant of DLKP, DLKP-A, also exhibited the ability to transfer lucifer yellow from scrape-loaded cells, in a calcium-free solution, to surrounding cells. This implied that they also possess active gap junctions (Figure 3.14.1.2). Two of the MDR clones isolated from DLKP-A, DLKPA5F and DLKPA2B and the cell line generated by mixing clones DLKPA5F, DLKPA2B, DLKPA11B and DLKPA6B in the ratio 2:1:1:1, respectively (Section 3.13), was also observed to possess functional gap junctional intercellular communications (Figure 3.14.1.3 - 3.14.1.5).

The literature has cited many cases of a decrease or absence of functional gap junctions in cancer cells (Klann *et al.*, 1989; Borek *et al.*, 1969). However, functional gap junctions were observed in all variants of DLKP *i.e.* the parental cell line, the adriamycin-selected MDR variant, the clones of the MDR variant and the cell line generated by mixing the clones. To determine if functional gap junctions existed in other lung cell lines, cell lines established from other tumours and in other MDR cell lines established by adriamycin-selection of the parental cells, a range of cell lines were examined to determine if they also possessed the ability to intercellularly transfer lucifer yellow.

No detectable lucifer yellow intercellular transfer was noted in the human lung squamous cell carcinoma, SKMES1. Fluorescence was confined to cells damaged by the scrape

(3.14.1.6). The human colon (HT29), bladder (T24) and ovarian (OAW42) cell lines also exhibited no transfer of the fluorescent dye from damaged to intact cells, with which they were in physical contact (Figures 3.14.1.6 and 3.14.1.7). Therefore, no functional gap junctions were detectable in these cells. When the human epidermoid carcinoma of the larynx, Hep-2, and its adriamycin-selected MDR variant, Hep-2A (Redmond *et al.*, 1990) were examined to determine if they contained active gap junctional intercellular communications, no lucifer yellow transfer, from the scrape-loaded cells, was observed (Figure 3.14.1.7).

The ability of variants of DLKP to transfer adriamycin from scrape-loaded cells to surrounding cells, with which they were in physical contact, was also examined. The adriamycin was loaded into the cells by scraping a confluent monolayer of cells, in the presence of 100μM adriamycin in a calcium-free solution and incubating the cells for 10 minutes at 37°C, to allow transfer of the drug *via* active gap junctions. Gap junction transfer was than stopped by washing the cells in a calcium-containing solution. When the cells were examined by fluorescent microscopy, only cells containing adriamycin fluoresced.

DLKP cells bordering the scrape were observed to fluoresce strongly, with weaker background fluorescence also noted, implying that, unlike lucifer yellow, adriamycin readily diffused into the cells (Figure 3.14.1.1). Physically damaged cells fluoresced more intensely than the background intact cells, but this stronger fluorescence was confined to cells bordering the scrape and was not transferred to neighbouring cells. This implied that the adriamycin was not being transferred intercellularly, *via* active gap junctions, that have been shown to exist by lucifer yellow intercellular transfer, even though the molecular size of adriamycin would, theoretically, allow gap junctional transfer. As was observed for DLKP, the MDR cell line DLKP-A, its clones DLKPA5F and DLKPA2B and the cell line consisting of mixed DLKP-A clones, did not intercellularly transfer adriamycin *via* active gap junction communication (Figure 3.14.1.2 - 3.14.1.5). The intensity of the background fluorescence, due to adriamycin diffusing into, and remaining in the cells, was lower in all the MDR cell lines examined. Again, only the cells bordering the scrape fluoresced intensely.

As previously shown, all MDR variants of DLKP exhibit the characteristic of over-expressing P-glycoprotein (Section 3.9) which functions by pumping a broad range of chemotherapeutic drugs, including adriamycin, out of cells and therefore renders them more resistant to the drug. To ensure that the effect of drug efflux was not masking the gap junctional transfer of adriamycin in the MDR cell lines, the cellular localisation of the adriamycin, in the presence of 30μg/ml cyclosporin A, was examined. No intercellular transfer of adriamycin was observed following scrape loading of cells in the concurrent presence of both adriamycin and cyclosporin A. A similar fluorescence pattern, to that observed in the presence of 100μM adriamycin alone, was noted (Figure 3.13.1.1 - 3.14.1.5).

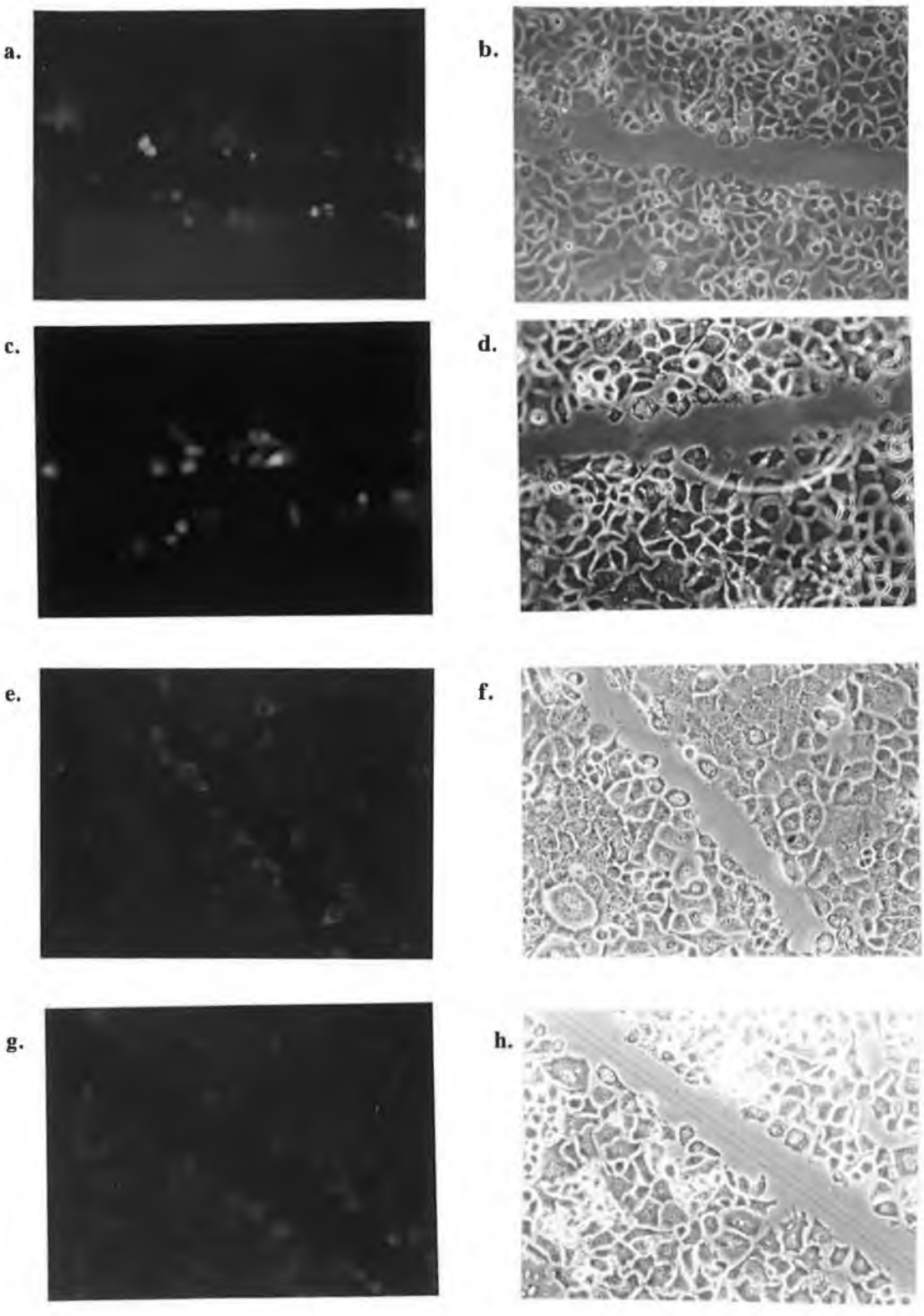


Figure 3.14.1.1 : Gap junction intercellular communication in DLKP, as determined by the transfer of scrape-loaded lucifer yellow in confluent monolayers (a). The intercellular transfer of lucifer yellow was blocked by the presence of the gap junction inhibitor, heptanol, at a concentration of 1.5mM (c). Intercellular transfer of adriamycin was not observed (e) when the cells were scrape-loaded in the presence of 100μM adriamycin. When the cells were co-incubated with adriamycin and 30μg/ml cyclosporin A, a drug efflux circumventing agent, gap junctional mediated intercellular transfer of the drug was not observed (g). Figures b, d, f and h are the light microscope views of Figure a, c, e and g, respectively.

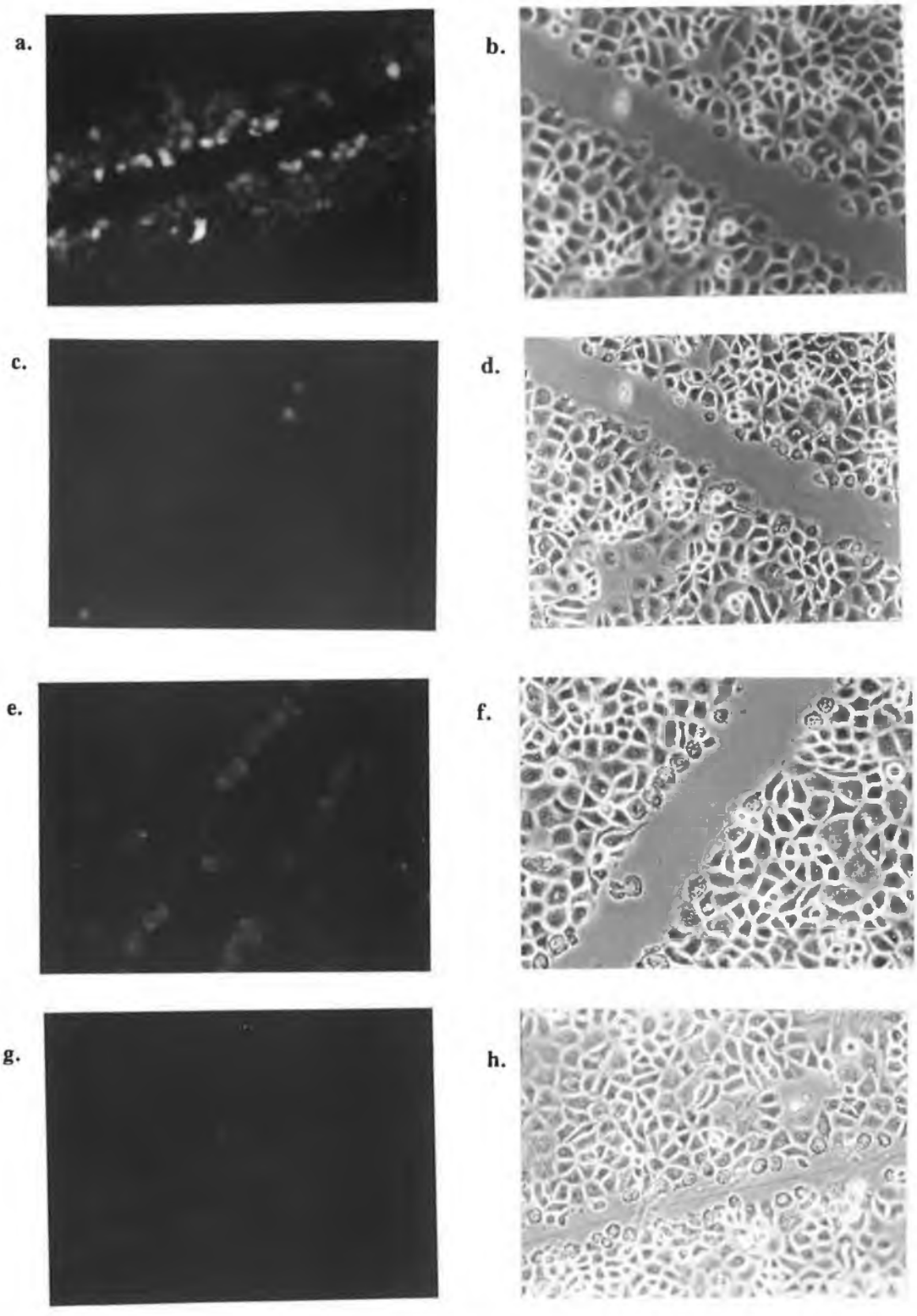


Figure 3.14.1.2 : Gap junction intercellular communication in DLKP-A, as determined by the transfer of scrape-loaded lucifer yellow in confluent monolayers (a). The intercellular transfer of lucifer yellow was blocked by the presence of the gap junction inhibitor, heptanol, at a concentration of 1.5mM (c). Intercellular transfer of adriamycin was not observed (e) when the cells were scrape-loaded in the presence of 100μM adriamycin. When the cells were co-incubated with adriamycin and 30μg/ml cyclosporin A, a drug efflux circumventing agent, gap junctional mediated intercellular transfer of the drug was not observed (g). Figures b, d, f and h are the light microscope views of Figure a, c, e and g, respectively.

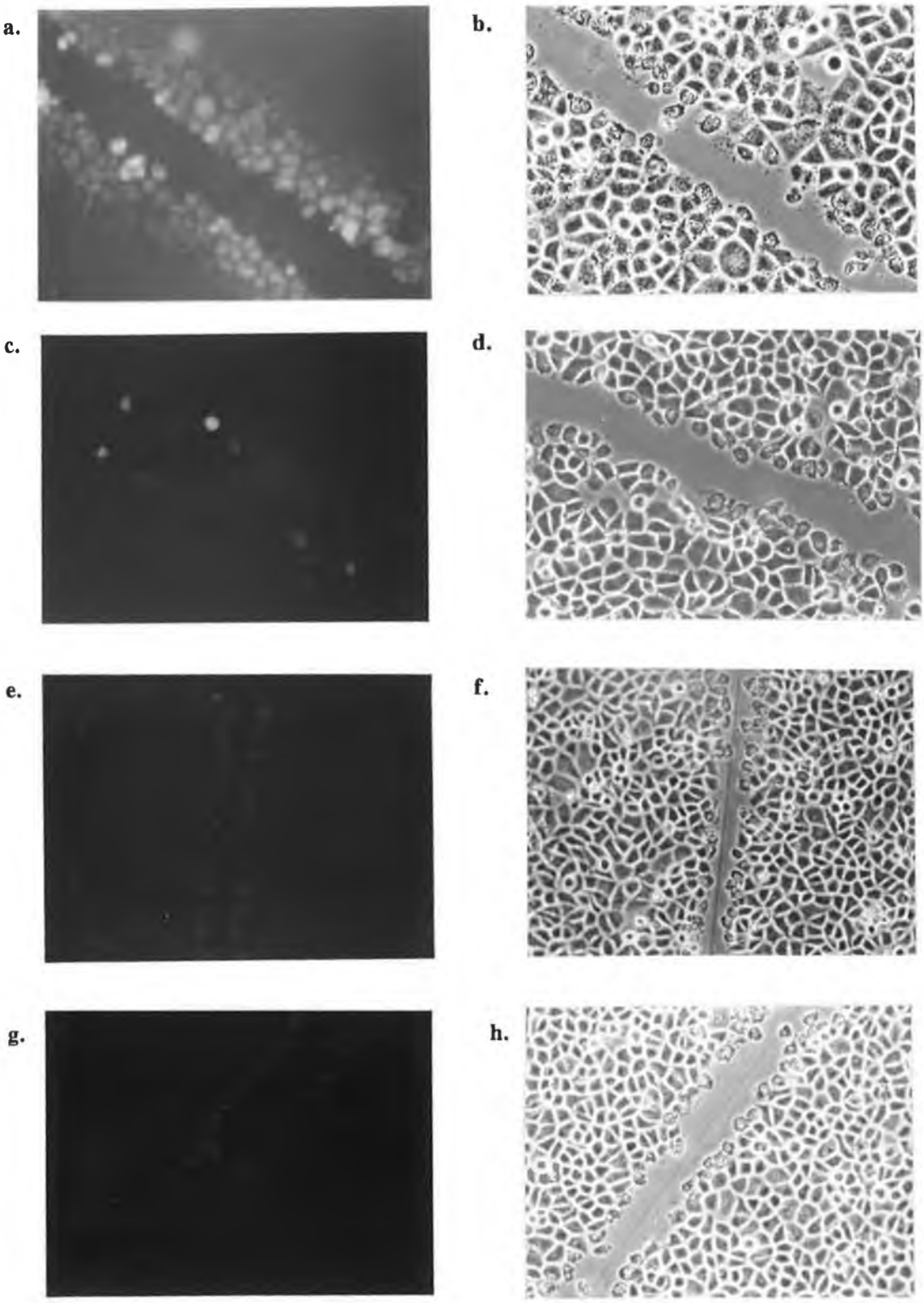


Figure 3.14.1.3 : Gap junction intercellular communication in DLKPA2B, as determined by the transfer of scrape-loaded lucifer yellow in confluent monolayers (a). The intercellular transfer of lucifer yellow was blocked by the presence of the gap junction inhibitor, heptanol, at a concentration of 1.5mM (c). Intercellular transfer of adriamycin was not observed (e) when the cells were scrape-loaded in the presence of 100μM adriamycin. When the cells were co-incubated with adriamycin and 30μg/ml cyclosporin A, a drug efflux circumventing agent (g), gap junctional mediated intercellular transfer of the drug was not observed. Figures b, d f and h are the light microscope views of figure a, c e and g, respectively.

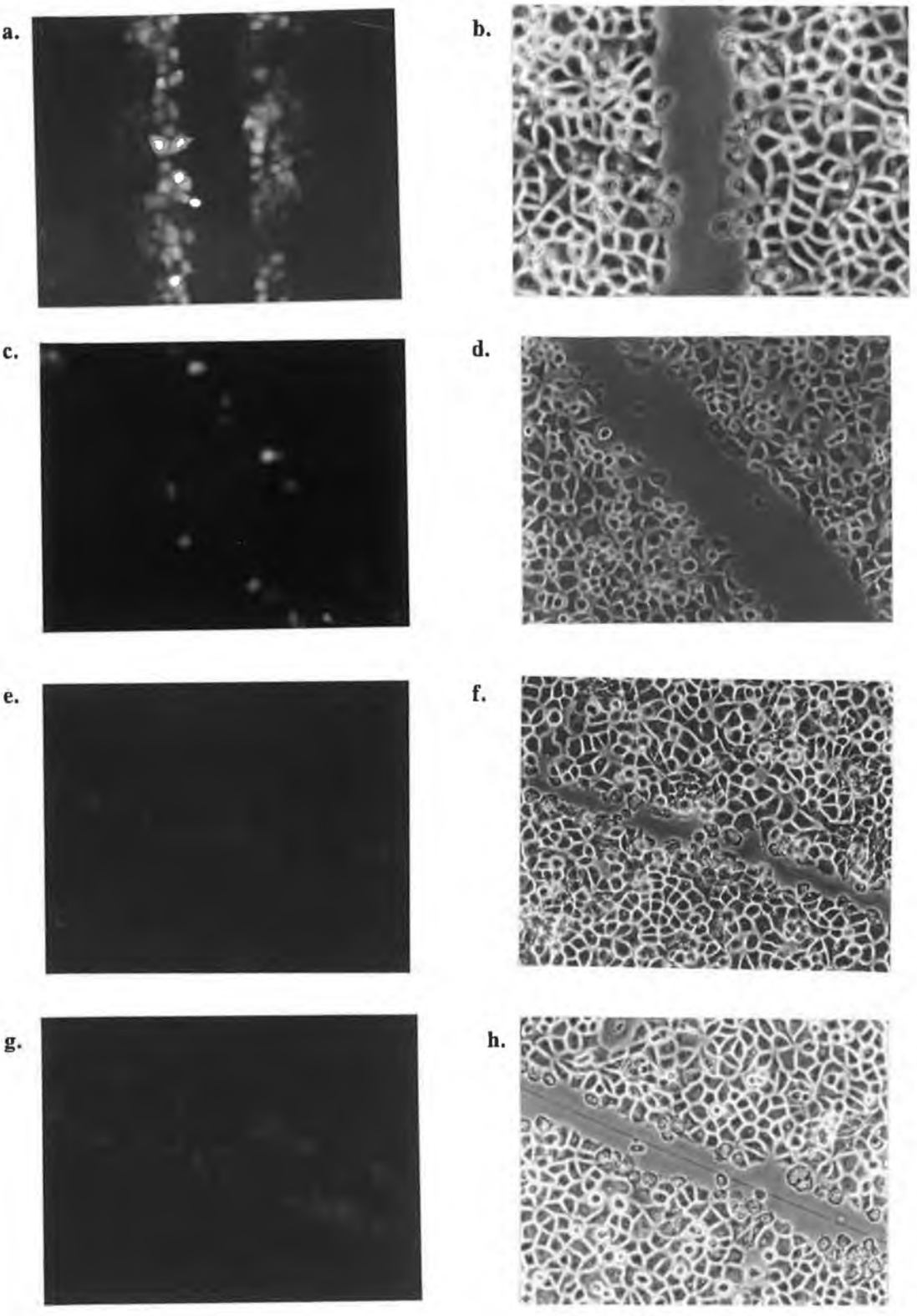


Figure 3.14.1.4 : Gap junction intercellular communication in DLKPA5F, as determined by the transfer of scrape-loaded lucifer yellow in confluent monolayers (a). The intercellular transfer of lucifer yellow was blocked by the presence of the gap junction inhibitor, heptanol, at a concentration of 1.5mM (c). Intercellular transfer of adriamycin was not observed (e) when the cells were scrape-loaded in the presence of 100μM adriamycin. When the cells were co-incubated with adriamycin and 30μg/ml cyclosporin A, a drug efflux circumventing agent, gap junctional mediated intercellular transfer of the drug was not observed (g). Figures b, d, f and h are the light microscope views of Figure a, c, e and g, respectively.

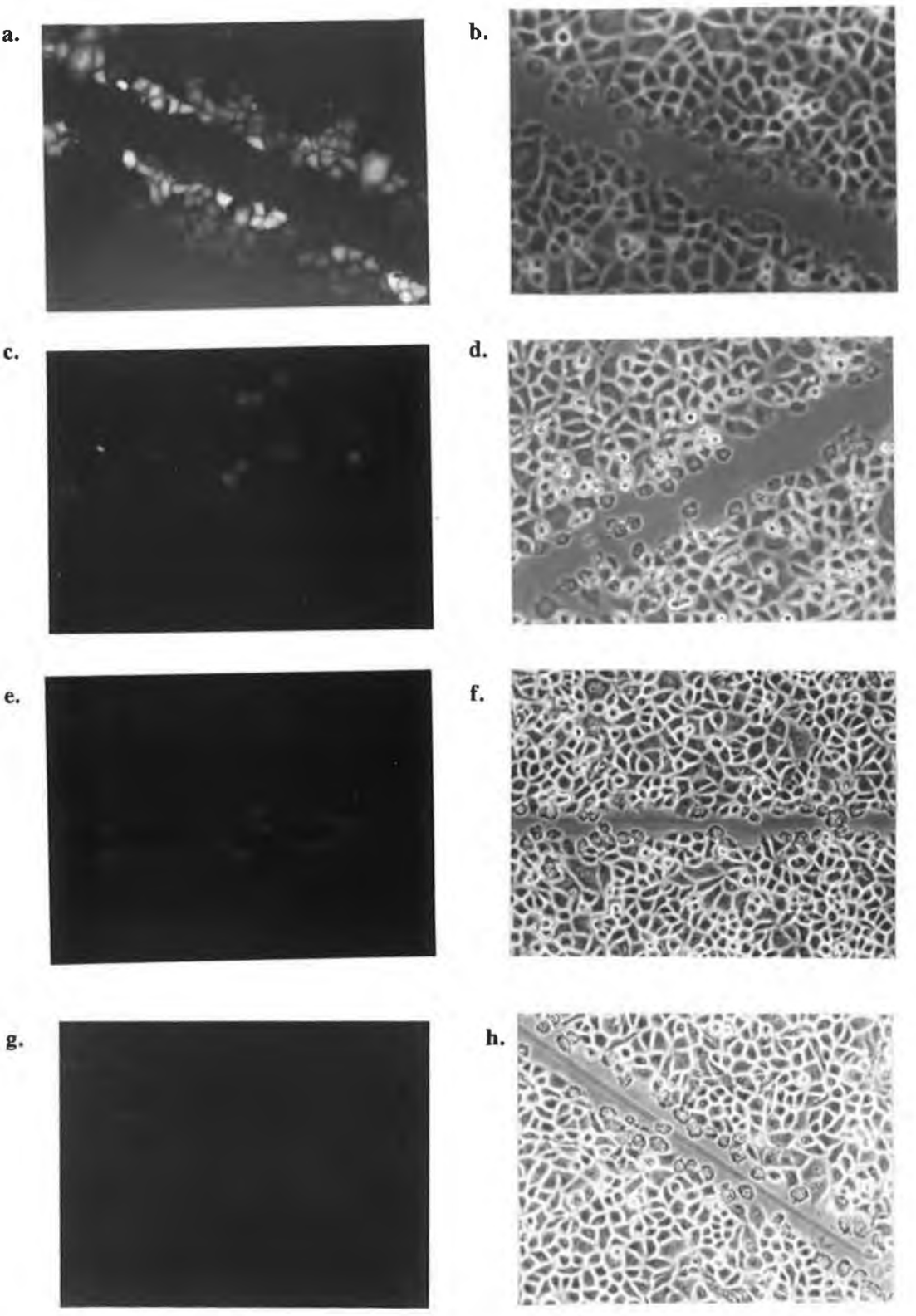


Figure 3.14.1.5 : Gap junction intercellular communication in the cell line generated by mixing the DLKP-A clones, as determined by the transfer of scrape-loaded lucifer yellow in confluent monolayers (a). The intercellular transfer of lucifer yellow was blocked by the presence of the gap junction inhibitor, heptanol, at a concentration of 1.5mM (c). Intercellular transfer of adriamycin was not observed (e) when the cells were scrape-loaded in the presence of 100μM adriamycin. When the cells were co-incubated with adriamycin and 30μg/ml cyclosporin A, a drug efflux circumventing agent, gap junctional mediated intercellular transfer of the drug was not observed (g). Figures b, d, f and h are the light microscope views of figure a, c, e and g, respectively.

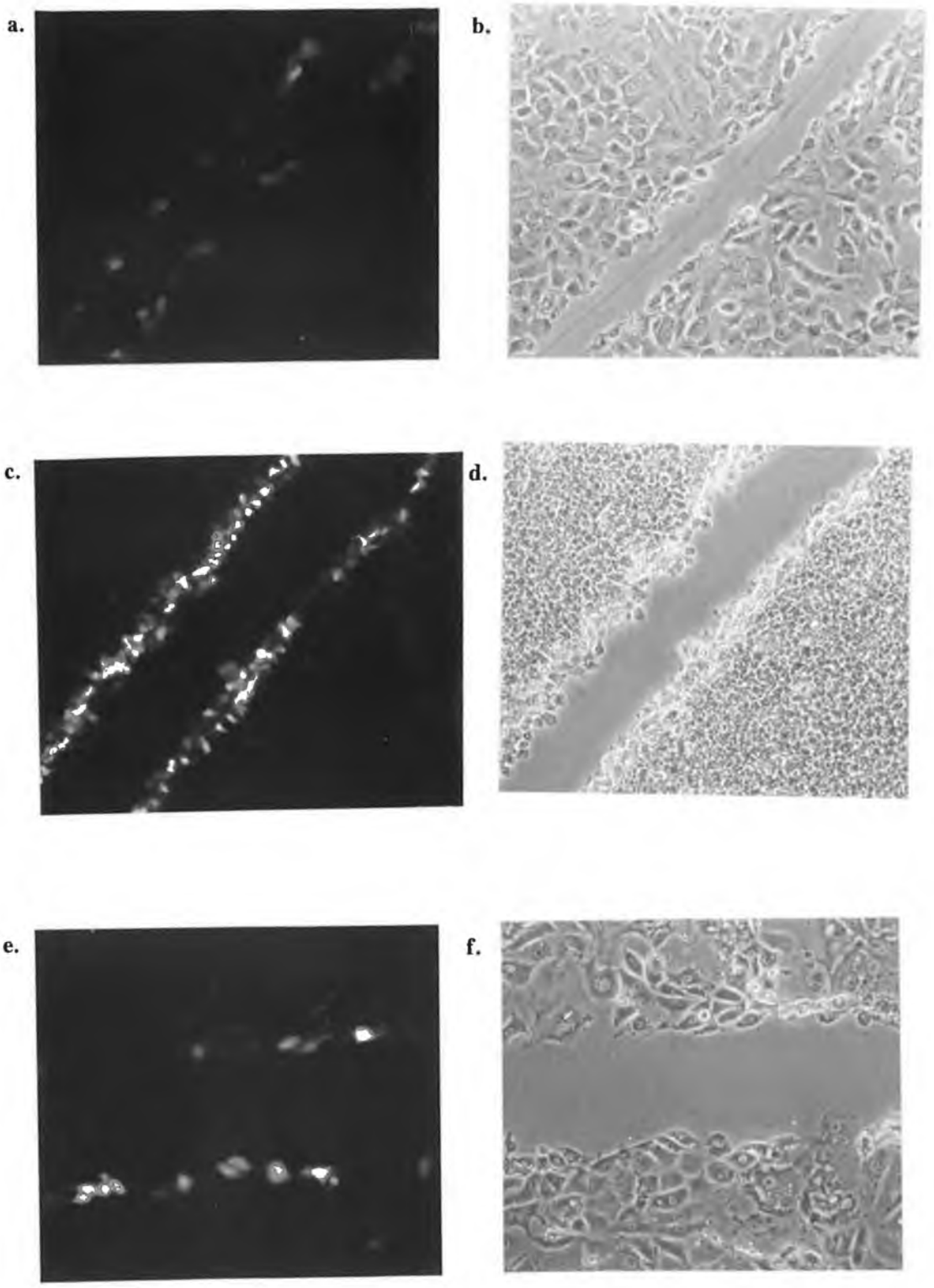


Figure 3.14.1.6 : Gap junction intercellular communication in cell lines, as determined by the transfer of scrape-loaded lucifer yellow, in confluent monolayer cultures. The intercellular transfer of lucifer yellow in the human squamous lung carcinoma, SKMES1 (a), the human colon HT29 cell line (c) and the human bladder cell line, T24 (e), was examined. Figures b, d, and f are the light microscopic view of Figures a, c and e, respectively.

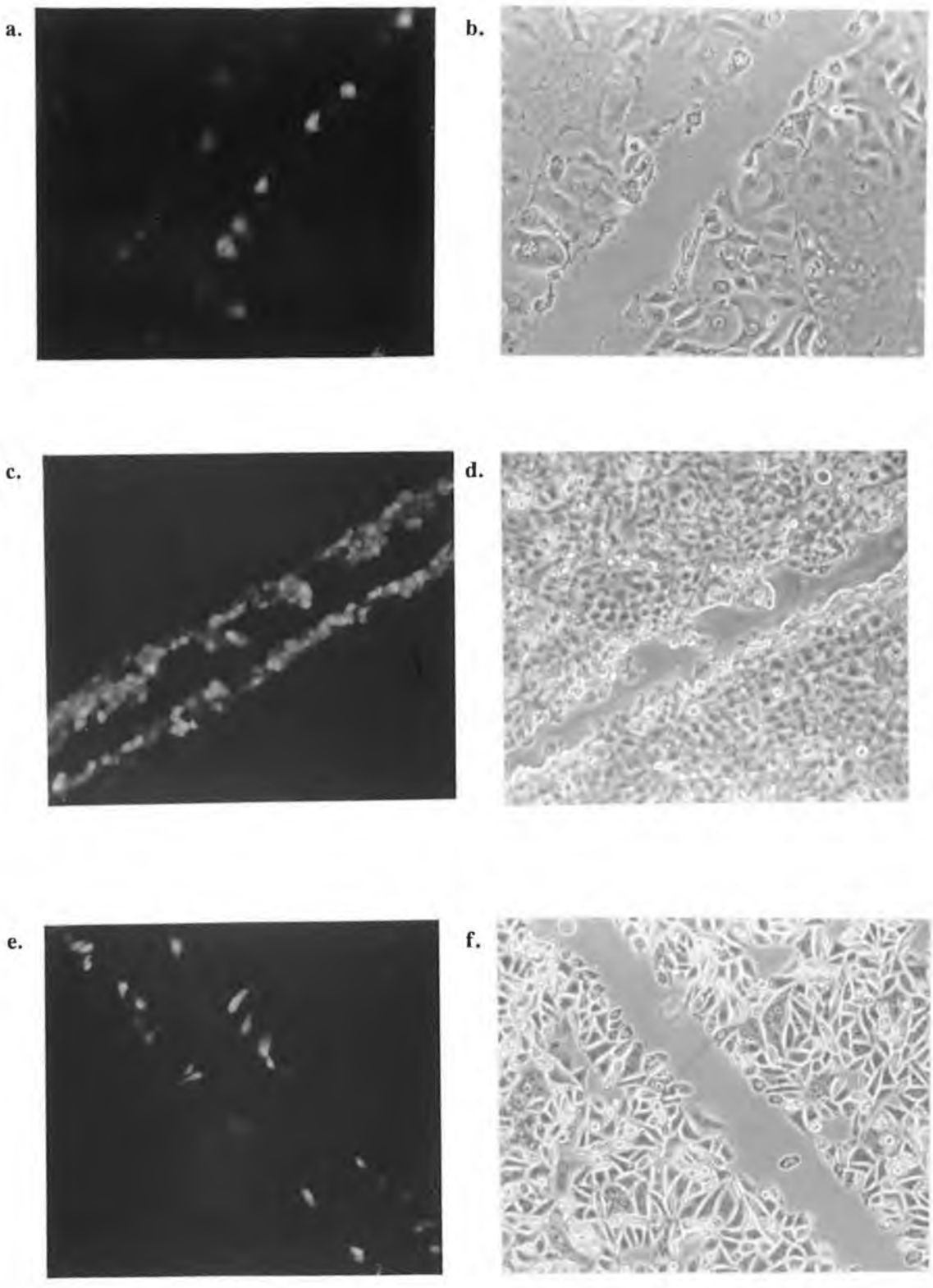


Figure 3.14.1.7 : Gap junction intercellular communication in cell lines, as determined by the transfer of scrape-loaded lucifer yellow, in confluent monolayer cultures. The intercellular transfer of lucifer yellow in the human ovarian carcinoma, OAW42 (a), the human epidermoid carcinoma of the larynx, Hep-2 (c) and its adriamycin-selected MDR variant, Hep-2A (e), was examined. Figures b, d, and f are the light microscopic view of Figures a, c and e, respectively.

3.14.2 Inhibition of gap junctional communication

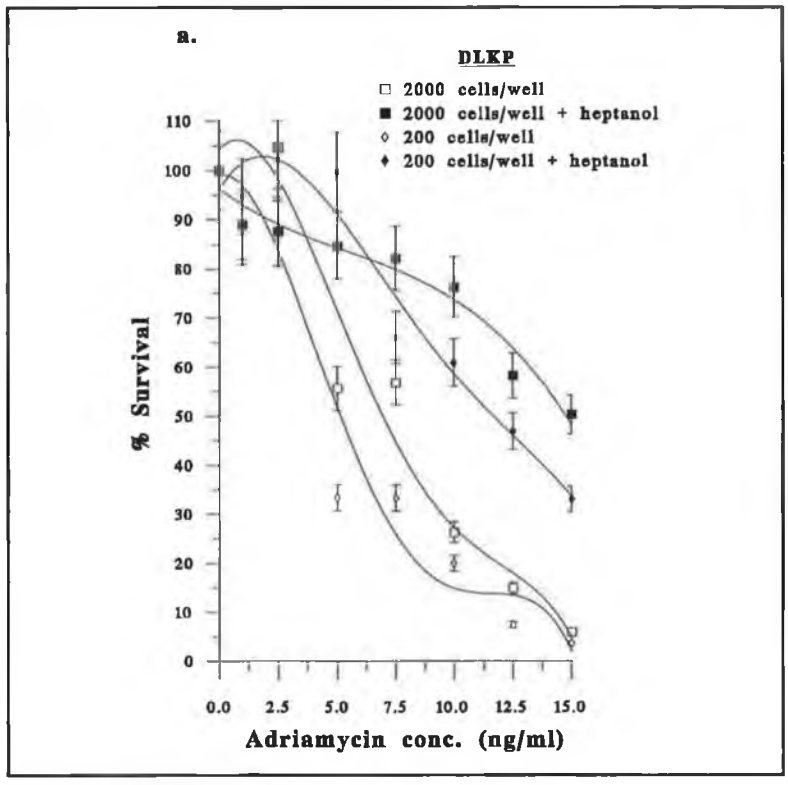
Gap junctional communication was blocked in all DLKP variants by pre-incubation of the cell with 1.5mM heptanol for 1.5h. and the continual presence of 1.5mM heptanol during scrape loading of the fluorescent marker (Figure 3.14.1.1 - 3.14.1.5). When lucifer yellow was loaded into the cells in the presence of the gap junctional communication inhibitor, fluorescence was only observed in cells bordering the scrape and so, the lucifer yellow was not transported *via* active gap junctions to surrounding cells, as had been observed previously. The presence of 1.5mM heptanol, under similar conditions, did not alter the cellular fluorescent staining patterns generated by adriamycin, from that originally observed for cells scrape loaded with adriamycin alone. The adriamycin was confined to damaged cells and was not transferred to intact cells (photographs not shown).

3.14.3 Effect of gap junction inhibition on adriamycin toxicity

The effect of blocking intercellular communication, *via* gap junctions, on the toxicity of adriamycin to a range of DLKP variants was examined, by performing an adriamycin toxicity assay, at high and low plating density, in the presence of 1.5mM heptanol. The assay was carried out on the DLKP cell line, its adriamycin-selected MDR variant DLKP-A, the DLKP-A clones DLKPA2B and DLKPA5F and on the cell line generated by mixing the clones. The assay was carried out in duplicate with triplicate repeats performed in each duplicate. The graphs shown represent the data obtained from one of the repeats.

The concentration of heptanol which resulted in complete inhibition of gap junctional communication (1.5mM) also resulted in 50% cell kill under toxicity assay conditions. Therefore the cells were set up at twice the cell density required, if heptanol was to be added, as outlined in Section 2.11.6. The inhibition of gap junctional communication in cell lines did not, in general, enhance their sensitivity to adriamycin. The presence of heptanol resulted in a slight increase in cell survival in the presence of adriamycin, but alone did not appear to stimulate cell growth. In both the parental and MDR cell line DLKP-A, heptanol appeared to protect the cells from the toxic effect of adriamycin, at both high and low densities (Figure 3.14.3.1). In the cell clone DLKPA5F, heptanol did not significantly alter the adriamycin toxicity profile generated by the cells at both high and low plating density (Figure 3.14.3.2(b)). With respect to DLKPA2B, the least resistant clone, heptanol enhanced the toxicity of adriamycin at high cell density and reduced its toxicity at clonal density (Figure

3.14.3.2(a)). When the clones were mixed to regenerate a DLKP-A-like cell line and the cells exposed to adriamycin in the presence of heptanol, the toxicity profile was not significantly different from that observed when the cells were exposed to adriamycin alone, at the respective cell plating density (Figure 3.14.3.2(c)).



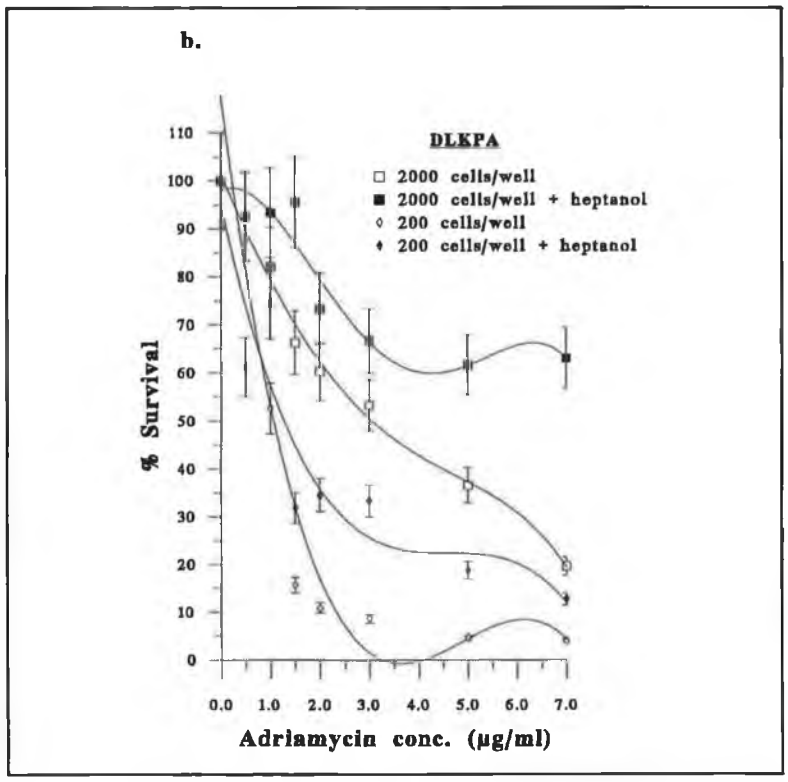
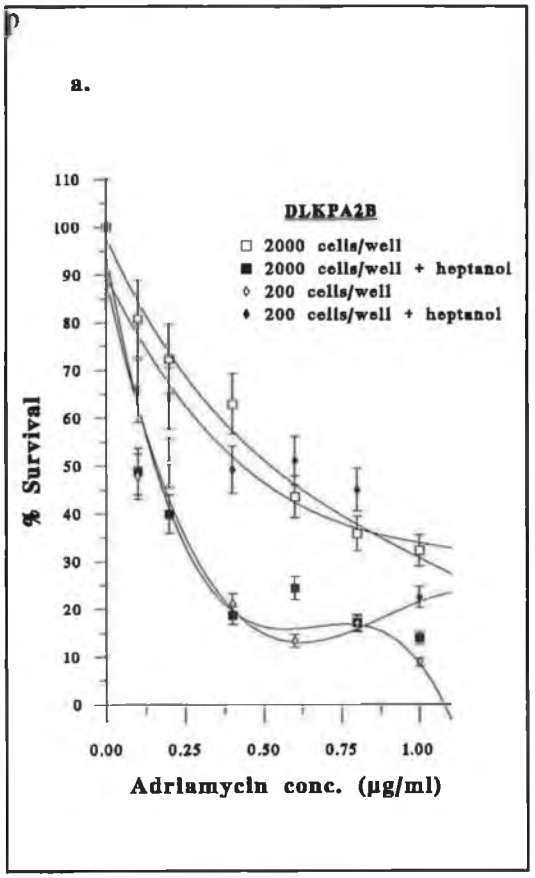
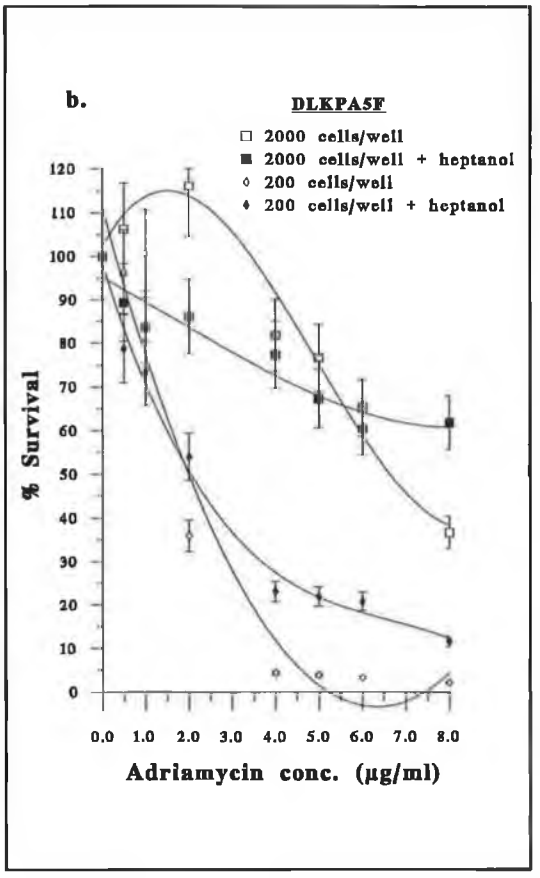


Figure 3.14.3.1 : The effect of pre-treatment with and continuous exposure to 1.5mM heptanol on adriamycin toxicity to DLKP (a) and DLKP-A (b) when cells are plated at 2000 cells/well and 200 cells/well. The error bars represent the standard deviation on the mean of three replicas.





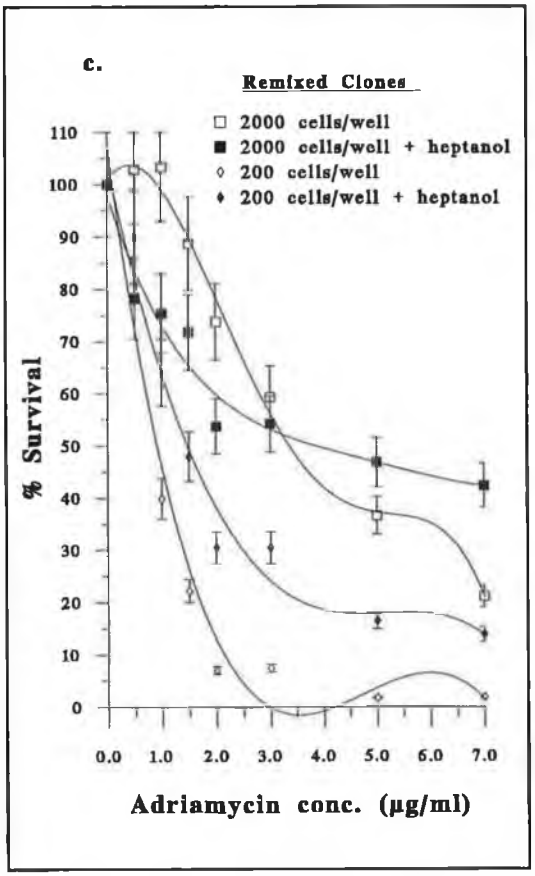


Figure 3.14.3.2 : The effect of 1.5mM heptanol on adriamycin toxicity to DLKPA2B (a), DLKPA5F (b) and the mixed clones, generating a DLKP-A-like population (c), at a plating density of 2000 cells/well. The error bars represent the standard deviation on the mean of three replicas.

3.15 Effect of conditioned media on adriamycin toxicity to DLKPA2B

A preliminary experiment was carried out to investigate whether the protective effect observed at high cell plating densities, with respect to adriamycin toxicity, was due to a physical or non-physical method of protection. The effect of conditioned media from DLKPA5F and DLKPA2B, generated both in the presence and absence of 2.5μg/ml adriamycin for 24h. (Table 3.15), on adriamycin toxicity to DLKPA2B at high and low cell density was investigated. The assay was performed once, with triplicate intra-assay repeats.

Description of conditioned medium, as pertaining to Figures 3.15.1 and 3.15.2	**Preparation of the Conditioned Medium.**
Fresh medium and adriamycin	Adriamycin was added to fresh ATCC medium, to a final concentration of 2.5μg/ml. Serial dilutions of the drug-containing medium were made, using fresh ATCC medium.
Cond. medium from DLKPA2B exposed to 2.5μg/ml adr.	ATCC medium, containing 2.5μg/ml adriamycin, was exposed to DLKPA2B for 24h. at 37°C. The conditioned medium was collected, filter-sterilised using a low protein-binding filter, and the adriamycin content of the medium determined by fluorometric analysis. Fresh adriamycin was then added to the medium to bring it to a final concentration of 2.5μg/ml and this drug-containing conditioned medium diluted with drug-free conditioned medium.
Cond. medium from DLKPA5F exposed to 2.5μg/ml adr.	ATCC medium, containing 2.5μg/ml adriamycin, was exposed to DLKPA5F for 24h. at 37°C. The conditioned medium was collected, filter-sterilised using a low protein-binding filter, and the adriamycin content of the medium determined by fluorometric analysis. Fresh adriamycin was then added to the medium to bring it to a final concentration of 2.5μg/ml and this drug-containing conditioned medium diluted with drug-free conditioned medium.
Cond. medium from DLKPA2B with fresh adriamycin	Fresh ATCC medium was exposed to DLKPA2B cells for 24h. at 37°C. The conditioned medium was collected and filter-sterilised through a low protein-binding filter. Adriamycin was added to an aliquot of the conditioned medium, to bring it to a final concentration of 2.5μg/ml. Serial dilutions were performed on the drug-containing conditioned medium, using the drug-free conditioned medium.
Cond. medium from DLKPA5F with fresh adriamycin	Fresh ATCC medium was exposed to DLKPA5F cells for 24h. at 37°C. The conditioned medium was collected and filter-sterilised through a low protein-binding filter. Adriamycin was added to an aliquot of the conditioned medium, to bring to a final concentration of 2.5μg/ml. Serial dilutions were performed on the drug-containing conditioned medium, using the drug-free conditioned medium.

Table 3.15 : Protocol followed in generating adriamycin-free and adriamycin-containing conditioned medium and in determining the level of adriamycin present in the medium.

As previously observed (Section 3.13.1), adriamycin toxicity to DLKPA2B was cell density-dependent. At high plating density the IC_{50} value obtained for adriamycin toxicity was approximately 0.75μg/ml (Figure 3.15.1) but this was decreased to approximately 0.15μg/ml adriamycin (Figure 3.15.2) when the cells were plated at 200 cells/well. At high plating density, *i.e.* when the cells were plated at 2000 cells/well, conditioned media from both DLKPA2B and DLKPA5F, generated both in the presence and absence of 2.5μg/ml adriamycin, partially protected DLKPA2B cells from the toxic effect of adriamycin (Figure 3.15.1). However, at low plating density, only the conditioned media generated by DLKPA5F in the presence of 2.5μg/ml adriamycin exerted a protective effect on DLKPA2B cells (Figure 3.15.2).

The final amount of adriamycin present in the conditioned media, generated by exposing DLKPA2B and DLKPA5B to 2.5μg/ml adriamycin for 24h., was determined by the level of the media's fluorescence. Any fluorescence was assumed to be active adriamycin. This did not account for any deactivation of adriamycin that may have occurred in the 24h. period, which was not reflected by a decrease in the drug's fluorescence. When DLKPA2B was plated at high density, the level of cell kill caused by the presence of adriamycin, in the conditioned media from both DLKPA5F samples was similar, whether the adriamycin had been exposed to DLKPA5F for 24h. at 37°C and the conditioned medium then supplemented with fresh adriamycin or whether fresh adriamycin was added to drug-free conditioned medium from DLKPA5F, immediately prior to use in the assay. This suggests that equal levels of active adriamycin and/or its active metabolites, were present in both samples. Therefore, the lower level of cell kill observed in DLKPA2B when exposed to conditioned medium from DLKPA5F, which had been exposed to adriamycin for 24h. and then supplemented with fresh adriamycin, was not due to a lesser amount of active drug being present in that sample.

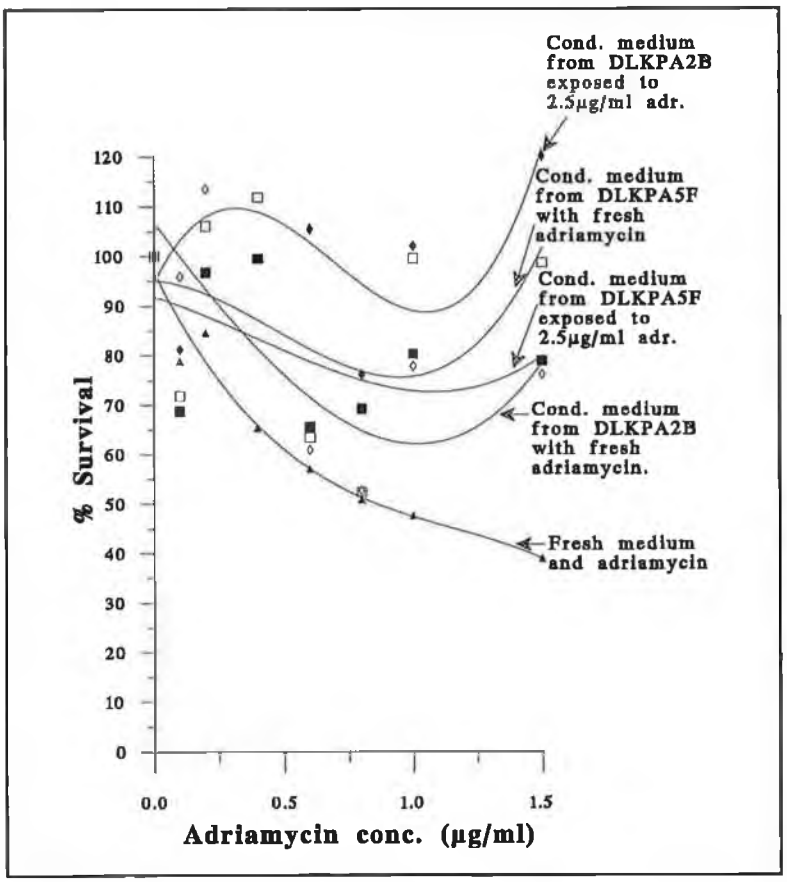


Figure 3.15.1 : Effect of conditioned media from DLKPA2B and DLKPA5F, on adriamycin toxicity to DLKPA2B, at a cell plating density of 2000 cells/well.

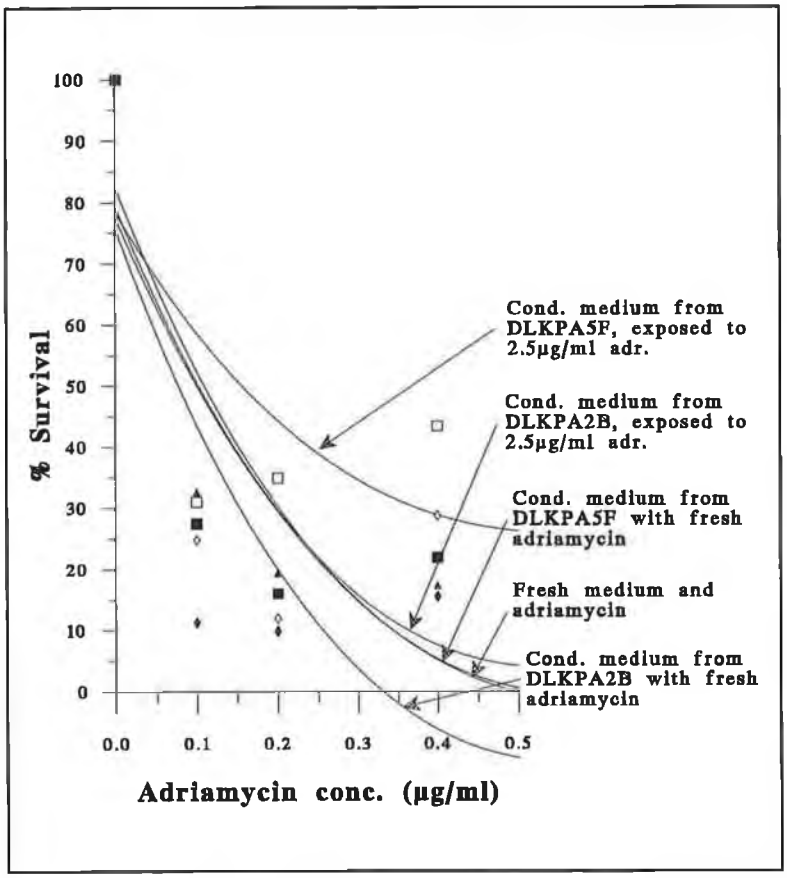


Figure 3.15.2 : Effect of conditioned media from DLKPA2B and DLKPA5F on adriamycin toxicity to DLKPA2B, at a cell plating density of 200 cells/well.

4. Discussion

4.0 Discussion

The existence of the phenomenon of tumour cell heterogeneity has been widely documented in the literature (Owens *et al.*, 1982; Heppner and Miller, 1989) and is responsible for variations in the characteristics of tumour cells to a range of attributes, from growth properties (Fried *et al.*, 1993) to sensitivity to chemotherapeutic drugs (Dolfini *et al.*, 1993; Tsuruo and Fidler, 1981). Clonal variation has also been demonstrated in MDR cell lines (Yang *et al.*, 1993) and to further investigate this phenomenon, clonal cell lines have been isolated from the P-glycoprotein overexpressing, adriamycin-selected MDR variant of DLKP, DLKP-A. VP-16-selected MDR variants of the squamous cell carcinoma of the lung have also been isolated and characterised, and the mechanisms underlying their resistance profile have been explored.

4.1 Confirmation that MDR cell lines are variants of DLKP

The majority of cell lines used in the course of this study are variants of DLKP. Cross contamination of the cells with other cell lines could result in an alteration of the characteristics of the cells under investigation. DNA fingerprint analysis, performed by Cellmark Diagnostics, confirmed that all variants of DLKP used in this study possessed DNA fingerprints homologous to DLKP (Section 3.3), and so were confirmed as having been derived from DLKP cells. The variants of DLKP share 56 identical bands in their fingerprints. DLKP, DLKP-A and DLKPA6B possess one additional band at different positions within their fingerprints. DLKP/VP-3 and DLKPA11B have two additional bands, one of which is shared by both cell lines. Cellmark Diagnostics concluded that all DLKP variants shared a common origin and that additional bands to those shared by all cells within the group may be due to slight clonal variation as a result of random mutational events.

4.2 Resistance Profile of MDR cell lines

Following selection with VP-16, the DLKP variants DLKP/VP-3 and DLKP/VP-8 exhibited stably expressed MDR profiles. The cells were highly cross-resistant to vincristine (1088.98 $\pm$ 161.08 and 1737.42 $\pm$ 347.00-fold resistance, respectively), adriamycin (90.10 $\pm$ 13.24 and 272.42 $\pm$ 38.07-fold resistance, respectively), VP-16 (58.23 $\pm$ 2.31 and 101.24 $\pm$ 6.54-fold resistance, respectively), hyper-sensitive to cisplatin (0.32 $\pm$ 0.17 and 0.22

± 0.06-fold resistance, respectively), with no significant alteration in 5-fluorouracil toxicity (0.47 ± 0.38 and 0.90 ± 0.43-fold resistance, respectively) (Table 3.1.2.2). This profile mirrors the cross resistance profile, except with respect to the cisplatin sensitivity, obtained for DLKP-A, the adriamycin-selected MDR variant of the squamous cell carcinoma (Clynes *et al.*, 1992).

When the VP-16-selected cell line, DLKP/VP-3, was exposed over a period of 3 months, to higher concentrations of VP-16 (up to 8.0μg/ml for DLKP/VP-8), the cross resistance profile was altered. The resistance of the cells to VP-16, vincristine and adriamycin was increased and the ratio of fold resistance, between the three drugs, was altered. When the fold resistance to adriamycin was taken as one, the ratio of fold resistance to adriamycin, vincristine and VP-16 was 1:12.09:0.65 for DLKP/VP-3 and 1:6.38:0.37 for DLKP/VP-8 (Section 3.1.2). However, the relative fold resistance exhibited by the VP-16 selected cell lines to vincristine and VP-16 remained approximately constant. When the fold resistance to VP-16 was taken as one, DLKP/VP-3 possessed a ratio of vincristine fold resistance to VP-16 fold resistance of 18.60:1, whereas DLKP/VP-8 exhibited a ratio of 17.24:1. The relative sensitivity to cisplatin and 5-fluorouracil exhibited by the VP-16-selected cell lines was not significantly altered by exposing the cells to VP-16, at a higher concentration and for a longer time period.

Similarities also existed between DLKP-A and DLKP/VP-8 in relation to their relative cross resistance ratios towards vincristine, adriamycin and VP-16 (Tables 3.1.2.2 and 3.2.2.2). While DLKP/VP-8 possessed the aforementioned fold resistance ratio of 1:6.38:0.37 to adriamycin, vincristine and VP-16, respectively, DLKP-A cells exhibited a ratio of 1:5.92:0.24. Allowing for the standard deviations observed within the IC_{50} calculations, both cell lines exhibited similar cross-resistance profiles. Neither DLKP-A, DLKP/VP-3 nor DLKP/VP-8 were most resistant to their specific selective agents, adriamycin for DLKP-A and VP-16 for the remaining cells. All possessed similar resistance profiles: most resistant to vincristine; followed by adriamycin; least resistant to VP-16; no cross resistance to 5-fluorouracil and only the VP-16-selected cells exhibiting sensitivity to cisplatin. This suggests that the cross resistance profile obtained for the MDR variants of the DLKP cell line, when selected with MDR associated drugs, was dictated by more than one mechanism of resistance. However, the resistance to adriamycin, vincristine and VP-16 exhibited by the DLKP-A and DLKP/VP-8 cells may be due to one mode of resistance, while the collateral sensitivity to cisplatin, exhibited by VP-16-selected cells, may be due to a second mechanism, not displayed by DLKP-A cells.

The altered toxicity profiles exhibited by the MDR cells may be due to the selective agents used to derive the drug-resistant cell lines. The two chemotherapeutic drugs used in the selective process share some common modes of action. Adriamycin, as depicted

in Figure 4.1(a), produced by a species of *Streptomyces*, is a four ring anthracycline molecule, linked to a sugar, daunosamine. It has a wide range of clinical activities for many types of solid tumours and leukaemia. A number of mechanisms contribute to the cytocidal effect of adriamycin (Speth *et al.*, 1988; Booser and Nortobagyi, 1994). It intercalates with DNA causing partial unwinding of DNA, single- and double-strand breaks (adriamycin is a topoisomerase II inhibitor) and inhibition of DNA and RNA synthesis. It undergoes metabolism of its quinone ring to a semiquinone radical, which in turn reacts with oxygen to yield superoxide, leading to oxidative damage of cell membranes and DNA. It binds to cell membranes and kills cells through membrane-related effects (Tritton and Yee, 1982). VP-16 (Figure 4.1(b)) is a semi-synthetic glycoside derivative of podophyllotoxin, which is an antimitotic derivative of the mandrake plant. It is used for the treatment of small cell lung carcinoma, testicular cancer and lymphomas (O'Dwyer *et al.*, 1985). The drug arrests cells in late S or early G_2 phase of the cell cycle. It is a potent topoisomerase II inhibitor, causing DNA strand breaks. It also inhibits nucleoside transport into cells and nucleoside incorporation into RNA and DNA. However, although adriamycin functions as a topoisomerase II inhibitor by binding to the DNA in the cleavable complex formed by the DNA, enzyme and drug, VP-16 blocks topoisomerase II action by binding to the enzyme. Exposure of cells to both drugs has also resulted in the overexpression of the P-glycoprotein drug efflux pump, as a mechanism of resistance (Twentyman *et al.*, 1986; Takigawa *et al.*, 1992).

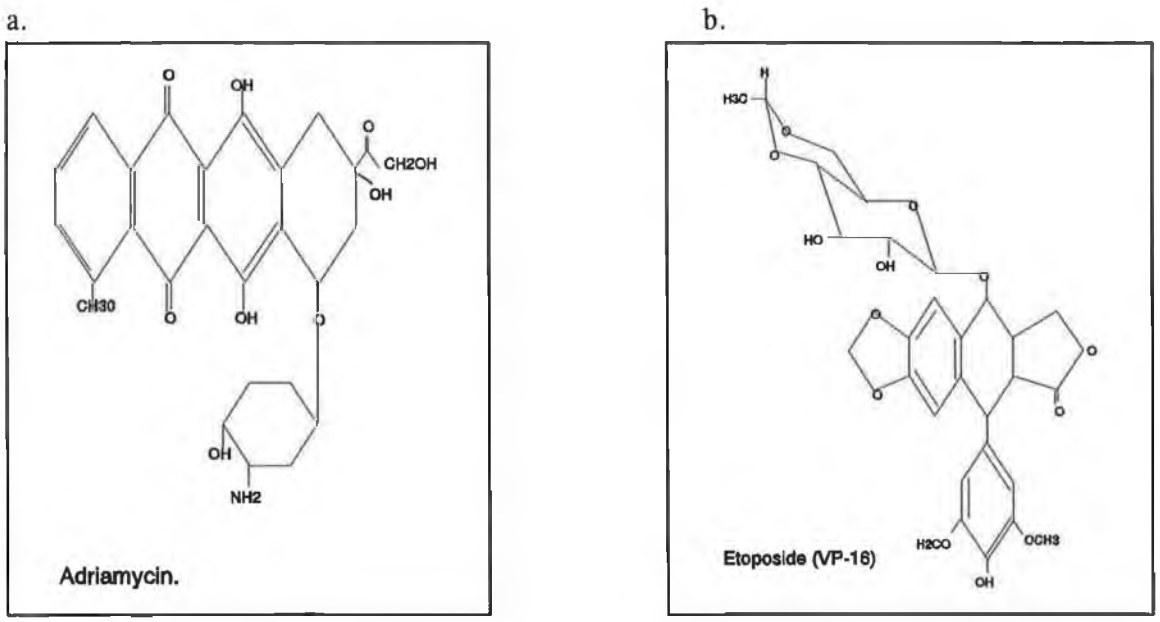


Figure 4.1 : The structures of adriamycin (a) and VP-16 (b).

Cell line	Cell type	Selective drug fold resistance	ADR.	DAUN.	VCR.	VBL.	VP-16	VM-26	5-FU	CDDP	References
NCIH69/Dau8	SCLC	DAUN 9.5X	*		*		*	*	#	#	Jensen *et al., 1989*
H69/LX4	SCLC	ADR 5X			*		*				Twentyman *et al.*, 1986a
H69AR	SCLC	ADR 35X		*	*	*	*		NC		Mirski *et al.*, 1987
GLC_4/ADR	SCLC	ADR 44X			5.7		37.5			2.1	Zijlstra *et al.*, 1987
U1690-40	SCLC	ADR 5.5X			40.1		16.3			1.4	Nygren *et al.*, 1991
U1690-150	SCLC	ADR 7.0			245.6						Nygren *et al.*, 1991
U1285/100	SCLC	ADR 3.4X			4.9		8.2			3.63	Nygren *et al.*, 1991
U1285/250	SCLC	ADR 3.1X			16.7						Nygren *et al.*, 1991
COR L23/R	LCLC	ADR 16.9X		9.9	25.5		12.1				Twentyman *et al.*, 1986a
SW1573/50	SqCLC	ADR 10X		3.6	5.5		23			0.6	Keizer *et al.*, 1989
SW1573/500	SqCLC	ADR 250X		70	540		270			0.25	Keizer *et al.*, 1989
SW1573/10000	SqCLC	ADR 2000X					750			0.7	Keizer *et al.*, 1989
H69/VPR-2	SCLC	VP-16 20X		5.5	20				1.5	1	Glisson and Alpeter, 1992
HCIH69/VP	SCLC	VP-16 17X	6.3		7.9						Jensen *et al.*, 1992
SBC-3/ETP	SCLC	VP-16 52.1X	39.5		172			147	1	1	Takagawa *et al.*, 1992
A549(VP)28	Adenocarc.	VP-16 8X		8.1	3.1					0.08	Long *et al.*, 1991
A549(VM)28	Adenocarc.	VM-26 8X	8.4		3.1					0.35	Long *et al.*, 1991
OC-NYH/VM	SCLC	VM-26 10.7X	2.9		0.9		12.0				Jensen *et al.*, 1991,1993
H69/CDDP	SCLC	CDDP 10.9X	1.01		1		0.7				Hong *et al.*, 1988
A549/CTP	Adenocarc.	CTP 1.8X	2.3				2.1				Sugimoto *et al.*, 1990(a,b)

Cross resistance (*) and collateral sensitivity (#) was observed, but the inherent heterogeneity of the cells makes exact quantification of the degree of resistance difficult.
SCLC = Small cell lung carcinoma, SqCLC = Squamous cell carcinoma of the lung, Adenocarc.= Adenocarcinoma of the lung, NC = No change,
ADR.= Adriamycin, DAUN.= Daunorubicin, VCR.= Vincristine, VBL.= Vinblastine, 5-FU = 5-Fluorouracil, CDDP = Cisplatin, CTP = Camptothecin.

Table 4.2 : Resistance profiles of MDR cell lines, established by exposing lung cell lines to chemotherapeutic drugs.

Table 4.2 presents the cross-resistance profile of drug-selected MDR human lung cancer cell lines. When the cell lines shown in the table are compared to MDR variants of DLKP-A, similarities and differences in their resistance profiles are evident. Long *et al.* (1991) established a MDR variant of the human lung adenocarcinoma cell line A549 by selection with VP-16. The resulting MDR cell line was 8-fold resistant to VP-16, cross resistant to adriamycin (3.1-fold) and hypersensitive to cisplatin (0.08-fold resistance). In the work described in this thesis, selection by VP-16 also conferred sensitivity to cisplatin on MDR variants of DLKP, but the adriamycin-selected MDR variant exhibited no alteration in its cisplatin sensitivity. However, adriamycin-selected variants of the squamous cell carcinoma of the lung, SW1573, also exhibited collateral sensitivity to cisplatin. Takigawa *et al.* (1992) found that selection of a MDR variant of a human small cell lung carcinoma, SBC-3, with increasing concentrations of VP-16, resulted in a cell line with cross resistance to a similar range of drugs and in a similar rank order, with respect to adriamycin and vincristine, as DLKP/VP-3 and DLKP/VP-8, but the sensitivity to cisplatin was unaltered. Although the fold resistance to VP-16 (52.1-fold) was similar to that exhibited by DLKP/VP-3 cells (58.23-fold), the relative levels of resistance to vincristine and adriamycin were substantially lower than that observed for DLKP/VP-3. Mirski *et al.* (1993) found that the MDR cell line resulting from the H209 human small cell lung carcinoma's selection with VP-16, produced a similar MDR profile to that obtained by Takigawa *et al.* (1992).

Both the adriamycin- and VP-16-selected DLKP cells exhibited high levels of cross resistance to vincristine. Generally, such high levels of resistance to vincristine result following selection by that drug, or where an equally high level of resistance to the selective agent is noted (Nielsen and Skovsgaard, 1992; Beck and Danks, 1991). However, a number of MDR cell lines have been isolated by selection with drugs other than vincristine, which also exhibit high levels of cross-resistance to vincristine and where the resistance to the selective agent is substantially lower. Keizer *et al.* (1989) found that the adriamycin-selected human squamous lung carcinoma, SW-1573/500, was 540-fold cross resistant to vincristine. However, the magnitude of resistance, in comparison to adriamycin resistance (250-fold), was not as high as that observed in the DLKP variants or by Nygren *et al.* (1991). They noted that the cell lines, resulting from the exposure of the small cell lung carcinoma cell line, U1690, to varying concentrations of adriamycin, were cross-resistant to vincristine at a higher magnitude than their resistance to adriamycin. While the cell line U1690-40 was 5.5-fold resistant to adriamycin, it was 40.1-fold resistant to vincristine and 16.3-fold resistant to VP-16 and although U1690-150 was only 7-fold resistant to adriamycin it was 245.6-fold resistant to vincristine. Benard *et al.* (1989) have noted a similar intensity, and rank order, in relative resistance levels, of a vincristine-selected MDR variant of a human ovarian carcinoma, IGORVI, to that observed in

the DLKP variants. The cell line described by Benard *et al.* (1989) was 750-fold resistant to vincristine, 30-fold resistant to adriamycin and 20-fold resistant to VP-16, by comparison with their drug sensitive parental cell line.

4.3 Resistance profile of clones

Marked variation existed within the DLKP-A clones, with respect to their sensitivity to adriamycin-induced cell kill (Section 3.2.2). Characterisation of the clonal variants of the DLKP-A cell line, with respect to their drug sensitivity profiles, indicated that cells within the MDR population all possessed individual cross resistance profiles. All clones were highly resistant to the selective agent, adriamycin, and also to vincristine and VP-16 and all possessed a similar rank order of resistance to these drugs, as that exhibited by DLKP-A. The clones were all most resistant to vincristine, next to adriamycin and less so to VP-16. Most exhibited no, or slight, alteration in their cross resistance profiles to 5-fluorouracil or cisplatin, by comparison with their toxicity to either DLKP or DLKP-A. DLKPA11B exhibited collateral sensitivity to 5-fluorouracil, by comparison with DLKP-A, but no significant alteration in its 5-fluorouracil resistance level, when compared to DLKP. DLKPA5D was the only subpopulation isolated from DLKP-A which exhibited differential resistance to cisplatin, by comparison with both DLKP and DLKP-A (it exhibited a 2-fold resistance to cisplatin, by comparison with DLKP). Although cross-resistance to 5-fluorouracil and cisplatin is not a characteristic of MDR, most MDR cells exhibit slight alterations in their resistance to the two drugs. The MDR small cell lung carcinoma isolated by exposing U1285 cells to adriamycin (Nygren *et al.*, 1991) was 3.63-fold resistant to cisplatin and the GLC_4/ADR MDR cells, derived by exposing a small cell lung cancer cell line to adriamycin (Zijlstra *et al.*, 1987) exhibited an increase in its cisplatin resistance (2.1-fold). The VP-16-selected variant of NCI H69, isolated by Glisson and Alpeter (1992) exhibited slight resistance to 5-fluorouracil (1.5-fold).

A 9-fold variation in adriamycin resistance existed between the most and least resistant clone, which spanned the level of adriamycin resistance exhibited by DLKP-A. As DLKPA5F maintained a stable resistance profile throughout extensive subculturing and cryopreservation (Section 3.4.2), it is unlikely that the resistance profiles of the other DLKP-A clones were not stably maintained. This implies that the variation in the resistance profile of DLKP-A clones is a characteristic of the DLKP-A population and did not arise due to their isolation and being maintained separately and in the absence of adriamycin, whereby instability in the cells' resistance to adriamycin would have led to a decline in their resistance levels.

Therefore, within the DLKP-A cell line, subpopulations are existing which possess individual resistance profiles. Some subpopulations are more resistant to the chemotherapeutic drugs than the DLKP-A cell line, while some subpopulations possess substantially reduced resistance profiles.

A notable feature, common to all variants of DLKP, was the substantial variation in their resistance to a given chemotherapeutic drug noted in replicate assays. This variation was most notable in the case of the cells' sensitivity to vincristine, where up to 50% variation was observed in day-to-day repeats. When MDR variants of the small cell lung carcinoma, NCI H69, were selected by exposing the cells to daunomycin (Jensen *et al.*, 1989) and adriamycin (Twentyman *et al.*, 1986a; Mirski *et al.*, 1987), the resulting cell lines all exhibited cross resistance to a range of chemotherapeutic drugs. However, exact quantification of the degree of resistance was hindered by the high level of variation in the cells. This variation in resistance levels was possibly due to the inherent heterogeneity of the uncloned cell lines. Heterogeneity within the DLKP, DLKP-A, DLKP/VP-3 and DLKP/VP-8 cell lines may explain the high variation in resistance levels in these uncloned cell lines, but this cannot be the cause of the variation observed in the cloned variants of DLKP-A. The low intra-assay variability and the good correlation between repeat assays performed simultaneously (results not shown), argues against the assay method being the cause of the variability.

As no one clone accurately mirrored the toxicity profile of DLKP-A with respect to the range of chemotherapeutic drugs, it implied that it was the combined characteristics of the clones which were responsible for the MDR profile of the DLKP-A cells. However, the drug resistance of the remixed clones could not completely account for the toxicity profile of the parental cells, DLKP-A. At low adriamycin concentrations, the toxicity profile generated by the mixed clones and the DLKP-A cell line deviated significantly (Section 3.7). This implied that a factor, or factors, which was important in determining the cells ability to survive in the presence of adriamycin, was absent in the mixed clones. This missing factor(s) may be the extra degree of resistance provided by an additional clone, either characterised in this study or still unknown, or may by due to the interaction of the clones within the DLKP-A population.

4.4 Clonal Heterogeneity

Two possibilities as to the origin of drug resistance in cells are that it is an inherent property of the cells or that it may be acquired following exposure to drug. The chemotherapeutic drugs may therefore act as mutagens, causing genetic changes which result

in MDR cells and/or as selective agents, eliminating the sensitive cells from the population. DLKP was established from a primary human squamous lung carcinoma and exhibited high levels of cytogenetic variability (Law *et al.*, 1992). DLKP-A was established by exposing the uncloned DLKP cell line to increasing concentrations of adriamycin over a prolonged time period (Clynes *et al.*, 1992). Consequently, the potential existed for the drug resistant cells to have been selected from a pre-existing resistant population or to have been acquired following exposure to adriamycin. DLKP-A exhibit cytogenetic aberrations, consistent with mutagenic events associated with the acquisition of an MDR phenotype (Clynes *et al.*, 1992).

Heterogeneity within tumours, with respect to drug sensitivity, has been widely documented in the literature (Allalunis-Turner *et al.*, 1993; Tanigawa *et al.*, 1984; Trope 1982). Trope (1982) detailed a study in which eighteen mouse sarcoma tumours were quartered and each section used to prepare a cell suspension. When the cell suspensions were tested *in vitro* for sensitivity to melphalan, the level of sensitivity varied markedly between individual tumours. As regards individual tumours, some appeared nearly homogeneous, while others showed high levels of intra-tumour variation in their sensitivities. Dolfini *et al.* (1993) isolated two clones of the colon adenocarcinoma cell line, LoVo. While clone LoVo C1.7 was intrinsically resistant to adriamycin, clone LoVo C1.5 showed the same resistance level as the mixed population. In an investigation of clonal variation within an MDR cell line, Yang *et al.* (1993) isolated two distinct subclones from a cisplatin-resistant human colon carcinoma cell line, LoVo, as distinguished by morphology. It was found that the clones exhibited varying drug resistance levels and cross-resistance profiles, to a variety of anticancer agents. Both clones were resistant to the selective agent, cisplatin and also to 5-fluorouracil and mustargen, but to varying levels in each clone. The most resistant clone, with respect to cisplatin toxicity, was also cross resistant to adriamycin and vincristine, whereas the least resistant clone exhibited a slightly enhanced sensitivity to the two chemotherapeutic drugs.

However, research has shown that the sensitivity of mixed cell populations to chemotherapeutic drugs is dictated by an additional factor, other than the sensitivity of the individual clones. Miller *et al.* (1989) observed that when subpopulations of a murine mammary tumour (with varying intrinsic sensitivity to methotrexate), were mixed and injected into nude mice, the resulting tumours exhibited altered sensitivity to methotrexate. The tumours resulting from the mixture of cell lines 66 and 4TO7, tended to be as sensitive as tumours arising from homogenous 4TO7 cells (these were the most sensitive tumours). However, tumours arising following injection of mixtures of 168 and 4TO7 cells tended to be more resistant than tumours derived from 4TO7 cells. Alterations in the melphalan sensitivity of tumours derived from mixtures of this murine mammary cell lines, have also been documented (Miller *et al.*, 1991).

Heterogeneity within the DLKP-A cell line was not limited to the cells' sensitivity to a range of chemotherapeutic agents. Variation within the growth rates of subpopulations of DLKP-A was also observed. Although the doubling time of DLKP-A was determined to be 31.72h., the doubling time of clones of DLKP-A ranged from 15.60h. for DLKPA2D to 32.91h. for DLKPA6B. Wilson *et al.* (1993) observed intra-tumoural heterogeneity in the doubling time in 30 colorectal cancers. Miller *et al.* (1989, 1991) also observed variation, not only in the drug sensitivity of a murine mammary cell line, but also in the growth rate (Miller *et al.*, 1988) and noted that the growth rate of the clonal cell lines was modified when clonal subpopulations were grown in contact with other sub-populations.

When DLKP-A clones were compared in terms of their growth rate, it is clear that cells with a low doubling time (DLKPA2D, DLKPA10C) should outgrow cells which possess longer growth rates (DLKPA2B, DLKPA6B, DLKPA5F). However, this did not occur in the DLKP-A population. While the ratio of the cell mixes in the DLKP-A cell line is unknown, it is clear that subpopulations of various growth rates are co-existing. This may be due to modification of the growth rates of the clonal subpopulations (as a result of sub-population interactions), as observed by Miller *et al.* (1988).

However, it must also be noted that cells which were existing in the DLKP-A population were also undergoing selective pressure, due to the presence of adriamycin. Therefore, in mixed populations, cells may not only be modified to alter their growth rates, but modification of the cells' sensitivities to adriamycin may be occurring consecutively. However, no apparent association existed between the resistance of the cells to drugs and their doubling time. Both the most and least resistant cell line (with respect to adriamycin toxicity), DLKPA2B and DLKPA5F, respectively, exhibited similar doubling times (31.74h. and 32.86h., respectively), while DLKPA3C, a cell line which exhibited comparable adriamycin-resistance as DLKPA5F, possessed a much shorter doubling time (20.63h.).

4.5 Irradiation

The literature is divided in relation to the correlation between resistance to chemotherapeutic drugs and radiation. Tanio *et al.* (1990) have described cells which are inherently drug and radiation resistant, and drug resistance has been acquired by cells following exposure to radiation (Mattern *et al.*, 1991; Hill *et al.*, 1990(a, b)), implying shared or related mechanisms of resistance. However, drug resistant cells have also been characterised which possess unaltered sensitivity to radiation (Sognier *et al.*, 1992; Mitchell *et al.*, 1988).

When DLKP cells were exposed to increasing doses of radiation, with the cells

allowed to recover between each exposure, the resulting cell line exhibited neither cross resistance nor collateral sensitivity, to a range of chemotherapeutic drugs (Section 3.5.1). The IC_{50} values obtained for adriamycin, vincristine, VP-16, cisplatin and 5-fluorouracil toxicity to radiation exposed DLKP cells, did not deviate significantly, from those observed in DLKP.

Exposure of the human ovarian carcinoma JA-T and SK-OV-3 cell lines to radiation resulted in cells resistant to VP-16 and vincristine but which exhibited unaltered sensitivity to adriamycin and irradiation. The JA-T cells were collaterally sensitive to cisplatin, 5-fluorouracil and methotrexate but the SK-OV-3 cells were resistant to cisplatin (Dempke *et al.*, 1992(a, b); 1993). Hill *et al.* (1988) noted that radiation exposure of the lymphoblasts, L5178Y, the breast carcinoma cells, MCF-7 and the squamous cell carcinoma of the tongue, HN-1, resulted in the acquisition by the cells of resistance to vincristine and VP-16. They were collaterally sensitive to cisplatin, and adriamycin sensitivity was unaltered. In contrast, radiation exposure of the testicular teratoma cell line, SuSa, resulted in cells with a similar resistance profile (resistant to vincristine and VP-16 with unaltered sensitivity to adriamycin), except the cells were also resistant to cisplatin. Eichholtz *et al.* (1993) observed that radiation exposed murine fibrosarcoma cells, SSIL, exhibited transient resistance to cisplatin.

In general, when radiation exposure of cells resulted in the acquisition of resistance to chemotherapeutic drugs, resistance to a range of drugs associated with an MDR profile was observed, with the exception of resistance to adriamycin. Mattern *et al.* (1991) found that exposure of the human epidermoid lung carcinoma xenograft, HXL-55, to radiation, resulted in the acquisition of resistance to vincristine and overexpression of P-glycoprotein. When Hill *et al.* (1990) exposed the Chinese hamster ovary cell line, AuxB1, to radiation, the resulting cell line possessed an MDR profile and overexpressed P-glycoprotein but sensitivity to adriamycin was unaltered. This suggests that radiation-induced cross resistance to chemotherapy is mediated by P-glycoprotein, but if so, the lack of resistance to adriamycin is difficult to explain. However, Gros *et al.* (1991) have reported that a single mutation in mdr1, changing the $serine_{941}$ to phenylalanine in P-glycoprotein, altered the capability of the gene to confer drug resistance. It retained the ability to confer vinblastine-resistance but lost the ability to confer resistance to adriamycin and colchicine. Devine *et al.* (1992) and Dhir *et al.* (1993) have also reported the modulation of drug resistance profiles due to sequence alterations in the P-glycoprotein gene. Therefore, mutations within the mdr1 gene, whether specifically introduced or randomly induced, as a result of exposing the cells to drug or radiation, may alter the proteins ability to recognise and transport specific substrate. McClean and Hill (1994) reported that the MDR cell line that resulted following radiation exposure of Chinese hamster cells overexpressed P-glycoprotein, but again, adriamycin sensitivity was unaltered. When these cells were then exposed to vincristine, the level of P-glycoprotein expression was further

increased and the resulting cell line was resistant to adriamycin. This implies that the P-glycoprotein overexpressed following radiation exposure, does not possess the same functions as the protein overexpressed following chemotherapeutic drug exposure, in that it is incapable of supporting resistance to adriamycin.

The radiation sensitivity of the adriamycin-selected MDR squamous cell carcinoma of the lung, DLKP-A, was unaltered, with respect to the parental cell line (Section 3.5.2). The sensitivity of DLKP and its drug-resistant variants to radiation, was comparable to that observed in the literature for other human cells. A large number of cell survival curves have been derived for malignant and normal cell populations, following exposure to radiation. These demonstrate that D_0 values (see Appendix B for a definition) for most mammalian cells are quite similar, about 1-2 Gy. Although DLKP-A exhibited unaltered radiation sensitivity, with respect to DLKP, within the DLKP-A cell line, clones existed which possessed altered sensitivities to radiation. DLKPA5F was resistant to radiation, DLKPA5D exhibited collateral sensitivity and the radiation sensitivity of the remaining clones was unaltered, by comparison with DLKP-A and DLKP. Tumour cell heterogeneity with respect to radiation sensitivity has been documented. Allam *et al.* (1993) observed significant intra-tumoural heterogeneity in the radiation sensitivity of malignant gliomas and Powell and McMillan (1991) noted clonal variation in response to ionising radiation, in a human glioma cell line, IN859. Allalunis-Turner *et al.* (1993) concurrently established two cell lines from a single specimen from a patient with glioblastoma, and the two subpopulations differed in their sensitivity to radiation and chemotherapeutic agents.

No obvious correlation existed between the drug resistance level or the growth rate of the clones and their sensitivity to radiation. DLKPA5F was the most resistant clone, with respect to both drug- and radiation-induced cell kill. However, the ranking order of clones, in terms of radiation resistance and resistance to any of the chemotherapeutic drugs, does not correlate. DLKPA5F and DLKP-A possessed comparable sensitivities to cisplatin but had significantly differing radiation sensitivities. In this study, no correlation appeared to exist between adriamycin-induced drug-resistance and radiation-resistance and it is probable that, as the literature has shown, the link between adriamycin-resistance and radiation-resistance is weak. Louie *et al.* (1985) noted that the radiation sensitivity of the adriamycin-resistant variant of the human ovarian carcinoma cell line, A2780, was not altered, with respect to the parental cells. Belli (1989) showed that adriamycin-resistant variants of the Chinese hamster cell line, V79, had increased sensitivity to radiation, but the degree of sensitivity did not relate to their level of adriamycin resistance. However, Lehnert *et al.* (1989) noted that adriamycin-selected MDR variants of the breast cell line, MCF-7, exhibited resistance to radiation and adriamycin-resistant HT-1080 cells were also cross resistant to radiation (Miller *et al.*, 1992). When Tanio

et al. (1990) investigated the chemosensitivity and radiosensitivity of three small cell lung carcinomas, established from biopsy samples, a correlation was observed. Cells which appeared resistant to radiation also exhibited resistance to chemotherapeutic drugs, including adriamycin.

In this study, the cells' sensitivity to radiation did not correlate with their doubling time. It has been documented that cells exhibit a cell cycle-associated sensitivity to radiation. Cells were most resistant to radiation during the S-phase of the cell cycle (Iliakis and Okayasu, 1990) and eliminating the S-phase cells from a cell population increased the radiation sensitivity of the population (Nothdurft *et al.*, 1992). Quiet *et al.* (1991) observed that a radioresistant squamous cell carcinoma of the head and neck had twice the number of cells in the S-phase of the cell cycle, by comparison with a radiosensitive cell line, while Gillies McKenna *et al.* (1991) noted that cell lines which were resistant to radiation underwent a significantly longer arrest in the G_2-phase of the cell cycle after radiation exposure, than the radiation sensitive cells. However, though the DLKP variants exhibited differing doubling times, implying perturbed cell cycle, no correlation was apparent between their growth rate and their ability to survive following exposure to radiation.

4.6 Altered biochemical properties of MDR cells

Differential expression of P-glycoprotein and topoisomerase II are two of the main mechanisms of MDR exhibited by a broad range of cells. The VP-16-selected variants of DLKP, overexpressed P-glycoprotein and exhibited a reduction in their topoisomerase II levels, by comparison with their drug-sensitive parents. DLKP-A has been shown to overexpress P-glycoprotein and to possess cytogenetic aberrations associated with gene amplification (Clynes *et al.*, 1993). In the course of this research work, DLKP-A cells have also been shown to possess reduced topoisomerase II levels. The clonal populations, derived from DLKP-A, exhibited variable levels of P-glycoprotein and topoisomerase II. All clones overexpressed the P-glycoprotein drug efflux pump and possessed altered levels of nuclear topoisomerase II. Cell lines with multiple mechanisms of resistance have previously been detailed in the literature. Takigawa *et al.* (1992) described a VP-16-selected MDR variant of the human small cell carcinoma line SBC-3, which overexpressed both mdr1 mRNA and its protein product, P-glycoprotein and had reduced topoisomerase II activity. Hoban *et al.* (1992) showed that an MDR Chinese hamster ovary cell line, derived by exposure to increasing concentrations of adriamycin, exhibited several mechanisms of resistance, including mdr1 and GST overexpression and a reduction in transcripts coding for topoisomerase II.

4.6.1 P-glycoprotein levels

A general characteristic of many MDR cells is the overexpression of both mdr1 mRNA and P-glycoprotein (Lemontt *et al.*, 1988; Bradley *et al.*, 1989; Reeve *et al.*, 1989; Baas *et al.*, 1990). P-glycoprotein levels (determined by Western blot analysis using the C219 antibody) and the mRNA levels coding for the protein (determined by RT-PCR) were overexpressed in all MDR cell lines examined. Neither the mRNA nor the protein was detected in the drug sensitive parental cell line, DLKP (Section 3.8 and 3.9). The level of mRNA detected in the drug resistant cell lines did not correlate precisely with the level of protein detected. No significant differences in mdr1 mRNA levels were noted between any of the MDR cell lines, except in the case of DLKPA6B. Western blot analysis of the cell line, using the C219 monoclonal antibody, showed that these cells possessed levels of P-glycoprotein comparable to the other three clones of DLKP-A, which had been analyzed. However, RT-PCR analysis of the cells' mdr1 mRNA showed that the cell line exhibited reduced levels of the mdr1 homologous transcript. The VP-16-selected MDR cell lines expressed significantly higher levels of P-glycoprotein, by comparison with the adriamycin-selected cell lines, but this difference was not reflected in their mdr1 mRNA levels.

The level of P-glycoprotein expressed by DLKP-A cells was low, by comparison with the VP-16-selected cells and the clonal variants of DLKP-A. However, the densitometrical analysis of P-glycoprotein levels in DLKP-A cells gave a substantially lower reading for P-glycoprotein in DLKP-A cells, than other cell lines. Through visual analysis, a difference in the level of P-glycoprotein in DLKP-A and the VP-16-selected cells could be seen, but the difference did not appear as great as that shown by the densitometrical analysis. In all cases, mdr1 mRNA levels were determined by RT-PCR analysis and so detected the presence of a 157bp fragment of mRNA, specific to mdr1 (O'Driscoll *et al.*, 1993). As such, it is unclear whether full length mRNAs were present and/or truncated mRNA fragments were detected, which in turn may have coded for a non-immunodetectable protein. The PCR reaction was allowed to proceed for 30 cycles. It is not known if the reaction was still undergoing exponential growth at the end of this number of cycles. Therefore, the level of PCR product may not be a true reflection of the level of template present in the cells. The levels of template can only be correlated to product during the exponential phase of the PCR reaction. Post-translational modifications, alterations in the location of the protein and/or partial protein degradation, may also account for the discrepancy between the implied P-glycoprotein level (as determined from mRNA analysis) and the detected P-glycoprotein level (as determined by the immunological reactivity of the protein).

P-glycoprotein levels present in the VP-16-selected cell lines inversely correlated with their level of adriamycin accumulation (Section 3.10). DLKP/VP-3, the less resistant variant, expressed higher levels of mdr1 mRNA and immunodetectable P-glycoprotein and accumulated less adriamycin than the more resistant DLKP/VP-8 cells, which possessed lower levels of mdr1 mRNA and P-glycoprotein. The levels of immunodetectable P-glycoprotein in the clones, established from DLKP-A, did not correlate with their toxicity profiles or their level of adriamycin accumulation (I. Cleary, personal communication). Although the clones DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F exhibited approximately equal levels of P-glycoprotein, the rank order of their drug accumulation levels, was inversely related to the rank order of their adriamycin sensitivity, neither of which correlated with their levels of detectable P-glycoprotein or mdr1 mRNA.

4.6.2 Topoisomerase II levels

In general, the level of topoisomerase II and its α subunit are reduced in MDR cell lines resistant to topoisomerase II poisons (Deffie *et al.*, 1989a; deJong *et al.*, 1990; Mirski *et al.*, 1993; Per *et al.*, 1987). As has been noted in the literature, the reduction in immunodetectable topoisomerase II in DLKP variants was generally mirrored by a reduction in the α form of the enzyme (Section 3.9.1). Topoisomerase II levels varied in the cell lines, depending on whether the level of the enzyme present in the isolated nucleus or whole cell extract, was examined. In general, the level of the enzyme was reduced in resistant cells, with respect to the sensitive parents. The levels of both topoisomerase II and its α subunit detected in nuclear and total cell extracts, were reduced in DLKP-A and DLKP/VP-8, by comparison with DLKP. Although both cell lines exhibited similar resistance to adriamycin, vincristine and VP-16, DLKP-A had the lowest levels of both topoisomerase II and its α subunit while DLKP/VP-8 had higher levels of P-glycoprotein (Section 3.9.2). DLKP/VP-3 possessed levels of topoisomerase II and its α subunit similar to that detected in DLKP, as determined from their total cell extracts, but the level of enzyme confined to the nucleus in DLKP/VP-3 cells, was reduced to 50-60% that detected in DLKP. The amount of topoisomerase II present in DLKPA6B was reduced to 55-70% of that observed in DLKP, in both nuclear and total cell extracts and the ratio of topoisomerase II-α to total topoisomerase II protein was similar, in both nuclear and total cell extractions of DLKPA6B. The level of topoisomerase II present in total cell lysates of the remaining clones, DLKPA2B, DLKPA11B and DLKPA5F was similar to that present in DLKP, but the level of the α subunit was reduced. However, in nuclear extracts, all of these cell lines exhibited a reduction in both topoisomerase II and its α subunit, by

comparison to DLKP. The rank order of topoisomerase II levels in the nuclear extracts of DLKPA2B, DLKPA11B and DLKPA5F was mirrored by that observed in the total cell extracts. DLKPA2B, the most adriamycin-sensitive clone, had levels of nuclear topoisomerase II and topoisomerase II-α similar to that observed for DLKP-A. However, both DLKPA2B and DLKP-A possessed reduced levels of the enzyme by comparison with DLKP, yet the cell lines DLKPA2B and DLKP-A exhibited significantly different resistances to drugs associated with topoisomerase II-mediated cell kill.

Using antiserum to topoisomerase II, Berrios *et al.* (1985) found that topoisomerase II was differentially localised in *Drosophila* larva, during various stages of the cell cycle. Though generally localised in the nucleus, during mitosis the enzyme was distributed diffusely throughout the cell. Petrov *et al.* (1993) localised both the topoisomerase II-α and the ß subunits to the nucleus, in Chinese hamster fibroblasts, using polyclonal antibodies described by Chung *et al.* (1989), but they also found that during mitosis, both forms were detected in the cytoplasm. In this research work, the cells from which topoisomerase II was isolated were in the exponential phase of growth, but were not synchronised. It may, therefore, be assumed that a small percentage of the cells were undergoing mitosis, and so cytoplasmic topoisomerase II would be present.

Both a 180 kDa. and a 150 kDa. immunoreactive protein was detected using the topoisomerase II-ß specific monoclonal antibody (Section 3.9). The 150 kDa. form of topoisomerase II-ß was only detected in total cell extract and not in nuclear extracts (Figures 3.9.1.1.2 and 3.9.1.2.2), implying that it is cytoplasmic associated. When Coutts *et al.* (1993) used the topoisomerase II-ß antibody raised by Negri *et al.* (1992) to study topoisomerase II levels in non-small cell lung cancer cell lines, they detected a 150 kDa. form of topoisomerase II-ß, which was assumed to be due to protein degradation. Proteolytic degradation of the 180 kDa. form of topoisomerase II had previously been described by Holden *et al.* (1992), who detected a 180 and 160 kDa. form of topoisomerase II-ß using the polyclonal antibody described by Drake *et al.* (1987). However, in this research, the polyclonal antibody, which detects both the α and ß forms of the topoisomerase II enzyme, detected only the 180 kDa. form of topoisomerase II-ß, whereas the topoisomerase II-ß monoclonal antibody detected either both a 180 kDa. and a 150 kDa. form, or only the 150 kDa. form of the enzyme, in the same cell extract as examined with the polyclonal antibody. All topoisomerase II isolations were carried out on ice, in the presence of proteolytic inhibitors (PMSF, benzamidine, soybean trypsin inhibitor, leupeptin, pepstatin A and aprotinin) and all Western blotting procedures were carried out immediately following protein isolation. The fact that no degradation of the 170 kDa. form of topoisomerase II was observed, that no degradation of the 180 kDa. form of topoisomerase II in the nuclear extract was noted and that the polyclonal antibody which detects both

topoisomerase II-α and -ß, only detected the 180 kDa. form of topoisomerase II-ß in total cell extract, all argue against random proteolytic degradation, due to the isolation procedure. Therefore, either the 150 kDa. form was a specific precursor or breakdown product of the 180 kDa. form of the protein and the polyclonal and monoclonal antibodies recognise different epitopes on the 180 kDa. form of the protein, thereby differentiating between various forms of the topoisomerase II-ß enzyme, or the monoclonal antibody directed against topoisomerase II-ß was cross-reacting with a homologous protein. When Chung *et al.* (1989) raised antibodies to topoisomerase II-ß using a synthetic dodecapeptide homologous to a section of the topoisomerase II-ß protein (starting at amino acid 748 and consisting of the following sequence: NVRRMLDGLDPH), the resulting antibody recognised both a 180 kDa. protein and a protein of approximately 145 kDa. However, a second topoisomerase II-ß antibody (raised against the synthetic peptide beginning at amino acid residue 785 and reading EIFVVDRNTVEIT) selectively recognised the 180 kDa. form of the enzyme, suggesting the first antibody cross-reacted with a homologous protein.

Therefore, the presence of no detectable 150 kDa. form of topoisomerase II-ß in the nuclear extract may have resulted from either the isolation procedure, or its cytoplasmic location. As the nucleus was isolated from the cytoplasm, prior to lysis in a high salt solution, cytoplasmic- and cytoplasmic organelle-associated proteolytic enzymes were removed before the topoisomerase II was exposed to them. The high salt solution and the presence of proteolytic inhibitors may have exerted an inhibitory effect on any remaining proteolytic enzyme activity, further protecting the topoisomerase II enzyme from degradation. However, differing cell location-associated forms of topoisomerase II-α have been described in the literature. Feldhoff *et al.* (1994) demonstrated that the human small cell lung cancer cell line, H209 contained the 170 kDa. form of topoisomerase II-α, which was located in the nucleus but its VP-16-selected variants contained a 160 kDa. cytoplasmic-located form of the enzyme. The cytoplasmic form exhibited similar catalytic activity as the 170 kDa. form, suggesting that the missing sequence was only necessary for nuclear localisation of the enzyme. Perhaps a mutated form of topoisomerase II-ß is present in the DLKP variants, and the mutation caused a differential cellular localisation of the enzyme.

As previously noted in relation to P-glycoprotein and its mRNA levels, gene transcript levels for topoisomerase II enzymes do not correlate to protein expression (Sections 3.8, 3.9). Both DLKP and DLKP-A had similar levels of topoisomerase II mRNA, but DLKP-A had vastly reduced levels of the immunodetectable protein, in both its total cell extract and nuclear extract. Alhough DLKP/VP-3 and DLKP/VP-8 also had similar levels of topoisomerase II mRNA, the protein level was lower in DLKP/VP-8. The level of immunodetectable topoisomerase II present in total cell extracts of DLKPA6B was only 50% of that present in the

other clones of DLKP-A, yet all of the cell lines had similar transcript levels. This lack of correlation between protein and mRNA levels was also observed for topoisomerase II-α and topoisomerase II-ß. This variation may be due to the fact that different detection methods were employed to examine protein and gene transcript levels. The mRNA levels of the genes of interest were determined by RT-PCR analysis, whereby only a small but specific fragment of the mRNA (O'Driscoll *et al.*, 1993) was detected. It was assumed that this section was located on a stretch of RNA, which coded for the complete sequence for immunodetectable protein, and was not a truncated mRNA. Deffie *et al.* (1989a) showed that topoisomerase II levels were reduced in adriamycin-resistant P388 leukaemia cells and that northern blot analysis (1989b) revealed two mRNA splices: a 6.6kb transcript in sensitive cells, which was reduced 7-fold to 8-fold in resistant cells and accounted for the decrease in protein levels and a second shorter transcript, only present in the resistant cells. Post-translational modifications which may alter the immunodetectability of the protein were not taken into account *e.g.* partial degradation or the possibility of protein cross-reactivity by the antibody used for detection. Deffie *et al.* (1992) noted that topoisomerase II levels were reduced in adriamycin-selected Chinese hamster ovary cells and in mitoxantrone-resistant HeLa cells, in comparison with the parental populations, though no differences were observed in the levels of complete mRNA, as determined by northern analysis, implying post-translational modifications of the protein. In the course of this research, PCR analysis of topoisomerase II mRNA, and that of its α and ß subunits, was carried out using 30 cycles of PCR and it is not known if the reaction is still exponentially amplifying the template at the end of this number of cycles. Therefore, the mRNA levels can only be assumed to be semi-quantitative.

4.7 Correlation of resistance profiles to biochemical properties of the cells

The adriamycin and VP-16 resistance of DLKP/VP-3 and DLKP/VP-8, may be explained by their combined levels of P-glycoprotein and topoisomerase II. DLKP/VP-8 was the more resistant cell line, with respect to the two drugs mentioned above, and although it had slightly lower levels of P-glycoprotein than DLKP/VP-3, it had substantially reduced levels of immunodetectable topoisomerase II and its α subunit. The level of P-glycoprotein expression by the cells can not fully explain their resistance profile to vincristine, implying other mechanisms of resistance must be present. DLKP/VP-8 was approximately 1.75-fold more resistant to vincristine than DLKP/VP-3, yet it possessed slightly lower levels of both P-glycoprotein and mdr1 mRNA. Vincristine is not associated with topoisomerase II-mediated toxicity. Therefore, the level of vincristine resistance exhibited by the cells should be mirrored

by their P-glycoprotein levels, if P-glycoprotein was the only mechanism of vincristine resistance associated with the cells. Alterations other than that of P-glycoprotein and topoisomerase II, must have occurred in DLKP/VP-8, which account for its extra resistance to vincristine. Cole *et al.* (1991) found that an adriamycin-selected MDR NCI-H69 cell line, which was cross resistant to vinca alkaloids, had reduced levels of topoisomerase II but did not overexpress P-glycoprotein, in comparison with its parental cell line. The adriamycin-selected MDR variants derived from the human small cell lung cancer cell lines, U1690 and U1285, by Nygren *et al.* (1991) also exhibited cross resistance to vincristine but overexpressed neither mdr1 mRNA nor P-glycoprotein. Twentyman *et al.* (1986a) described resistance to vincristine without P-glycoprotein or mdr1 mRNA overexpression in the adriamycin-selected MDR variant of the large cell lung carcinoma, COR L23.

A range of proteins, other than P-glycoprotein, have been detailed in the literature, whose expression have been altered in drug-resistant cells by comparison with their sensitive counterparts. Bhalla *et al.* (1985), Marsh *et al.* (1986, 1987) and McGrath and Center (1988) have detailed the presence of a membrane-associated 150 kDa. protein in resistant cells. Proteins with sequence homology to P-glycoprotein have also been observed in drug-resistant cells. Overexpression of the multidrug resistant-associated protein (MRP) has been noted in the small cell lung carcinoma H69/AR cells (Cole *et al.*, 1992b) and Krishnamachry and Center (1992) have observed overexpression of a 150 kDa. and 130 kDa. protein in the leukaemia cell line HL69/ADR. The overexpression of a 110 kDa. cytoplasmic protein in the lung cell lines SW1573/2R120 and GLC_4/R has been detailed by Versantvoort *et al.* (1992).

Although both DLKP-A and DLKP/VP-8 exhibit similar resistance to adriamycin, vincristine and VP-16, DLKP-A had lower levels of P-glycoprotein than DLKP/VP-8, it also had substantially reduced levels of topoisomerase II. Therefore, although both cells possessed similar levels of resistance to similar drugs, differing biochemical alterations were responsible for conferring the MDR profile. An analysis of MDR variants of NCI-H69 selected by exposure to the anthracyclines, both adriamycin and daunomycin, by Jensen *et al.* (1989, 1993), Mirski *et al.* (1987) and Twentyman *et al.* (1986a), showed that while populations of multidrug resistant cells may possess similar cross resistance profiles, the underlying mechanism of resistance may vary. The resulting MDR cell lines, in these cases, exhibited similar cross resistance profiles but the inherent heterogeneity of the MDR variant of NCI-H69 made exact quantitation of the degree of cross resistance difficult. The cell lines selected by Jensen *et al.* (1989) and Twentyman *et al.* (1986) overexpressed the drug pump, P-glycoprotein, while the NCI-H69 variant derived by Mirski *et al.* (1987) had no detectable overexpression of P-glycoprotein (Reeve *et al.*, 1989). The clones of DLKP-A all exhibited differential expression of P-glycoprotein and topoisomerase II and both its subunits. Therefore, the DLKP-A cell line

is not only heterogeneous in terms of its chemotherapeutic drug and radiation resistance levels and its cell growth rates, but also in respect to its mechanisms of drug resistance. When Yang *et al.* (1993) characterised cell clones of the MDR variant of LoVo, one of the two distinct subpopulations overexpressed P-glycoprotein, while the other displayed no alteration.

4.8 Effect of topoisomerase II poisons on the toxicity of topoisomerase I inhibitors

Increased resistance to topoisomerase II inhibitors, exhibited by MDR variants of DLKP, could, in part, be accounted for by alterations in the level of the topoisomerase II enzyme. Decreased immunodetectable topoisomerase II was noted in nuclear extracts of all MDR cell lines. In order to maintain the biological activity of the topoisomerase enzymes at a constant level, alterations in the level of topoisomerase II have often been paralleled by an opposite alteration in topoisomerase I (Ferguson *et al.*, 1988; Sugimoto *et al.*, 1990(a, b); Lefevre *et al.*, 1991). However, some researchers have reported alterations in topoisomerase II levels independently of topoisomerase I (Matsuo *et al.*, 1990; de Jong *et al.*, 1990). In general, acquisition of an MDR profile by cells is not associated with modification of topoisomerase I (reviewed by Pessina, 1993). In this study, cells that were highly resistant to topoisomerase II inhibitors, exhibited minimal alterations in their sensitivity to topoisomerase I poisons, suggesting alterations in topoisomerase II levels were not balanced by an opposite alteration in topoisomerase I levels. Although DLKP/VP-8 was 272-fold resistant to adriamycin and 101-fold resistant to VP-16, it was only a maximum of 2-fold more sensitive to camptothecin than the drug sensitive parental cell line, DLKP. This implies that the topoisomerase I levels may be increased 2-fold, to compensate for the 2-fold to 3-fold decrease in topoisomerase II levels observed in these cells (Section 3.9). When both the MDR and sensitive cell lines were exposed to combinations of a topoisomerase II and topoisomerase I inhibitors (adriamycin and camptothecin respectively), no enhanced toxicity was observed over the product of their individual toxicities. This suggests that the two classes of drug acted in an independent, non-synergistic manner and that exposure of cells to topoisomerase II poisons does not render DLKP variants more susceptible to cell kill by a topoisomerase I inhibitor, with the converse also holding true.

4.9 Drug accumulation

Decreased drug accumulation has been found to be a property of some drug resistant cells (Toffoli *et al.*, 1991; Zijlstra *et al.*, 1987; Kuiper *et al.*, 1990). The level of adriamycin accumulated by VP-16-selected MDR cells was decreased by comparison with the drug sensitive DLKP cell line (Section 3.10). However, the level of adriamycin present within the cells did not present a true indication of the cells' resistance, suggesting multiple mechanisms of resistance to adriamycin were operating. DLKP/VP-3, the least resistant of the VP-16-selected MDR cell lines, surprisingly accumulated less adriamycin than the more resistant DLKP/VP-8 cell line. The level of adriamycin accumulated by both cell lines was reflected in their levels of immunodetectable P-glycoprotein. DLKP/VP-3 expressed higher levels of mdr1 mRNA, immunodetectable P-glycoprotein and accumulated less adriamycin than DLKP/VP-8. However, in order to achieve 50% cell kill in DLKP/VP-8, the cells required exposure to nearly twice the concentration of adriamycin as that required to achieve the same level of kill in DLKP/VP-3, implying that extra mechanisms of resistance are present in the DLKP/VP-8 cell line. It has been shown that topoisomerase II levels were reduced in DLKP/VP-8 by comparison with DLKP/VP-3. Adriamycin is a topoisomerase II poison, exerting its toxicity by blocking the triplex cleavable complex formed by adriamycin, topoisomerase II and DNA and resulting in DNA strand breaks. Therefore, reduced levels of topoisomerase II in DLKP/VP-8, even in the presence of higher levels of intracellular adriamycin, may result in an extra protective effect towards the cells, with respect to adriamycin toxicity.

Alternatively, although adriamycin accumulation is higher in DLKP/VP-8 cells, in comparison with DLKP/VP-3 cells, altered cellular distribution of the adriamycin may result in the cells possessing the capability to survive in the presence of higher drug concentrations, than their total intercellular drug levels would imply. As adriamycin exerts its toxicity by intercalating with DNA, the nucleus is the prime target for the drug. Therefore, exclusion of the adriamycin from the nucleus, may result in a cell protective effect. Keizer *et al.* (1989) investigated a range of MDR variants of the human non-small cell lung cell line, SW-1573 and found that with increasing adriamycin resistance, cellular adriamycin accumulation decreased and the subcellular adriamycin distribution shifted from mainly nuclear to a mainly cytoplasmic pattern. When Schuurhuis *et al.* (1991) examined the ratio of nuclear to cytoplasmic fluorescence in the MDR variants, it was noted that the ratio was reduced in both P-glycoprotein overexpressing and non-P-glycoprotein overexpressing MDR cell lines, by comparison with the drug sensitive parental cells. Gervasoni *et al.* (1991) found that exposure of the cell lines HL-60, P388 and MCF-7 and their MDR counterparts to daunorubicin resulted in rapid drug distribution from the plasma membrane to the perinuclear region. This was

followed by redistribution of the drug, in the sensitive cells, to the nucleus and cytoplasm. In the MDR variants, redistribution of the daunorubicin occurred to vesicles distinct from the nucleus, in an energy dependent manner. Reduced levels of drug accumulation in the nucleus of MDR cells, by comparison with their sensitive parental cells, was also noted by Coley *et al.* (1993), Boiocchi *et al.* (1992) and Weaver *et al.* (1991).

Alterations in the level of adriamycin metabolising enzymes could also account for increased resistance to adriamycin, in the presence of intracellular drug. Vasanthakumar and Ahmed (1985) showed that although a range of human myeloid leukaemia cell lines, erythroleukemia cell lines and myeloblasts from two untreated patients with acute myelogenous leukaemia accumulated similar levels of daunorubicin, their metabolism of daunorubicin to daunorubicinol and their levels of reductase activity were extremely variable. Therefore, possible alterations in the level of drug metabolising and deactivation enzymes in VP-16-selected MDR cells may partially account for their altered drug resistance.

Adriamycin accumulation by the MDR clones of DLKPA, DLKPA2B, DLKPA6B, DLKPA11B and DLKPA5F, was decreased, by comparison with DLKP (I. Cleary, personal communication). The cell line most resistant to MDR-associated chemotherapeutic drugs, DLKPA5F, accumulated least adriamycin, in comparison to the remaining clones and DLKP. DLKPA5F cells accumulated one fifth to one quarter the amount of drug accumulated by DLKP. Of the cloned cell lines, the least resistant clone, DLKPA2B, accumulated highest levels of adriamycin, which was slightly lower than the level of adriamycin accumulated by DLKP (DLKPA2B accumulated approximately 75% of the adriamycin accumulated by DLKP). DLKPA6B and DLKPA11B exhibited similar, but intermediate, levels of adriamycin resistance, by comparison to DLKPA5F and DLKPA2B. Both cell lines also accumulated an intermediate level of adriamycin, by comparison with the two extreme clones. However, DLKPA6B accumulated slightly higher levels of adriamycin than DLKPA11B.

In contrast to the VP-16-selected MDR cell lines, where their levels of drug accumulation reflected the level of immunodetectable P-glycoprotein, and not the cells' resistance to adriamycin, adriamycin accumulation by the DLKP-A clones reflected their level of drug resistance *i.e.* the most resistant clone accumulated the least adriamycin and the least resistant clone accumulated the highest level of adriamycin. Adriamycin accumulation by the clonal subpopulations of DLKP-A did not reflect their individual levels of P-glycoprotein. No significant variation existed within the four clones, with respect to immunodetectable P-glycoprotein, but a 3-fold reduction in adriamycin accumulation in DLKPA5F, in comparison to DLKPA2B, was observed.

In the VP-16-selected MDR cell lines, the presence of circumvention agents reverted the level of adriamycin accumulated to that observed in the drug sensitive DLKP cell

line. Both verapamil and cyclosporin A caused an increase in adriamycin accumulation, to levels comparable with DLKP, and in the case of DLKP/VP-8, the presence of verapamil resulted in higher adriamycin accumulation than that observed in the parental cells. Verapamil and cyclosporin A have been widely used *in vitro* to overcome drug resistance (Larsson and Nygren, 1990; Tsuruo *et al.*, 1983) and with both circumventing agents, the increase in the cells' sensitivity to the drug of interest has been paralleled by an increase in drug accumulation (Van de Vrie *et al.*, 1993; Ross *et al.*, 1993). In this study, both verapamil and cyclosporin A enhanced the toxicity of adriamycin to DLKP/VP-8 and DLKP/VP-3 (Section 3.11). This increase in sensitivity was, at least partially, due to increased adriamycin accumulation.

4.10 Circumvention of drug resistance

Both verapamil and cyclosporin A proved to be potent enhancers of adriamycin toxicity in drug sensitive and MDR variants of DLKP (Section 3.10). Both compounds were less toxic to DLKP than its MDR variants. Schuurhuis *et al.* (1990) also observed that calcium channel blockers, including verapamil, were more toxic to several MDR cell lines than to their parental cells, and Twentyman *et al.* (1986b) noted that the growth inhibitory effect of verapamil was greater in MDR variants of the small cell lung cell line, NCI-H69, than in the parental cell line. In the research described in this thesis, both circumvention agents were observed to potentiate the toxicity of adriamycin in DLKP cells, but it must occur by a mechanism other than increased drug accumulation, as neither cyclosporin A nor verapamil altered the level of adriamycin accumulated by the cells (Section 3.10). This suggests that in the drug sensitive cells, P-glycoprotein is neither the target for verapamil or cyclosporin A nor the mode by which they sensitise the cells. Cole *et al.* (1989) noted that verapamil enhanced adriamycin toxicity in non-P-glycoprotein overexpressing MDR variants of the fibrosarcoma cell line, HT-1080 and the small cell lung cancer cell line, NCI-H69. Twentyman *et al.* (1986b) also found that verapamil was capable of sensitising MDR variants of the large cell lung carcinoma, COR-L23, to adriamycin toxicity even though the cells did not overexpress P-glycoprotein. Cyclosporin A and verapamil both possess P-glycoprotein binding activities (Foxwell *et al.*, 1989; Qian and Beck, 1990), which may be responsible, at least in part, for their ability to enhance drug toxicity in the MDR cells.

Both cyclosporin A and verapamil appeared to modulate adriamycin and vincristine toxicity to a greater extent than that of VP-16 and both circumventing agents enhanced adriamycin and vincristine toxicity in DLKP cells to a greater degree than in the MDR cells. Cyclosporin A enhanced the toxicity of adriamycin, vincristine and VP-16 in all variants

of DLKP but the toxicity of VP-16 was modified to a lesser extent than that of either adriamycin or vincristine. Verapamil also enhanced adriamycin- and vincristine-induced cell kill in all DLKP variants. However, the effect exerted by verapamil, on the enhancement of the toxicity of VP-16, was cell line dependent. Verapamil enhanced the toxicity of VP-16 to the parental cell line, DLKP and also to DLKP-A and DLKPA11B. A slight enhancement of the toxicity of VP-16 to DLKPA5F, by the presence of verapamil, was observed, while the presence of verapamil decreased VP-16 toxicity to DLKPA2B, DLKPA6B and, most notably, to DLKP/VP-3 and DLKP/VP-8. In no instance was the magnitude of the effect of verapamil on VP-16 toxicity in the order of that observed for verapamil with vincristine or adriamycin or when cyclosporin A was combined with VP-16. Larsson and Nygren (1990) also noted that neither verapamil nor cyclosporin A potentiated the toxicity of VP-16 to the same degree as that observed for vincristine. Chao *et al.* (1990) observed that verapamil was less effective than cyclosporin A at enhancing VP-16 toxicity to a human leukaemic cell line and to normal bone marrow cells.

In cases where verapamil appeared to diminish VP-16 toxicity in this study, verapamil was toxic to the cells in its own right and the level of apparent reduction in VP-16 toxicity was most notable in cells to which verapamil alone was most toxic. Whether VP-16 was decreasing the toxicity of verapamil towards the cell lines or whether verapamil was modulating VP-16's toxicity, or a combination of both, was unclear. Glisson and Alpeter (1992) noted that both cyclosporin A and verapamil enhanced the accumulation of VP-16 in a VP-16-selected MDR variant of the small cell lung cancer cell line, NCI-H69, yet only cyclosporin A was effective at enhancing VP-16 toxicity to the cell line, relative to its parental cells. Although the effect of verapamil on VP-16 accumulation by the cells was not investigated in this work, verapamil caused increased adriamycin accumulation in DLKP/VP-3 cells, to levels comparable with the parental cell line and resulted in even higher intracellular levels of adriamycin in DLKP/VP-8 cells. This implied that verapamil was active at blocking adriamycin efflux *via* the P-glycoprotein pump, probably by competitive inhibition, in DLKP/VP-3 and DLKP/VP-8 cell lines and it may be assumed to have a similar effect on VP-16 accumulation.

As previously mentioned, verapamil was highly toxic to all the cell lines where its combination with VP-16 resulted in an antagonistic effect, with respect to cell kill. The degree of antagonism observed was relative to the toxicity of verapamil to the individual cells. In these cells, exposure to VP-16 and low concentrations of verapamil, resulted in an apparent potentiation of VP-16 toxicity. An increase in the concentration of verapamil resulted in no alteration in toxicity, other than that due to the toxicity of verapamil and VP-16 alone, while a further increase in verapamil caused an antagonistic effect between VP-16 and verapamil, with respect to cell kill. Therefore, the masking of toxicity was dependent on the concentration of

verapamil and paralleled verapamil toxicity to the cells.

The pH of the extracellular environment is capable of influencing the toxicity of drugs to cells and the effectiveness of toxicity modulating agents. Hamilton *et al.* (1993a) noted that acidic extracellular media reduced the toxicity of adriamycin to drug resistant variants of HT-29 and SW-420 cells. Decreased pH values also diminished the ability of verapamil and cyclosporin A to enhance drug toxicity and cellular uptake of verapamil was also reduced. In all circumvention assays carried out in this research project, the chemotherapeutic drug and the circumvention agent were added to cells, after they were 24h. in culture. Therefore, the pH of the growth media would be reduced, due to cell metabolism. The addition of verapamil, especially at the higher concentrations, would further decrease the pH (verapamil in solution is acidic, a 0.1% aqueous solution has a pH of 5.25 (Merck Index)), possibly to a level capable of modulating the toxicity of the drug and the effectiveness of the chemotherapeutic agent. As verapamil was extremely toxic to the cells in which antagonistic combinations of VP-16 and verapamil were noted, any reduction in the intracellular concentration of the circumventing agent may enhance cell survival. An increase in the concentration of the circumventing agent to which the cells were exposed, may result in a decrease in the extracellular pH, resulting in a reduction in verapamil uptake and toxicity, and a reduction in the enhancement of VP-16 toxicity, as was noted in these cases. However, this does not fully explain the effect observed in the cells in this study, whereby at low verapamil concentrations an enhancement of VP-16 toxicity was noted. This implies that at low concentrations, verapamil was acting as an effective VP-16-toxicity enhancing agent.

It has been shown extensively that verapamil circumvents drug resistance by competing with the drug for efflux *via* the P-glycoprotein pump, resulting in increased cellular accumulation of the drug and cell kill (Tsuruo *et al.*, 1981; Harker *et al.*, 1986; Sikic *et al.*, 1989; Barrand *et al.*, 1993). However, an optimum concentration of the circumventing agent generally resulted in maximum drug accumulation-associated cell kill. In this case, perhaps maximum enhancement of VP-16 toxicity was produced by a low intercellular concentration of verapamil. This would account for the synergistic effect observed between VP-16 and verapamil at low concentrations of the circumventing agent. At higher intercellular levels of the circumventing agent, the beneficial effects exerted regarding enhancement of drug toxicity may be balanced by the toxicity exerted by the verapamil itself, resulting in no apparent enhancement of VP-16's toxicity. At high verapamil concentrations, the acidity of the media would affect the cells' accumulation of verapamil and modulate the effectiveness of verapamil, resulting in the increased cell survival observed.

However, the toxicity of VP-16, in the presence of verapamil, may also be modulated *via* its action as a topoisomerase II poison. Alterations in the function of

topoisomerase II, caused by verapamil, may alter cell survival in the presence of VP-16. Verapamil is a calcium antagonist, which alters the level of intracellular Ca^{2+}. Therefore, as the concentration of verapamil increased, the level of intracellular Ca^{2+} may have decreased. Topoisomerase II activity requires the presence of divalent cations (Osheroff, 1986; 1987) and Ca^{2+} has been shown to be capable of supporting topoisomerase II activity (Osheroff and Zechiedrich, 1987). If calcium levels were decreased, the levels of enzyme activity may be reduced, resulting in decreased VP-16 toxicity to the cells. Meech and Thomas (1977) have shown that an increase in intracellular Ca^{2+} levels resulted in a decrease in the intracellular pH of cells and a decrease in intracellular pH has also been associated with verapamil-mediated enhanced cell kill (Hamilton *et al.*, 1993a; Keizer *et al.*, 1989). Therefore, if the converse holds true, a decrease in intracellular Ca^{2+} would result in a decreased intracellular acidification and increased cell survival in the presence of drug.

Adriamycin and vincristine have alternative modes of toxicity, other than intercalating with the topoisomerase II cleavable complex. Therefore, verapamil may enhance the toxicity of these drugs by increasing their presence in the cell and thereby allowing them to induce a topoisomerase II-independent mode of cell kill. Cyclosporin A is not a calcium antagonist and therefore would not affect the topoisomerase II-mediated toxicity of VP-16.

In this research work, it has been shown that the combination of VP-16 and verapamil enhanced toxicity in some of the lung cell lines and diminished toxicity in others. An alternative explanation for this phenomenon, other than that outlined above, is that the topoisomerase II enzyme was altered in the cell lines where diminished toxicity, resulting from the combination of the chemotherapeutic drug and the circumventing agent, was observed. A mutated topoisomerase II enzyme has been described in MDR cells that requires increased ATP to sustain equal topoisomerase II activity as that observed in the sensitive cell line (Danks *et al.*, 1989). In relation to the modification of VP-16 toxicity in this study, perhaps the topoisomerase II enzyme is altered in DLKP/VP-8, DLKP/VP-3, DLKPA2B and DLKPA6B such that higher concentrations of divalent cation are required for its activity. Therefore, topoisomerase II-mediated toxicity by chemotherapeutic drugs would be decreased in the presence of agents that decrease levels of intracellular Ca^{2+}.

However, this does not explain why adriamycin toxicity is enhanced by verapamil, in the same cell lines. Perhaps the adriamycin toxicity is mediated by a mechanism other then as a topoisomerase II poison in these cell lines, when co-exposed to verapamil (as suggested previously). To further elucidate the phenomenon that is occurring between VP-16 and verapamil in these cell lines, the toxicity of various combinations of verapamil and other drugs (both topoisomerase II poisons *e.g.* VM-26, amsacrine and non-topoisomerase II-mediated toxins *e.g.* vinblastine) could be studied.

4.11 Cell density effects

The toxicity of chemotherapeutic drugs to variants of DLKP was observed to be a function of the density at which the cells were exposed to the drug (Section 3.13). All cells investigated in this work were more sensitive to adriamycin when exposed at low density than at higher densities. Cell density dependent adriamycin toxicity was also noted in the VP-16-selected cells and VP-16 toxicity was subject to the cells' plating density in both DLKP and DLKP-A cell lines. This implies that a cell density dependent mechanism of resistance, or enhanced sensitivity, existed in the cell lines.

This alteration in the cells' sensitivity to chemotherapeutic drugs may have resulted from a physical and/or biochemical modification of the cells and/or their environment. Decreased adriamycin toxicity to cells, at high cell densities, may have resulted from decreased intracellular accumulation, thus allowing the adriamycin sensitive cells to survive in the presence of drug levels which would cause cell kill, when analyzed under the non-confluent conditions associated with the toxicity assays. Dimanche-Boitrel *et al.* (1992) observed a confluency-dependent resistance to adriamycin, vincristine and VP-16, in the human colon cancer cell line, HT-29. The decrease in sensitivity was reflected in a lower intracellular level of drug and the altered drug penetration with confluency could be related to a decrease in plasma membrane fluidity. Pelletier *et al.* (1990) also noted that both the cytotoxic efficacies of adriamycin, and the nuclear drug concentration, progressively decreased with increasing confluency, in the human and rat colon cell line HT-29 and DHD/K12/TRb, respectively.

During routine maintenance of the DLKP and DLKP-A cell lines in culture, it was noted that better growth occurred when the serum-supplemented medium was left on the cells for a number of days, as opposed to daily changes of the media (results not shown). At the stage of the initial isolation of the DLKP-A clones, it was also observed that better cell growth occurred when the cells, plated at the low density required to isolate clones, were partially fed with conditioned medium produced by the DLKP-A population (50% conditioned medium and 50% fresh medium). This suggests that DLKP variants may be producing an autocrine growth stimulating factor (or factors). This factor may also be involved in altering their sensitivity to adriamycin. Huang and Wright (1994) observed that NIH3T3 cells transfected with ßFGF, a non-oncogene member of the fibroblastic growth factor family, exhibited a significant elevation in N-(phosphonacetyl)-L-aspartate resistance, while Zuckier and Tritton (1983) demonstrated a drug-dependent increase in the capacity of HeLa and 3T3 cells, grown in the presence of lethal and sub-lethal concentrations of adriamycin, to bind EGF. Shin *et al.* (1991) noted the simultaneous amplification of EGF receptor and mdr1 genes, in a poorly differentiated adenocarcinoma of the human lung. Meyers *et al.* (1993) have also observed an

increase (3-fold) in the number of EGF receptors in MDR variants of the Chinese hamster lung DC-3F cell line, which overexpresses P-glycoprotein.

The pH of the extracellular environment is capable of influencing the toxicity of drugs to cells. Hamilton *et al.* (1993a) noted that acidic extracellular media reduced the toxicity of adriamycin to drug resistant variants of HT-29 and SW-420 cells. At high densities, cells would modify the pH of the growth medium to a greater extent than comparable cells at low cell densities, due to the breakdown of nutrients present in the medium and the production of waste products. Therefore, at high cell plating densities, adriamycin may be less effective at inducing cell kill, due to modification of the extracellular environment.

Alterations in drug toxicity to cells, in a density dependent manner, may also be associated with intercellular transfer of protective compounds by cells in contact, *via* gap junctions. Loewenstein (1979) has shown that thioguanine sensitivity can be transferred from thioguanine sensitive cells to resistant cells, by a mechanism involving cell contact, suggesting that the modification may be due to intercellular transport of toxic compounds, *via* gap junctions. If cell contact can increase the cells' sensitivity to a toxin, then perhaps the converse holds true, whereby cell contact may allow the exchange of compounds which would protect sensitive cells from toxins.

4.12 Gap junctional intercellular communication

Gap junctions are intercellular communication ports, through which small organic molecules, inorganic ions and electrical signals pass (Gilula *et al.*, 1972). Gap junctions occur where the cell surface membrane of adjacent cells are separated by a gap of approximately 15nm (Darnell *et al.*, 1986) and the interior of the two cells are linked as a result of the joining of two structural units, one on each cell membrane. The structural unit of the gap junction, termed connexon, is a collection of six protein subunits (connexin), spanning the plasma membrane, which are paired, end to end, with an identical hexamer on the adjacent cell, forming a cylindrical channel of approximately 1.5-2.0 nanometers in inner diameter, across the intercellular gap (Revel *et al.*, 1986). Hydrophilic amino acid residues line the inner channel of the gap junction and the channel is insulated by peripherally organised hydrophobic amino acid residues (Goodenough and Revel, 1970), allowing the intercellular flow of hydrophilic molecules of molecular weights less than approximately 1000, by passive diffusion.

The gap junction may be composed of mixes of different proteins (Nicholson *et al.*, 1987) and a wide range of connexin proteins have been identified in the same and in different tissues (Table 4.3). The naming system of the connexin proteins consist of the use of the word

connexin in conjunction with the predicted molecular weight of the protein.

Protein	Tissue	Reference
Connexin 70	Lens Fibres	Kistler *et al.*, 1988
MP 70	Lens Fibres	Gruijters *et al.*, 1987
Connexin 56	Lens	Rup *et al.*, 1993
Connexin 43	Heart Lung Pancreas Bone Marrow	Beyer *et al.*, 1987 Chaudhuri *et al.*, 1993 Meda *et al.*, 1991 Dorshkind *et al.*, 1993
Connexin 40	Heart	Beyer *et al.*, 1992
Connexin 37	Blood vessel endothelial cells	Reed *et al.*, 1993
Connexin 32	Liver	Paul, 1986
Connexin 28	Liver	Heynkes *et al.*, 1986
Connexin 26	Liver	Zhang and Nicholson, 1989
Major Intrinsic Protein 26	Lens	Gorin *et al.*, 1984
Connexin 16	Liver Heart	Finbow *et al.*, 1985

Table 4.3 : Tissue distribution of putative gap junction proteins.

Heterologous cells in culture, from differing tissues, can form gap junctions (Epstein and Gilula, 1977). Connexons can exhibit both functional (open) and non-functional (closed) states and studies have produced evidence that cells joined by gap junctional structures may not be functionally coupled (Flagg-Newton and Loewenstein, 1979). Gap junction function is regulated by Ca^{2+} and closure of the intercellular communication port is induced by increased levels of intercellular Ca^{2+}. Spray *et al.* (1982) found that both H^+ and Ca^{2+} could act as independent gap junctional blockers in blastomere cell pairs. Intercellular gap junction communication may also be blocked by other cellular secondary messengers, such as cAMP (Murray and Fletcher, 1984) and the phosphoinositide signal pathway compounds (Yada *et al.*, 1985). Certain anaesthetics also inhibit gap junction mediated intercellular communication (Mantz *et al.*, 1993; Bohrmann and Haas-Assenbaum, 1993; Meda *et al.*, 1990). The down-regulation of functional gap junctions has been detected as a conformational change, whereby the connexon straightens and slides radially, in a shutter-like system (Unwin and Ennis, 1984). However, Loewenstein and Rose (1978) observed that functional uncoupling of gap junctions may

result from increased intracellular concentrations of Ca^{2+} in the vicinity of the junction, without detectable alterations of the structure of the gap junctions.

The literature has shown that various cancer cells have lost or decreased gap junction intercellular communication capacities (Klann *et al.*, 1989; Boreli *et al.*, 1969), but not all tumorigenic or transformed cells exhibit this decreased intercellular communication (Yamasaki *et al.*, 1987; Katoh and Yamasaki, 1991). When Chaudhuri *et al.* (1993) examined gap junctional intercellular communication in non-transformed and transformed mouse lung epithelial cells and in a non-transformed cell line treated with tumour promoters, a correlation was observed between the *in vitro* inhibition of gap junctional intercellular communication and lung tumour promotion.

Heptanol is a long chain alcohol which reversibly closes gap junctions by physically reacting with the gap junction plasma membrane assembly, abolishing cell to cell coupling (Nathanson and Burgstahler, 1992; Perez-Armendariz *et al.*, 1991; Christ *et al.*, 1991). Bastiaane *et al.* (1993) suggested that a heptanol-induced decrease of gap junctional-intercellular communication in rat cardiac cells was mediated by a decrease in the fluidity of the cholesterol-rich domain of the membrane, in which the gap junction channels were embedded. Heptanol did not perturb intracellular pH or Ca^{2+} (Meda *et al.*, 1990) and did not cause detectable changes in the actual structure and size of the gap junctions (Meda *et al.*, 1986).

To determine if gap junction intercellular communication was occurring in the squamous cell carcinoma of the lung, DLKP, and its drug-resistant variants, the intercellular transfer of lucifer yellow was investigated (El-Fouly *et al.*, 1987). In the course of the research described in this thesis (Section 3.14), functional gap junctional intercellular channels were observed in DLKP and its MDR variants, as determined by lucifer yellow transfer. When the cells were pre-incubated with 1.5mM heptanol for 1.5h. and continually exposed to heptanol during detection of active gap junctions, no intercellular communication was observed, implying that heptanol inhibited the gap junctional intercellular communication previously noted in DLKP variants.

Although active intercellular communication was observed in all cell lines, the intercellular transfer of fluorescent adriamycin was not observed in any DLKP variant. In the course of assaying for intercellular communication in DLKP variants, adriamycin was observed to diffuse into all variants of DLKP when the cells were incubated in a calcium-free solution containing 100μM adriamycin, for 10 minutes. When the cells were examined by fluorescent microscopy, DLKP cells were observed to possess a brighter background than the drug-resistant variants, suggesting that the DLKP cells accumulate more adriamycin than the resistant cells. The protocol followed to detect the presence of active intercellular transport of adriamycin did not allow the exact intracellular localisation of the adriamycin within the cells to be ascertained.

As all MDR variants of DLKP have been shown to overexpress P-glycoprotein, the decreased background fluorescence in the drug-resistant cells could be explained by a higher level of drug efflux from the cells. However, cells damaged by the scrape-loading of the adriamycin fluoresced much more brightly than the background fluorescence, in all cell lines, and this more intense fluorescence was not transferred to neighbouring cells. In the presence of cyclosporin A, which would block the rapid efflux of adriamycin from the cells *via* P-glycoprotein in the MDR cells, an absence of intercellular transfer of adriamycin was still found. Therefore, the lack of detectable intercellular transfer of adriamycin, in the MDR cells, was not due to a masking of the effect by the drug's P-glycoprotein-mediated drug efflux. This implies that adriamycin is not transferred from cell to cell *via* gap junctions, even though it is a water soluble compound and within the size range of molecules transferred by gap junctions. However, the ability of a molecule to be transferred intercellularly also depends on it's shape (P. Meda, personal communication). A molecule must have a diameter of less than 1.8nm to enable it to be transferred *via* gap junctions. An extensive literature survey has revealed a lack of scientific papers detailing gap junctional intercellular transfer of chemotherapeutic drugs. In this study, the intercellular transfer of any non-fluorescent adriamycin degradation products produced by the cell or of small molecules or inorganic ions which may have resulted from the presence of adriamycin in the cells, was not detectable by the experimental procedures.

Gap junction intercellular communication can only occur in cells which are in contact. At plating density routinely used in assays, cells were not in contact, resulting in no gap junctional communication and any cells which formed contacts under these circumstances are generally derived from the same single cell, resulting in gap junctional communication between homologous cells. For gap junctional intercellular communication to function in potentiating or diminishing the toxicity of chemotherapeutic drugs to cells, functional gap junctions must exist between heterologous cells, with respect to their toxicity to adriamycin, allowing toxic, or protective, molecules to be transferred from sensitive to resistant cells, or *vice versa*. Heptanol can only be assumed to alter the toxicity of adriamycin to cells *via* a gap junctional mediated mode of action, if a differential toxicity change is observed in the cell line in both the presence and absence of heptanol, and at both a high and low plating density. Heptanol induced alterations in adriamycin toxicity occurring at low plating density may be assumed to be mediated by a mechanism, or mechanisms, other than gap junctional communication.

The inhibition of gap junctional intercellular communication by heptanol neither potentiated nor diminished the toxicity of adriamycin to DLKP, its MDR variant DLKP-A, the cloned cell line DLKPA5F or the cell line generated by mixing the clones, at high density, over that observed at low plating density. Exposure of the DLKPA5F and the cell line generated by mixing the clones, to both adriamycin and heptanol resulted in no significant alteration to

adriamycin-associated toxicity, by comparison with that observed in the absence of heptanol. In both the parental DLKP cell line and its MDR variant DLKP-A, heptanol exhibited a protective effect on the cells, with respect to adriamycin toxicity. This effect was observed at both high and low plating density, implying that the heptanol associated decrease in adriamycin toxicity, was by a means other than the gap junctional intercellular communication pathway.

Heptanol appeared to enhance the toxicity of adriamycin to DLKPA2B at high cell density and reduced its toxicity at low plating density, which implies that gap junctional intercellular communication may alter the cells' susceptibility to kill by adriamycin. At low cell plating density, heptanol exhibited a protective effect towards DLKPA2B with respect to adriamycin. The effect also seen in DLKP and DLKP-A at both high and low cell plating densities implying that it may have mediated a cellular mechanism other than gap junctional communication, which in turn resulted in reduced cell sensitivity to adriamycin.

Intercellular transfer of adriamycin *via* gap junctional communication ports was not observed in DLKPA2B, implying that any gap junction associated alterations in adriamycin-induced cell kill, were mediated by other small molecular weight compounds. Gap junctional transfer of H^+ has been widely documented in the literature (Hertzberg *et al.*, 1981) and alterations in intercellular pH has been associated with enhanced cell kill. Barry *et al.* (1993) observed that VP-16-induced apoptosis in human HL-60 cells was associated with intracellular acidification. MDR cells have been reported to exhibit an increase in intracellular pH (Boscoboinik *et al.*, 1990) and circumvention agents have been shown to decrease the cytosolic pH (Hamilton *et al.*, 1993(a, b); Keizer and Joenje, 1989), resulting in increased drug accumulation (Simon *et al.*, 1994) and therefore, cell kill. Therefore, any modification in the H^+ intercellular transfer pathway may result in alterations in cell kill. Perhaps H^+ was transported from sensitive cells to more resistant variants, thereby increasing the resistance of the sensitive cells. However, if gap junctional transfer of protective agents to sensitive cells, or of toxins away from the sensitive cells is occurring, the molecule may be other than H^+. For gap junctional intercellular transfer of cell-protective compounds to occur in DLKPA2B, but not in DLKP-A or the other clones derived from it, the cell line must be assumed to be a very different variant of DLKP-A and must also be composed of a sub-population of cells which exhibit altered capabilities to survive in the presence of adriamycin. However, the cell line DLKPA2B was established as a cloned cell line of DLKP-A. The fact that heptanol enhanced adriamycin toxicity to DLKPA2B implies that the drug's toxicity is being masked by a gap junction associated function. Alternatively, heptanol may be acting *via* a hitherto undiscovered mechanism. DLKPA2B was also one of the cell lines where the combination of VP-16 and verapamil resulted in diminished overall toxicity, in the circumventing assays, a phenomenon which may also be associated with alterations in intracellular pH and Ca^{2+}.

4.13 Cell contact independent modification of adriamycin toxicity

Cell contact-mediated intercellular communication could not fully explain the differential adriamycin toxicity profiles obtained at high and low cell plating densities. Therefore, the effect of intercellular communication by means other than direct cell contact, on the cells' sensitivity to adriamycin, was investigated in a preliminary assay, which was only performed once.

Conditioned media generated by the more resistant cell clone DLKPA5F, during its exposure to adriamycin, modified the adriamycin sensitivity of the least resistant clone DLKPA2B. The decrease in the sensitivity of DLKPA2B to adriamycin toxicity, when exposed in the presence of conditioned media produced by DLKPA5F, was observed at high and low DLKPA2B plating densities. When the adriamycin sensitivity of DLKPA2B was examined at high cell density, the conditioned media from both DLKPA2B and DLKPA5F, generated in the presence and absence of adriamycin, caused a slight increase in the ability of DLKPA2B to survive in the presence of adriamycin.

Therefore, it appears that both DLKPA2B and DLKPA5F secrete a compound, or compounds, that modifies adriamycin toxicity to DLKPA2B and this product is more active, or is secreted to higher concentrations, by DLKPA5F, when the cells are grown in the presence of adriamycin. Interactions between the two cell populations affected the sensitivity of the most sensitive cell line to the chemotherapeutic agent, adriamycin, and the interaction was mediated by a means independent of cell contact.

As previously mentioned, it was noted that better growth occurred in DLKP and DLKP-A cells when the medium was left on the cells for a number of days, as opposed to being changed daily and that better cell growth occurred when the cells (plated at the low density required to isolate clones), were fed with conditioned medium produced by the DLKP-A population. These observations imply that DLKP variants may be producing an autocrine growth stimulating factor (or factors), which may also be involved in altering their sensitivity to adriamycin. Zuckier and Tritton (1983) noted that cells grown in the presence of adriamycin possessed increased EGF binding capacities. Meyers *et al.* (1993) and Shin *et al.* (1991) have associated the expression of EGF receptors and P-glycoprotein. However, Kwok and Sutherland (1991) found that EGF increased the sensitivity of a human squamous carcinoma cell line, A431, to adriamycin. The relative enhancement of sensitivity by EGF was greater in two adriamycin-resistant variants, which also possessed increased numbers of EGF receptors, in comparison with the parental cells. When Ogawa *et al.* (1993) investigated the relationship between EGF receptor and cisplatin sensitivity in 84 lung carcinomas, the expression of EGF receptors and proliferating

cell nuclear antigen was significantly lower in cisplatin-sensitive non-small cell carcinomas and squamous cell carcinomas. Alterations in TGF-ß levels have also been associated with the sensitivity of cells to chemotherapeutic drugs (Su *et al.*, 1991; Huang and Wright, 1994).

The literature has shown that cells interact and modify the drug sensitivity of other cells. This has been widely demonstrated when cells of differing sensitivity are grown in contact (Miller *et al.*, 1989; 1991), but the phenomenon has also been noted when cells are grown physically separated but sharing the same host. Miller *et al.* (1981b) observed that the presence of a sensitive subpopulation of a mouse mammary tumour, enhanced the cyclophosphamide sensitivity of a more resistant subpopulation, when the two populations were located on opposite flanks of male BALB/cfC3H mice, implying a cell-contact independent mechanism of enhancement of cyclophosphamide toxicity.

4.14 Co-existence of populations of differing adriamycin sensitivity in DLKP-A

Clones have been isolated from the DLKP-A population and the toxicity profiles of these clones imply that cells of varying adriamycin sensitivity are existing within the DLKP-A cell line. When the clones were examined in isolation, some of the cell lines were sensitive to the concentration of adriamycin to which the DLKP-A cell line has been selected. Yet, in the cell mixture that makes up DLKP-A, the cells survive just as well as clones that possess higher adriamycin resistance. Cells which possess a level of adriamycin resistance greater than the concentration of adriamycin to which DLKP-A has been exposed, survive because they have not been exposed to the level of toxin required to cause cell kill. However, the clones which are sensitive to the level of adriamycin to which DLKP-A has been exposed, can only have survived due to assistance from other cells and this assistance may have taken a number of forms.

The DLKP-A cell line was established by exposing the uncloned squamous cell carcinoma of the lung, DLKP, to increasing concentrations of adriamycin over a prolonged time period, resulting in stable genetic alterations, associated with the acquisition of an MDR phenotype (Clynes *et al.*, 1992). Therefore, the possibility exists that the drug sensitive DLKP cells consist of a heterogenous population, with respect to their adriamycin sensitivity. Variation with respect to drug sensitivity within tumours has been widely documented in the literature (Dolfini *et al.*, 1993; Tanigawa *et al.*, 1984; Allalunis-Turner *et al.*, 1993). Consequently, the potential existed for the drug resistant cell line, DLKP-A, to have been selected from one pre-existing resistant cell or to have been induced following exposure to adriamycin. In both cases, adriamycin exposure may have caused mutations, which could alter the resistance of either an initially homologous population, or a population which originally exhibited heterogeneity in its

adriamycin resistance profile.

During the initial selection process, by which the MDR cell line was derived, the DLKP cells were grown at high density, approximately 50-70% confluent, prior to the initial drug exposure. The initial drug concentration, to which the cells were exposed, reduced the cell population to approximately 5%. However, the cells remaining were generally clumped in small colonies and did not consist of single, dispersed cells. These colonies were then grown, at that drug concentration, until they had attained high cell numbers and appeared healthy. At this stage, the adriamycin concentration was doubled (A. Redmond, Ph.D. thesis, Dublin City University, 1991). During selection of the DLKP-A cell line for resistance to adriamycin, the cells also underwent a change in their growth patterns, from monolayer sub-confluent growth to a state where areas of confluency occurred, in a dispersed manner. Therefore, during the induction of an MDR phenotype in DLKP areas of confluency constantly existed. Consequently, some less resistant cells may have been protected from adriamycin-induced cell kill, by having their sensitivity to adriamycin altered, by the physical protection of a layer of surrounding cells.

DLKP-A is a cell line composed of a mixture of cells, with respect to their level of resistance to chemotherapeutic drugs, mode of resistance to these drugs and cell doubling times. Yet the subpopulations were stably co-existing within the MDR cell line. Therefore, it must be assumed that intercellular communication is occurring, whether mediated by a cell contact independent means *e.g.* growth factor- or hormone-mediated communication, and/or a cell contact means *e.g.* intercellular gap junction communication, as outlined previously.

The literature has revealed a vast supply of information on intercellular interactions, resulting in the modification of the characteristics of the individual cells involved in the interactions and the generation of a population with its own distinctive attributes. These interactions have modified growth properties of the cells (Leith *et al.*, 1987; 1988) and the cells' drug sensitivity (Miller *et al.*, 1981b; 1989), and these interactions are a probable means by which cells in the DLKP-A population, that are individually not capable of surviving, can grow in the presence of specific adriamycin concentrations. Decreased adriamycin toxicity to cells, in areas of confluency, may have resulted from decreased intracellular accumulation. This may have allowed sensitive cells to survive at drug levels which should cause cell kill, when analyzed under the sub-confluent conditions associated with the toxicity assays. Dimanche-Boitrel *et al.* (1992) noted confluency-dependent resistance to a range of chemotherapeutic drugs, in human colon cancer cells. The decrease in the cells' sensitivity to adriamycin, vincristine and VP-16 was reflected in a lower intracellular level of drug. The altered drug penetration with confluency could be related to a decrease in plasma membrane fluidity. Pelletier *et al.* (1990) also noted that both the cytotoxicity of adriamycin, and the concentration of drug located in the nucleus, progressively decreased with increasing confluency, in the human and rat colon cell lines, HT-29

and DHD/K12/TRb, respectively.

The density at which DLKP-A were exposed to both adriamycin and VP-16 affected the toxicity of drugs to a greater extent than the density-associated alterations in drug toxicity observed in the sensitive cell line, DLKP or in the DLKP-A cell clones (Section 3.13). As greater cell survival resulted at higher cell densities, in the presence of drug, it implies that the heterogeneity of the DLKP-A cells allowed the more sensitive cells to survive, especially at high cell density. Although gap junction intercellular transfer of adriamycin does not appear to occur, transfer of toxicity-related small molecules may be occurring, as previously discussed. Gap junctional-associated modifications to adriamycin toxicity to DLKP-A, however, were not detected. As drug toxicity has been linked with intracellular acidification (Hamilton *et al.*, 1993(a, b); Boscoboinik *et al.*, 1990), gap junctional transfer of ions could modulate the toxicity of individual cells, generating an apparent uniform population, with respect to adriamycin toxicity, at high cell density or areas of confluency. In the DLKP-A cell line, during selection with adriamycin, the more sensitive cells may have survived if a cell population, or populations, existed which could modify the drugs' toxicity to the sensitive cells. Therefore, if the degree of adriamycin toxicity to DLKP-A is associated with the intracellular pH of the cells, the transfer of ions *e.g.* H^+, Ca^{2+}, from less resistant to more resistant cells, *via* gap junctions, in areas of confluency, may have allowed the sensitive cells *e.g.* DLKPA2B, to survive at adriamycin concentrations which, individually, would cause cell kill.

Modifications to the extracellular environment may also have allowed the more sensitive cells to be maintained in the DLKP-A population. During the adriamycin selection process, DLKP-A cells were exposed to fresh drug only when cells were over 50% confluent and appeared healthy. Therefore, the cells were producing and secreting metabolites and so, were constantly altering the extracellular pH, changing it to a more acidic environment. Adriamycin accumulation by cells is not only dependent on intracellular pH but also on the extracellular pH. A decrease in the pH of the extracellular environment has been shown to protect cells from cytotoxicity (Jahde *et al.*, 1990). Consequently, acidification of the extracellular media, alone or in conjunction with the possible intracellular alterations previously mentioned, may have enabled the more sensitive cells to survive.

Adriamycin-sensitive cells may also survive in the presence of toxic concentrations of the drug, in the DLKP-A population, due to modification of the medium in which the cells are grown, by the production of specific compounds by subpopulations within DLKP-A. The research work presented in this thesis, showed that conditioned medium, produced by DLKPA5F, when grown in the presence of adriamycin, partially protected DLKPA2B cells from the toxic effects of the drug (Section 3.15). Miller *et al.* (1981) have also shown that factor(s) produced by cells, not growing in contact, can modify each others sensitivity

to drugs. Therefore, as factor(s) produced by DLKPA5F can modify the sensitivity of DLKPA2B to adriamycin, it would follow that factor(s) produced by the DLKPA5F population, or other subpopulations of DLKP-A, while in the DLKP-A cell line, would also modify the sensitivity of cell subpopulations. This would alter the sensitivity of the overall DLKP-A population to adriamycin, and so, cells may be surviving in the presence of concentrations of adriamycin which, if the cell 'protective' factor(s) were removed, would cause cell kill. The factor(s) produced by DLKP-A subpopulations may be growth stimulatory factors, growth inhibitory factors or other, as yet undefined, factors. Growth factors have been shown to modify the sensitivity of cells to drugs, both enhancing their sensitivity (Kwok and Sutherland, 1991) and resistance (Huang and Wright, 1994). Overexpression of growth factor receptors has also been associated with MDR (Shin *et al.*, 1991; Meyers *et al.*, 1993; Ogawa *et al.*, 1993). Growth inhibitory factors would decrease the growth rate of the cell subpopulations, and so alter their sensitivity to chemotherapeutic drugs. The sensitivity of cells to a range of chemotherapeutic drugs is correlated to their growth rate. Cell undergoing active proliferation are more sensitive to drug-induced cell kill, than quiescent cells (Chambers *et al.*, 1984).

4.15 Implications for clinical therapy of cancer.

The acquisition by cancer cells of resistance to a broad range of chemotherapeutic drugs, or their inherent resistance, is a major problem in the effective treatment of cancer. Much research has been carried out in developing mechanisms of overcoming this resistance and improving patient therapy. However, the characteristics of the individual cancer cells themselves are not the only important factors to be considered when devising effective treatment regimes, the extracellular environment, the structural organisation of the tumours and cell heterogeneity and interactions must also be considered.

Tumour heterogeneity has been widely documented in the literature and has been reflected by cell variations observed in established cell lines, derived from tumour samples. Perhaps the most important considerations of tumour cell heterogeneity is the variability in drug and radiation resistance, growth properties and tumourogenicity exhibited by clonal subpopulations (Dolfini *et al.*, 1993; Allam *et al.*, 1993; Fried *et al.*, 1993; Okabe *et al.*, 1983). To effectively treat cancer, the most resistant subpopulation must be killed, otherwise the tumour may regrow, with altered properties, giving rise to not only a regenerated tumour, but possibly metastasis, with altered properties.

The existence of variation within MDR cell lines (Yang *et al.*, 1993 and as shown in this thesis, with respect to DLKP-A) may add an extra variable to the problem of treating drug-resistant cancers. The ability of subpopulations to interact and modify each other's drug sensitivity, when grown either in contact or separately, may further complicate the situation. Cell interactions have been shown to enhance the sensitivity of cells to chemotherapeutic agents, when the cells are grown both in contact and separately (Miller *et al.*, 1981; 1988; 1991), thereby exhibiting an optimistic prospect for cancer chemotherapy, especially that of combination chemotherapy, whereby cells which are resistant to one drug may be sensitive to another drug. However, in this study, interactions between subpopulations of the MDR cell line DLKP-A, have been shown to enhance the resistance of the most sensitive subpopulation. Therefore, the efficacy of drugs to cells may be decreased by the presence of surrounding, or distant, cells.

Before chemotherapeutic drugs can be effective in the treatment of cancers, they must be capable of reaching their cellular target. Most drugs used in cancer treatment possess cell cycle specific targets, so that the drug is more effective against growing cells than those in quiescence. Therefore, the target is generally the cell nucleus and factors which enhance drug accumulation by the cells, or alter cell growth, may act as chemotherapeutic agents.

Solid tumours consist of clumps of cells, in which the cells at the centre of the tumour are either necrotic or quiescent and those at the outer edges undergo cell division.

Therefore, the inability of drugs to penetrate into the inner cells results in these cells not being exposed to chemotherapeutic drugs. Erlichman and Vidgan (1984) investigated the cytotoxic activity of adriamycin on the human bladder carcinoma cell line, MGH-U1, growing as monolayers, as spheroids and as xenografts in nude mice. The cells growing as spheroids were much more resistant to adriamycin than when treated as monolayers and adriamycin fluorescence was demonstrated only in the outer two layers of cells forming the spheroids, suggesting limited drug penetration. Durand (1981) observed that in Chinese hamster V79 cells grown as spheroids, a gradient of adriamycin uptake existed, with only the one or two outer layers brightly fluorescing after 1h. exposure. Growth of cells as spheroids seemed to impart an additional degree of drug resistance relative to cells grown as monolayers, in that equal toxicity required greater intracellular fluorescence for spheroids. Cells grown as spheroids are in general more resistant to a range of drugs than their counterparts, growing as monolayer cultures (Wilson *et al.*, 1981; Nederman, 1984; Sasaki *et al.*, 1984). Lack of drug penetration in tumour masses may be due to the inability of cells to intercellularly transfer the drug, *via* gap junctions. Gap junctional communication in some cancer cells has been decreased or abolished (Klann *et al.*, 1989; Borek *et al.*, 1969), but even in the DLKP-A cells, where functional gap junctional communication was demonstrated, intercellular transfer of adriamycin was not observed. Therefore, any agent that would enhance the intercellular transfer of chemotherapeutic drugs, may also be effective enhancers of cell toxicity and may be of use in the treatment of cancers.

Solid tumours consist of densely packed cells, with only the outer layers dividing. Confluent cells have been shown to accumulate lower levels of drugs (Dimanche-Boitrel *et al.*, 1992; Pelletier *et al.*, 1990) and confluent, non-proliferating cells are less sensitive to chemotherapeutic agents than proliferating cells (Chambers *et al.*, 1984; Drewinko *et al.*, 1981). In the course of research undertaken for this thesis, it was noted that adriamycin and VP-16 toxicity to variants of DLKP was cell density dependent. This cell density dependent toxicity of chemotherapeutic drugs may also be due to physiological alterations that have occurred to the extracellular environment. Drug accumulation is decreased at reduced pH values (Hamilton *et al.*, 1993b) and cell growth, both in culture and tumours, may result in the production of waste products which would alter the pH of the surrounding environment. A decrease in the pH of the extracellular environment may therefore result in decreased drug accumulation and enhanced cell survival.

In culture, and therefore possibly in tumours, the effectiveness of chemotherapeutic agents in inducing cell kill is dependent not only on the sensitivity of individual cells, but on the ability of the cell populations to alter the chemosensitivity of surrounding cells and to modify the environment in which the cells exist. Intercellular interactions, whether mediated *via* cell contact-independent or -dependent mechanisms, are also capable of modulating the cells' characteristics. Therefore, when analyzing the toxicity of chemotherapeutic drugs to

cells in culture, interactions capable of altering the cells' sensitivity must be considered, when correlating the findings of cell culture systems to *in vivo* situations.

5. Conclusion

This project investigated mechanisms by which subpopulations of the human squamous cell lung carcinoma, DLKP, exert their drug-resistance phenotypes. The existence of heterogeneous cell populations within the adriamycin-selected MDR variant of DLKP was studied, by isolating and characterising subpopulations of the cell line. The ability of cells of varying characteristics to exist as a cell line was investigated, by examining possible cell interaction mechanisms.

Variants of DLKP, selected by exposure to VP-16, exhibited an MDR phenotype, in that the cell lines were resistant to VP-16, adriamycin and vincristine, but they exhibited hyper-sensitivity to cisplatin. The drug resistance of the cell lines was mediated by the over-expression of P-glycoprotein, reduced drug accumulation and decreased levels of topoisomerase II. Cyclosporin A and verapamil reversed the accumulation of adriamycin to levels observed in the sensitive parental cells and caused partial reversal of the cells' resistance to adriamycin and vincristine. Verapamil proved to be highly toxic to the VP-16-selected MDR cell lines and the simultaneous exposure of VP-16 and verapamil to the cells resulted in less cell kill than their individual toxicities would indicate.

Clonal subpopulations of the adriamycin-selected MDR cell line, DLKP-A, all exhibited an MDR profile. The resistance to adriamycin, displayed by the clonal subpopulations, spanned a 9-fold range, from less resistant than DLKP-A cells to more resistant. The MDR profile observed in the clonal populations was mediated, at least in part, by alterations in the levels of both P-glycoprotein and topoisomerase II, with each cell clone possessing an individual profile. Further characterisation of the clones revealed that the DLKP-A cell line was also heterogeneous in terms of its drug- and radiation-sensitivity and with respect to its cell doubling time. Exposure of the DLKP cells to adriamycin and the conferring of a P-glycoprotein and topoisomerase II mediated MDR profile, did not alter the combined (DLKP-A) cells' sensitivity to radiation. Similarly, irradiation of the DLKP cell line did not confer drug resistance on the cells. Even though the DLKP-A cell line exhibited a similar cell doubling time and radiation sensitivity as the parental DLKP cell line, clones of DLKP-A showed individual cell doubling times and radiation sensitivity.

All variants of the DLKP cell line possessed functional gap junctional communication, but adriamycin was not transferred intercellularly. The inhibition of gap junctional communications did not result in an enhancement of the cells' sensitivity to adriamycin, suggesting other toxic or protective molecules were not being transferred intercellularly *via* gap junctions. However, cell density-dependent toxicity was observed in all cell lines; cells at high density were more resistant to the cytocidal and/or cytostatic effects of chemotherapeutic drugs than the same cells when exposed at low cell densities. A substance produced by the most resistant subpopulation of DLKP-A and secreted into the growth medium,

was apparently capable of masking the toxicity of adriamycin to DLKPA2B cells.

Although the primary characterisation of the VP-16-selected MDR variants of DLKP has been carried out during the course of this research work, the knowledge attained, pertaining to the mechanisms of resistance exhibited by the cells, may have many implications. The most striking of these relates to what is happening when the MDR cells are exposed to verapamil and VP-16, simultaneously. Both verapamil and VP-16 were highly toxic to the cell lines, but the combination of both was less toxic than was expected. Whether this was as a result of less accumulation of one or both of the toxins or was due to an alternative modification, could be investigated. The level of accumulation of verapamil and VP-16, by the VP-16-selected cells and their effect on the accumulation of each other, could be determined by using radiolabelled analogues of the drugs.

Other interesting future areas of research include the cellular localisation of adriamycin and other drugs within the cells. Circumventing agents have been shown to enhance the toxicity of adriamycin to DLKP cells while the same agents exhibited no alteration on the level of adriamycin accumulation by the cells. In contrast, the same circumventing agents reverse the adriamycin accumulation by MDR cells to the level observed in the drug-sensitive parents, but drug resistance was not completely reversed. The first case implies that the circumventing agents possess the ability to enhance drug toxicity in a manner independent from a drug accumulation mechanism. The second case implies that an enhancement of drug accumulation is not paralleled by an increase in toxicity. In order to determine if altered drug localisation is a mechanism of resistance displayed by the cells, the intracellular location of adriamycin (determined by fluorescent microscopic analysis), in the absence and presence of toxicity-enhancers, should be studied.

The existence of alternative mechanisms of resistance (*e.g.* GST-π, MRP) could be determined by RT-PCR and/or Western blotting analysis. This would result in a more complete profile of the mechanisms of resistance by the cells and would help to explain the cross-resistance profiles more completely.

Gap junctional communication has been demonstrated in cell variants of DLKP. However, intercellular transfer of adriamycin was not noted. This technique involved placing confluent cell populations in an adriamycin-containing Ca^{2+}-free solution, scoring through some centrally located cells with a blade and monitoring the transfer of adriamycin from these scrape loaded cells to surrounding cells. Adriamycin diffuses into cells. Hence, this an indefinitive experiment as the level of fluorescence in cells boardering the scrape was being compared with the background fluorescence. Further work, using micro-injection techniques to insert a fluorescent probe into an individual cell and examining the surrounding cells for fluorescence, which would have resulted from the intercellular transfer of the fluorescent probe, would result

in more definitive answers regarding gap junctional transfer of adriamycin. This would also act as a more exact quantification of the level of gap junctional transfer of lucifer yellow between cells.

The final, and perhaps most interesting aspect of the results, which could lead to further research, pertains to the conditioned medium experiment. Firstly, an expanded assay could be performed to establish whether, in addition to the conditioned medium produced by DLKPA5F modulating the drug-sensitivity of DLKPA2B cells, the converse also holds true. An expanded range of cell lines could be examined, not only for the production of a toxicity modulating factor (or factors) but for their responsiveness to this factor. The expression of growth factor receptors by the cells and the effect of growth factors on the toxicity of adriamycin to the cells should also be studied and may indicate whether the unknown adriamycin toxicity modulating factor is a growth factor or another type of compound. Partial, or full purification of the factor from the conditioned medium (by filtration through molecular weight cut-off membranes, gel filtration and/or high performance liquid chromatography purification), followed by the analysis and identification of the factor, would be important in determining its significance as a factor in modulating drug toxicity. An analysis of other drug-sensitive and drug-resistant cell lines could then be performed to determine the extent of production of this molecule by cells, either naturally or when stimulated by exposure to chemotherapeutic drugs.

6. Bibliography

Aabo K., Vindelov L.L., Skovsgaard T. and Spang-Thomsen M. (1987)
Interaction among two subpopulations of Ehrlich ascites tumor *in vitro*: evidence of a contact mediated immune response.
Acta Path. Microbiol. Immunol. Scand. 95 : 325-332

Ackerman P., Glover C.V.C. and Osheroff N. (1985)
Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity *in vitro*.
Proc. Natl. Acad. Sci. USA 84 : 3164-3168

Adachi Y., Luke M. and Laemmil U.K. (1991)
Chromosome assembly *in vitro*: Topoisomerase II is required for condensation.
Cell 64 : 137-148

Ahmed N.K. and Vasanthakumar G. (1987)
Characterization of daunorubicin resistance in K562 leukemia cells lacking daunorubicin reductase activity.
Eur. J. Cancer Res. Clin. Oncol. 23 : 1329-1336

Allam A., Taghiam A., Gioioso D., Duffy M., Suit H.D. (1993)
Intratumoral heterogeneity of malignant gliomas measured *in vitro*.
Int. J. Radiat. Oncol. Biol. Phys. 27 : 303-308.

Allanlunis-Turner M.J., Barron G.M., Bay III R.S., Dobler K.D. and Mirzayans R. (1993)
Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs.
Radiation Res. 134 : 349-354

Austin C.A., Sng J.H., Patel S. and Fisher M. (1993)
Novel HeLa topoisomerase II is the IIß isoform: complete coding sequence and homology with other type II topoisomerases.
Biochim. Biophys. Acta 1172 : 283-291

Baas F., Jongsma A.P.M., Broxterman H.J., Arceci R.J., Housman D., Scheffer G.L., Riethorst A., Van Groenigen M., Nieuwint A.W.M. and Joenje H. (1990)
Non-P-Glycoprotein mediated mechanism for multidrug resistance preceedes P-glycoprotein expression during *in vitro* selection for doxorubicin resistance in a human lung cancer cell line.
Cancer Res. 50 : 5392-5398

Barcellos-Hoff M.H., Linfoot P.A., Marton L.J. and Deen D.F. (1992)
Production of stable phenotypes from 9L rat brain tumor multicellular spheroids treated with 1,3-bis(2-chloroethyl)-1-nitrosurea.
Int. J. Cancer. 52 : 409-413

Barranco S.C., Townsend C.M., Jenkins V.K.Jr., Koester S.K., Ho B.Y and Reumont K.J. (1988)
Treatment-induced changes in sensitivity in a multiclonal human tumor mixture model *in vitro*.
Cancer Res. 48 : 2749-2755

Barrand M.A., Rodes T., Center M.S. and Twentyman P.R. (1993)
Chemosensitisation and drug accumulation effects of cyclosporin A, PSC-833 and verapamil in human MDR large cell lung cancer cells expressing a 190k membrane protein distinct from P-glycoprotein.
Eur. J. Cancer 29A : 408-415

Barry M.A., Reynolds J.E. and Eastman A. (1993)
Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification.
Cancer Res. 53 : 2349-2357

Baskin F., Rosenbery R.N. and Dev V. (1981)
Correlation of double-minute chromosomes with unstable multidrug cross-resistance in uptake mutants of neuroblastoma cells.
Proc. Natl. Acad. Sci. USA 78 : 3654-3658

Beck W.T. and Danks M.K. (1991)
Characteristics of multidrug resistance in human tumor cells
In: Molecular and cellular biology of multidrug resistance in tumor cells. 3-55
Ed: Roninson I.B.

Becker I., Becker K.F., Meyermann R. and Hollt V. (1991)
The multidrug-resistance gene MDR-1 is expressed in human glial tumors.
Acta Neuropathol. 82 : 516-519

Belli J.A. (1989)
Interaction between radiation and drug damage in mammalian cells IV. Radiation response of adriamycin-resistant V79 cells
Radiat. Res. 119 : 88-100

Benard J., Da Silva J., Teyssier J.-R. and Riou G. (1989)
Over-expression of MDR_1 gene with no DNA amplification in a multiple-drug-resistant human ovarian carcinoma cell line.
Int. J. Cancer 43 : 471-477

Berendsen H.H., De Leij L., De Vries E.G.E., Mesander G., Mulder N.H., De Jong B., Buys C.H.C.M., Postmus P.E., Poppema S., Sluiter H. J. and The H.T. (1988)
Characterization of three small cell lung cancer cell lines established from one patient during longitudinal follow-up.
Cancer Res. 48 : 6891-6899

Berens M.E., Bjotvedt G., Levesque D.C., Rief M.D., Shapiro J.R. and Coons S.W. (1993)
Tumorigenic, invasive, karyotypic, and immunocytochemical characteristics of clonal cell lines derived from a spontaneous canine anaplastic astrocytoma.
In Vitro Cell. Dev. Biol. 29A : 310-318

Berrios M., Osheroff N. and Fisher P.A. (1985)
In situ localization of DNA topoisomerase II, a major polypetide component of the *Drosophila* nuclear matrix fraction.
Proc. Natl. Acad. Sci. USA 82 : 4142-4146

Beyer E.C., Reed K.E., Westphale E.M., Kanter H.L. and Larson D.M. (1992)
Molecular cloning and expression of rat connexin 40, a gap junction protein expressed in vascular smooth muscle
J. Membr. Biol. 127 : 69-76

Beyer E.C. (1993)
Gap junctions.
Int. Rev. Cyto. 137C : 1-37

Beyer E.C., Payl D.L. and Goodenough D. (1987)
Connexin 43: a protein from the rat heart homologous to a gap junction protein from liver
J. Cell Biol. 105 : 2621-2629

Bhalla K., Hindenburg A., Taub R.N. and Grant S. (1985)
Isolation and characterisation of an anthracycline-resistant human leukemic cell line
Cancer Res. 45 : 3657-3662

Biedler J.L., Melera P.W., Peterson R.H.F. and Spengler B.A. (1980)
Marker chromosome with a homogeneously staining region (HSR) in vincristine-resistant cells.
Proc. Am. Assoc. Cancer Res. 21 : 292

Binaschi M., Capranico G., De Isabella P., Mariani M., Supino R., Tinelli S. and Zunino F. (1990)
Comparison of DNA cleavage induced by etoposide and doxorubicin in two human small-cell lung cancer lines with different sensitivities to topoisomerase II inhibitors.
Int. J. Cancer 45 : 347-352

Binaschi M., Giaccone G., Gazdar A.F., De Isabella P., Astaldi Ricotti G.C.B., Capranico G. and Zunino F. (1992)
Characterization of a topoisomerase II gene rearrangement in a human small-cell lung cancer cell line.
J. Natl. Cancer. Inst. 84 : 1710-1716

Boege F., Kjeldsen E., Gieseler F., Alsner J. and Biersack H. (1993)
A drug-resistant variant of topoisomerase IIα in human HL-60 cells exhibits alterations in catalytic pH optimum, DNA binding and sub-nuclear distribution.
Eur. J. Biochem. 218 : 575-584

Bohrmann J. and Haas-Assenbaum A. (1993)
Gap junctions in ovarian follicles of *Drosophila melanogaster:* Inhibition and promotion of dye-coupling between oogyte and follicle cells.
Cell Tissue Res. 273 : 163-173

Boiocchi M., Tumiotto L., Giannini F., Viel A., Biscontin G., Sartor F. and Toffoli G., (1992)
P-glycoprotein but not topoisomerase II and glutathione-S-transferase-pi accounts for enhanced intracellular drug-resistance in LoVo MDR human cell lines.
Tumori 78 : 159-166

Booser D.J. and Nortobagyi G.N. (1994)
Anthracycline antibiotics in cancer therapy: Focus on drug resistance.
Drugs 47 : 223-258

Borek C., Higashino S. and Loewenstein W.R. (1969)
Intercellular communication and tissue growth IV. Conductance of membrane junctions of normal and cancerous cells in culture
J. Membrane Biol. 1 : 274-293

Boscoboinik D., Gupta R.S. and Epand R.M. (1990)
Investigation of the relationship between altered intracellular pH and multidrug resistance in mammalian cells
Br. J. Cancer 61 : 568-572

Bradley G., Naik M. and Ling V. (1989)
P-glycoprotein expression in MDR human ovarian carcinoma cell lines
Cancer Res. 49 : 2790-2796

Bronson D.L., Andrews P.W., Solter D., Cervenka J., Lange P.H. and Fraley E.E. (1980)
Cell line derived from a metastasis of a human testicular germ cell tumor.
Cancer Res. 40 : 2500-2506.

Bugg B.Y., Danks M.K., Beck W.T. and Suttle D.P. (1991)
Expression of a mutant DNA topoisomerase II in CCRF-CEM human leukemic cells selected for resistance to teniposide.
Proc. Natl. Acad. Sci. USA 88: 7654-7658

Burden D.A., Goldsmith L.J. and Sullivan D.M. (1993)
Cell-cycle-dependent phosphorylation and activity of Chinese-hamster ovary topoisomerase II.
Biochem. J. 293 : 297-304

Campain J.A., Padmanabhan R., Hwang J., Gottesman M.M. and Pastan I. (1993)
Characterization of an unusual mutant of human melanoma cells resistant to anticancer drugs that inhibit topoisomerase II.
J. Cell. Physiol. 155 : 414-425

Capranico G., Kohn K.W. and Pommier Y. (1990)
Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin.
Nucl. Acids Res. 18 : 6611-6619

Chambers A.F., Hill, R.P. and Ling V. (1981)
Tumor heterogeneity and stability of the metastatic phenotype of mouse KHT sarcoma cells.
Cancer Res. 41 : 1368-1372

Chambers S.H., Bleehen N.M. and Watson J.V. (1984)
Effect of cell density on intracellular adriamycin concentration and cytotoxicity in exponential and plateau phase EMT6 cells.
Br. J. Cancer 49 : 301-306

Chan V.T.W., Ng S.W., Eder J.P. and Schnipper L.E. (1993)
Molecular cloning and identification of a point mutation in the topoisomerase II cDNA from an etoposide-resistant Chinese hamster ovary cell line.
J. Biol. Chem. 268 : 2160-2165

Chao N.J., Aihara M., Blume K.G. and Sikic B.I. (1990)
Modulation of etoposide (VP-16) cytotoxicity by verapamil or cyclosporin in multidrug-resistant human leukemia cell lines and normal bone marrow.
Exp. Hematol. 18 : 1193-1198

Chao Yeh G., Lopaczynska J., Poore C.M., Phang J.M. (1992)
A new functioal role for P-glycoprotein: efflux pump for banzo(alpha)pyrene in human breast cancer MCF-7 cells.
Cancer Res. 52 : 6692-6695

Chapuis J.C., Keng P.C. and Siemann D.W. (1992)
Activity of etoposide (VP-16) and teniposide (VM-26) in exponential and plateau phase human tumor cell cultures.
Anti-Cancer Drugs 3 : 245-252

Chaudhuri R., Sigler K., Dupont E., Trosko J., Malkinson A. and Ruch R. (1993)
Gap junctional intercellular communication in mouse lung epithelial cell lines: effect of cell transformation and tumor promoters
Cancer Lett. 71 : 11-18

Chen G.L., Yang L., Rowe T.C., Halligan B.D., Tewey K. and Liu L.F. (1984)
Non-intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II
J. Biol. Chem. 259 : 13560-13566

Chen Y-N., Mickley L.A., Schwartz A.M., Acton E.M., Hawang J. and Fojo A.T. (1990b)
Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein.
J. Biol. Chem. 265: 10073-10080

Chen C., Clark D., Ueda K., Pastan I., Gottesman M.M. and Roninson I.B. (1990a)
Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins.
J. Biol. Chem. 265 : 506-514

Chen C-J., Chin J.E., Veda K., Clark D.P., Pastan I., Gottesman M.M. and Roninson I.B. (1986)
Internal duplication and homology with bacterial tranport protein in the mdr1 (P-glycoprotein) gene from Multidrug-Resistant human cells.
Cell 47 : 381-389

Chin J.E., Soffir R., Noonan K.E., Choi k. and Roninson I.B. (1989)
Structure and expression of the human mdr (P-glycoprotein) gene family.
Mol. Cell. Biol. 9 : 3808-3820

Choi K., Chen C.J., Kriegler M. and Roninson I.B. (1988)
An altered pattern of cross resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene.
Cell 53 : 519-529

Chou T-C. and Talalay P. (1984)
Dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors.
Adv. Enz. Regul. 22 : 27-55

Chow K.C. and Ross W.E. (1987)
Topoisomerase-specific drug sensitivity in relation to cell cycle progression.
Mol. Cell. Biol. 7 : 3119-3123

Christ G.J., Moreno A.P., Parker M.E., Gondre C.M., Valcic M., Melman A. and Spray D.C. (1991)
Intercellular communication through gap junctions: A potential role in pharmacomechanical coupling and tissue contraction in vascular smooth muscle isolated from the human corpus cavernosum
Life Sci. 49 : 195-200

Chung I.K. and Muller M.T. (1991)
Aggregates of oligo(dG) bind and inhibit topoisomerase II activity and induce formation of large networks.
J. Biol. Chem. 266 : 9508-9514

Chung T.D.Y., Drake F.H., Tan K.B., Per S.R., Crooke S.T. and Mirabelli C.K. (1989)
Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes.
Proc. Natl. Acad. Sci. USA 86 : 9431-9435

Clynes M., Redmond A., Moran E. and Gilvarry U. (1992)
Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A.
Cytotechnology 10 : 75-89

Clynes M., Heenan M. and Hall K. (1993)
Human cell lines as models for multidrug resistance in solid tumours
Cytotechnology 12 : 231-256

Cole S.P.C., Downes H.F and Slovak M.L. (1989)
Effect of calcium antagonists on the chemosensitivity of two multidrug resistant humna tumor cell lines which do not overexpress P-glycoprotein.
Br. J. Cancer 59 : 42-46

Cole S., Downes H., Mirski S. and Clements D. (1990a)
Alteration in glutathione and glutathione-related enzymes in a multidrug-resistant small cell lung cancer cell line.
Mol. Pharmacol. 37 : 192-197

Cole S.P.C., Bhardwaj G., Gerlach J.H., Mackie J.E., Grant C.E., Almquist K.C., Stewart A.J., Kurz E.U., Duncan A.M.V. and Deeley R.G. (1992b)
Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.
Science 258 : 1650-1654

Cole S.P.C. and Deeley R.G. (1993)
Multidrug resistance-associated protein: Sequence correction
Science 260 : 879

Cole S.P.C., Chanda E.R., Dicke F.P., Gerlach J.H., Mirski S.E.L (1991)
Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: Evidence for descreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II.
Cancer Res. 51 : 3345-3352

Cole S.P.C. (1990)
Patterns of cross-resistance in a multidrug-resistant small-cell lung carcinoma cell line
Cancer Chemother. Pharmacol. 26 : 250-256

Cole S.P.C., Pinkoski M.J., Bhardwaj G. and Deeley R.G. (1992a)
Elevated expression of Annexin II (Lipocortin II, p36) in a multidrug resistant small cell lung cancer cell line.
Br. J. Cancer. 65 : 498-502

Coley H.M., Amos W.B., Twentyman P.R. and Workman P. (1993)
Examination by laser scanning confocal fluorescence imaging microscopy of the subcellular localisation of anthracyclines in parent and multidrug resistant cell lines.
Br. J. Cancer 67 :1316-1323.

Connolly D.T., Knight M.B., Harakas N.K., Wittwer A.J. and Feder J. (1986)
Detemination of the number of endothelial cells in culture using an acid phosphatase assay.
Anal. Biochem. 152 : 136-140.

Cordon-Cardo C., O'Brien J.P., Casals D., Rittman-Graver L., Biedler J.L., Melamed M. R. and Bertino J.R. (1989)
Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood brain barrier sites.
Proc. Natl. Acad. Sci. USA 86 : 695-698

Cordon-Cardo C., O'Brien J.P., Boccia J., CasalsD., Bertino J.R. and Melamed M. R. (1990)
Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues.
J. Histochem. Cytochem. 38 : 1277-1287.

Coutts J., Plumb J.A., Brown R. and Kieth W.N. (1993)
Expression of topoisomerase II alpha and beta in an adenocarcinoma cell lines carrying amplified topoisomerase II alpha and retinoic acid receptor alpha genes.
Br. J. Cancer 68 : 793-800

Croop J.M., Raymod M., Haber D., Devault A., Arceci R.J., Gros P. and Housman D.E. (1989)
The three mouse multidrug resistance (mdr) genes are expressed in a tissue-specific manner in normal mouse tissues.
Mol. Cell. Biol. 9 : 1346-1350

Cunningham J.M., Francis G.E., Holland M.J., Pirollo K.F. and Chang E.H. (1991)
Aberrant DNA topoisomerase II activity, radioresistance and inherited susceptibilily to cancer
Br. J. Cancer 63 : 29-36

Currier S., Veda K., Willingham M., Pastan I. and Gottesman M. (1989)
Deletion and insertion mutants of the multidrug transporter.
J. Biol. Chem. 264 : 14376-14381

Danks M.K., Schmidt C.A., Deneka D.A. and Beck W.T. (1989)
Increased ATP requirement for activity of and complex formation by DNA topoisomerase II from human leukemic CCRF-CEM cells selected for resistance to teniposide.
Cancer Comm. 1 : 101-109

Danks M.K., Schmidt C.A., Cirtain M.C., Suttle D.P. and Beck W.T. (1988)
Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26.
Biochemistry 27 : 8861-8869

Danks M.K., Warmoth M.R., Frische E., Granzen B., Bugg B.Y., Harker G., Zwelling L.A., Futscher B. W., Suttle D.P. and Beck W.T. (1993)
Single-strand conformational polymorphism analysis of the Mr1700,000 isozyme of DNA topoisomerase II in human tumor cells.
Cancer Res. 53 : 1373-1379

Darnell J., Lodish H. and Baltimore D. (1986)
The plasma membrane
In: Molecular Cell Biology Scientific American Books.

Davis S.L., Jenkins J.R. and Hickson I.D. (1993)
Human cells express two differentially spliced forms of topoisomerase IIß mRNA.
Nucl. Acids Res. 21 : 3719-3723

De Vries E.G.E., Meijer C., Timmer-Bosscha H., Berendsen H.H., De Leij L., Scheper R.J. and Mulder N.H. (1989)
Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up.
Cancer Res. 49 : 4175-4178

De Jong S., Kooistra A.J., De Vries E.G.E. Mulder N.H. and Zijlstra J.G. (1993)
Topoisomerase II as a target for VM-26 and 4'-(-9-acridinylamino)methanesulfon-*m*-aniside in atypical multidrug resistant human small cell lung carcinoma cells.
Cancer Res. 53 : 1064-1071

De Jong S., Zijlstra J.G., De Vries E.G.E. and Mulder N.H. (1990)
Reduced DNA topoisomerase II activity and drug-induced DNA clevage activity in an adriamycin-resistant human small cell lung carcinoma cell line.
Cancer Res. 50 : 304-309

De Isabella P., Capranico G., Binaschi M., Tinelli S. and Zunnio F. (1990)
Evidence of DNA topoisomerase II-dependent mechanisms of multidrug resistance in P388 leukemia cells.
Mol. Pharmacol. 37 : 11-16

Deffie A.M., Bosman D.J. and Goldenberg G.J. (1989b)
Evidence for a mutant allele of the gene for DNA topoisomerase II in adriamycin-resistance P388 murine leukemia cells.
Cancer Res. 49 : 6879-6882

Deffie A.M., Bastra J.K. and Goldenberg G.J. (1989a)
Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines.
Cancer Res. 49 : 58-62

Deffie A.M., McPherson J.P., Gupta R.S., Hedley D.W. and Goldenberg G.J. (1992)
Multifactorial resistance to antineoplastic agents in drug-resistant P388 murine leukemia, Chinese hamster ovary, and human HeLa cells, with emphasis on the role of DNA topoisomerase II.
Biochem. Cell Biol. 70 : 354-364

Dempke W.C.M., Whelan R.D.H. and Hill B.T. (1992a)
Expression of resistance to etoposide and vincristine *in vitro* and *in vivo* after X-irradiation of ovarian tumor cells
Anti-Cancer Drugs 3 : 395-399

Dempke W.C.M., Shellard S.A., Hosking L.K., Fichtinger-Schepam A.M.J. and Hill B.T. (1992b)
Mechanisms associated with the expression of cisplatin resistance in a human ovarian tumor cell line following exposure to fractionated X-irradiation *in vitro*
Carcinogenesis 13 : 1209-1215

Dempke W.C.M., Hosking L.K. and Hill B.T. (1992c)
Expression of collateral sensitivity to cisplatin, methotrexate and fluorouracil in a human ovarian carcinoma cell line following exposure to fractionated X-irradiation *in vitro*
Semin. Oncol. 19 : 66-72

Devine S.E., Ling V. and Melera P. W. (1992)
Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance.
Proc. Natl. Acad. Sci. USA 89 : 4564-4568

DeVita V.T., Hellman S. and Rosenbery S.A. (1985)
In: Cancer: Principles and practices on oncology. Lippiscott J.B. Philadelphia, P.A.

DeVore R.F., Corbett A.H. and Osheroff N. (1992)
Phosphorylation of topoisomerase II by casein kinase II and protein kinase C: Effects on enzyme-mediated DNA clevage/regligation and sensitivity to the antineoplastic drugs etoposide and 4'-(9-acridinylamino)methane-sulfon-m-anisidide.
Cancer Res. 52 : 2156-2161

Dhir R., Grizzuti K., Kajiji S. and Gros P. (1993)
Modulatory effects on substrate specificity of independant mutations at the serine$^{939/941}$ position in predicted transmembrane domain 11 of P-glycoproteins.
Biochemistry 32 : 9492-9499

Dimanchi-Boitrel M.T., Pelletier H., Genne P., Petit J.M., Le Grimellec C., Canal P., Ardiet C., Bastian G. and Chauffert B. (1992)
Confluence-dependent resistance in human colon cancer cells: Role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells.
Int. J. Cancer 50 : 677-682

Dolfini E., Dasdia T., Perletti G., Romagnoni M. and Piccinini F. (1993)
Analysis of calcium-dependent Protein Kinase-C isoenzymes in intrinsically resistant cloned lines of LoVo cells: Reversal of resistance by Kinase inhibitor 1-(5-isoqinolinylsulfonyl)2-methylpiperazine.
Anticancer Res. 13 : 1123-1128

Dorshkind K., Green L., Godwin A. and Fletcher W. (1993)
Connexin-43-type gap junctions mediate communication between bone marrow stromal cells.
Blood 82 : 38-45

Doyle L.A., Kaufman S.H., Fojo A.T., Bailey C.L. and Gazdar A.F. (1993)
A novel 95 kilodalton membrane polypeptide associated with lung cancer drug resistance.
Lung Cancer 6 : 317-326

Drake F.H., Hofmann G.A., Bartus H.F., Mattern M.R., Crooke S.T. and Mirabelli C.K. (1989)
Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II.
Biochemistry 28 : 8154-8160

Drake F.H., Zimmerman J.P., McCabe F.L., Bartus H.F., Per S.R., Sullivan D.M., Ross W.E., Mattern M.R., Johnson R.K., Crooke S.T. and Mirabelli C.K. (1987)
Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells.
J. Biol. Chem. 262 : 16739- 16747

Drewinko B., Patchen M., Yan L.Y. and Barlogie B. (1981)
Differential killing efficacy of twenty antitumor drugs on proliferating and non-proliferating human tumor cells.
Cancer Res. 41 : 2328-2333

Durand R.E. (1981)
Flow cytometry studies of intracellular adriamycin in multicell spheroids *in vitro*.
Cancer Res. 41 : 3495-3498.

Earnshaw W.C., Halligan B., Cooke C.A., Heck M.M.S. and Liu L.F. (1985)
Topoisomerase II is a structural component of mitotic chromosome scaffolds.
J. Cell Biol. 100 : 1706-1715

Efferth T. and Volm M. (1993)
Modulation of P-glycoprotein-mediated multidrug resistance by monoclonal antibodies, immunotoxins or antisense oligodeoxynucleotides in kidney carcinoma and normal kidney cells.
Oncology 50 : 303-308

Efferth T., Mattern J. and Volm M. (1992)
Innumohistochemical detection of P-glycoprotein, glutathione-S-transferase and DNA topoisomerase II in human tumors.
Oncology 49 : 368-375

Eichholtz-Wirth H., Reidel G. and Hietel B. (1993)
Radiation-induced transient cisplatin resistance in murine fibrosarcoma cells associated with elevated metallothionein content.
Br. J. Cancer 67 : 1001-1006

Eijdems E.W.H.M., Borst P., Jongsma A.P.M., De Jong S., De Vries E.G.E., Van Groenigen M., Versantvoort C.H.M., Niewqint A.W.M. Baas F. (1992)
Genetic transfer of non-P-glycoprotien-mediated multidrug resistance (MDR) in somatic cell fusion: Dissection of a compound MDR phenotype.
Proc. Natl. Acad. Sci. USA 89 : 3498-3502

El-Fouly M.H., Trosko J.E. and Chang C.C. (1987)
Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication.
Exp. Cell Res. 168 : 422-430

Endicott J.A., Jurantia P.F., Sarangi F., Gerlach J. H., Denchars K.L. and Ling V. (1987)
Simultaneous expression of two P-glycoprotein genes in drug-sensitive Chinese hamster ovary cells.
Mol. Cell Biol. 7 : 4075-4081.

Epstein M.L. and Gilula N.B. (1977)
A study of communication specificity between cells in culture.
J. Cell Biol. 75 : 769-787

Erlichman C. and Vidgen E. (1984)
Cytotoxicity of adriamycin in MGH-U1 grown as monolayer cultures, spheroids and xenografts in immune-deprived mice.
Cancer Res. 44 : 5369-5375

Estay E., Adlakha R.C., Hittelman W.N. and Zwelling L.A. (1987)
Cell cycle stage dependent variations in drug-induced topoisomerase II mediated DNA cleavage and cytotoxicity.
Biochemistry 26 : 4338-4344

Evans D.P., Meyn R.E. and Tomasovic S.P. (1986)
Survival of rat mammary tumor cell clones and DNA strand damage following adriamycin treatment.
Cancer Chemother. Pharmacol. 18 : 137-139

Fairchild C.R., Moscow J.A., O'Brien E.E. and Cowan K.H. (1990)
Multidrug resistance in cells transfected with human genes encoding a varient P-glycoprotein and glutathione-S-transferase-π.
Mol. Pharmacol. 37 : 801-809

Fairchild C., Ivys., Kao-Shan C-S., Whang-Peng S., Rosen N., Israel M., Melera P., Cowan K. and Goldsmith M. (1987)
Isolation of amplifed and overexpressed DNA sequences form adriamycin-resistant human breast cancer cells.
Cancer Res. 47 : 5141-5148

Favrot M., Combaret V., Goillot E., Wagner J.P., Bouffet E., Mazingue R., Thyss A., Bordigoni P., Delsol G., Bailly C., Fontaisiere B. and Philip T. (1991)
Experssion of P-glycoprotein restricted to normal cells in neuroblastoma biopsies.
Br. J. Cancer 64 : 233-238

Feldhoff P.W., Mirski S.E.L., Cole S.P.C. and Sullivan D.M. (1994)
Altered subcellular distribution of topoisomerase 11α in a drug-resistant human small cell lung cancer cell line.
Cancer Res. 54 : 756-762

Ferguson P.J., Fisher M.H., Stephenson J., Li, D., Zhou B. and Cheng Y. (1988)
Combined modalities of resistance in etoposide-resistant human KB cell lines.
Cancer Res. 48 : 5956-5964

Fernandes D.J., Danks M.K. and Beck W.T. (1990)
Decreased nuclear matrix DNA topoisomerase II in human leukemia cells resistant to VM-26 and m-AMSA.
Biochemistry 29 : 4235-4241

Fey F.M., Simmermann A., Borisch B. and Tobler A. (1993)
Studying clonal heterogeneity in human cancers.
Cancer Res. 53 : 921

Finbow M.E., Eldridge T., Buultjens J., Kam E., Shuttleworth J. and Pitts J.D. (1985)
Comparison of the protein components present in vertebrate and anthropod gap junction preparations
In: Gap Junctions 77-89
Ed: Bennett M.V.L. and Spray D.C. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Flagg-Newton J. and Loewenstein W.R. (1979)
Experimental depression of junctional membrance permeability in mammalian cell culture. A study with tracer molecules in the 300 to 800 dalton range.
J. Membrane Biol. 50 : 65-100

Fojo A.T., Veda K., Salmon D.J., Poplack D.G., Gottesman M.M. and Pastan I. (1987)
Expression of a multidrug-resistance gene in human tumors and tissues.
Proc. Natl. Acad. Sci. USA 84 : 265-269

Foote S.J., Thompson J.K., Cowman A.F. and Kemp D.J. (1989)
Amplificaiton of the multidrug resistance gene in some chloroquinine-resistant isolates of *"P. falciparum"*.
Cell 57 : 921-930

Fosse P., Rene B., Le Bret M., Paoletti C. and Saucier J.M. (1991)
Sequence requirements for mannalian topoisomerase II mediated DNA cleavage stimulated by an ellipticine derivatie.
Nuc. Acids Res. 19 : 2861-2868

Friche E., Danks M.K., Schmidt C.A. and Beck W.T. (1991)
Decreased DNA topoisomerase II in daunourubicin-resistant ehrlich ascites tumor cells.
Cancer Res. 51 : 4213-4218

Fried A., Benayau D. and Wientroub S. (1993)
Marrow stroma-derived osteogenic clonal cell lines: Putative stages in osteoblastic differentiation.
J. Cell. Physiol. 115 : 472-482

Gekeler V., Frese G., Noller A., Handgretinger R., Wilisch A., Schmidt H., Muller C.P., Dopfer R., Klingebiel T., Diddens H., Probst H. and Niethemmer D. (1992)
Mdr/P-glycoprotein, topoisomerase, and glutathione-S-transferase π gene expression in primary and relapsed state adult and childhood leukaemias.
Br. J. Cancer 66 : 507-517

Gellert M., Mizuuchi K., O'Dea M.H. and Nash H.A. (1976)
DNA gyrase: An enzyme that introduces superhelical turns into DNA.
Proc. Natl. Acad. Sci. USA 73 : 3872-3876

Gerlach J.H., Endicott J.A., Juranka P.K., Henderson G., Sarangi F., Keuchars K.L. and Ling V. (1986)
Homology between P-glycoprotein and a bacterial haemolysin transport protein suggest a model for multidrug resistance.
Nature 324 : 485-489

Gervasoni J.E., Fields S.Z Jr., Krishna S., Baker M.A., Rosando M., Thuraisamy K., Hindenburg A.A. and Taub R.N. (1991)
Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy.
Cancer Res. 51 : 48955-4963

Giaccone G., Gazdar A.F., Beck H., Zunino F. and Capranico G. (1992)
Multidrug sensitivity phenotype of human lung cancer cells associated with topoismoerase II expression.
Cancer Res. 52 : 1666-1667

Gillies McKenna W., Iliakis G., Weiss M.C., Bernhard E.J. and Muschel R.J. (1991)
Increased G_2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc.
Radiat. Res. 125 : 283-287

Gilula N.B., Reeves O.R. and Steinbach. (1972)
Metabolic coupling, ionic coupling and cell contacts.
Nature 235 : 262-265

Giocanti N., Hennequin C., Balosso J., Mahler M. and Favaudon V. (1993)
DNA repair and cell cycle interactions in radiation sensitization by the topoisomerase II poison etoposide.
Cancer Res. 53 : 2105-2111

Glisson B.S. and Alpeter M.D. (1992)
Multidrug resistance in a small cell lung cancer line: Rapid selection with etoposide and differential chemosensitization with cyclosporin A.
Anti-Cancer Drugs 3 : 359-366

Glisson B., Gupta R., Smallwood-Kentro S. and Ross W. (1986)
Characterization of acquired epipodophyllotoxin resistance in a Chinese hamster ovary cell line: Loss of drug-stimulated DNA cleavage activity.
Cancer Res. 46 : 1934-1938

Goodenough D.A. and Revel J.P. (1970)
A fine structural analysis of intercellular junctions in the mouse liver.
J. Cell Biol. 45 : 272-290

Gorin M.B., Yancey S.B., Cline J., Revel J.P. and Norwitz J. (1984)
The major intrinsic protein (MIP) of the bovine lens fiber membrane : Characterization and structure based on cDNA cloning.
Cell 39 : 49-59

Grant C.E., Valdimarsson G., Hipfner D.R., Almquist K.C., Cole S.P.C. and Deeley R. (1994)
Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs.
Cancer Res. 54 : 357-361

Grenman R., Carey T.E., McClatchey K.D., Wagner J.G., Pekkola-Heino K., Schwartz D.R., Wolf G.T., Lacivita L.P., Ho L., Baker S.R., Krause C.J. and Lichter A.S. (1991)
In vitro radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck.
Cancer 67 : 2741-2747

Gros P., Croop J. and Housman D. (1986a)
Mammalian multidrug resistance gene: Complete cDNA sequence indicates strong homology to bacterial transport proteins.
Cell 47: 371-380

Gros P., Raymond M., Bell J. and Housenan D.E. (1988)
Cloning and characterization of a second member of the mouse mdr gene family.
Mol. Cell Biol. 8 : 2770-2778

Gros P., Croop J., Roninson I., Varshavsky A. and Housman D.E. (1986b)
Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells.
Proc. Natl. Acad. Sci. USA 83 : 337-341

Gros P., Dhir R., Croop J., and Talbot F. (1991)
A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr1 and mdr3 drug efflux pumps.
Proc. Natl. Acad. Sci. USA 88 : 7289-7293

Gruijters W.T.M., Kistler J., Bullivant S. and Goodenough D.A. (1987)
Immunolocalization of MP70 in lens fiber 16-17nm intercellular junctions.
J. Cell Biol. 104 : 565-572

Hamilton G., Cosentini E., Teleky B., Bischof G., Koperna T., Zacherl J., Schiessel R. and Wenzl E. (1993a)
Chemosensitisation effect of verapamil and cyclosporin A *in vitro* is reduced under acidic pH conditions
Eur. J. Cancer 29A : 1635

Hamilton G., Cosentini E.P., Teleky B., Koperna T., Zacheri J., Reigler M., Feil W., Schiessel R. and Wenzi E. (1993b)
The multidrug-resistance modifiers verapamil, cyclosporine A and tamoxifen induce an intracellular acidification in colon carcinoma cell lines *in vitro*.
Anticancer Res. 13 : 2059-2064

Harker W.G., Bauer D., Etiz B.B., Newman R.A. and Sikic B.I. (1986)
Verapamil-mediated sensitisation of doxorubicin-selected pleotropic resistance in human sarcoma cells selected for drugs which produce DNA scission.
Cancer Res. 46 : 2369-2373

Heck M.M.S., Hittelman W.N. and Earnshaw W.C. (1988)
Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle.
Proc. Natl. Acad. Sci. USA. 85 : 1086-1090

Heck M.M.S. and Earnshaw W.C. (1986)
Topoisomerase II: A specific marker for cell proliferation.
J. Cell Biol. 103 : 2569-2581

Heck M.M.S., Hittelman W.N. and Earnshaw W.C. (1989)
In vitro phosphorylation of the 170-kDa form of eukaroyotic DNA topoisomerase II.
J. Biol. Chem. 264 : 15161-15164

Heppner G.H. and Miller B.E. (1989)
Therapeutic implications of tumor heterogeneity.
Semin. Oncol. 16 : 91-105

Hertzberg E.L., Lawrence T.S. and Gilula N.B. (1981)
Gap junctional communication.
Ann. Rev. Physiol. 43 : 479-491

Heynkes R., Kozjek G., Traub O. and Willecke K. (1986)
Identification of a rat liver cDNA and mRNA coding for the 28kDa gap junction protein.
FEBS Lett. 205 : 56-60

Higgins C.F. and Gottesman M.M. (1992)
Is the multidrug transporter a flippase.
TIBS 17 : 18-21

Hildebrand-Zanki S.U. and Kern D.H. (1986)
A rapid bioassay to determine stabilities of anticancer agents under conditions of the clonogenic assay.
In vitro Cell. Develop. Biol. 22 : 247-252

Hill B.T., Deuchars K., Hosking L.K., Ling V. and Whelan R.D.H. (1990a)
Overexpression of P-glycoprotein in mammalian tumor cell lines after fractionated X irradiation *in vitro*.
J. Natl. Cancer Inst. 82 : 607-612

Hill B.T., Whelan R.D.H., Hosking L.K., Shellard S.A., Bedford P. and Lock R.B. (1988)
Interactions between antitumor drugs and radiation in mammalian tumor cell lines: differential drug response and mechanisms of resistance following fractioned X-irradiation or continuous drug exposure *in vitro*.
NCI Monogr. 6 : 177-181

Hill B.T., Whelan R.D.H., Hosking L.K., Bedford P., Dempke W.C.M. and Shellard S.A. (1990b)
Differential expression of drug resistance following *in vitro* exposure of human tumour cell lines to fractionated X-irradiation.
Cancer Treatment Reviews 17 : 21-26

Hill B. (1993)
Differing patterns of cross-resistance resulting from exposure to specific antitumour drugs or to radiation *in vitro*.
Cytotechnology 12 : 265-288

Hinds M., Deisserort K., Mayes J., Altschuler E., Jansen R.,Ledley F. and Zwelling L.A. (1991)
Identification of a point mutation in the topoismerase II gene from a human leukemia cell line containing an amsacrine-resistant form of topoisomerase II.
Cancer Res. 51 : 4729-4731

Hoban P.R., Robson C. N., Davies S.M., Hall A.G., Cattan A.R., Hickson I.D. and Harris A.L. (1992)
Reduced topoismoerase II and elevated α class glutathione-S-transferase expression in a multidrug resistant CHO cell line highly cross-resistant to mitomycin C.
Biochem. Pharmacol. 43 : 685-693

Holden J.A., Rolfson, D.H. and Wittwer C.T. (1992)
The distribution of immunoreactive topoisomerase II protein in human tissues and neoplasms.
Oncol. Res. 4 : 157-166

Holden J.A., Dresler S.L. and Low R.L. (1990a)
Identification of DNA topoisomerase-II activity in terminally differentiated mammalian organs and in non-growing cultured cells.
Enzyme 43 : 197-206

Holden J.A., Rolfson D.H. and Wittwer C.T. (1990b)
Human DNA topoisomerase II: Evaluation of enzyme activity in normal and neoplastic tissues.
Biochemistry 29 : 2127-2134

Holland B., Possot O., Blight M. and Yue K. (1991)
Bacterial haemolysin and mammalian P-glycoprotein.
Biochem. Soc. Trans. 19 : 252-255

Holzymayer T.A., Hilsenbeck S., Von Hoff D.D. and Roninson I.B. (1992)
Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas.
J. Natl. Cancer Inst. 84 : 1486-1491

Hong W.S., Saijo N., Sasaki Y., Minato K., Nakano H., Nakagawa K., Fujiwara Y., Nomura K. and Twentyman P. R. (1988)
Establishment and characterization of cisplatin-resistant sublines of human lung cancer cell lines.
Int. J. Cancer 41 : 462-467

Hsiang Y-H, Wu H-Y. and Liu L.F. (1988)
Proliferation-dependant regulation of DNA topoisomerase II in cultured human cells.
Cancer Res. 48 : 3230-3235

Hsu S.I., Lothstein L. and Horwitz S.B. (1989)
Differential overexpression of the three mdr gene family members in multidrug-resistant 3774.2 mouse cells.
J. Biol. Chem. 264 : 12053-12062

Huang A. and Wright J.A. (1994)
Fibroblast growth factor mediated alterations in drug resistance and evidence of gene amplification.
Oncogene 9 : 491-499

Huang H.W., Jaung J.K. and Liu H.J. (1992)
The recognition of DNA cleavage sites by porcine spleen topoisomerase II
Nuc. Acids Res. 20 : 467-473

Hyde S.C., Emsley P., Hartshorn M.S., Mimmack M.M., Gileadi V., Pearce S.R., Gullagher M.P., Gill D.R., Hubbard R.E. and Higgins C.F. (1990)
Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport.
Nature 346 : 362-365

Iliakis G.E. and Okayasu R. (1990)
Radiosensitivity throughout the cell cycle and repair of potentially lethal damage and DNA double-strand breaks in an X-ray-sensitive CHO mutant.
Int. J. Radiat. Biol. 57 : 1195-1211

Jahde E., Glusenkamp K-H. and Rajewsky M.F. (1990)
Protection of cultured malignant cells from mitoxantrone cytotoxicity by low extracellular pH: A possible mechanism for chemoresistance *in vivo*.
Eur. J. Cancer 26 : 101-106

Jelinek D.F., Ahamann G.J., Greipp P.R., Jalal S.M.,Westendorf J.J., Katzmann J.A., Kyle R.A. and Lust J.A. (1993)
Coexistence of aneuploid subclones within a myeloma cell line that exhibits clonal immunoglobulin gene rearrangement: Clinical implication.
Cancer Res. 53 : 5320-5327

Jenkins J.R., Ayton P., Jones T., Davies S.L., Simmons D. L., Harris A.L., Sheer D. and Hickson I.D. (1992)
Isolation of cDNA clones encoding the ß isoform of human DNA topoisomerase II and localisation of the gene to chromosome 3p24.
Nuc. Acids Res. 20 : 5587-5592

Jensen P.B., Roed H., Sehested M., Demant E.J.F., Vindelov L., Christensen I.J. and Hansen H.H. (1992)
Doxorubicin sensitivity pattern in a panel of small-cell lung-cancer cell lines: Correlation to etoposide and vincristine sensitivity and inverse correlation to carmustine sensitivity.
Cancer Chemother. Pharmacol. 31 : 46-52

Jensen P.B., Vindelov L., Roed H., Demant E.J.F., Sehested M., Skovsgaard T. and Hansen H.H. (1989)
In vitro evaluation of the potential of aclarubicin in the treatment of small cell carcinoma of the lung (SCLL).
Br. J. Cancer 60 : 838-844

Jensen P.B., Jensen P.S., Demant EIJ.F., Friche E., Sorensen B.S., Sehested M., Wassermann K., Vindelov L., Westergaard O. and Hansen H.H. (1991)
Antagonistic effect of aclarubicin on daunorubicin-induced cytotoxicity in human small cell lung cancer cells: Relationship to DNA integrity and topoisomerase II.
Cancer Res. 51 : 5093-5099.

Jensen P.B., Christensen I.J., Sehested M., Hansen H.H. and Vindelov L. (1993)
Differential cytotoxicity of 19 anticancer agents in wild type and etoposide resistant small cell lung cancer cell lines.
Br. J. Cancer 67 : 311-320

Juliano R.L. and Ling V. (1976)
A surface glycoprotein modulating drug permeability in Chinese hamster overy cell mutant.
Biochim. Biophys. Acta 455 : 152-162

Kamath N., Grabowski D., Ford J., Kerrigan D., Pommier Y. and Ganapathi R. (1992)
Overexpression of P-glycoprotein and alterations in topoisomerase II in P388 mouse leukemia cells selected *in vivo* for resistance to mitoxantrone.
Biochem. Pharmacol. 44 : 937-945

Kas E. and Laemmli U.K. (1992)
In vivo topoisomerase II cleavage of the *Drosophila* histone and satellite III repeats: DNA sequence and structural characteristics.
EMBO J. 11 : 705-716

Kasahara K., Fujiwara Y., Nishio K., Ohmori T., Sugimoto Y., Komiya K., Matsuda T and Saijo N. (1991)
Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to Cisplatin.
Cancer Res. 51 : 3237-3242

Katoh F. and Yamasaki H. (1991)
Regulation of gap-junction intercellular communication in transformation-sensitive and transformation-resistant BALB/c 3T3 cell variants.
Carcinogensis 12 : 1923-1926

Kaufmann S.H. (1991)
Antagonism between camptothecin and topoisomerase II-directed chemotherapeutic agents in a human leukemia cell line.
Cancer Res. 51 : 1129-1136

Kaufmann W.K., Boyer J.C., Estabrook L.L. and Wilson S.J. (1991)
Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes
Mol. Cell Biol. 11 : 3711-3718

Keith W.N., Douglas F., Wishart G.C., McCallum H.M., George W.D., Kaye S.B. and Brown R. (1993)
Co-amplification of erbB2, topoisomerase II α and retinoic acid receptor α genes in breast cancer and allelic loss at topoisomerase I on chromosome 20.
Eur. J. Cancer 29A : 1469-1475

Keizer H.G., Schuurhuis G.J., Broxterman H. J., Lankelma J., Schoonen W.G.E.J., Van Rijn J., Pinedo H.M. and Joenje H. (1989)
Correlation of multidrug resistance with decreased drug accumulation, altered subcellular drug distribution, and increased P-glycoprotein expression in cultured SW-1573 human lung tumur cells.
Cancer Res. 49 : 2988-2993

Keizer H.G. and Joenje H. (1989)
Increased cytosolic pH in multidrug-resistant human lung tumor cells: Effect of verapamil.
J. Natl. Cancer Inst. 81 : 706-709

Kim R., Hirabayashi N., Nishiyama M., Jinushi K., Toge T. and Okada K. (1992)
Factors contributing to adriamycin sensitivity in human xenograft tumors: The relationship between expression of the MDR1, GST-π and topoisomerase II genes and tumor sensitivity to adriamycin.
Anticancer Res. 12 : 241-246

Kim R., Hirabayashi N., Nishiyama M., Saeki S., Toge T. and Okada K. (1991)
Expression of MDR1, GSTπ and topoisomerase II as an indicator of clinical response to adriamycin.
Anticancer Res. 11 : 429-432

Kistler J., Christie D. and Bullivant S. (1988)
Homologies between gap junction proteins in lens, heart and liver.
Nature 331 : 721-723

Klann R.C., Fitzgerald J.D., Piccoli C., Slaga T.J. and Yamasake H. (1989)
Gap-junctional intercellular communication in epidermal cell lines from selected stages of SENCAR mouse skin carcinogensis.
Cancer Res. 49 : 699-705

Kobayashi H., Dorai T., Holland J.F. and Ohnuma T. (1994)
Reversal of drug sensitivity in multidrug-resistant tumor cells by an MDR1 (PGY1) ribozyme.
Cancer Res. 54 : 1271-1275

Kramer R.A., Zahher J. and Kiss G. (1988)
Role of the glutathione redox cycle in acquired and *de novo* multidrug resistance.
Science 241 : 694-697

Krishnamachary N. and Center M.S. (1993)
The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-KDa membrane bound glycoprotein.
Cancer Res. 53 : 3658-3661

Kuiper C.M., Broxterman H.J., Baas F., Schuurhuis G.J., Haisma H.J., Scheffer G.L., Lankelma J. and Pinedo H.M. (1990)
Drug transport variants without P-glycoprotein overpression from a human squamous lung cancer cell line after selection with doxorubicin.
J. Cell Pharmacol. 1 : 35-41

Kwok T.T. and Sutherland R.M. (1991)
Epidermal growth factor reduces resistance to doxorubicin.
Int. J. Cancer 49 : 73-76.

Lars Bastiaanse E.M., Jongsma H.J., Van Der Laarse A. Takens-Kwad B.R. (1993)
Heptanol-induced decrease in cardiac gap junctional conductance is mediated by decrease in the fluidity of membranous cholesterol-rich domains.
J. Membrane Biol. 136 : 135-145

Larsson R. and Nygren P. (1990)
Pharmacological modification of multi-drug resistance (MDR) *in vitro* detected by novel flurometric microculture cytotoxicity assay. Reversal of resistance and selective cytotoxic actions of cyclosporin A and verapamil on MDR lewkemia T-cells.
Int. J. Cancer 46 : 67-72

Lavoie L., Tremblay A. and Mirault M.E. (1992)
Distinct oxidoresistance phenotype of human T47D cells transfected by rat glutathione-S-transferase Yc expression vectors.
J. Biol. Chem. 267 : 3632-3636

Law E., Gilvarry U., Lynch V., Gregory B., Grant G. and Clynes M. (1992)
Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines.
Cancer Genet. Cytogenet. 59 : 111-118

Lee M.S., Wang J.C. and Beran M. (1992)
Two independent amsacrine-resistant human myeloid leukemia cell lines share and identical point mutation in the 170 kDA form of human topoisomerase II
J. Mol. Biol. 233 : 837-843

Lefevre D., Riou J.-F., Ahomadegbe J.C., Zhou D., Benard J. and Riou G. (1991)
Study of molecular markers of resistance to m-AMSA in a human breast cancer cell line; decrease in topoisomerase II and increase of both topoisomerase I and acidic glutathione-S-transferase.
Biochem. Pharmacol. 41 : 1967-1979

Lehnert S., Greene D. and Batist (1989)
Radiation response of drug-resistant variants of a human breast cancer cell line
Radiat. Res. 118 : 568-580

Leith J.T., Faulkner L.E., Bliven S.F. and Michelson S. (1988a)
Compositional stability of artificial heterogeneous tumors *in vivo*: Use of mitomycin C as a cytotoxic probe.
Cancer Res. 48 : 2669-2673

Leith J.T., Faulkner L.E., Bliven S.F. Michelson S. (1988b)
Tumor bed expression in xenografted artifical heterogeneous colon tumors.
Int. J. Radiat. Oncol. Biol. Phys. 15 : 151-158

Leith J.T., Michelson S., Faulkner L. E. and Bliven S.F. (1987)
Growth properties of artificial heterogeneous human colon tumors.
Cancer Res. 47 : 1045-1051

Lemontt J.F., Azzaria M. and Gros P. (1988)
Increased MDR gene expression and decreased accumulation in MDR human melanoma cells.
Cancer Res. 48 : 6348-6353

Leyland-Jones B.R., Townsend A.J., Tu C.P.D., Cowan K.H. and Goldsmith M.E. (1991)
Antineoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected with a human α class glutathione S-transferase gene.
Cancer Res. 51 : 587-594

Lincke C.R., Smit J.J.M., Van Der Velde-Kowets T. and Borst P. (1991)
Structure of the human *MDR*3 gene and physical mapping of the human *MDR* locus.
J. Biol. Chem. 266 : 5303-5310

Liu L.F. (1989)
DNA topoisomerase poisons as antitumor drugs.
Annu. Rev. Biochem. 58 : 351-375

Liu L.F., Rowe T.C., Yang L., Teweny K.M., and Chen G.L. (1983)
Cleavage of DNA by mammalian DNA topoisomerase II
J. Biol. Chem. 258 : 15365-15370

Liu L.F., Liu C.C. and Alberts B.M. (1980)
Type II DNA topoisomerases: Enzymes that can unknot a topologically knotted DNA molecule *via* a reversible double-strand break.
Cell 19 : 697-707

Loewenstein W.R. (1979)
Junctional intercellular communication and the control of growth.
Biochim. Biophys. Acta 560 : 1-65

Loewenstein W.R. and Rose B. (1978)
Calcium in (junctional) intercellular communication and a thought on its behavior in intracellular communication.
Ann. N.Y. Acad. Sci. : 284-307

Long B.H., Wang L., Lorico A., Wang R.C.C., Brattain M.G. and Casazza A.M. (1991)
Mechanisms of resistance to etoposide and teniposide in acquired resistant human colon and lung carcinoma cell lines.
Cancer Res. 51 : 5275-5284

Louie K.G., Behrens B.C., Kinsella T.J., Hamilton T.C., Grotzinger K.R., McKoy W.M., Winker M.A. and Ozols R.F. (1985)
Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoxaimine.
Cancer Res. 45 : 2110-2115

Lynch T.H., Anderson P., Wallace D.M.A., Kondratowicz G.M., Beaney R.P. and Vaughan A.T.M. (1991)
A correlation between nuclear supercoiling and the response of patients with bladder cancer to radiotherapy.
Br. J. Cancer 64 : 867-871

Maekawa R., Kishimoto Y., Sato K and Homma M. (1993)
Establisment of highly metastatic tumor clones derived from a skin squamous cell carcinoma (SqC-NH), D-1 and F-3, with distinct features of pulmonary metastases.
Clin. Exp. Metastasis 11 : 343-353

Makela T.P., Mattson K. and Alitalo K. (1991)
Tumour markers and oncogenes in lung cancer.
Eur. J. Cancer 27 : 1323-1327

Mantz J., Cordier J. and Giaume C. (1993)
Effects of general anesthetics on intercellular communications mediated by gap junctions between astrocytes in primary culture.
Anesthesiology 78 : 892-896

Marquardt D., McCrone S. and Center M.S. (1990)
Mechanisms of multidrug resistance in HL60 cells: Detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein.
Cancer Res. 50 : 1426-1430

Marsh W., Sicheri D. and Center M.S. (1986)
Isolation and characterisation of adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug.
Cancer Res. 46 ; 4053-4057

Marsh W and Center M.S. (1987)
Adriamycin resistance in HL60 cells and accompanying modification of a surface membrane protein contained in drug-sensitive cells.
Cancer Res. 47 : 5080-5086

Martin A. and Clynes M. (1991)
Acid phosphatase: Endpoint for *in vitro* toxicity tests.
In Vitro Cell. Dev. Biol. 27A : 183-184

Matsuo K., Kohno K., Takano H., Sato S., Kiue A. and Kuwano M. (1990)
Reduction of drug accumulation and DNA topoisomerase II activity in acquired teniposide-resistant human cancer KB cell lines.
Cancer Res. 50 : 5819-5824

Mattern J., Efferth T. and Volm M. (1991)
Overexpression of P-glycoprotein in human lung carcinoma xenografts after fractionated irradiation *in vivo*.
Radiat. Res. 127 : 335-338

McClean S. and Hill B.T. (1994)
Modified multiple drug resistance phenotype of Chinese hamster ovary cells selected with X-rays and vincristine *versus* X-rays only.
Br. J. Cancer 69 : 711-716

McGrath J.P. and Varshavsky A. (1989)
The yease STEG gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein.
Nature 340 : 400-404

McGrath T and Center M.S. (1988)
Mechanisms of multidrug resistance in HL-60 cells: Evidence that a surface membrane protein distinct from P-glycoprotein contributes to reduced cellular accumulation of drug.
Cancer Res. 48 : 3959-3963

McPherson J.P., Brown G.A. and Goldenberg G.J. (1993)
Characterization of a DNA topoisomerase IIα gene rearrangement in adriamycin-resistant P388 leukemia: Expression of a fusion messenger RNA transcript encoding topoisomerase IIα and the retinoic acid receptor α locus.
Cancer Res. 53 : 5885-5889

Meda P., Bosco D., Chanson M., Giordano E., Vallar L., Wollheim C. and Orci L. (1990)
Rapid and reversible secretion changes during uncoupling of rat insulin-producing cells.
J. Clin. Invest. 86 : 759-768

Meda P., Chanson M., Pepper M., Giordano E., Bosco D., Traub O., Willecke K., El Aoumari A., Gros D., Beyer E.C., Orci L. and Spray D.C. (1991)
In vivo modulation of connexin 43 gene expression and junctional coupling of pancreatic B-cells.
Exp. Cell Res. 192 : 469-480

Meda P., Bruzzone R., Knodel S. and Orci L. (1986)
Blockage of cell-to-cell communicatio within pancreatic acini is associatied with increased basal release of amylase.
J. Cell Biol. 103 : 475-483

Meech R.W. and Thomas R.C. (1977)
The effect of calcium injection on the intracellular sodium and pH of snail neurones.
J. Physiol. 265 : 867-879

Mehle C., Ljungberg B., Stenling R. and Roos G. (1993)
DNA fingerprinting of renal cell carcinoma with special reference to tumor heterogeneity.
Genes Chromosomes Cancer 6 : 86-91

Meijer C., Mulder N.H., Timmer-Bosscha H., Zijlstra J.G. and De Vries E.G.E. (1987)
Role of free radicals in an adriamycin-resistant human small cell lung cancer cell line.
Cancer Res. 47 : 4613-4617

Meyers M.B., Yu P. and Mendelsohn J. (1993)
Crosstalk between epidermal growth factor receptor and P-glycoprotein in actinomycin D resistant Chinese hamster lung cells.
Biochem. Pharmacol. 46 : 1841-1848

Meyers M.B., Spengler B.A., Chang T.D., Melera P.W. and Biedler J.L. (1985)
Gene amplification-associated cytogenetic aberrations and protein changes in vincristine-resistant Chinese hamster, mouse and human cells.
J. Cell Biol. 100 : 588-597

Meyers M.B., Merluzzi V.J., Spengler B.A. and Biedler J.L. (1986)
Epidermal growth factor receptor is increased in multidrug-resistant Chinese hamster and mouse tumor cells
Proc. Natl. Acad. Sci. USA 83 : 5521-5525

Michelson S., Leith J.T. and Glicksman A.S. (1987)
A technique to analyse the emerging zonality of heterogeneous solid tumours.
Cell Tissue Kenet. 20 : 499-506

Miller B.E., Miller F.R. and Heppner G.H. (1989)
Therapeutic perturbation of the tumor ecosystem in reconstructed heterogeneous mouse mammary tumors.
Cancer Res. 49 : 3747-3753.

Miller B.E., Machemer T., Lehotan M. and Heppner G.H. (1991)
Tumor subpopulation interactions affecting melphalan sensitivity in palpable mouse mammary tumors.
Cancer Res. 51 : 4378-4389.

Miller B.E., Miller F.R. and Heppner G.H. (1985)
Factors affecting growth and drug sensitivity of mouse mammary tumor lines in collagen gel cultures.
Cancer Res. 45 : 4200-4205

Miller B.E., McInerney D., Jackson D. and Miller F.R. (1986)
Metabolic cooperation between mouse mammary tumor subpopulations in three-dimensional collagen cell cultures.
Cancer Res. 46 : 89-93

Miller P.R., Hill A.B., Slovak M.L. and Shimm D.S. (1992)
Radiation resistance in a doxorubicin-resistant human fibrosarcoma cell line
Am. J. Clin. Oncol. 15 : 216-221

Miller K.G., Liu L.F. and England P.T. (1981a)
A homogeneous type II DNA topoisomerase from HeLa cell nuclei.
J. Biol. Chem. 256 : 9334-9339

Miller B.E., Miller F.R., Wilburn D.J. and Heppner G.H. (1987)
Analysis of tumour cell composition in tumours composed of paired mixtures of mammary tumour cell lines.
Br. J. Cancer 56 : 561-569.

Miller B.E., Miller F.R., Wilburn D. and Heppner G.H. (1988)
Dominance of a tumor subpopulation line in mixed heterogeneous mouse mammary tumors.
Cancer Res. 48 : 5747-5753

Miller B.E., Miller F.R. and Heppner G.H. (1981b)
Interactions between tumor subpopulations affecting thier sensitivity to the antineoplastic agents cyclophosphamide and methotrexate.
Cancer Res. 41 : 4378-4381.

Milroy R., Plumb J.A., Batstone P., Macclay A., Wishart G.C., Hay F.G., Candlish W., Adamson R., Khan M.Z., Banham S. and Kaye S.B. (1992)
Lack of expression of P-glycoprotein in 7 small cell lung cancer cell lines established both from untreated and from treated patients.
Anticancer Res. 12 : 193-200

Minato K., Kanzawa F., Nishio K., Nakagawa K., Fujiwara Y. and Saijo N. (1990)
Characterization of an etoposide-resistant human small-cell lung cancer cell line.
Cancer Chemother. Pharmacol. 26 : 313-317

Mirski S.E.L., Gerlach J.H. and Cole S.P.C. (1987)
Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin.
Cancer Res. 47 : 2594-2598.

Mirski S.E. and Cole S.P. (1989)
Antigens associated with MDR in H69AR, a small cell lung cancer cell line.
Cancer Res. 49 : 5719-5724

Mirski S.E.L., Evans C.D., Almquist K.C., Slovak M.L. and Cole S.P.C. (1993)
Altered topoisomerase IIα in a drug-resistant small cell lung cancer cell line selected in VP-16.
Cancer Res. 53 : 4866-4873

Mirski S.E.L. and Cole S.P.C. (1991)
Multidrug resistance-assocaited antigens on drug-sensitive and -resistant human tumor cell lines.
Br. J. Cancer 64 : 15-22

Mitchell J.B., Gramson J., Russo A., Friedman N., DeGraff W., Carmichael J. and Glatstein E. (1988)
Chinese hamster pleiotropic multidrug-resistant cells are not radioresistant.
NCI. Monogr. 6 : 187-191

Miura M. and Sasaki T. (1991)
Role of glutathione in the intrinsic radioresistance of cell lines from a mouse squamous cell carcinoma.
Radiat. Res. 126 : 229-236

Mousseau M., Chauvin C., Nissou M.F., Chaffanet M., Plantaz D., Pasquier B., Schaerer R. and Benabid A. (1993)
A study of the expression of four chemoresistance-related genes in human primary and metastatic brain tumours.
Eur. J. Cancer 29A : 753-759

Mulder S.H., Van Grondelle R., Westerhoff H.V. and Lankelma J. (1993)
A plasma membrant 'vacuum cleaner' for daunorubicin in non-P-glycoprotein multidrug-resistant SW-1573 human non-small cell lung carcinoma cell.
Eur. J. Biochem. 218 : 871-882

Murray S.A. and Fletcher W.H. (1984)
Hormone-induced intercellular signal transfer dissociates cyclin AMP-dependent protein kinase.
J. Cell Biol. 98 : 1710-1719

Nahon E. Best-Belpomme M. and Saucier J.M. (1993)
Analysis of the DNA topoisomerase-II-mediated cleavage of the long terminal repeat of *Drosophila* 1731 retrotransposon.
Eur. J. Biochem. 21 : 95-102

Nakagawa M.M., Schneider E., Dixon H., Horton J., Kelly K., Morrow C. and Cowan K. H. (1992)
Reduced intracellular drug accumulationin the absence of P-glycoprotein (*mdr*1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells.
Cancer Res. 52 : 6175-6181

Nathanson M.H. and Burgstahler A.D. (1992)
Coordination of hormone-induced calcium signals in isolated rat hepatocyte couplets: Demonstration with confocal microscopy.
Mol. Biol. Cell 3 : 113-121

Nederman T. (1984)
Effects of vinblastine and 5-fluorouracil on human glioma and thyroid cancer cell monolayers and spheroids.
Cancer Res. 44 : 254-258

Negri C., Chiesa R., Cerino A., Bestagno M., Sala C., Zini N., Maraldi N. and Astaldi-Riccotti G. (1992)
Monoclonal antibodies to human DNA topoisomerase I and two isoforms of DNA topoisomerase II: 170- and 180- KDa. isozymes.
Exp. Cell Res. 200 : 452-459

Nelson W., Cho K., Hsiang Y-H., Liu L. and Coffey D. (1987)
Growth-related elevations of DNA topoismerase II levels found in Dunning R3327 rat prostatic adenocarcinomas.
Cancer Res. 47 : 3246-3250

Nelson E., Tewey K. and Liu L. (1984)
Mechanism of antitumor drug action: Poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-acridinylamino)-methanesulfon-m-anisidide.
Proc. Natl. Acad. Sci. USA 81 : 1361-1365

Ng., W.F., Darangi F., Zastawny R.L., Veinot-Drebot L. and Ling V. (1989)
Identification of members of the P-glycoprotein multigene family.
Mol. Cell. Biol. 9: 1224-1232.

Nguyen H.N., Sevin B-U., Averette H.E., Ramos R., Ganjei P. and Perras J. (1993)
Evidence of tumor heterogeneity in cervical cancers and lymph node metastases as determined by flow cytometery.
Cancer 71 : 2543-2550.

Nicholson B., Dermietzel R., Teplow D., Traub O., Willecke K. and Revel J.P. (1987)
Two homologous protein components of hepatic gap junctions.
Nature 329 : 732-734

Nielsen D. and Skovsgaard T. (1992)
P-glycoprotein as multidrug transporter: A critical review of current multidrug resistant cell lines.
Biochim. Biophys. Acta 1139 : 169-183

Nieuwint A.W.M., Baas R., Wiegant J. and Joenje H. (1992)
Cytogenetic alterations associated with P-glycoprotein- and non-P-glycoprotein-mediated multidrug resistance in SW-1573 human lung tumor cell lines.
Cancer Res. 52 : 4361-4371

Noguchi S., Motomura K., Inaji H., Imaoka S. and Koyama H. (1993)
Clonal analysis of fibroadenoma and phyllodes tumor of the breast.
Cancer Res. 53 : 4071-4074

Noguchi S., Motomura K., Inaji H., Imaoka S. and Koyama H. (1992)
Clonal analysis of human breast cancer by means of the polymerase chain reaction.
Cancer Res. 52 : 6594-6597

Noonan K.E., Beck C., Holzmayer T.A., Chin J.E., Wunder J.S., Andrulis I.L., Gazdar A.R., Willman C.L., Griffith B., Von Hoff D.D and Roninson I.B. (1990)
Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction.
Proc. Natl. Acad. Sci. USA 87 : 7160-7164

Nothdurft W., Baltschukat K. and Selig C. (1992)
Differences in radiation response between cells in S-phase and non-S-phase cells of the granulocyte/macrophage progenitor (GM-CFC) compartment.
Int. J. Radiat. Oncol. Biol. Phys. 23 : 345-351

Nygren P., Larsson R., Gruber A., Peterson C. and Bergh J. (1991)
Doxorubicin selected multidrug-resistant small cell lung cancer cell lines characterised by elevated cytoplasmic Ca^{2+} and resistance modification by verapamil in absence of P-glycoprotein overexpression.
Br. J. Cancer 64 : 1011-1018

O'Driscoll L., Daly C., Saleh M. and Clynes M. (1993)
The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug-resistant cells.
Cytotechnology 12 : 289-314

O'Dwyer P.J., Leyland-Jones B., Alonso M.T., Marsoni S. and Wittes R.E. (1985)
Drug therapy: Etoposide (VP-16-312), current status of an active anticancer drug.
New Eng. J. Med. 312 : 692-700

Ogawa J-I., Iwazaki M., Inoue H., Koide S. and Shohtsu A. (1993)
Immunohistochemical study of glutathione-related enzymes and proliferative anigens in lung cancer.
Cancer 71 : 2204-2209

Okabe T., Suzuki A., Hirono M., Tamaoki N., Oshiura M. and Takaku F. (1983)
Establishment of different clonal strains from a human sarcoma of the stomach: Tumorigenic heterogeneity in athymic nude mice.
Cancer Res. 43 : 5456-5461

Okamura S., Chechik, B.E., Lee C., Gelfand E.W. Mak T.W. (1981)
Heterorgeneity of human thymocytes and malignant T-lymphoblast cell line, MOLT-3.
Cancer Res. 41 : 1664-1668

Olive P.L., Banath J.P. and Evans H.H. (1993)
Cell killing and DNA damage by etoposide in Chinese hamster V79 monolayers and spheroids: Influence of growth kinetics, growth environment and DNA packaging.
Br. J. Cancer 67 : 522-530

Osheroff N., Shelton E.R. and Brutlag D.L. (1983)
DNA topoisomerase II from *Drosophila melanagaster*.
J. Biol. Chem. 258 : 9536-9543

Osheroff N. and Zechiedrich E.L. (1987)
Calcium-promoted DNA cleavage by eukaryotic topoisomerase II: Trapping the covalent enzyme-DNA complex in an active form.
Biochemistry 26 : 4303-4309

Osheroff N., Zechiedreich E.L. and Gale K.C. (1991)
Catalytic function of DNA topoisomerase II.
BioEssays 13 : 269-275

Osheroff N. (1989)
Biochemical basis for the interactions of type I and type II topoisomerases with DNA.
Pharmacol. Ther. 41 : 223-241

Osheroff N. (1987)
Role of the divalent cation in topoisomerase II mediated reactions.
Biochemistry 26 : 6402-6406

Osheroff N. (1986)
Eukaryotic topoisomerase II: Characterization of enzyme turnover.
J. Biol. Chem. 261 : 9944-9950

Owens A.H., Coffey D.S. and Baylin S.B. (editors) (1982)
In: Tumor cell heterogeneity: origins and implications. Academic Press Inc.

Paul D.L. (1986)
Molecular cloning of cDNA for rat liver gap junction protein.
J. Cell Biol. 103 : 123-134

Pelletier H., Millot J.-M., Chauffert B., Manfait M., Genne P. and Martin F. (1990)
Mechanisms of resistance of confluent human and rat colon cancer cells to anthracyclines: Alterations of drug passive diffusion.
Cancer Res. 50 : 6626-6631

Per S.R., Mattern M.R., Mirabelli C.K., Drake F.H., Johnson R.K. and Crooke S.T. (1987)
Characterization of a subline of P388 leukemia resistant to amsacrine: Evidence of altered topoisomerase II function.
Mol. Pharmacol. 32 : 17-25

Perez-Armendariz M., Roy C., Spray D.C. and Bennett M. V.L. (1991)
Biophysical properties of gap junctions between freshly dispersed pair of mouse pancreatic beta cells.
Biophys. J. Biophys. Soc. 59 : 76-92

Pessina A. (1993)
Topoisomerase I in multiple drug resistance.
Cytotechnology 12 : 127-136

Petrov P., Drake F.H., Loranger A., Haung W. and Hancock R. (1993)
Localization of DNA topoisomerase II in Chinese hamster fibroblasts by confocal and electron microscopy.
Exp. Cell Res. 204 : 73-81

Pommier Y., Capranico G., Orr A. and Kohn K.W. (1991b)
Distribution of topoisomerase II cleavage sites in simian virus 40 DNA and the effects on drugs.
J. Mol. Biol. 222 : 909-924

Pommier Y., Orr A., Kohn K.W. and Riou J.F. (1992)
Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in the human c-myc protooncogene.
Cancer Res. 52 : 3125-3130

Pommier Y., Capranico G., Orr A. and Kohn K.W. (1991a)
Local base sequence preferences for DNA cleavage by mammalian topoisomerase II in the presence of amsacrine or teniposide.
Nuc. Acids Res. 91 : 5973-5980

Poste G., Tzeng J., Doll J., Greig R., Rieman D. and Ziedman I. (1982)
Evolution of tumor heterogeneity during progressive growth of individual lung metastases.
Proc. Natl. Acad. Sci. USA 79 : 6574-6578

Poste G., Doll J. and Fidler I.J., (1981)
Interactions among clonal subpopulations affect stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells.
Proc. Natl. Acad. Sci. USA. 78 : 6226-6230

Powell S. and McMillan T.J. (1991)
Clonal variation of DNA repair in a human glioma cell line.
Radiother. Oncol. 21 : 225-232

Prosperi E., Sala E., Negri C., Oliani C., Supino R., Astraldi-Ricotti G.B.C. and Bottiroli G. (1992)
Topoisomerase II α and ß in human tumor cells grown *in vitro* and *in vivo*.
Anticancer Res. 12 : 2093-2100

Quiet C.A., Weichselbaum R.R. and Grdina D.J. (1991)
Variation in radiation sensitivity during the cell cycle of two human squamous cell carcinomas.
Int. J. Rad. Onc. Biol. Phys. 20 : 733-738

Quin X. and Beck W.T. (1990)
Binding of an optically pure photoaffinity analogue of verapamil, LU-49888, to P-glycoprotein from multidrug-resistant human leukemic cell lines.
Cancer Res. 50 : 1132-1137

Quinn L.A., Moore G.E., Morgan R.T. and Woods L.K. (1979)
Cell lines from human colon carcinoma with unusual cell products double minutes, and homogeneously staining regions.
Cancer Res. 39 : 4914-4924

Ramachandran C., Sany T.S., Huang X.L., Yuan K.Z. and Krishan A. (1993)
Doxurubicin-induced DNA breaks, topoisomerase II activity and gene expression in human melanoma cells.
Biochem. Pharmacol. 45 : 1367-1371

Raymond M., Rose E., Housman D. and Gros P. (1990)
Physical mapping, amplification, and overexpression of the mouse mdr gene family in multidrug-resistant cells.
Mol. Cell Biol. 10 : 1642-1651.

Redmond A., Law E., Gilvarry U. and Clynes M. (1990)
Establishment of two new multi-drug resistant variants of the human tumour line Hep-2.
Cell Biol. Tox. 6 : 293-302

Reed K.E., Westphale E.M., Larson D.M., Wang H.-Z., Veenstra R.D. and Beyer E.C. (1993)
Molecular cloning and functional expression of human connexin 37, an endothelial cell gap junction protein.
J. Clin. Invest. 91 : 997-1004

Reeve J.G., Rabbitts P.H. Twentyman P.R. (1990)
Non-P-glycoprotein-mediatied multidrug resistance with reduced EGF receptor expression in a human large cell lung cancer cell line.
Br. J. Cancer 61 : 851-855

Reeve J.G., Rabbitts P.H. and Twentyman P.R. (1989)
Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69.
Br. J. Caner 60 : 339-342

Revel J.P., Yancey S.B. and Nicholson B.J. (1986)
The gap junction proteins.
TIBS 11 : 375-377

Ritke M.K., Roberts D., Allan W.P., Raymond J., Bergoltz V.V. and Yalowich J.C. (1994)
Altered stability of etoposide-induced topoisomerase II-DNA complexes in resistant human leukaemia K562 cells.
Br. J. Cancer 69 : 687-697

Rivoltini L., Colombo M.P., Supino R., Ballinari D., Tsuruo T. and Parmiani G. (1990)
Modulation of multidrug resistance by verapamil or mdr1 anti-sense oligodeoxynucleotide does not change the high susceptibilty to lymphokine-activated killers in mdr-resistant human carcinoma (LoVo) line.
Int. J. Cancer 46 : 727-732

Robertson S.M., Ling V. and Stanners C.P. (1984)
Co-amplification of double minute chromosomes, multiple drug resistance, and cell surface P-glycoprotein in DNA-mediated transformants of mouse cells.
Mol. Cell. Biol. 4 : 500-506

Robinson M.J., Martin B.A., Gootz T.D., McGuirk P.R., Moynihan M., Sutcliffe J.A. and Osheroff N. (1991)
Effects of quinolone derivatives on eukaryotic topoisomerase II: A novel mechanism for enhancement of enzyme-mediated DNA cleavage.
J. Biol. Chem. 266 : 14585-14592

Robinson R.G., Rapp L., Bowdish K.J., Graham M.A., Huff A.C. and Coughlin S.A. (1993)
Isolation and characterization of monoclonal antibodies to a recombinant human topoisomerase II polypeptide.
Hybridoma 12 : 407-415

Robinson M.J. and Osheroff N. (1990)
Stabilization of the topoismerase II-DNA cleavage complex by antineoplastic drugs: Inhibition of enzyme-mediated DNA religation by 4'-(9-acridinylamino)methanesulfon-m-anisidide .
Biochemistry 29 : 2511-2515.

Robson C.N., Hoban P.R., Harris A.L. and Hickson I.D. (1987)
Cross-sensitivity to topoisomerase II inhibitors in cytotoxic drug-hypersensitive Chinese hamster ovary cell lines.
Cancer Res. 47 : 1560-1565

Roca J. and Wang J.C. (1992)
The capture of a DNA double helix by an ATP-dependent protein clamp: A key step in DNA transport by type II DNA topoisomerases.
Cell 71 : 833-840

Roninson I. B., Chin J.E., Choi K., Gros P., Houseman D.E., Fajo A., Shen D., Gottesman M.M. and Pastan I. (1986)
Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells.
Proc. Natl. Acad. Sci. USA 83 : 4538-4542

Roninson I.B., Pastan I., Gottesman M.M. (1991)
Isolation and chacterisation of the human MDR (P-glycoprotein) gene.
In: Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. 91-106
Ed: Roninson I.B. Plenum Press, New York.

Ross W., Rowe T., Glisson B., Yalowich J. and Liu L. (1984)
Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage.
Cancer Res. 44 : 5857-5860

Ross D.D., Wooten P.J., Sridhara R., Ordonez J., Lee E.J. and Schiffer C.A. (1993)
Enhancement of doxorubicin accumulation, retention and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukemia.
Blood 82 : 1288-1299

Rowe T.C., Wang J.C. and Liu L.F (1986b)
In vivo localization of DNA topoisomerase cleavage sites on *Drosophila* heat shock chromatin.
Mol. Cell Biol. 6 : 985-992

Rowe T.C., Chen G.L., Hsiang Y.H. and Liu L.F. (1986a)
DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II.
Cancer Res. 46 : 2021-2026

Rup D.M., Veenstra R.D., Wang H.-Z., Brink P.R. and Beyer E.C. (1993)
Chick connexin-56, a novel lens gap junction protein.
J. Biol. Chem. 268 : 706-712

Safa A. R., Stern R.K., Choi K., Agresti M., Tamai I., Mehta N.D. and Roninson I.B. (1990)
Molecular basis of prefential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185 to Val-185 substitution in P-glycoprotein.
Proc. Natl. Acad. Sci. USA 87 : 7225-7229

Safa A.R., Glover C.J., Sewell J.L., Meyers M.B., Biedler J.L. and Felsted R.L. (1987)
Identification of the multidrug-resistance-related membrane glycoprotein as an acceptor for calcium channel blockers.
J. Biol. Chem. 262 : 7884-7888

Saito Y., Nakada Y., Hotta T., Mikami T., Kurisu K., Yamada K., Kiya K., Kawamoto K. and Uozumi T. (1991)
Glutathione and cellular response of ACNU-resistant rat glioma sublines to drugs and radiation.
Eur. J. Cancer 48 : 861-865

Sander M. and Hsien T. (1983)
Double strand DNA cleavage by type II DNA topoisomerase from *Drosophila melanogaster*.
J. Biol. Chem. 258 : 8421-8428

Sasaki T., Yamamoto M. and Kuwahara K. (1984)
Lethal effect of bleomycin and peplomycin on HeLa cells in multicell tumor spheroids.
Cancer Res. 44 : 1374-1379

Scagliotti G.V., Michelotto F., Kalikatzaros G., Leonardo E., Cappia S., Gubetta L., Bosario P. and Possi E. (1991)
Detection of multidrug resistance associated P-170 glycoprotein in previously untreated non small cell lung cancer.
Anticancer Res. 11 : 2207-2210

Scheper R.J., Broxterman H.J., Scheffer G.L., Kaaijk P., Dalton W.S., Van Heijningen T.H.M., Van Kalken C.K., Solvak M.L., De Vries E.G.E., Van Der Valk P., Meijer C.J.L.M. and Pinedo M. (1993)
Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance.
Cancer Res. 53 : 1475-1479

Schinkel A.H., Roelofs M.E.M. and Borst P. (1991)
Characterization of the human MDR3 P-glycoprotein and its recognition by P-glycoprotein-specific monoclonal antibodies.
Cancer Res. 51 : 2628-2635

Schlag P. and Schremi W. (1982)
Heterogeneity in growth pattern and drug sensitivity of primary tumor and metastases in the human tumor colony-forming assay.
Cancer Res. 42 : 4086-4089

Schneider E., Horton J.K., Yang C.H., Nakagawa M. and Cowan K.H. (1994)
Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance.
Cancer Res. 54 : 152-158

Schuurhuis G., Pinedo H., Broxterman H., van Kalkan C., Kuiper C. and Lankelma J. (1990)
Differential sensitivity of multidrug-resistant and -sensitive cells to resistance modifying agents and the relation with reversal of anthracycline resistance.
Int. J. Cancer 46: 330-336

Schuurhuis G.J., Broxterman H.J., De Lange J.H.M., Pinedo H. M., Van Heijningen T.H.M., Kuiper C.M., Scheffer R.J., Van Kalken C.K., Baak J.P.A. and Lankelma J. (1991)
Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein.
Br. J. Cancer 64 : 857-861

Shapiro J.R., Yung W-K. A. and Shapiro W.R. (1981)
Isolation, karyotype, and clonal growth of heterogeneous subpopulations of human mulignant gliomas.
Cancer Res. 41 : 2349-2359

Shimm D.S., Olson S. and Hill A.B. (1988)
Radiation resistance in a multidrug resistant human T-cell leukemia line.
Int. J. Rad. Oncol. Biol. Phys. 15 : 931-936

Shin H.J., Shin D.M. Grant G., Hong W. K. and Pathak S. (1991)
Simultaneous amplification of epidermal growth factor-receptor and multidrug-resistance genes in a newly established human lung cancer cell line.
Anticancer Res. 11 : 214-248

Shin H.J.C., Lee J.S., Hong W.K. and Shin D.M. (1992)
Study of multidrug resistance (mdr1) gene in non-small-cell lung cancer.
Anticancer Res. 12 : 367-370

Sikic B.I., Scudder S.A. and Evans T.L. (1989)
Multidrug (pleotropic) resistance in the human sarcoma cell line MES-SA.
In: Resistance to antineoplastic drugs. 37-47
Ed: Kessel D.

Simon S., Roy D. and Schindler M. (1994)
Intracellular pH and the control of multidrug resistance.
Proc. Natl. Acad. Sci. USA 91 : 1128-1132

Slovak M.L., Hoeltge G.A. and Trent J.M. (1987)
Cytogenetic alterations associated with the acquisition of doxorubicin resistance possible significance of chromosome 7 alterations.
Cancer Res. 47 : 6646-6652

Slovak M.L., Mirski S.E.L., Cole S.P.C., Gerlach J.H., Yohem K.H. and Trent J.M. (1991)
Tumourigenic multidrug-resistant HT1080 cells do not overexpress receptors for epidermal growth factor.
Br. J. Cancer 64 : 296-298

Sognier M.A., Zhang Y., Eberle R.L. and Belli J.A. (1992)
Characterization of adriamycin-resistant and radiation-sensitive Chinese hamster cell lines.
Biochem. Pharmacol. 44 : 1859-1868

Speth P.A.J., van Hoesel Q.G.C.M. and Haanen C. (1988)
Clinical pharmacokinetics of doxorubicin.
Clin. Pharmacokinetics 15 : 15-31

Spray D.C., Stern J.H., Harris A.L. and Bennett M.V.L. (1982)
Gap junctional conductance: Comparison of sensitivities to H and Ca ions.
Proc. Natl. Acad. Sci. USA 79 : 441-445

Spritzner J.R. Chung I.K. and Muller M.T. (1990)
Eukaryotic topoisomerase II preferentilly cleaves alternating purine-pyrimidine repeats.
Nuc. Acids Res. 18 : 1-11

Su I-J., Chang I-C. and Cheng A-L. (1991)
Expression of growth-related genes and drug-resistance genes in HTLV-I-positive and HTLV-I-negative post-thymic T-cell malignancies.
Ann. Oncol. 2 : 151-155

Sugawara I., Kataoka I., Morishita Y., Hamada H., Tsuruo T., Itoyama S and Mori S. (1988)
Tissue distribution of P-glycoprotein encoded by a multidrug resistant gene as revealed by a monoclonal antibody, MRK16.
Cancer Res. 48 : 1926-1929

Sugawara I., Hamada H., Tsuruo, T. Mori S. (1990)
Specialized locaization of P-glycoprotein recognized by MRK16 monoclonal antibody in endothelial cells of the brain and the spinal cord.
Jpn. J. Cancer Res. 81 : 727-730

Sugimoto Y., Hamada H., Tsukahara S., Noguch K., Yamaguchi K., Sato M. and Tsuruo T. (1993)
Molecular cloning and characterization of the complementary DNA for the M_r 85,000 protein overexpressed in adriamycin-resistant human tumor cells.
Cancer Res. 53 : 2538-2543

Sugimoto Y., Tsukahara S., Oh-hara T., Isoe T. and Tsuruo T. (1990b)
Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody.
Cancer Res. 50 : 6925-6930

Sugimoto Y., Tsukahara S., Oh-hara T., Liu L.F. and Tsuruo T. (1990a)
Elevated expression of DNA topoisomerase II in camptothecin-resistant human tumor cell ines.
Cancer Res. 50 : 7962-7965

Swarts S.G., Nelson G.B., Wallen C.A. and Wheeler K.T. (1990)
Radiation-induced cytotoxicity, DNA damage and DNA repair: Implications for cell survival theory.
Radiat. Environ. Biophys. 29 : 93-102

Takagawa N., Ohnoshi T., Ueoka H., Kiura K. and Kimura I. (1992)
Establishment and characterization of an etoposide-resistant human small cell lung cancer cell line.
Acta Med. Okayama 46 : 203-212

Takano H., Kohno, K., Ono, M., Uchida, Y. and Kuwano M. (1991)
Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells.
Cancer Res. 51 : 3951-3957

Tan K.B., Dorman T.E., Falls K.M., Chung T.D.Y., Mirabelli C.K., Crooke S.T. and Mao J. (1992)
Topoisomerase IIα and topoisomerase IIß genes: Characterization and mapping to human chromosomes 17 and 3, respectively.
Cancer Res. 52 : 231-234

Tan W.B., Mattern M.R., Eng W.K., McCabe F.L., Johnson R.K. (1989)
Nonproductive rearrangement of DNA topoisomerase I and II genes: Correlation with resistance of topoisomerase inhibitors.
J. Natl. Cancer Inst. 81 : 1732-1735

Tanigawa N., Mizuno Y., Hashimura T., Honda K., Satomura K., Hikasi Y., Niwa O., Sugahara T., Yoshida O., Kern D.H. and Morton D.L. (1984)
Comparison of drug sensitivity among tumor cells within a tumor, between primary tumor and metastases, and between different metastases in the human tumor colony-forming assay.
Cancer Res. 44 : 2309-2312

Tanio Y., Watanabe M., Inoue T., Kawase I., Shirasaka T., Ikeda T., Hara H., Masuno T., Saito S., Kawano K., Kitamura H., Kubota K., Kodama N., Kawahara M., Sakatani M., Furuse K., Yamamoto S. and Kishimoto S. (1990)
Chemo-radioresistance of small cell lung cancer cell lines derived from untreated primary tumors obtained by diagnostic bronchofiberscopy.
Jpn. J. Cancer Res. 81 : 289-297

Tatsuta T., Naito M., Oh-hara T., Sugawara I. and Tsuruo T. (1992)
Functional involvement of P-glycoprotein in blood-brain barrier.
J. Biol. Chem. 267 : 20383-20391

Tewey K.M., Rowe T.C., Yang L., Halligan B.D., Liu L.F. (1984)
Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II.
Science 226 : 466-468

Thiebaut F., Tsuruo T., Hannada H., Bottesman M.M., Pastan I. and Willinghara M.C. (1989)
Immunohistochemical localisation in normal tissue of different epitopes in the multidrug transport protein P170: Evidence for localization in brain capillaries and cross reactivity of one antibody with a muscle protein.
J. Histochem. Cytochem. 37 : 159-164

Thiebaut F., Tsuruo T., Hamada H., Gottesman M.M., Pastan I. and Willingham M.C. (1987)
Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues
Proc. Natl. Acad. Sci. USA 84 : 7735-7738

Toffoli G., Viel A., Tumitto L., Biscontin G., Rosse C. and Boiocchi M. (1991)
Pleiotrophic-resistant phenotype is a multifactorial phenomenon in human colon carcinoma cell lines.
Br. J. Cancer 63 : 51-56

Tritton T.R. and Yee G. (1982)
The anticancer agent adriamycin can be actively cytotoxic without entering cells.
Science 217 : 248-250

Trope C. (1982)
Different susceptibilities of tumor cell populations to cytotoxic agents and therapeutic consequences.
In: Tumor cell heterogeneity : origins and implications. 147-168
Ed : Owens A.H., Coffey D.S. and Baylin S.B.

Tsai-Pflugfelder M., Liu L.F., Liu A.A., Tewey K.M., Whang-Peng J., Knutsen T., Huebner K., Croce C.M. and Wang J.C. (1988)
Cloning and sequencing of cDNA encoding human DNA topoisomerase II localization of the gene to chromosome region 17q21-22.
Proc. Natl. Acad. Sci. USA 85 : 7177-7181

Tsuruo T., Iida H., Tsukagoshi S. and Sakurai Y (1981)
Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil.
Cancer Res. 41 : 1967-1972

Tsuruo T. and Fidler I.J. (1981)
Differences in drug sensitivity among tumor cells from parental tumors, selected variants, and spontaneous metastases.
Cancer Res. 41 : 3058-3064

Tsutsui K., Tsutsui K., Okada S., Watanabe M., Shohmori T., Seki S. and Inoue Y. (1993)
Molecular cloning of partial cDNAs for rat DNA topoisomerase II isoforms and their differential expression in brain development.
J. Biol. Chem. 268 : 19076-19083

Twentyman P.R., Wright K.A. and Rhodes T. (1991)
Radiation response of human lung cancer cells with inherent and acquired resistance to cisplatin.
Int. J. Radiat. Oncol. Biol. Phys. 20 : 217-220

Twentyman P.R., Fox. N.E., Wright K.A. and Bleehen N.M. (1986a)
Derivation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells.
Br. J. Cancer 53 : 529-537.

Twentyman P.R., Fox N.E. and Bleehen N.M. (1986b)
Drug resistance in human lung cancer cell lines: Cross-resistance studies and effects of the calcium transport blocker, verapamil.
Int. J. Rad. Oncol. Biol. Phys. 12 : 1355-1358

Unwin P.N.T. and Ennis P.D. (1984)
Two configurations of a channel-forming membrane protein.
Nature 307 : 609-613

Van der Bliek A.M., Kooiman P.M., Schneider C. and Borst P. (1988)
Sequence of *mdr3* cDNA encoding a human P-glycoprotein.
Gene 71 : 401-411

Van de Vrie W., Gheuens E.E.O., Durante N.M.C., De Bruijn E.D. Marquet R.L., Van Oosterom A.T. and Eggermont A.M.M. (1993)
In vitro and *in vivo* chemosensitizing effect of cyclosporin A on an intrinsic multidrug-resistant rat colon tumour.
J. Cancer Res. Clin. Oncol. 119 : 609-614

Van der Zee A.G.J., De Jong S., Keith W.N., Hollema H., Boonstra H. and De Vries E.G.E. (1994)
Quantitative and qualitative aspects of topoisomerase I and II α and ß in untreated and platinum/cyclophosphamide treated malignant ovarian tumours.
Cancer Res. 54 : 749-755.

Vasanthakumar G. and Ahmed N. (1985)
Uptake and metabolism of daunorubicin by human myelocytic cells.
Cancer Chemother. Pharmacol. 15 : 35-39

Versantvoort C.H.M., Broxterman H.J., Pinedo H.M., De Vries E.G.E., Feller N., Kuiper C.M. and Lankelma J. (1992)
Energy-dependent precesses involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoproterin expression.
Cancer Res. 52 : 17-23

Vindelov L.L., Hansen H.H., Christensen I.J., Spang-Thomsen M., Hirsch F.R., Hansen M. and Nissen N.I. (1980)
Clonal heterogeneity of small-cell anaplastic carcinoma of the lung demonstrated by flow-cytometric DNA analysis.
Cancer Res. 40 : 4295-4300

Volm M., Mattern J. and Samsel B. (1992a)
Relationship of inherent resistance to doxorubicin, proliferative activity and expression of P-glycoprotein 170, and glutathione S-transferase-π in human lung.
Cancer 70 : 764-769

Volm M., Mattern J., Efferth T. and Pommerenke E.W. (1992b)
Expression of several resistance mechanisms in untreated human kidney and lung carcinomas.
Anticancer Res. 12 : 1063-1068.

Volm M., Kastel M., Mattern J. and Efferth T. (1993)
Expression of resistance factors (P-glycoprotein, glutathione S-transferase-π, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas.
Cancer 71 : 3981-3987

Volm M., Pommerenke E.W. Efferth T., Lohrke H. and Mattern J. (1991)
Overexpression of P-glycoprotien and GSTπ in resistant nonsmall cell lung carcinomas of smokers.
Br. J. Cancer 64 : 700-704.

Von Hoff D.D., Clark G.M., Forseth B.J. and Cowan J.D. (1986)
Simultaneous *in vitro* drug sensitivity testing on tumors from different sites in the same patient.
Cancer 58 : 1007-1013.

Wang J.C. (1985)
DNA topoisomerases.
Ann. Rev. Biochem. 54 : 665-697

Wang J.C. (1969)
Interaction between DNA and an *Escherichia coli.* protein *w.*
J. Mol. Biol. 43 : 263-272

Warters R.L., Lyons B.W., Li T.M. and Chen D.J. (1991)
Topoisomerase II activity in a DNA double-strand break repair deficient Chinese hamster ovary cell line.
Mut. Res. DNA Repair. 254 : 167-174

Weaver J.L., Pine S.P., Aszalos A., Schoenlein P.V., Currier S.J., Padmanabhan R. and Gottesman M.M. (1991)
Laser scanning and confocal microscopy of daunorubicin, doxorubicin and rhodamine 123 in multidrug-resistant cells.
Exper. Cell Res. 196 : 323-329.

Wilson W.R., Whitmore G.F. and Hill R.P. (1981)
Activity of 4'-(9-acridinylamino)methanesulfon-*m*-anisidide against Chinese hamster cells in multicellular spheroids.
Cancer Res. 41 : 2817-2822

Wilson M.S., West C.M.L., Wilson G.D., Roberts S.A., James R.D. and Schofield P.F. (1993)
Intra-tumoral heterogeneity of tumour potential doubling times (T_{pot}) in colorectal cancer.
Br. J. Cancer 68 : 501-506

Woessner R.D., Mattern M.R., Mirabelli C.K., Johnson R.K. and Drake F.H. (1991)
Proliferation- and cell cycle-dependant differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells.
Cell Growth Diff. 2 : 209-214

Wolverton J.S., Danks M.K., Granzen B. and Beck W.T. (1992)
DNA topoisomerase II immunostaining in human leukemia and rhabdomyosarcoma cell lines and their responses to topoisomerase II inhibitors.
Cancer Res. 52 : 4248-4253

Worland S.T. and Wang. J.C. (1989)
Inducible overexpression, purification, and active site mapping of DNA topoisomerase II from the yeast *Saccharomyces cerevisiae*.
J. Biol. Chem. 264 : 4412-4416.

Worsham M.J., Carey T.E., Benninger M.S., Gasser K.M., Kelker W., Zarbo R.J. Van Dyke D.L. (1993)
Clonal cytogenetic evolution in a squamous cell carcinoma of the skin from a xeroderma pigmentosum patient.
Genes, Chromosomes & Cancer 7 : 158-164

Wyckoff E., Natalie D., NOlan J.M., Lee M. and Hsieh T.S. (1989)
Structure of *Drosophila* DNA topoisomerase II gene nucleotide sequence and homology among topoisomerases II.
J. Mol. Biol. 205 : 1-13

Yada T., Rose B. and Loewenstein W.R. (1985)
Diacylglycerol downregulates junctional membrane permeability: TMB-8 blocks this effect.
J. Membrane Biol. 88 : 217-232

Yamasaki H., Hollstein M., Mesnil M., Martel N. and Aguelon A-M. (1987)
Selective lack of intercellular communication between transformed and nontransfromed cells as a common property of chemical and oncogene transformation of BALB/c 3T3 cells.
Cancer Res. 47 : 5658-5664

Yang L-Y and Trujillo J.M. (1990)
Biological characterization of multidrug-resistant human colon carcinoma sublines induced/selected by two methods.
Cancer Res. 50 : 3218-3225

Yang L., Would M.S., Li J.J., Kelly. T.J. and Liu L.F. (1987)
Roles of DNA topoisomerase in simian virus 40 DNA replication *in vitro*.
Proc. Natl. Acad. Sci. USA 84 : 950-954

Yang L-Y., Trujillo J.M., Siciliano M.J., Kido Y., Siddik Z.H. and Su Y-Z. (1993)
Distinct P-glycoprotein expression in two subclones simultaneously selected from a human colon carcinoma cell line by cis-diamminedichloroplatinum (II).
Int. J. Cancer 53 : 478-485

Zaman G.J.R., Versantvoort C.H.M., Smit J.J.M., Eijdems E.W.H.M., De Haas M., Dmith A.J., Broxterman H.J., Mulder N.H., De Vries E.G.E., Baas F. and Borst P. (1993)
Analysis of the expression of *MRP*, the gene for new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines.
Cancer Res. 53 : 1747-1750

Zangwill B.C., Balsara G., Dunton C., Varello M., Rebane B.A., Hernandez E. and Atkinson B.F. (1993)
Ovarian carcinoma heterogeneity as demonstrated by DNA ploidy.
Cancer 71 : 2261-2267

Zechiedreich E.L. and Osheroff N. (1990)
Eukaryotic topoisomerases recognize nucleic acid topology by preferentially interacting with DNA crossovers.
EMBO J. 9 : 4555-4562

Zhang J.L. and Nicholson B.J. (1989)
Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA.
J. Cell Biol. 109 : 3391-3401

Zijlstra J.G., De Vries E.G.E. and Mulder N.H. (1987)
Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line.
Cancer Res. 47 : 1780-1784

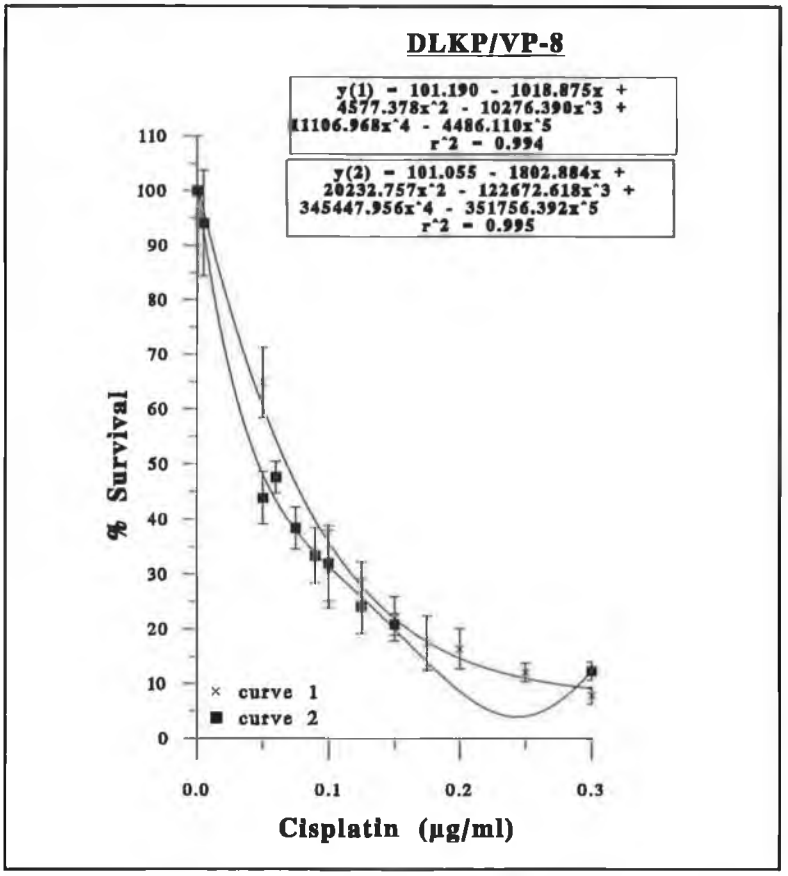
Zini N., Martelli A., Sabatelli P., Sarti S., Negri C., Astaldi Ricotta G., Maraldi N. (1992)
The 180-KDa isofrom of topoisomerase II is localized in the nucleolus and belongs to the structural elements of the nuclear remmant.
Exp. Cell Res. 200 : 460-466

Zuckier G. and Tritton T.R. (1983)
Adriamycin causes up regulation of epidermal growth factor receptors in actively growing cells.
Exper. Cell Res. 148 : 155-161

Zwelling L.A., Slovak M.L., Doroshow J.H., Hinds M., Chan D., Parker E., Mayes J., Sie K.L., Meltzer P.S. and Trent J.M. (1990)
HT1080/DR4: A P-glycoprotein-negative human fibrosarcoma cell line exhibiting resistance to topoisomerase II-reactive drugs despite the presence of a drug-senstive topoisomerase II.
J. Natl. Cancer Inst. 82 : 1553-1561

Appendix A

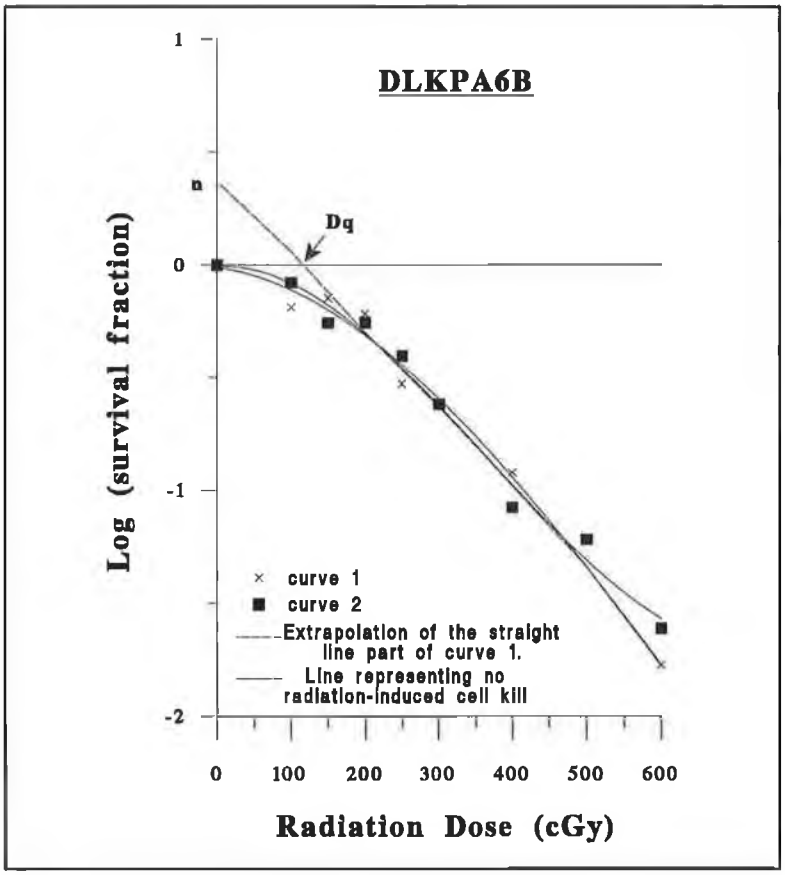
Determination of the IC_{50} value.



The polynomial equations illustrated in the above diagram, present the best correlation between cell survival, on the Y-axis and the drug concentration, on the X-axis. This equation has been obtained from the graphical curve fitting carried out by the Cricket Graph computer software package. The correlation coefficient, r, conveys information about the state of the fitness of the equation, in relation to the data obtained from the assay. Thus, the drug concentration corresponding to any percentage of the cell survival, can be calculated by solving the polynomial best fit curve. The determination of the IC_{50}, which denotes the drug concentration corresponding to 50% cell survival, has been carried out by taking Y = 50 and solving the best fit curve equation, with respect to Y. The Newton-Raphson method for numerical solution of *n*th. order polynomial equations has been employed for this determination. A computer program was developed in-house, by Dr. Mohamad Saleh, to execute and perform the calculation. The IC_{50} values, from replica assays, were determined in this manner and used to ascertain the average IC_{50} value and the standard deviation on the average value.

Appendix B

Description of D_o, D_q and *n* values.



The above diagram represents a typical survival curve for cells treated with radiation. The survival is plotted on a logarithmic scale against dose, plotted on a linear scale. The survival curve is characterised by a shoulder region, followed by a terminal exponential region, the slope of which is defined by a D_o value. D_o is referred to as the radiosensitivity of the cell or tissue under investigation, and is the dose required to reduce cell survival from any value *N* to 0.37*N*, in the straight-line region of the survival curve. The quasi-threshold dose, D_q, is the irradiation dose corresponding to the intercept of the extrapolated straight-line region of the survival curve with the 100% survival level. The extrapolation number, *n*, is defined by the intercept of the straight-line region, extrapolated back to the 0 dose axis. Both D_q and *n* define the width of the shoulder on the survival curve.

Appendix C

Abbreviations

amsacrine	4'-(-9-Acridinylamino)methanesulfin-*m*-anaiside
ATCC	American Tissue Culture Collection
ATP	Adenosine tri-phosphate
bp	base pair
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
DEM	Minimum essential medium eagle's
DMEM	Dulbeccos minimum eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene glycol-bis(ß-aminoethylether)-N,N,N',N'-tetraacetic acid
EGF	Epidermal growth factor
FCS	Foetal calf serum
GST	Glutathione-S-transferase
Gy	Gray
HCl	Hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
MDR	Multidrug resistant, Multiple drug resistant
$MgCl_2$	Magnesium chloride
mRNA	Messanger ribonucleic acid
MRP	Multidrug resistant related protein
$NaHCO_2$	Sodium bicarbonate
NaOH	Sodium Hydroxide
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodedecyl sulphate
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
Topoisomerase II	DNA Topoisomerase II enzyme
Tris	Tris(hydroxymethyl)aminomethane hydrochloride

Appendix D

Molecular weights of compounds used in this research

Adriamycin	580.0
Camptothecin	348.4
Cisplatin	300.0
5-Fluorouracil	130.1
Lucifer yellow	457.2
Verapamil	491.1
Vincristine	923.0
VP-16	588.6