# Drug Accumulation Studies in Multiple Drug Resistant Human Cell Lines

A thesis submitted for the degree of Ph.D.

by

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

*Signed:* Irene Cleary

*Date:* 2nd August 1995.

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### Abstract

Through continuous exposure to increasing concentrations of carboplatin, three novel platinum resistant variants of the human squamous carcinoma cell line, DLKP, were established. Cross resistance studies revealed that, while these variants exhibited resistance to the platinum analogues carboplatin and cisplatin, no significant resistance or sensitivity was observed against the classical MDR drugs, adriamycin and vincristine. Characterisation studies demonstrated that the resistant phenotype in the DLKPC variants did not appear to be to be either P-glycoprotein or topoisomerase mediated, nor did it appear to be related to alterations in either GST or glutathione levels. Metallothionein expression in two of the established variants, DLKPC 6.2 and DLKPC 14, was found to be increased, correlating with the degreee of platinum resistance. The increased level of metallothionein expression in the highest resistant variant, DLKPC 25, however, did not correlate with the platinum resistance level. This suggested the involvement of alternative mechanisms in mediating resistance in this variant, possibly through decreased intracellular platinum accumulation.

Drug accumulation studies with adriamycin and vincristine, were carried out on a number of human MDR cell lines, demonstrating reduced cellular accumulation of drug. The subsequent addition of verapamil or cyclosporin A, agents known to interact with P-glycoprotein, resulted in complete restoration of cellular drug levels in the MDR cell lines SKMES-1/ADR, T24A, T24V, OAW42-A and OAW42-A1, suggesting that the accumulation defect observed in these cell lines was P-glycoprotein mediated and that, accordingly, these cell lines exhibited characteristics consistent with classical MDR phenotype. In contrast, although P-glycoprotein appears to mediate a reduction in the cellular accumulation of drug in the DLKPA10 cell line, an alternative transport mechanism also appears to be present. Adriamycin subcellular distribution studies revealed the presence of an ATP-dependent, vesicular sequestration mechanism, involved in reducing adriamycin nuclear accumulation. This was demonstrated through ATP inhibition and disruption of the cytoplasmic vesicles, resulting in increased adriamycin accumulation and redistribution to the nucleus. In addition, studies indicated that membrane fluidity may be altered in these cells and that this may also contribute to a reduction in drug accumulation. An antibody was also raised against the DLKPA10 cells to determine if decreased drug accumulation was associated with either overexpression or down regulation of an antigen in the resistant cells. While overexpression of a 170-180 kDa antigen was observed in the DLKP parental cells, its role in mediating resistance remains to be clarified, although preliminary studies suggest that this antibody may play a role in cell adhesion or intercellular communication.

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# *1. Introduction*

## 1.1 General Introduction

Resistance to chemotherapy represents the single most important factor in the failure of many cancer treatments. While resistance to chemotherapeutic agents is inherent in many human tumours, other tumours, initially responsive to chemotherapy, also develop resistant variants. Tumours, inherently resistant to a variety of anticancer drugs, display a minimal, if any, response to chemotherapy. In most cases, the basis for this inherent resistance is undefined, although, probably involving a combination of kinetic and biochemical factors, such as inability to effectively transport the drug into the cell or to convert it into its active form. With acquired drug resistance, a population of cells, initially sensitive to the drug, develops resistant characteristics. Typically, while there is an initial decrease in tumour mass, tumour growth eventually resumes, despite continued treatment. Cells, either partially or completely resistant to an anticancer agent are, therefore, more likely to survive in a drug-containing environment. Combination chemotherapy was introduced in an attempt to overcome this problem. However, many tumours can also develop multidrug resistant (MDR) variants. Classical MDR is characterised by cross-resistance to a range of chemically unrelated drugs. In general, the cytotoxic agents involved are large molecules, containing hydrophobic and hydrophilic regions and include the anthracyclines, vinca alkaloids, epipodophyllotoxins, actinomycin D and colchicine (Clynes *et al.*, 1990; Nielsen and Skovsgaard, 1992; Clynes *et al.*, 1993; Prat *et al.*, 1994).

Given the widespread lack of sustained response to chemotherapy in human tumours, there is considerable interest in understanding the mechanisms of MDR. In attempting to identify the factors giving rise to MDR, a number of mechanisms have been described. These include an increase in drug efflux, relating with an overexpression of a membrane glycoprotein (Juliano and Ling, 1976), reduced expression of the enzyme, topoisomerase II, which is involved in DNA replication and repair (Hoffmann and Mattern, 1993), and an alteration in the metabolising enzymes, glutathione-S-transferases (Moscow and Dixon, 1993). The most frequent determinant of MDR in many tumour cell lines, however, appears to be the ability of the cells to greatly decrease the cellular accumulation of drug. This usually results from the overexpression of the *mdr1* gene, which encodes the transmembrane protein, P-glycoprotein. This protein acts as an ATP-dependent efflux pump for a variety of cytotoxic agents, reducing the cellular concentration of the drug (Dano, 1973; Fojo *et al.*, 1985; Willingham *et al.*, 1986).

## 1.2 Pharmacology of MDR drugs

### 1.2.1 The Anthracyclines

The anthracycline antibiotics are among the most important of the antitumour agents and are routinely used in the treatment of cancer. They are produced by the fungus, *Streptomyces peucetius* var. *caesius*. Daunorubicin was isolated independently by two groups in the early 1960s (DiMarco and Dubost) and adriamycin (doxorubicin) was isolated by Arcamone and coworkers in 1969. Although they differ, only slightly, in chemical structure, daunorubicin has been used primarily in the treatment of acute lymphocytic and acute granulocytic leukaemias, whereas adriamycin displays activity against a wide range of human cancers, including a variety of solid tumours. Used concurrently with other anticancer agents, adriamycin is used in the treatment of non-Hodgkins lymphoma, as well as carcinomas of the ovary, breast, bladder and lung. Adriamycin is also a useful agent in the treatment of metastatic thyroid carcinoma and carcinomas of the endometrium, testes, prostate, cervix and head and neck (Prat *et al.*, 1984). Unfortunately the clinical value of both agents is limited, due to their toxic side effects. These include, nausea, vomiting, loss of hair, myelosupression and local tissue necrosis. Damage to heart muscle is also a major toxicity of the anthracyclines, the incidence of which increases with increasing cumulative dosage of drug (Unverferth *et al.*, 1982). The drug induced cardiomyopathy presents a severe, rapidly progressive, congestive heart failure, with the classical signs of tachycardia, pulmonary edema and pleural effusion. Consequently, a large number of drugs are being developed in an effort to retain the antitumour therapeutic effects of the anthracyclines while reducing their toxicity.

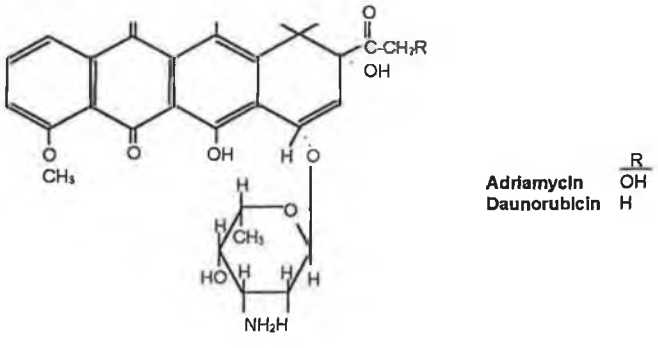


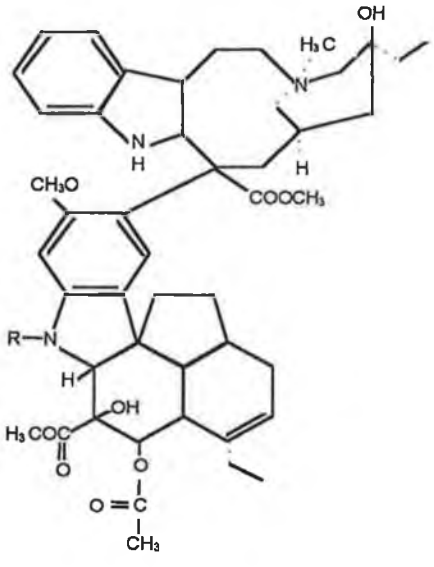

**Figure 1.2.1** Structure of adriamycin and daunorubicin

The antracycline antibiotics consist of tetracycline ring structures with an unusual sugar, daunosamine, attached by glycosidic linkages. Cytotoxic agents of this class all contain quinone and hydroquinone moieties on adjacent rings, permitting them to function as electron-accepting and -donating agents. The chemical structures of adriamycin and daunorubicin differ only by a single hydroxyl group, on $C^{14}$, as shown in figure 1.2.1. A number of mechanisms have been attributed to the action of the anthracyclines, which, as yet, remain to be clarified. Most of the available data relates to the cytotoxicity to binding of the drugs to DNA, by intercalating between the base pairs. The anthracyclines bind tightly to DNA, with their cytotoxicity appearing to be associated with this binding, although, the pathway leading to cytotoxicity remains to be confirmed. Studies have shown that the anthracyclines may induce single and double strand breaks in DNA, by interacting with the enzyme , topoisomerase II (section 1.6.1). It has also been proposed that the anthracyclines may undergo metabolism of its quinone ring to form a semiquinone radical that reacts with oxygen to form superoxide $O_2$- (Bachur *et al.*, 1977). The superoxide is known to undergo several reactions that can ultimately lead to cell death, including oxidative damage to cell membranes and DNA. Adriamycin and related drugs also bind to cell membranes and thus may exert its toxicity through membrane related effects. It has also been demonstrated that adriamycin can cause cell death without actually being transported into the cells (Tritton and Yee, 1982; Vichhi and Tritton, 1983). However, the transport of anthracyclines into other cells is important for toxicity since decrease in the cellular accumulation of drug is an important mechanism relating to acquired multidrug resistance (section 1.4).

Adriamycin and daunorubicin are usually administered intravenously and are subsequently widely distributed in the body. There is rapid uptake of the drugs in the heart, kidney, liver, lungs and spleen. Plasma clearance of the drugs is triphasic with a initial half life of approximately 12 minutes, an intermediate half life of approximately 3.3 hours, attributable to metabolism of the drug in the liver, and a final phase of approximately 30 hours in which the drug is released from tissue binding sites. Although both daunorubicin and adriamycin are metabolised in the liver and excreted in the bile, there is notable differences in the metabolism of the two compounds. Daunorubicin is metabolised primarily to daunorubicinol, while a lot of adriamycin is excreted unchanged and there appears to be multiple metabolites including adriamycinol which retains some cytotoxic activity.

### 1.2.2 The Vinca Alkaloids

The vinca alkaloids are compounds derived from extracts of the periwinkle plant, *Catharantus roseus G.Don.* Noble and coworkers (1958) observed granulocytopenia and bone-marrow suppression in rats treated with extracts of the plant, which led to purification of an active alkaloid. Other investigations showed activity of alkaloid fractions against acute lymphocytic neoplasm in mice (Johnson *et al.*, 1963; Johnson *et al.*, 1968). Fractionation of these extracts yielded four active dimeric alkaloids: vincristine, vinblastine, vinleurosine and vinrosidine. However, only two of these, vincristine and vinblastine, have to be clinically useful (Hodes *et al.*, 1960; Costa *et al.*, 1962; Gialani *et al.*, 1966; Nobel *et al.*, 1967). Chemically, the vinca alkaloids are very similar. They are asymmetrical dimers. The structure of vincristine and vinblastine are shown in figure 1.2.2.




| | R |
|---|---|
| Vincristine | CHO |
| Vinblastine | $CH_3$ |

**Figure 1.2.2** Structure of the vinca alkaloids

Minor differences in the structure of these large alkaloidal molecules result in notable differences in toxicity and antitumour activity of vincristine and vinblastine. Vinblastine is an important drug in combination chemotherapy (with cisplatin or bleomycin) of testicular cancer, while vincristine is the most clinically used drug for the treatment of childhood leukemia. Alterations in the structure of the molecules can result in decreased activity such as removal of the acetyl group at $C_4$ of one portion of vinblastine or

acetylation of the hydroxyl groups. Both of these effects can result in the destruction of its antileukemic activity. Hydrogenation of the double bond or reductive formation of carbinols also reduces or destroys the activity of the alkaloids. Serious side effects have been associated with both vinblastine and vincristine. Vinblastine causes major toxicity to bone marrow and its use also leads to gastrointestinal disturbances, loss of hair and neurotoxicity. The clinical toxicity of vincristine is mostly neurological with parenthesis, loss of deep tendon reflexes, muscle weakness and neuritic pain. The observation that minor structural differences were associated with marked pharmacological changes has stimulated much research on the development of semisynthetic derivatives, maintaining the antitumour activity, with less toxicity. A number of compounds have been entered in clinical trials and include, vindesine (Cersosimo et al 1983), vinorelbine (Depierre *et al.*, 1991), vinzolidine (Khayat *et al.*, 1992) and vintriptol (Oosterkamp *et al.*, 1991).

The vinca alkaloids are cell-cycle-specific agents, which block mitosis with metaphase arrest. They act by binding to the protein tubulin, thus inhibiting its polymerization, to form microtubules. Disruption of the microtubules results in the inhibition of mitotic spindle formation and, thus, cell division is arrested in metaphase. In the absence of intact mitotic spindles the chromosomes may either disperse throughout the cytoplasm or occur in unusual groupings. The inability to segregate chromosomes correctly, ultimately leads to cell death. Resistance to the vinca alkaloids has been reported to be associated with decreased membrane transport and decreased accumulation of the drug (Fojo *et al.*, 1987; Chao *et al.*, 1990; Shalinsky *et al.*, 1993). Resistance to drugs that cause microtubule depolymerization can also be due to alterations in tubulin structure. In some instances, these mutations affect drug binding to tubulin (Ling *et al.*, 1979) .

Vinca alkaloids, usually administered intravenously, are rapidly cleared from the blood. Their plasma clearance is described by triphasic curves, with an initial half life of of 2-5 minutes, an intermediate half life of approximately 1.5 hours and a terminal half life of approximately 25 hours and 85 hours for vinblastine and vincristine respectively. Studies, using radiolabelled drugs, show that the vinca alkaloids are primarily eliminated by a combination of hepatic metabolism and biliary excretion, with only a small amount of parent drug and metabolites recovered in the urine (Owellan *et al.*, 1977).

### 1.2.3 The Epipodophyllotoxins

Podophyllotoxin is an extract derived from the mandrake plant *Podophyllum peltatum*. It is an antimitotic agent, acting by binding to tubulin at a site different to the vinca alkaloids and preventing the formation of microtubules (Pratt and Ruddon, 1979). The podophyllotoxins also act by inhibiting the nuclear enzyme topoisomerase II, causing DNA damage (Ross *et al.*, 1984). Clinical studies were carried out in the early 1950s to evaluate the activity of extracts of podophyllotoxin. However, it was found that the extracts had poor antitumour activity and were extremely toxic, thereby curtailing any clinical use. A number of semisynthetic derivatives of podophyllotoxin have since been developed and two compounds in particular, VP16 (etoposide) and VM-26 (tenoposide) have been demonstrated to display antitumour activity. In recent years VP16 has been used increasingly for the treatment of various cancers including small-cell lung cancer, testicular cancer and lymphomas, leukaemia and Kaposis sarcoma (Issell *et al.*, 1984; van Mannen *et al.*, 1988). VM-26 has proved successful in the treatment of a wider range of cancers, including refractory acute lymphoblastic leukaemias, neuroblastoma, small-cell lung cancer, refractory Hodgkins and non-Hodgkins lymphoma and central nervous system malignancies. The dose limiting toxicity of the epipodophyllotoxins is to the bone marrow. Treatment with these agents can result in leukopenia and thrombocytopenia. Other side effects associated with epipodophyllotoxins include gastrointestinal toxicity and loss of hair.

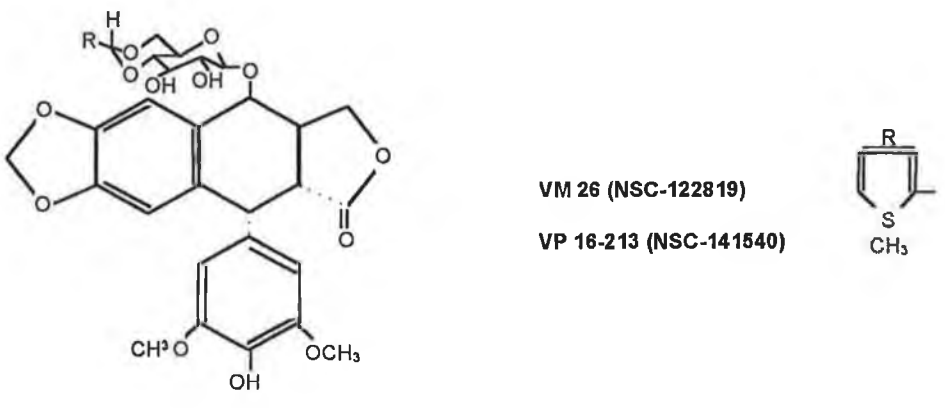



**Figure 1.2.3** Structure of the epipodophyllotoxins

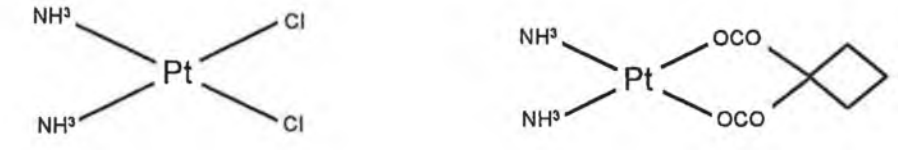
The epipodophyllotoxin family of drugs share a common multiringed structure, as shown in figure 1.2.3. This multiringed structure is referred to as epipodophyllotoxin, from which is derived the family name of these compounds. Although the podophyllotoxins bind tubulin, VP16 and VM-26 appear to have very little effect on microtubulin assembly or degradation. The drugs can inhibit nucleoside incorporation into RNA and DNA and also cause single strand breaks in DNA. The major mechanism underlying the antitumour activity of VP16 and VM-26 therefore appears to be mediated through topoisomerase II inhibition (van Mannen *et al.*, 1988). Resistance to the epipodophyllotoxins is associated with decreased activity of the topoisomerase enzyme and decreased accumulation of the drug due to the presence of the membrane based P-glycoprotein efflux pump.

VP16 and VM-26 are usually administered by intravenous injection and their plasma clearance is based on a biphasic model, with initial and terminal half lives of 3 hours and 15 hours respectively. Following administration, approximately 60% of the drug is excreted unchanged while the remainder is excreted in the biliary tract.

### 1.2.4 Cisplatin and platinum analogues

The platinum complexes are a class of cytotoxic agents, first discovered by Rosenberg and coworkers. Growth inhibition of *E. coli* was observed when an electric current was delivered to the bacteria, through platinum electrodes. The inhibitory effect on the bacteria was subsequently shown to be due to the formation of inorganic, platinum containing, compounds, in the presence of ammonium and chloride ions (Rosenberg *et al.*, 1965; Rosenberg *et al.*, 1967). The active compound was found to be cisplatin, which was shown to have cytotoxic activity against several tumours in mice (Rosenberg *et al.*, 1969). Cisplatin is currently one of the most effective anticancer agents, used in the treatment of ovarian, testicular, head and neck, non-small cell lung and brain tumours (Rosenberg, 1985). The major toxicity caused by cisplatin is dose related nephrotoxicity, causing cumulative impairment of renal tubule function, which may ultimately lead to renal failure. Other side effects include, nausea, vomiting, myelosupression, tinnitus and loss of hearing. The severity of the side effects has led to the synthesis of other platinum compounds, including carboplatin, iproplatin (Sessa *et*

*al.*, 1990), oxaliplatin (Extra *et al.*, 1990) and tetraplatin (Burchenal *et al.*, 1980). Carboplatin is now used frequently as a cytotoxic agent, in particular for the treatment of ovarian and lung carcinomas (Gore *et al.*, 1989; Raghaven *et al.*, 1994). It has considerably less nephrotoxicity than cisplatin. However, its antitumour activity spectrum and cross resistance pattern has been demonstrated to be very similar to cisplatin (Calvert *et al.*, 1982; Canetta *et al.*, 1985; Gore *et al.*, 1989).




**a.** **b.**

**Figure 1.2.4** Structure of cisplatin (a) and carboplatin (b)

Cisplatin and its analogues are inorganic water-soluble compounds, with relatively simple structural formulas, as presented in figure 1.2.4. They exert their toxicity by binding to base pairs of DNA, resulting in the formation of intrastrand cross-links and intrastrand adducts. This leads to the disruption and unwinding of the double helix of the DNA molecule. Therefore, the degree of toxicity correlates with the number of cross links formed between the DNA strands (Fichtinger-Schepman *et al.*, 1987). The leaving groups of the platinum complexes may be displaced directly by nucleophilic groups of DNA, or indirectly, after the leaving group is displaced by hydroxyl groups, through the reaction of the drug with water. Cisplatin and carboplatin bind to two sites on the DNA molecule, with the preferred sites for binding being the N-7 positions of guanine and adenine bases. Cellular resistance to cisplatin and cisplatin analogues has been associated with a number of mechanisms. These include decreased DNA damage or increased DNA repair (Lai *et al.*, 1988; Andrews and Howell, 1990; Johnson *et al.*, 1994), enhanced inactivation by the intracellular detoxication systems *ie* glutathione (Waxman, 1990) and metallothionein (Bahnson *et al.*, 1991) and reduced drug accumulation (Gately and Howell, 1993).

The platinum complexes are usually injected intravenously and following administration are rapidly and tightly bound to proteins with more than 90% of the free drug lost in the first two hours. The plasma clearance for the free drug is described as a two compartmental model. However the clearance for total drug (free and bound) may be described as a three compartmental model with a prolonged terminal half life of 58 to 73 hours. High concentrations of platinum compounds are found in the kidney, liver, intestines and testes, but there is poor penetration into the central nervous system. The platinum compounds are excreted mainly via the urine and approximately 15-30% of the administered drug is excreted during the first 24 hours.

## 1.3 P-glycoprotein in MDR

It was first reported by Kessel *et al.* (1968) that anthracycline-resistant cells accumulated less drug than the sensitive parental cell line. Further studies showed that cellular resistance to anticancer agents occurred in association with the activation of an energy dependent pump (Dano, 1973) which was subsequently identified by Juliano and Ling (1976) and was designated P-glycoprotein, P-gp or P-170 (figure 1.3). P-glycoprotein is an 170 kilodalton membrane associated protein of approximately 1280 amino acids in length. It contains 12 transmembrane domains and consists of a short highly charged cytoplasmic domain followed by three membrane loops and a large cytoplasmic domain. This cytoplasmic domain is followed by three additional membrane loops and another large cytoplasmic domain (Gros *et al.*, 1986a and Chen *et al.*, 1986). ATP binding sites are found in both of the large cytoplasmic domains (Walker *et al.*, 1982). Therefore, the P-170 molecule appears to comprise of two halves, each of similar structure. When the two halves are aligned a short segment called the linker region appears to bridge the amino and carboxy halves (Van der Bliek *et al.*, 1987). The linker regions of P-glycoproteins that convey drug resistance contain sequences for c-AMP and c-GMP-dependent protein kinase phosphorylation sites, however these sequences are not present in P-glycoproteins that are not capable of conveying drug resistance (Hsu *et al.*, 1989).

Clear homologies have been shown to exist between P-glycoprotein and several bacterial transport proteins, in particular with the conservation of the ATP binding domains. These proteins transport substances into bacterial cells and obtain their energy from the hydrolysis of ATP. Strong homology exists between P-glycoprotein and the Hly B protein of *Escherichia coli*, which exports $\alpha$ hemolysin from haemolytic bacteria. The carboxy terminal of the Hly B protein contains the ATP binding site and is identical to P-glycoprotein in 50% of its amino acid sequence (Juranka *et al.*, 1989). Other homologies of the P-glycoprotein include the protein ndvA, which is involved in $\beta$-(1-2)glycan transport in *Rhizobium Meliloti* (Stansfield *et al.*, 1988) and the leukotoxin secretion, Ikt, found in *Pasteurella Haemolytica* (Strathdee and Lo, 1989). In eukaryotes, one defined normal role for an MDR homologous gene has been found with the STE6 gene of yeast *Saccaromyces cerevisiae* (McGrath and Varshavsky, 1989). The product of this locus actively transports the $\alpha$ factor mating pheromone in a manner analogous to the efflux of cytotoxic drugs by the P-glycoprotein and shares approximately 57% of the amino acid sequence with P-

glycoprotein. The product of the cystic fibrosis gene also shows homology to P-gp (Riordan *et al.*, 1989), the polypeptide contains two repeat motifs each of which consists of six membrane spanning domains and a hydrophilic region containing the ATP binding sites. The two halves display approximately 21% amino acid identity to each other and 15% amino acid identity with the mammalian P-glycoproteins. A large highly charged cytoplasmic domain, termed the R group, contains a cluster of protein kinase A and protein kinase C phosphorylation sites and links the two halves of the molecule at the analogous position of the linker region in the P-glycoprotein.

P-glycoprotein is encoded by the *mdr-1* gene which is located on the long arm of chromosome 7 at position 21.1 and was sequenced and cloned in 1986 (Chen *et al.*, 1986; Gros *et al.*,1986b). Two promoter sites for *mdr* gene expression have been identified, one upstream and one downstream (Ueda *et al.*, 1987). The majority of resistant cells *in vitro* appear to utilize the downstream promoter, although both sites may become active (Kohno *et al.*, 1989). The *mdr* gene is translated as a 140 kilodalton precursor protein which undergoes post translational modification over a two to four hour period to form the 170 kDa P-glycoprotein. This process requires enzymatic phosphorylation by protein kinase C to achieve the active form (Anderson *et al.*, 1991). The *mdr* gene was first isolated from hamsters which were shown to have three homologes of the gene (Endicott *et al.*, 1987; Ng *et al.*, 1989). The mouse *mdr* gene family has also been shown to have three classes (Raymond *et al.*, 1990; Gros *et al.*, 1986b). While other species can display up to six mdr gene homologues, humans only have two classes (Chen *et al.*, 1986; Rononson *et al.*, 1986; Van der Bliek *et al.*, 1988; Chin *et al.*, 1989; Chen *et al.*, 1990). Table 1.1 represents the human, hamster and mouse *mdr* gene families. (Two terminologies are used in the literature for the mouse mdr gene family).

| Class | Human | Mouse | Hamster |
|---|---|---|---|
| I | *mdr1* | *mdr3*<br>*mdr1a* | pgp1 |
| II | | *mdr1*<br>*mdr1b* | pgp2 |
| III | *mdr2/3* | *mdr2*<br>*mdr2* | pgp3 |

**Table 1.1** Classification and nomenclature of the mammalian P-glycoproteins

In hamsters class I and II only are thought to be responsible for drug resistance. Only class I (*mdr1*) is believed to convey drug resistance in humans. This has been verified by transfection of the *mdr* genes into drug sensitive cells resulting in the establishment of multidrug resistant cell lines. Gros *et al.* (1986a) have shown that transfection of full length *mdr1* cDNA in the drug sensitive hamster cell line LZ, confered a complete multidrug resistant phenotype in the cells. In contrast Van der Bliek *et al.* (1988) demonstrated that transfection of the *mdr3* gene did not confer the resistant phenotype.

A variety of physical and chemical agents have been demonstrated to affect the expression of the *mdr* gene, predominantly through increased transcription of the gene. The differentiating agents dimethyl sulphoxide (DMSO), sodium butyrate and dimethyl formamide have all been shown to increase both *mdr1* mRNA and P-glycoprotein levels in a number of colon carcinoma cell lines (including the cell lines KB, SW-620 and HCT-15) as measured by *in situ* hybridization and immunoblotting (Mickley *et al.*, 1989). The renal carcinoma cell line, HTB 46, demonstrated a 7 to 8-fold increase in *mdr1* mRNA levels in response to heat shock, ethanol, sodium arsenite and cadmium chloride (Chin *et al.*, 1990). These findings were consistent with a role for P-glycoprotein as a stress inducible gene product following environmental insults. Phorbol esters, which increase the activity of protein kinase C have also been shown to increase *mdr* expression. Bates *et al.* (1989) have demonstrated the overexpression of *mdr1* gene in human neuroblastoma cells following exposure to retonic acid. The HIV virus can also increase the expression of P-glycoprotein of infected cells (Kohno *et al.*, 1989; Gupta *et al.*, 1990).

### 1.3.1 P-glycoprotein in normal tissues

To date, the physiological role of P-glycoprotein is unknown. Studies of nonmalignant tissues using immunohistochemical and *in situ* hybridization techniques have revealed that many normal tissues express high amounts of P-glycoprotein. The expression of P-glycoprotein occurs primarily in specialised epithelial cells with secretory or excretory functions. Generally the highest levels of P-glycoprotein have been found in the secretory cells of the lumina of the proximal tubules in the kidney, in the regions of the zona fasciculata and reticulata of the adrenal cortex, on the luminal surface of biliary hepatocytes and the mucosal surface of epithelial cells of the small and large intestine (Thiebaut *et al.*,

1987). Strong expression has also been demonstrated in endothelial cells of capillary blood vessels at blood tissue barrier sites, primarily capillaries of the central nervous system, testis and dermis (Thiebaut *et al.*, 1989). P-glycoprotein has also been detected in placenta tissue (Sugawara *et al.*, 1988) and circulating lymphocytes (Chaudhary *et al.*, 1992). Although the actual substrate of P-glycoprotein in these normal tissues is unknown, its presence in specialised tissues suggests that P-glycoprotein may have a protective function in the cells by actively effluxing toxic substances into the bloodstream, urine or bile. It has also been proposed that P-glycoprotein may act as a cellular transporter of endogenous substances including corticosteroids and bile (Van Kalken *et al.*, 1993).

### 1.3.2 P-glycoprotein in human tumours

Numerous studies have been preformed to determine the significance of *mdr1* expression in clinical tumour samples. Both measurement of RNA levels by *in-situ* hybridization and RT-PCR and immunocytochemistry have been employed. In general, tumours derived from tissue that naturally express high levels of P-glycoprotein show overexpression of the mdr gene. An extensive study on *mdr1* mRNA levels in over 400 human cancers demonstrated elevated expression of the *mdr1* gene in colon cancer, renal cell carcinoma, adrenotical carcinoma, hepatoma and pheochromoctoma, all of which naturally express mdr (Goldstein *et al.*, 1989). Messagner RNA levels were quantified using slot blot analysis comparing tumour samples to known drug sensitive (KB-3-1) and resistant (KB-8-5) cells. High P-glycoprotein expression has also been demonstrated in endometrial and gastric cancers (Schneider *et al.*, 1993; Robey-Cafferty *et al.*, 1991). Overexpression of the *mdr* gene has also been reported in leukemias, lymphomas and some other cancers derived from tissues that do not normally express the gene (Rischin and ling, 1993; Goasguen *et al.*, 1993). In a study on samples from patients with lymphoblastic leukaemia, 36 out of 104 samples screened were positive for P-glycoprotein expression (Sauerberg *et al.*, 1994). Several tumours have been demonstrated to express low levels of *mdr1* and are referred to as drug sensitive, including cancer of the ovary and breast and Wilm's tumour. Dixon and coworkers (1992) reported positive immunohistochemical staining with the monoclonal antibody C219 in a number of breast cancer samples. Veneroni *et al.* (1994) also demonstrated P-glycoprotein expression in breast cancer patients. In the same study, a number of ovarian samples were also found to be positive for P-glycoprotein expression.

However, other tumours also have low levels of the *mdr* gene but are drug resistant, such as adenocarcinomas of the lung and non-small cell lung carcinoma thus suggesting that other mechanisms of resistance are involved.

Many reports have indicated that overexpression of MDR is found more often in samples obtained from patients following treatment with chemotherapeutic agents, *ie* acquired drug resistance. In one study preformed on human soft tissue sarcoma samples, P-glycoprotein expression was found to be greater in samples obtained from patients following treatment with doxorubicin than in samples obtained from untreated patients (Toffoli *et al.*, 1992). Increased expression has also been noted in drug resistant leukemias, myelomas, ovarian cancers, breast cancers and neuroblastomas (Bourhis *et al.*, 1989; Dalton *et al.*, 1989a; Dalton *et al.*, 1989b; Goldstein *et al.*, 1989). The majority of studies so far have included relatively too few patients and consequently the clinical significance of MDR expression has been difficult to clarify. The lack of uniform testing for P-gp expression and the possible contamination by P-gp from normal tissue also makes it difficult to assess the contribution of P-glycoprotein to the clinical outcome. Findings from one group showed a significant relation between P-glycoprotein expression and poor survival in samples from patients with neuroblastoma, while another group using the same monoclonal antibody reported that positive staining of the neuroblastoma samples was restricted to the normal cells in the tumour and neuroblastoma cells were generally P-glycoprotein negative (Bates *et al.*, 1991; Chan *et al.*, 1991; Favrot *et al.*, 1991).

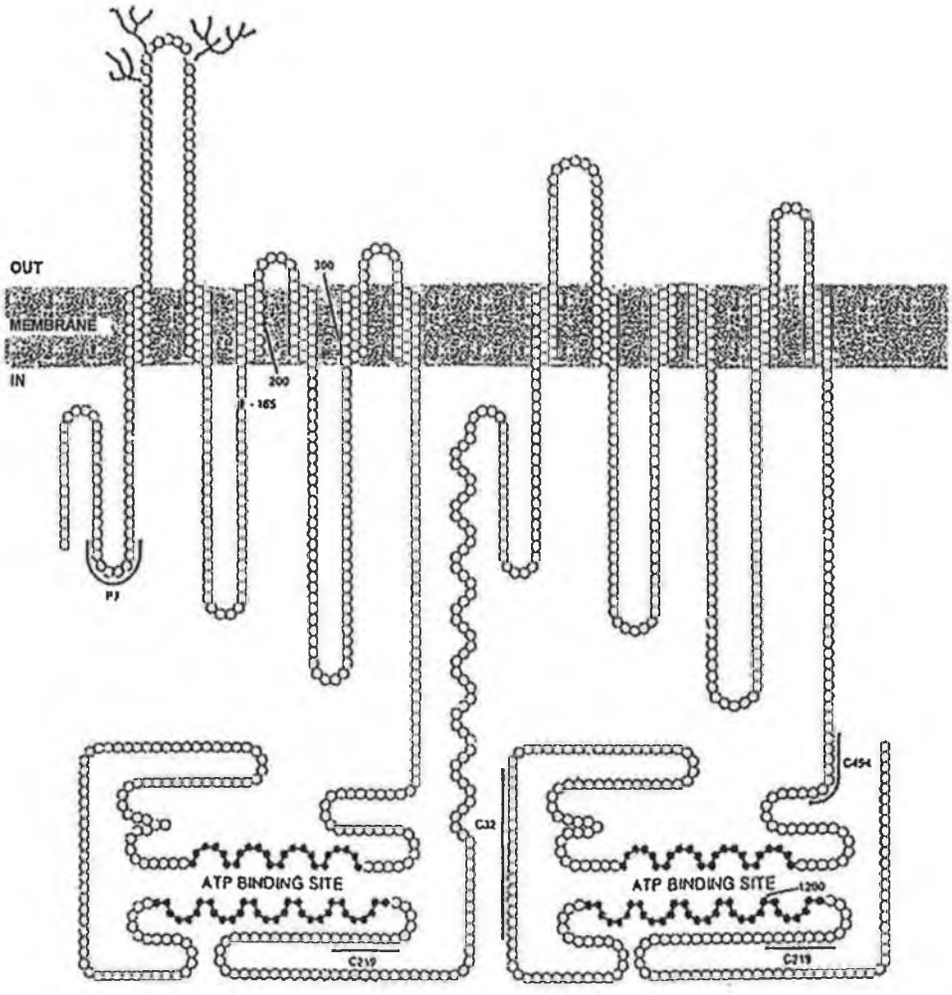



**Figure 1.3** Model of the MDR Transporter

## 1.4 P-glycoprotein in MDR cell lines defective in the accumulation of drug

P-glycoprotein expression has been studied in numerous MDR cell lines selected with various anticancer agents, including adriamycin, daunorubicin, vincristine, vinblastine, VP16, colchicine and taxol. It has frequently been demonstrated that the overexpression of P-glycoprotein in MDR cell lines is associated with a decrease in cellular accumulation of the selecting agent and other MDR related drugs. P-glycoprotein acts as an ATP-dependent efflux pump for a variety of cytotoxic agents, in this way reducing the cellular concentration of the drug. The majority of early studies on the accumulation of anticancer agents were carried out using rodent cell lines and Ehrlich ascites tumour cells. Most of the literature describes experiments on the uptake of the anthracyclines, in particular adriamycin and daunorubicin, as well as the vinca alkaloids. Various techniques have been employed to determine the cellular accumulation, retention and efflux of drugs including spectrofluorimetry, radiolabel studies, radioautography and more recently HPLC and flow cytometry studies. Decreased levels of anthracycline accumulation in resistant cells were first observed in resistant variants of chinese hamster ovary cells (Biedler and Riehm, 1969; Biedler and Riehm, 1970; Riehm and Biedler, 1971), in murine leukaemic cell lines (Chervinsky and Wang, 1972; Inaba and Johnson, 1978) and in Ehrlich ascitic cells (Skovsgaard, 1977; Skovsgaard, 1978a). Studies on the uptake of the vinca alkaloids have also revealed a reduction in the cellular accumulation of the drug in resistant variants, relative to the sensitive cells. Skovsgaard (1978b) demonstrated decreased cellular accumulation of the vinca alkaloid, vincristine, in the vincristine resistant cell line, EHR 2/VCR+, when compared to the parental wild type cells. A decrease in vincristine uptake was also observed in the vincristine resistant cell line, P388 (Tsurno *et al.*, 1981) and in a number of murine solid tumour Lewis Lung carcinoma cell lines resistant to vincristine (Tsuruo *et al.*, 1983).

Initial observations led to the suggestion that the reduction in drug accumulation in resistant cells was primarily due to differences in the cell membranes. It was postulated that alterations in the membrane of resistant cells resulted in decreased permeability and thus a reduction in the cellular accumulation of the drug. Studies also showed that intracellular pH has a marked effect on the net accumulation of the drugs. The $pK_a$ of the anthracyclines was found to be 8.15-8.45, suggesting that the uptake of the anthracyclines was determined by the permeability of the cell membrane to the un-ionized form of the molecule

(Skovsgaard, 1977). However, indications of an active outward transport of anthracyclines was obtained from studies by Dano in 1973, which demonstrated that the release of daunorubicin from resistant Ehrlich ascites tumour cells was reduced by metabolic inhibitors. Further evidence supporting an active outward efflux model was later reported by the same laboratory, from studies on the transport of the anthracyclines, adriamycin, daunorubicin and rubidazone in Ehrlich ascitic cells. The results obtained showed that treatment of the ascitic cells with the metabolic inhibitor, sodium azide, significantly enhanced the cellular accumulation of the three drugs in the resistant cells. A decrease in the cellular release of the drugs was also observed (Skovgaard, 1977). Inaba and coworkers (1979) demonstrated the presence of an active drug efflux mechanism in the murine leukaemia cell line, P388. Earlier reports had demonstrated reduced uptake of adriamycin and daunorubicin in anthracycline resistant variants of the P388 cells, when compared to the parental wild type cells (Inaba and Johnson, 1978) while these studies showed that the accumulation defect observed in the resistant cells could be abolished by the addition of metabolic inhibitors, including 2,4-dinitrophenol, sodium azide and valinomycin. These metabolic inhibitors enhanced daunorubicin accumulation in both sensitive and resistant cells, although daunorubicin uptake was enhanced to a much greater extent in the resistant P388 subline.

Initial drug accumulation studies on human epithelial cell lines were carried out by Fojo and coworkers in the mid 1980s. Detailed studies on the uptake, retention and efflux of six MDR related drugs in four resistant variant of the human colon carcinoma cell line, KB, were reported (Fojo *et al.*, 1985). The resistant mutants were selected in increasing concentrations of the anticancer drug, colchicine, and were found to be cross-resistant to adriamycin, vincristine, vinblastine and actinomycin A. Drug accumulation in the resistant cells was found to decrease with increasing levels of resistance and this relationship was most clearly observed with colchicine, vincristine, vinblastine and adriamycin, and to a lesser extent with actinomycin A. The accumulation of dexamethasone, an agent to which all lines were equally sensitive, was similar for the parental cell line and all four resistant variants. When drug efflux was investigated, it was found that a greater percentage of the drug was released in the more resistant variants. The rate of drug efflux was also found to be substantially higher in the resistant cells. A number of studies were also carried out on drug accumulation in human glioma cells. Merry *et al.* (1986) investigated the transport of adriamycin in three human glioma cell lines with different inherent sensitivities to

adriamycin while Kaba *et al.* (1985) studied the uptake and intracellular accumulation of vincristine in a number of glioblastoma derived cell lines.

### 1.4.1 Circumvention of MDR by interaction with P-glycoprotein

Several studies have since been published investigating the transport of anticancer agents in numerous human multidrug resistant cell lines. In general, a decrease in drug accumulation and an increase in the rate of drug efflux was observed in the resistant variants when compared to the sensitive parental cell lines. Many of the studies specifically investigated the effect of various compounds on drug accumulation and efflux in resistant cell lines. Several compounds belonging to various pharmacological families have been demonstrated to reverse the MDR phenotype *in vitro* (Steward and Evans, 1989; Ford and Hait, 1990). Among these compounds are a number of the calcium channel blockers, including verapamil and nifedepine, immunosuppressive agents such as cyclosporin A (and its more potent analogue, SDZ PSC 833) and calmodulin inhibitors, including the phenothiazines, trifluoroperazine and chloropromazine. These agents may modulate resistance by interacting with the chemotherapeutic drug binding sites of the P-glycoprotein molecule thereby preventing the drug from binding to the P-glycoprotein and being extruded from the cell.

#### 1.4.1.1 Calcium Antagonists

Although a number of calcium channel antagonists have been demonstrated to enhance drug accumulation in MDR cell lines, verapamil has been the most widely studied calcium channel blocker in the area of MDR circumvention. Many reports have suggested that calcium channel antagonists reverse resistance to most chemotherapeutic agents by inhibiting extrusion of the drug and consequently increasing the net accumulation of the drug. The exact mechanism of action of verapamil is unknown, although there is substantial evidence to suggest that the effect is not directly related to either alterations in calcium flux (Ramu *et al.*, 1984; Kessel and Wilberding, 1984; Robinson *et al.*, 1985; Naito and Tsuruo, 1989) or to a direct effect on calcium channels (Robert *et al.*, 1987). Photoaffinity labelling studies have shown that verapamil binds directly to the P-glycoprotein molecule (Safa *et al.*,

1988) but an indirect role via other effects on cell membranes has not yet been ruled out. Early studies on verapamil showed that non-toxic doses of the drug enhanced the cytotoxicity of vincristine and vinblastine in murine P388 leukaemia and its vincristine resistant subline P388/VCR. An increase in vincristine accumulation of approximately 10-fold was observed in the P388/VCR cells following treatment with verapamil, with the cellular concentration of vincristine comparable to the level observed in the parental cells (Tsuruo *et al.*, 1981). Studies have consistently shown that verapamil is an effective agent at reversing P-glycoprotein mediated resistance. Research into the effect of verapamil on drug accumulation in MDR cell lines has been extensively carried out and includes studies on adriamycin resistant and vincristine resistant variants of the murine leukaemia cell line, P388 (Tsuruo *et al.*, 1981; Tsuruo *et al.*, 1982; Ramu *et al.*, 1984; Kessel and Wilberding, 1984; Kessel and Wilberding, 1985), Ehrlich ascitic tumour cells (Friche *et al.*, 1987; Sehested *et al.*, 1990), rat hepatocyte primary culture cells (Le Bot *et al.*, 1994), human glioma cell lines (Kaba *et al.*, 1985; Merry *et al.*, 1986), resistant variants of the human breast cell line, MCF-7 (Fine *et al.*, 1988; Ford and Hait., 1990; Julia *et al.*, 1994), human T-lymphoblastoid cell lines (Boer *et al.*, 1994), and human lung cancer cell lines (Twentyman *et al.*, 1986).

The antiarrhythmic agent, quinidine, has also been shown to reverse chemotherapy induced resistance in rodent and human cells. A number of reports have demonstrated an increase in drug accumulation, following treatment with non-toxic levels of the drug. Klohs and coworkers (1986) illustrated that the addition of quinidine resulted in a marked increase in adriamycin accumulation in resistant P388 murine leukaemia cells. Studies by Tsuruo *et al.* (1984) also demonstrated enhanced cellular accumulation of adriamycin and vincristine in resistant variants of the P388 cell line. These studies also showed increased drug accumulation in adriamycin resistant K562 leukaemia cells following treatment with quinidine. Quinidine has also been shown to enhance drug accumulation in human KB cells (Willingham *et al.*, 1986) and in lymphoblastoid cell lines (Boer *et al.*, 1994). Other calcium channel antagonists that have proven effective at enhancing the cellular retention of drugs include the quinoxaline derivative, caroverine (Tsuruo *et al.*, 1982), nifedipine (Kessel and Wilberding, 1984; Tsuruo *et al.*, 1984; Onada *et al.*, 1989) and nicardipine (Tsuruo *et al.*, 1986).

### 1.4.1.2 Calmodulin Inhibitors

A number of studies have shown that treatment with various calmodulin inhibitors, including trifluoroperazine, chloropromazine and fluxpentixol resulted in a decrease in resistance in MDR cell lines. The decrease in resistance appeared to be associated with enhanced cellular accumulation of the drug. The specific mechanism of action of calmodulin inhibitors in enhancing drug accumulation in resistant cells is not clear. Calmodulin has a number of complex roles in cell physiology; hence, calmodulin inhibitors could potentially augment chemotherapy cytotoxicity in a variety of ways. Calmodulin has a high affinity for ionized calcium, and is an activator of enzymes regulated by calcium. It also acts as a receptor for drugs, stimulates phospholipase A (which hydrolyzes membranous phospholipids, thus increasing membrane permeability and calcium flux), has been shown to alter intracellular binding of vincristine and adriamycin and appears to play a role in DNA repair pathways (Helson, 1984; Hait and Lazo, 1986). A number of studies have been carried out to investigate the effectiveness of calmodulin inhibitors as circumvention agents. Non-toxic concentrations of the antipsychotic agent, trifluoroperazine, were shown to cause a 5-fold increase in vincristine accumulation and a 2-fold increase in adriamycin accumulation in P388/VCR and P388/ADM cells respectively (Tsuruo *et al.*, 1982). Studies have also demonstrated a 2-fold increase in daunorubicin accumulation in P388/DOX cells following treatment with this agent (Ganapathi *et al.*, 1984). Studies by Kessel and Wilberding (1985) have also shown an increase in drug accumulation in resistant variants of the P388 cell line, when treated with trifluoroperazine. A range of other calmodulin inhibitors have also proven to be effective circumvention agents, including thioridazine (Akiyama *et al.*, 1986), chloropromazine (Ford *et al.*, 1989) and cis- and trans-fluxpentixol (Ford *et al.*, 1989; Ford and Hait, 1990).

### 1.4.1.3 Cyclosporins

Cyclosporin A, an antifungal undecapeptide immunosuppressive agent, has been reported to act as a chemosensitiser in murine and human MDR cell lines. Studies have also shown this agent to be more effective than verapamil at reversing MDR in a number of cell lines (Coley *et al.*, 1989; Le Bot *et al.*, 1994). The mechanism of action of cyclosporin A is unclear, although, as is the case with the calcium channel antagonists, it is thought to

modulate MDR by competitively interacting with drug binding sites of P-glycoprotein. Reports have shown that cyclosporin A effectively inhibits the binding of anticancer drugs to P-glycoprotein and consequently has attracted a great deal of attention, as an effective P-glycoprotein modulator (Slater *et al.*, 1986; Twentyman *et al.*, 1988). A photoreactive analogue of cyclosporin A has been shown to label P-glycoprotein in living cells. However, this labelling was inhibited by cyclosporin A, cyclosporin H and verapamil, suggesting competition for common binding sites (Foxwell *et al.*, 1989). Binding studies, using membrane vesicles of MDR cells, demonstrated that cyclosporin A competitively interacted with a common binding site of P-glycoprotein, to which vinca alkaloids and verapamil also bind (Tamai and Safa, 1990). A study by Saeki *et al.* (1993) using LLC-$PK_1$ cells derived from epithelial cells of porcine kidney tubules, illustrated that cyclosporin A was also a substrate for the P-glycoprotein molecule and consequently that the cellular accumulation of cyclosporin A was reduced by the pumping action of P-glycoprotein. Studies by Slater *et al.* (1986) demonstrated that cyclosporin A could reverse daunorubicin resistance in Ehrlich ascites carcinoma cells. Treatment with cyclosporin A was also shown to decrease vincristine resistance in human lung cancer cells (Twentyman *et al.*, 1987). Cyclosporin A has also been found to be very effective at enhancing drug accumulation in chinese hamster ovary cells (Chambers *et al.*, 1989), mouse mammary EMT6 tumour cells (Coley *et al.*, 1989; Twentyman and Wright, 1991), murine hepatoma 129 cells (Meador *et al.*, 1987), rat hepatocytes (Le Bot *et al.*, 1994) and in the human CCRF-CEM T-lymphoblastoid cell line (Boer *et al.*, 1994). Enhanced drug accumulation has also been reported in a number of sensitive cell lines, following treatment with cyclosporin A (Chambers *et al.*, 1989; Gaveriaux *et al.*, 1989) although its effectiveness is greater in resistant cell lines. Other cyclosporins have also been investigated for their ability to circumvent resistance. Although cyclosporin C, cyclosporin G and cyclosporin H have all been found to alter drug accumulation, the activity of these agents was found to be substantially lower than cyclosporin A, even at elevated concentrations of drug (Twentyman *et al.*, 1987).

A non-immunosuppressive cyclosporin D analogue, SDZ PSC 833, has been shown to reverse drug resistance in a number of MDR cell lines at concentrations lower than that used for verapamil or cyclosporin A (Gaveriaux *et al.*, 1991). Using a short term retention assay SDZ PSC 833 was found to enhance intracellular daunorubicin accumulation in murine monocytic leukaemia MDR cells. Full restoration of daunorubicin retention was

obtained at a substantially lower concentration of SDZ PSC 833 than with cyclosporin A. Verapamil could only partially restore the accumulation defect in this cell line (Boesch *et al.*, 1991). SDZ PSC 833 has also been shown to induce a stronger and more stable inhibition of P-glycoprotein than other resistance modifying agents, including verapamil and cyclosporin A (Boesch and Loor, 1994).

## 1.5 Non-P-glycoprotein MDR in cell lines defective in the cellular accumulation of drug

Over the past number of years evidence has indicated that cell lines treated with a cytotoxic agent can develop multiple drug resistance, resulting from reduced drug accumulation, despite the absence of detectable levels of the P-glycoprotein efflux pump. Cells exhibiting a drug accumulation defect and which subsequently were shown to be negative for P-glycoprotein expression were first described in the mid 1980s. In 1985, Bhalla and coworkers isolated an adriamycin resistant variant of the human promyelocytic leukaemia cell line, HL60, that demonstrated a 2-fold decrease in adriamycin accumulation relative to the sensitive cells. While SDS gel electrophoresis revealed no detectable levels of P-glycoprotein, two prominent glycoproteins were detected in the resistant HL60/AR cells, with molecular weights of approximately 110 and 160 kilodaltons respectively (Bhalla *et al.*, 1985). Studies carried out on the HL60/ADR resistant variant of the HL60 cells also revealed reduced adriamycin accumulation in the cells. Drug accumulation studies illustrated that uptake of adriamycin or daunorubicin was essentially the same throughout a 60 minute incubation period in the sensitive and resistant cells. However, after this time period, the level of drug in the resistant cells began to decline, whereas in the sensitive cell line drug levels continued to increase (March *et al.*, 1986). A reduction in the cellular drug concentration was also observed in a vincristine variant of the parental HL60 cells. However, in contrast to the HL60/AR and HL60/ADR resistant variants, overexpression of P-glycoprotein was detected in this cell line by the C219 monoclonal antibody (McGrath and Center, 1988).

Twentyman *et al.* (1986) established an adriamycin resistant variant of the human large cell lung cancer cell line, COR-L23, which displayed a reduction in drug accumulation relative to the parental cells. Further studies revealed that the resistant variant did not overexpress the *mdr1* gene, as determined by Northern blotting, or its product P-glycoprotein, as determined by Western blotting and immunocytochemistry with the C219 antibody. The resistant cells did, however, show reduced expression of the EGF receptor (Kartner *et al.*, 1985; Reeve *et al.*, 1990). Studies by Barrand *et al.* (1993) later demonstrated that the COR-L23 resistant line overexpressed a membrane protein slightly larger in size than P-glycoprotein. This 190 kDa protein contained a short amino acid sequence, antigenically similar to a region of P-glycoprotein close to one of its nucleotide binding sites. Further

analysis of the two HL60 adriamycin resistant variants and the COR-L23/R cells demonstrated that all three cell lines contained an energy dependent efflux mechanism distinct from P-glycoprotein that probably contributed to the reduced intracellular drug levels observed in these cells (Hindenburg *et al.*, 1989; Coley *et al.*, 1991). Adriamycin resistant variants of the human small cell carcinoma cell line, GLC4 and the human non-small cell lung cancer cell line, SW1573/50 were also shown to exhibit decreased intracellular adriamycin levels (Zijlstra *et al.*, 1987; Keizer *et al.*, 1989). Initial results suggested that the resistant SW1573 cells were positive for P-glycoprotein. Further studies, however, revealed that while P-glycoprotein expression was not detected during the initial selection stages (SW1573/2R), the protein was expressed at a later stage (Kuiper *et al.*, 1990). No detectable levels of P-glycoprotein were observed in the GLC4 resistant line, although altered levels of topoisomerase II were observed in both the GLC4/ADR and SW1573/2R resistant lines, which may have contributed to the MDR phenotype in these cells. Additional studies by Eijdems and coworkers (1992), using somatic fusion experiments, demonstrated that genetic lesions contributed to the reduced drug accumulation and altered topoisomerase II levels in the SW1573/2R cells.

Slovak *et al.* (1988) isolated an adriamycin resistant subline of the human fibrosarcoma cell line, HT1080, which also exhibited a reduction in drug accumulation in the absence of P-glycoprotein. Drug efflux studies revealed that the retention of drug in the resistant cell line was substantially less than in the sensitive cell line, thereby suggesting that the resistance observed in the cell line may be related to an alternative transport mechanism, capable of altering drug efflux. A number of studies have also been carried out on resistant variants of the human breast carcinoma cell line, MCF-7. Taylor *et al.* (1991) studied drug accumulation in two resistant variants. The MCF-7/DOX and MCF-7/Mitox were established by exposure to adriamycin and mitoxantrone respectively. The adriamycin resistant cells were shown to display a classic multidrug resistance phenotype, with overexpression of P-glycoprotein, decreased drug accumulation relative to the parental line and reversal of resistance by verapamil. The mitoxantrone resistant cells also displayed resistance to multiple drugs, but in contrast to the MCF-7/DOX cells, did not overexpress P-glycoprotein, as determined by immunoblotting and RNA blot analysis. Net drug accumulation was decreased in the MCF-7/Mitox cells, although no selective modulation of drug accumulation was observed by the addition of verapamil (Taylor *et al.*, 1991). Nakagawa and coworkers (1992) also established a mitoxantrone resistant MCF-7 variant,

MCF-7/MX, exhibiting approximately 4000-fold resistance to mitoxantrone. Decreased drug accumulation was demonstrated in this resistant variant in the absence of P-glycoprotein overexpression. The accumulation defect was reversed in the presence of the metabolic inhibitors, sodium azide and 2-dinitrophenol, suggesting the involvement of an an energy dependent process distinct from P-glycoprotein.

A vincristine resistant variant of the human non-small cell lung cancer, PC-9, was established which was also defective in the cellular accumulation of drug. DNA Southern and RNA Northern blot hybridization analysis revealed that the PC-9/VCR cell line did not contain amplified *mdr* genes or overexpress P-glycoprotein. Further studies illustrated that the addition of ascorbic acid enhanced drug uptake and partially reversed the resistance observed in this cell line. These findings suggested the presence of an alternative, ascorbic acid sensitive, drug uptake mechanism which may be associated with the non-P-glycoprotein resistance observed in the cells (Chiang *et al.*, 1994). A number of leukaemia blast cell lysates from patients with acute myeloid leukaemia have recently been described. The samples were analysed for P-glycoprotein expression, using the C219 monoclonal antibody, and all were found to be negative. Out of the 46 patient samples studied, 16 were shown to have reduced daunorubicin accumulation compared with the negative marrow samples. These samples were also shown to express a 95 kilodalton membrane protein as detected by Western blot analysis. Although the role of this protein is unknown, it may function as an efflux transporter, representing an alternative mechanism associated with anthracycline resistance in acute myeloid leukaemia (Doyle *et al.*, 1995). Overexpression of this p95 protein had previously been detected in two human small cell lung cancer cell lines, NCL-H1688 and NCL-H660, which displayed intrinsic multidrug resistance. These cell lines did not overexpress P-glycoprotein and exhibited an atypical pattern of resistance (Doyle *et al.*, 1993).

### 1.5.1 Protein changes in non-P-glycoprotein MDR

Although protein changes have been detected in a number of non-P-glycoprotein MDR cell lines, the exact role which these alterations play in the resistant phenotype has yet to be clarified. Extensive studies have been carried out to investigate cellular changes occurring in HL60 cells isolated for resistance to adriamycin. Studies in the mid 1980s detected a 160

kDa membrane protein in the HL60/AR resistant variant which was absent in the sensitive cell line (Bhalla *et al.*, 1985). March *et al.* (1986) also demonstrated the presence of a 150 kDa membrane associated protein in the HL60/ADR resistant variant. When membrane fractions were isolated from the HL60/ADR cells and incubated with a protein phosphorylation system containing $^{32}$P-ATP, two proteins with molecular weights of 120 and 150 kDa respectively were found to be phosphorylated (March *et al.*, 1986). Additional studies by March and Center (1987) demonstrated that the level of phosphorylation of the 150 kDa protein, which was present in resistant cells, increased with increasing levels of resistance. However, they also found no significant difference in the levels of the protein in the sensitive and resistant cells, suggesting that the adriamycin resistance in the HL60/ADR cells may be related to a modified form of the protein present in the sensitive cells.

Another cellular protein which has been shown to be overexpressed in non-P-glycoprotein MDR cell lines and essentially absent in drug sensitive cells is a 190 kDa protein (p190). Overexpression of this protein has been demonstrated in a number of MDR cell lines including, HL60/ADR cells, COR-L23/R cells and GLC4/R cells (McGrath *et al.*, 1989; Marquardt *et al.*, 1990; Barrand *et al.*, 1992; Barrand *et al.*, 1993). The p190 protein appears to be localised primarily in the endoplasmic reticulum and is capable of binding ATP (Marquardt *et al.*, 1990). The contribution of p190 to MDR is unknown, although Barrand *et al.* (1992) demonstrated that antiserum (ASP-14) raised against a synthetic peptide of P-glycoprotein, also detected the p190 protein present in non-P-glycoprotein cell lines. The peptide sequence occurred in a region of the P-glycoprotein molecule close to the nucleotide binding site near the C-terminal. This sequence was found to be highly conserved amongst ATPase proteins throughout the animal kingdom (Riordan *et al.*, 1989). Very little is actually known about the amino acid sequence or the molecular structure of p190, although the similarity of its known region with regions found in ATP-dependent transport proteins suggests that it may also be a pump and subsequently may be involved either directly or indirectly with drug efflux.

Scheper *et al.* (1993) have detected overexpression of a 110 kDa protein (lung resistance protein, p110) in non-P-glycoprotein MDR cell lines. Initial studies were carried out on the human non-small cell lung cancer cell line, SW1573, and its adriamycin resistant variant, SW1573/2R. Using a p110 specific monoclonal antibody (LRP-56), strong

immunoreactivity was observed in the SW1573/2R cells, compared to the parental cell line. The p110 protein did not appear to be localised in the plasma membrane, since staining of the LRP-56 was detected primarily in the outer cytoplasmic zone, in a granular fashion suggesting that it reacted with molecules closely associated with endoplasmic reticular/lysosomal structures. A number of other non-P-glycoprotein MDR cell lines were also studied for p110 expression, including the GLC4/ADR small cell ling cancer cell line, the MCF-7/Mitox human breast cell line, the fibrosarcoma cell line, HT1080/DR4 and the myeloma cell line, 8226/MR40. Positive immunoreactivity with LRP-56 was observed in each of the resistant cell lines with cytoplasmic granular staining similar to the SW1573/2R cell line. Earlier studies, by SDS polyacrylamide gel electrophoresis, also demonstrated the expression of a 110 kDa glycoprotein in HL60/AR cells not detected in the sensitive cells (Bhalla *et al.*, 1985).

A polyclonal antibody, ASP-14, has been used to detect membrane protein changes in MCF-7 variants. Western blot analysis of the parental cells, the adriamycin resistant variant MCF-7/Adr and the mitoxantrone resistant variant MCF-7/MX illustrated that, as expected, the antibody recognized the 180 kDa P-glycoprotein present in the MCF-7/Adr cells. There was, however, no cross reactivity of the antibody with high molecular weight (160-180 kDa) proteins in either the parental or MCF-7/MX cells. In addition, the antibody was found to cross react with two proteins of molecular weights 42 kDa and 85 kDa respectively in the MCF-7/MX variant that were absent in the sensitive cells. These two proteins may contain sequence homology with P-glycoprotein and thus contribute to the enhanced drug efflux system of the MCF-7/MX variant (Nakagawa *et al.*, 1992).

Studies by Mirski and Cole (1991) reported the expression of a 36 kDa protein in the H69/AR non-P-glycoprotein MDR cell line, which was later identified as a member of the annexin/lipocortin family of $Ca^{2+}$ and phospholipid binding proteins. This phosphorylated protein was found to be membrane associated but not present on the external surface of the H69/AR cells. Immunoprecipitation studies showed that 5- to 6-fold more of the 36 kDa antigen was immunoprecipitated from the translation products of H69/AR mRNA, relative to the parental H69 cells (Cole *et al.*, 1992a). Studies by Cole *et al.* (1992b) also demonstrated that the H69/AR cells overexpressed a multidrug resistant related protein (MRP) which had sequence homology with P-glycoprotein and other proteins which may be involved in the drug transport process. Although the H69/AR cells are not defective in

drug accumulation, it has been suggested that the MRP protein may sequester drug into certain organelles and, thus, away from potential target sites (Cole *et al.*, 1991). Studies have also suggested that MRP mRNA may encode the 190 kDa protein detected in several non-P-glycoprotein cell lines (Twentyman *et al.*, 1994). This protein has also been demonstrated to confer the MDR phenotype in cells transfected with the MRP gene (Grant *et al.*, 1994). Chen and coworkers (1990) described a 95 kDa protein which was overexpressed in an adriamycin resistant MCF-7 cell line but was almost undetectable in the parental line. Polyclonal antibodies, raised against the protein, demonstrated a positive relation between the level of p95 expression and cellular adriamycin resistance. Immunohistochemical studies illustrated the localisation of the protein on the surface of resistant cells. Further studies by Doyle *et al.* (1993) demonstrated the expression of the p95 protein in two early passage small cell lung cancer cell lines from untreated patients and also in blast cell lysates from acute myeloid leukaemia (Doyle *et al.*, 1993; Doyle *et al.*, 1995).

### 1.5.2 Circumvention of non-P-glycoprotein MDR

To date, very little information has been obtained on agents capable of reversing the accumulation defect observed in non-p-glycoprotein MDR. The most extensive study investigated the effect of the calcium channel antagonist, verapamil, an agent which has been shown to sensitize P-glycoprotein containing MDR cells, in a non-P-glycoprotein MDR cell line (Tsuruo *et al.*, 1981). Studies have shown that, while verapamil can reverse resistance in some MDR lines, it is ineffective in other cells. This may indicate that a number of different mechanisms are involved in non-P-glycoprotein MDR. Verapamil also appears to be specific for resistant cells, since results have shown that this agent has very little effect on the parental cell lines. A number of reports have demonstrated the effect of verapamil on the HL60 adriamycin resistant variants, HL60/AR and HL60/ADR. Treatment of both lines with verapamil enhanced the cellular accumulation and retention of adriamycin and daunorubicin (Bhalla *et al.*, 1985; McGrath *et al.*, 1989). These studies did not detect any significant alteration in drug accumulation in the parental cell line. Other non-P-glycoprotein MDR lines have also been shown to respond to verapamil treatment, although the effect was substantially less than that observed in the HL60 resistant variants. Studies on the large cell lung carcinoma cell line, COR-L23, revealed that verapamil was capable of partially restoring the accumulation defect in the resistant variant. Daunorubicin

accumulation was unchanged in the parental cell line, while it was increased by approximately 60% in the COR-L23/R cells (Coley *et al.*, 1991). Slovak and coworkers (1988) also found a slight increase in the net accumulation of adriamycin in HT1080/DR4 cells, following treatment with verapamil. A number of investigators, however, have shown that verapamil has essentially no effect in circumventing resistance in other non-P-glycoprotein MDR lines, particularly in cell lines selected for resistance to mitoxantrone. Taylor *et al.* (1991) found that there was no selective modulation of drug accumulation or drug resistance by the addition of verapamil in the mitoxantrone resistant variant of the breast line MCF-7.

The immunosuppressive agent cyclosporin A and its analogue SDZ PSC 833 have also been shown to be capable of enhancing drug accumulation in non-P-glycoprotein cell lines. In a study by Barrand *et al.* (1993), both cyclosporin A and PSC 833 caused a slight, but significant, increase in the accumulation of daunorubicin and vincristine in the resistant COR-l23 cell line. The net accumulation, however, was less than that observed in the sensitive parental cells. In the same study, verapamil was shown to be more effective than both agents. Enhancement of daunorubicin accumulation by cyclosporin A was also noted in the p95 positive MCF-7/AdrVp subline, while no effect was observed in the parental cells (Doyle *et al.*, 1995).

The glutathione inhibitor, buthionine sulfoximine (BSO), has also been shown to be capable of enhancing drug accumulation and retention in HL60/AR cells. Treatment of the HL60 cells with BSO resulted in a significant increase in daunorubicin uptake in the resistant cells but had no effect on accumulation in the sensitive cell line. The mechanism by which BSO exerts its effect on drug accumulation, however, is unknown (Lutzky *et al.*, 1989).

More recently, a number of additional compounds have been studied for circumvention of resistance in non-P-glycoprotein MDR and these include the protein kinase inhibitors, genistein and staurosporine, the proton ionophores, monensin and nigericin, the Golgi disrupting agent, brefeldin A and the lysosomotropic agents, chloroquine and methylamine. An extensive study was carried out on the effect of genistein and staurosporine on a number of P-glycoprotein and non-P-glycoprotein MDR cell lines. The results obtained illustrated that while staurosporine was very effective at enhancing drug uptake in P-glycoprotein MDR cells (SW1573/2R160), it did not significantly alter drug accumulation in the non-P-

glycoprotein MDR cell lines, GLC4/ADR and SW1573/2R120. In contrast, genistein was shown to cause a dose dependent increase in daunorubicin accumulation in both GLC4/ADR and SW1573/2R120 but to have no effect on accumulation in P-glycoprotein MDR cell lines (Versantvoort *et al.*, 1993).

Marquardt and Center (1991) reported that adriamycin accumulation could be restored in the HL60/ADR cell line by treatment with the vacuolar ATPase inhibitors, bafilomycin A1 and 7-chloro-4-nitrobenz-2-oxa,1,3-diazole (NBD). These agents, in particular NBD, were also shown to be effective in the COR-L23/R resistant cell line at reversing the accumulation defect in the resistant cells (Rhodes *et al.*, 1994). It has also been demonstrated that brefeldin A causes a dose dependent increase in daunorubicin accumulation in both sensitive and resistant COR-L23 cell lines (Rhodes *et al.*, 1994). However, studies on the effect of brefeldin A in HL60/ADR cells revealed that it did not significantly alter the cellular accumulation of daunorubicin in these cells. Further studies on the HL60/ADR cells showed that the lysosomotropic agents, methylamine, chloroquine and ammonium chloride had no effect on drug uptake or efflux, while the ionophores monensin and nigericin induced a significant increase in drug accumulation and inhibited drug efflux. Nigericin has consistently been found to be more active than monensin and in this study it induced a complete block in daunorubicin efflux from the HL60/ADR cells (Marquardt and Center, 1992).

## 1.6 Subcellular distribution of anticancer agents in MDR cell lines

Numerous studies have reported that, in addition to a reduction in drug accumulation, many MDR cell lines also exhibit altered distribution, together with compartmentalisation of drug in vesicles away from the target sites. Consequently, the subcellular localisation of anticancer agents has frequently been studied in wild type sensitive and multidrug resistant cell lines. Many quantitative studies on the accumulation of drug, in intact cells, have used radioactively labelled drugs and spectrofluorometric techniques. These methods, however, do not allow examination of the intracellular compartmentalisation of drug in living cells. Fluorescence microscopy has therefore been employed to determine the cellular localisation of drugs, since a number of the anthracyclines are inherently fluorescent and can easily be visualized. Studies on a number of the anthracyclines revealed that adriamycin and daunorubicin were localised primarily in the nucleus of living cells (Silvestrini *et al.*, 1970; Egorin *et al.*, 1974). In contrast, a large number of anthracyclines appeared to be localised in the cytoplasm, often in discrete granules. Included in this group of anthracycline drugs were the N-acetyl and N,N-dibenzyl derivatives of daunorubicin, 4-demethoxydaunorubicin and carinomycin. Other anthracyclines, including N-formyl-adriamycin and 13-amino-daunorubicin, were not preferentially localised in either the nucleus or cytoplasm appearing to be equally distributed in both regions (Egorin *et al.*, 1980).

Cytofluorescent studies on the localisation of adriamycin in MDR cells were first described by Chauffert and coworkers in 1984 in adriamycin resistant rat colon cancer cells. Fluorescent microscopy demonstrated that adriamycin was distributed in two cellular compartments. Initially, adriamycin accumulated rapidly in the nucleus. However, when the cells were exposed to the drug for longer incubation periods, no nuclear fluorescence was observed. Adriamycin was also shown to accumulate in cytoplasmic granules, which occurred slowly and persisted long after the drug was removed from the medium. This work indicated that the inherently resistant colon cancer cells were capable of removing adriamycin from the nucleus, its main site of action, and through this permanent efflux mechanism, the cells were able to survive and grow in drug containing medium. Studies by Willingham *et al.* (1986) demonstrated daunorubicin distribution in drug sensitive and multidrug resistant human KB cells. The fluorescence pattern showed a very bright signal in the nucleus, the Golgi region and in lysosomes of the sensitive cells. A small amount of fluorescence was also detected on the plasma membrane and in the cytoplasm. In

contrast, resistant cells incubated with daunorubicin showed a significantly reduced amount of daunorubicin fluorescence in their nuclei. However, fluorescence in the Golgi region and in the lysosomes was as prominent as in the sensitive cells. The distribution of adriamycin and daunorubicin in the HL60 and HL60/AR cell lines was first described in 1987 by Hindenberg and coworkers. The localisation of the anthracyclines was studied by digitized video fluorescence microscopy and the results demonstrated different drug distribution patterns in the sensitive and resistant cells. Following exposure to drug, nuclear fluorescence was clearly visible in the HL60 parental cells. However, the fluorescence pattern observed in the HL60/AR cells was similar to the lysosomal fluorescence seen in both HL60 and HL60/AR cells exposed to acridine orange, a dye which preferentially stains the lysosomes, thus suggesting the intralysosomal distribution of the drug in resistant cells. Further studies showed that the initial fluorescence observed in the HL60/AR resistant cells was associated with the Golgi apparatus, although with longer incubation the fluorescence of the Golgi apparatus faded and lysosomal fluorescence was visible (Hindenburg *et al.*, 1989). Numerous studies have since described the distribution of drugs in parental, P-glycoprotein and non-P-glycoprotein MDR cell lines. In general, nuclear fluorescence was prominent in parental cell lines, while the fluorescent staining observed in resistant lines was predominately cytoplasmic.

The initial results obtained for the subcellular distribution of anticancer drugs agreed with the proposal that the decreased ability of MDR cells to retain certain drugs was due to the active efflux pump, P-glycoprotein. However, this could not explain the drug distribution patterns observed in the non-P-glycoprotein MDR HL60/AR cell line. Subsequently, a new hypothesis was independently proposed by Beck (1987) and Sehested *et al.* (1987). This hypothesis suggested that the lysosomal system in MDR cells was involved in drug efflux and that drugs such as the anthracyclines and vinca alkaloids, which are weak bases, can become trapped in these acidic compartments by protonation. Thus, the acidic compartments may act as a reservoir to sequester drugs, reducing their concentrations in the cytoplasm and nucleus. According to this proposal, the lysosomal vesicles then migrate to the plasma membrane, where they fuse with the membrane and extrude their contents to the outside. Neutralization of lysosomes has been shown to alter adriamycin resistance in the HCT116-VM46 resistant cell line by a factor of 14 over the sensitive strain, thus supporting the hypothesis of the involvement of lysosomes in MDR lines (Dubowchik *et al.*, 1994). Further evidence supporting this theory has been obtained from studies by Sognier

and coworkers (1994) on multidrug resistant chinese hamster cells. A highly resistant variant of the LZ chinese hamster cell line was established (LZ-100), which was shown to be the most adriamycin resistant line of the LZ series. The increased level of resistance in the LZ-100 variant did not appear to be a consequence of either overexpression of P-glycoprotein or more efficient drug efflux. Altered drug distribution was, however, observed in the cells. Adriamycin was largely excluded from the nucleus and appeared to be sequestered into vesicles within the cytoplasm. Drug distribution was investigated in a number of cell lines from the LZ series and the results demonstrated that drug sequestration occured rapidly, following exposure to drug and was observed in cells exhibiting different levels of resistance. It was also shown that the number of cytoplasmic vesicles observed was related to the level of drug resistance achieved by the LZ line studied. Studies by Moriyama *et al.* (1994) on constituted acidic organelles revealed that while no specific transporters appeared to be involved in drug uptake into the acidic vesicles the uptake was driven by pH changes established by vacuolar-type ATPases.

In addition to cytotoxic drugs, a number of other chemicals act as substrates for P-glycoprotein. These include a number of fluorescent dyes which have been used as molecular probes in the study of MDR. Early studies showed that adriamycin and daunorubicin resistant leukaemia variants accumulated the fluorescent mitrochondrial dye rhodamine 123 much less effectively than sensitive cells (Tapiero *et al.*, 1984). This led to a number of studies being carried out using fluorescent dyes. An extensive study was carried out on djungarian hamster fibroblasts, to test the ability of different fluorescent dyes to stain sensitive and resistant cells. While rhodamine 123 and berberin brightly stained mitochondria in sensitive cells, no staining was observed in the colchicine resistant variant, even with prolonged incubation periods. Slight fluorescence was, however, noted at higher concentrations of the dyes. All other tested dyes stained both the sensitive and resistant cells, but with different intensity of staining. The fluorescence intensity was found to be substantially greater in the sensitive cells (Neyfakh, 1988). The reduced staining related well with the multidrug resistant phenotype of the cells and thus fluorescent dyes proved to be a useful tool for the investigation of different aspects of multidrug resistance. Additional work by Neyfakh and coworkers (1989) demonstrated the role of rhodamine 123 as a detector of cells with a high MDR transport system. Experiments with B-lymphoma cell lines with different degrees of MDR showed that rhodamine 123 fluorescence was indeed inversely proportional to the degree of resistance. The subcellular distribution of rhodamine

123 has recently been used as a tool to study the MDR phenotype in a number of other resistant cell lines, including the murine mammary tumour cell line EMT6/AR1.0, chinese hamster lung fibroblasts, the human large cell lung cancer cell line COR-L23/R, uterine epithetial carcinomas and human leukaemia cell lines (Twentyman *et al.*, 1994; Altenberg *et al.*, 1994; Lizard *et al.*, 1994; Nare *et al.*, 1994).

### 1.6.1 Agents that alter drug distribution in MDR cell lines

Studies have shown that a number of agents have the ability to modulate the subcellular distribution of anthracyclines within MDR cell lines. Early reports showed that verapamil was effective at restoring nuclear fluorescence in resistant rat colon cancer cells and also at enhancing the toxicity of adriamycin in the cells (Chauffert *et al.*, 1984). Willingham and coworkers (1986) also demonstrated restoration of nuclear fluorescence in resistant human KB cells, to a level comparable with the sensitive cells, following treatment with verapamil. Verapamil has since been demonstrated to increase nuclear fluorescence in numerous multidrug resistant human cell lines, including the adriamycin resistant non small cell lung carcinoma cell line, SW1573 (Versantvoort *et al.*, 1993), the human ovarian A2780/AD cell line (Schuurhuis *et al.*, 1993) and the human leukaemia cell line, K562R (Sebille *et al.*, 1994). The mechanism by which verapamil exerts its effect on drug distribution is unclear, although early results suggested that verapamil altered drug distribution by inhibiting the efflux across the plasma membrane and thus increasing the cellular concentration of drug (Willingham *et al.*, 1986). The protein responsible for accelerated efflux was later identified as the membrane associated P-glycoprotein pump, and therefore inhibition of P-glycoprotein was thought to be involved in modulating drug distribution patterns.

This hypothesis, however, could not explain the effect of verapamil in non-P-glycoprotein MDR cell lines. Verapamil has been shown to alter subcellular drug distribution in non-P-glycoprotein MDR cell lines, although its effectiveness is not as great as in P-glycoprotein MDR cells. Studies by Versantvoort *et al.* (1993) demonstrated an increase in nuclear fluorescence, following exposure to verapamil in the non-P-glycoprotein MDR human lung cell lines GLC4/ADR and SW1573/2R. Verapamil also proved to be effective at restoring nuclear fluorescence in the human leukaemia HL60/AR cell line (Hindenburg *et al.*, 1987)

and in the human large cell lung cancer cell line, COR-L23/R (Barrand *et al.*, 1993). It is generally believed that verapamil reverses resistance in non-P-glycoprotein MDR cell lines by altering intracellular drug localisation. The exact mechanism by which this is achieved remains unknown. Hindenburg and coworkers (1987) proposed that verapamil could circumvent resistance in the HL60/AR cells by altering the hydrophobic/hydrophilic solubility of anthracyclines in the resistant cell, causing the drug to redistribute and thereby increasing its cytotoxicity.

The immunosuppressive agent cyclosporin A and its more potent analogue PSC 833 have also been reported to alter subcellular distribution of drug in MDR cell lines. Photoaffinity labelling studies have shown that, similar to verapamil, cyclosporin A may increase nuclear fluorescence by binding to the drug efflux pump, P-glycoprotein, in such a way as to reduce its ability to expel cytotoxic drugs from the resistant cells. Supporting this hypothesis, Sebille *et al.* (1994) reported that, in the presence of cyclosporin A, the intracellular concentration of adriamycin in the human P-glycoprotein MDR leukaemia cell line, K562/R, was approximately six times higher than without the modulator. Cyclosporin A and PSC 388 have also been demonstrated to alter drug distribution patterns in non-P-glycoprotein MDR cell lines, although generally the effect was substantially less than in P-glycoprotein cell lines. The way in which this is achieved is unknown. Reports have shown that these agents increase nuclear fluorescence in the non-P-glycoprotein cell lines, HL60/AR and COR-L23/R (Hindenburg *et al.*, 1987; Barrand *et al.*, 1993).

The ionophores, monensin and nigericin, have also been shown to increase drug accumulation and to alter the subcellular distribution of drug in MDR cell lines. These agents are thought to act by elevating intravesicular pH and by disrupting acidic vesicular traffic within the cell (Tartakoff, 1983). The ionophores are also capable of altering cytosolic pH (Pressman, 1976) and may in this way alter cellular distribution and block the efflux pathway. Sehested and coworkers (1988) demonstrated an increase in daunorubicin toxicity in resistant Ehrlich ascites tumour cells, following treatment with both monensin and nigericin. An increase in the subcellular accumulation of daunorubicin and a decrease in drug efflux was also observed in the cells. In studies by Klohs and Steinkampf (1988) these agents were also shown to potentiate adriamycin toxicity and inhibit adriamycin efflux in the resistant leukaemia cell line, P388R. A number of other agents known to interact with acidic compartments, including chloroquine and methylamine, were found to have the

same effect. More recent studies have shown that, in addition to increasing drug accumulation, the ionophores are capable of altering the distribution of drug. Marquardt and Center (1992) reported that treatment with monensin and nigericin induced an increase in adriamycin accumulation and inhibited adriamycin efflux in HL60/ADR cells. Studies were also carried out to examine the effect of nigericin on the subcellular distribution of adriamycin. Using fluorescent microscopy techniques, a complete retention of drug was found in the nuclei of resistant cells, in the presence of nigericin. The uptake of the fluorescent dye, rhodamine 123, in sensitive and resistant murine RFLC3 leukaemia cells was also investigated in the presence of nigericin and the results obtained showed that the increase in rhodamine 123 accumulation was accompanied by a redistribution of the dye throughout the cell (Canitrot *et al.*, 1994). Nigericin was also demonstrated to increase daunorubicin nuclear fluorescence in BNLCL.2 cells. Further studies revealed that decreased accumulation of adriamycin and daunorubicin within acidic organelles of the BNLCL.2 cells, following treatment with nigericin, was associated with an increase in the internal pH of the organelles (Moriyama *et al.*, 1994). Overall, these results show that the ionophores can modulate the cytotoxicity of anthracyclines by altering the subcellular distribution of the drug. The exact mechanism by which this is achieved is unknown, although evidence would suggest that these agents inhibit the sequestration of drug into the acidic vesicles and/or cause disruption of the vesicles, thus increasing the nuclear concentration of the drug. Other lysosomotropic agents which have also been demonstrated to increase drug retention and nuclear drug accumulation in resistant cells include chloroquine, methylamine and quinacrine (Zamora and Beck, 1986; Shiraishi *et al.*, 1986a; Klohs and Steinkampf, 1988). These agents have been found to alkalinize the lysosomal compartment in several cell lines (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981).

The fungal antibiotic, brefeldin A, has also been identified as a modulator of intracellular distribution of anthracyclines in MDR cell lines. In a study on the distribution of adriamycin in the MDR cell line, COR-L23/R, the addition of brefeldin A resulted in a shift of the drug from cytoplasmic to nuclear regions (Rhodes *et al.*, 1994). Earlier studies had shown that brefeldin A protected the murine leukaemia L1210 cell line from adriamycin cytotoxicity (Vichi and Tritton, 1993). Brefeldin A has been shown to disassemble the Golgi complex thus interfering with the vesicular trafficking between the Golgi apparatus and the endoplasmic reticulum (Misumi *et al.*, 1986; Fujiwara *et al.*, 1988). Any effect of

brefeldin A may therefore be related to its function as a Golgi inhibitor.

Studies have also been carried out on the tyrosine kinase inhibitor, genistein, to investigate its effect on the distribution of drug in MDR cell lines. Reports have shown that genistein can reverse the drug accumulation defect in a number of cell lines which overexpress the 190 kDa protein, while it appears to have little effect in P-glycoprotein mediated MDR cell lines. Takeda and coworkers (1994) demonstrated an increase in nuclear concentration of adriamycin in the non-P-glycoprotein MDR human leukaemia cell line, K562/TPA, following cotreatment with genistein. Versantvoort *et al.* (1993) reported an increase in the nuclear/cytoplasmic distribution ratio in the GLC4/ADR and SW1573/2R120 cell lines. In contrast, genistein had no effect on adriamycin distribution in the P-glycoprotein MDR cell lines SW1573/2R160, MCF-7/DOX and KB8-5.

The vacuolar $H^+$-Adenosine triphosphatase inhibitors, bafilomycin $A_1$ and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD), have also proved effective at modifying the intracellular distribution of anthracyclines. The vacuolar $H^+$-ATPase is a proton pump which maintains low internal pH within cells (Forgac, 1989). It has been suggested that these proton pumps play a pivotal role in acidification and protein degradation within lysosomes in cultured cells (Yoshimori *et al.*, 1991). Studies have shown that the addition of bafilomycin $A_1$ to cultured cells results in increased intralysosomal pH, as a consequence of the inhibition of the vacuolar proton pump (Yoshimori *et al.*, 1991). Therefore, the modifying effect of bafilomycin $A_1$ and NBD on intracellular drug distribution may be due to inhibition of lysosomal formation. Marquardt and Center (1991) reported an increase in both cellular and nuclear accumulation of daunorubicin in the non-P-glycoprotein MDR cell line, HL60/ADR, in the presence of bafilomycin $A_1$ and NBD. These agents were also shown to increase daunorubicin accumulation in the P-glycoprotein cell line HL60/Vinc, although bafilomycin $A_1$ did not compete with [$^3$H]azidopine binding to P-glycoprotein thus suggesting that bafilomycin $A_1$ does not act as a substrate for P-glycoprotein. A shift of drug from cytoplasmic to nuclear regions was also observed by laser-scan confocal microscopy in COR-L23/R cells, following treatment with bafilomycin $A_1$ and NBD. These agents were found to modify the distribution in the resistant cells towards that observed in the sensitive parental cells (Rhodes *et al.*, 1994).

Radiation exposure has recently been shown to alter drug distribution patterns in adriamycin resistant chinese hamster LZ-100 cells. Adriamycin was found to be largely excluded from the nucleus and appeared to be sequestered into vesicles within the cytoplasm of the resistant cells. Radiation exposure immediately preceeding adriamycin treatment was shown to decrease the number of cytoplasmic vesicles formed and also to alter the subcellular distribution of adriamycin, from cytoplasmic to a more homogenous nuclear/cytoplasmic distribution. The inhibitory effect of radiation on vesicle formation was also found to increase with radiation dose. The mechanism by which radiation treatment redistributes the drug is unknown, although it does not appear to result from radiation inhibition of P-glycoprotein (Sognier *et al.*, 1994).

## 1.7 Alternative mechanisms of MDR

### 1.7.1 Topoisomerase II

Topoisomerases are $Mg^{2+}$- and ATP-dependent nuclear enzymes which play a role in DNA replication, chromosome scaffold formation, chromosomal segregation and possibly recombination and gene transcription (Wang, 1985; Liu, 1989). Use of the topoisomerases as novel targets for cancer chemotherapy was first described in the early 1980s and study is ongoing to determine their mechanisms of action and to develop inhibitors which could prove to have therapeutic use in cancer chemotherapy (Liu *et al.*, 1980; Liu, 1983; Hsieh and Brutlag, 1980). A number of clinically useful antitumour agents have been found to inhibit the topoisomerase enzymes, including the DNA intercalators, adriamycin and daunorubicin, amsacrine, ellipticine, camptothecin and its related compound topotecan and the epipodophylotoxins, etoposide and tenoposide. Many of the topoisomerase II inhibitors such as the anthracyclines (adriamycin and daunorubicin) and the epipodophylotoxins (etoposide and teniposide) are exported from the cell by the high levels of the P-glycoprotein membrane associated efflux pump expressed in the "classic" or "typical" multidrug resistant cell lines (Ling, 1992). However, a second mechanism of MDR involving reduced or mutated topoisomerase II has been termed "atypical" MDR (Beck, 1989). Eukaryotic topoisomerases have been categorized into two types. The type I enzymes make transient single-strand breaks in DNA, whereas type II topoisomerases make transient double-strand breaks (Wang, 1985). Many of the functions of one type of topoisomerase can, in theory, be carried out by the other class, although it is likely that the topoisomerase I enzyme does not perform functions requiring double-strand DNA scission (Wang, 1985). Topoisomerase II was first isolated from human cells by Miller *et al.* (1981). The gene encoding the human topoisomerase II has been sequenced and found to be localised on chromosome 17 at position 21-22 (Tsai-Pflugfelder *et al.*, 1988). The cDNA encodes a 6.2 kilobase mRNA which codes for a 1530-amino acid protein of deduced molecular mass of 174 kilodaltons.

The mechanism of action of the eukaryotic topoisomerase II has been described by Osheroff (1986) as a five step process. The first step involves the recognition and binding of the topoisomerase enzyme to DNA. The second step involves the cleavage of the first double-stranded segment of the DNA, resulting in a covalent bond between the 5'-terminus of each

strand and a topoisomerase II polypeptide tyrosyl subunit. This step is followed by the passage of a second double strand of DNA through the break and this in turn is followed by the religation of the strand-cleaved DNA. The final step involves the ATP-dependent turnover of the topoisomerase II enzyme. The topoisomerase II inhibitors are thought to exert their inhibitory action by binding to the topoisomerase II enzyme, forming a cleavable compound, which then binds to the DNA forming a potentially lethal ternary complex in the cells (Takano *et al.*, 1992; Lui, 1989). The ternary complex halts the catalytic activity of the topoisomerase II enzyme prior to the religation of the cleaved DNA intermediate (Robinson and Osheroff, 1990). The stabilized intermediate complexes form barriers to DNA fork progression and so become lethal lesions when cells attempt to utilize these regions as templates (Liu, 1989). The topoisomerase II inhibitors therefore cause cell kill by stabilizing the enzyme-DNA complex and thus preventing DNA replication.

Protein, chemical and molecular cloning analyses have revealed two distinct isoforms of the topoisomerase II enzyme, with molecular weights of 170 kilodaltons and 180 kilodaltons respectively. The two isoforms are also referred to as topoisomerase II$\alpha$ and topoisomerase II$\beta$ (Drake *et al.*, 1987; Chung *et al.*, 1989; Tsutsui *et al.*, 1993). The isoforms have been shown to have different intranuclear localisation and tissue distribution (Capranico *et al.*, 1992; Zini *et al.*, 1992: Tsutsui *et al.*, 1993), suggesting their differential roles in cell function with special regard to cell proliferation. The level of topoisomerase II has been shown to vary throughout the stages of the cell cycle (Estey *et al.*, 1987: Woessner *et al.*, 1991). Topoisomerase II$\alpha$ is expressed primarily during the proliferative phases of growth and is downregulated at plateau phase. The level of topoisomerase II$\alpha$ increases at the onset of DNA replication and continues to increase throughout the S and $G_2$ phases. The level reaches a plateau in the late $G_2$ to M phase of the cell cycle and then decreases after mitosis has occurred (Heck *et al.*, 1988). Immunoblotting studies have demonstrated that, unlike the topoisomerase II$\alpha$ isoform, the level of topoisomerase II$\beta$ does not appear to vary greatly throughout the cell cycle (Woessner *et al.*, 1991).

The regulation of topoisomerase II during the cell cycle may be relevant in identifying which phase of the cycle is most sensitive to the effect of topoisomerase II inhibitors. Drake *et al.* (1987) have shown that the $\alpha$ isoform of the topoisomerase enzyme is the better target for the topoisomerase II inhibitors and this may explain why some of the inhibitors are proliferation-specific with regard to their toxicity. Chow and Ross (1987)

demonstrated that following serum stimulation of BALB-c 3T3 cells, actively proliferating cells showed much greater sensitivity to etoposide than quiescent cells. The increase in sensitivity commenced during the S phase of the cell cycle and reached a peak just before mitosis, with a maximal 2.5-fold increase in drug sensitivity. Modulation of the topoisomerase II enzyme during the cell cycle may involve phosphorylation of the enzyme. It has previously been shown that topoisomerase II is phosphorylated at specific serine residues, resulting in an increase in enzyme activity, with dephosphorylation resulting in inactivation of the enzyme (Fry and Hickson, 1993). A second mechanism of regulating topoisomerase II activity may be to vary the cellular content and localisation (compartmentalisation) of the enzyme. It is not fully understood how compartmentalisation is achieved, although it is known that topoisomerase II is a nuclear matrix protein and thus changes in the components of the nuclear matrix during the cell cycle may result in alteration in the accessibility of the enzyme to DNA (Beck and Danks, 1991).

#### 1.7.1.1 Topoisomerase II and drug resistance

A number of the classical MDR cell lines that overexpress P-glycoprotein also exhibit cross-resistance to topoisomerase II inhibitors. However, no alteration in the cellular content of the topoisomerase II enzyme is detected in these cells, since these compounds act as substrates for the P-glycoprotein and are exported from the cells (Beck and Danks, 1991). Various studies have been carried out demonstrating the "atypical" multidrug resistance phenotype in numerous cell lines. Characteristically, the cell lines exhibit cross resistance to the full range of anti-topoisomerase II drugs, but do not show cross resistance to the vinca alkaloids, which are classical MDR drugs. Two types of "atypical" MDR have been described, the type being defined according to whether there is a reduction in the level of topoisomerase II or an alteration in the enzyme that renders it resistant to the topoisomerase II inhibitor. Examples of resistant cell lines with reduced levels of topoisomerase II include the leukaemia cell line, P388/AMSA, selected for resistance to amsacrine (Johnson and Howard, 1982) and the adriamycin resistant small cell lung cancer cell line, H69AR (Cole *et al.*, 1991). Lower levels of immunoreactive topoisomerase II were observed in both cell lines compared to their respective parental cells.

A number of mechanisms of resistance, resulting in the alteration of the topoisomerase II enzyme have been postulated and include the presence of point mutations and hypermethylated sites on the topoisomerase II enzyme. A number of investigators have detected the presence of mutated topoisomerase II in "atypical" MDR cell lines. The cell lines, HL60/AMSA, $Vpm^R$-5 and KBM3/AMSA, have all been found to contain point mutations in a single specific region of the topoisomerase II$\alpha$ gene and all mutations were G to A transitions (Hinds *et al.*, 1991; Chan *et al.*, 1993; Lee *et al.*, 1992). Several studies have also demonstrated the presence of hypermethylated sites on the topoisomerase II enzyme which may be of significance in regulating the transcription of non-mutant alleles. Work by Tan *et al.* (1989) revealed that in the amsacrine resistant cell line, P388/AMSA, the $\alpha$ isoform of topoisomerase II was rearranged in one of two alleles and that the enzyme was hypermethylated in a number of sites. Both of these findings would be expected to be associated with a decrease in the cellular content of functional topoisomerase II, as was observed by Johnson and Howard (1982). Another mechanism of resistance to the topoisomerase II inhibitors has been attributed to the upregulation of the topoisomerase I enzyme. An increase in topoisomerase I levels has previously been demonstrated in "atypical" MDR cell lines. The level of topoisomerase I may increase as a compensatory effect for the down regulation of the topoisomerase II enzyme (Gellert *et al.*, 1982). The topoisomerase I enzyme may be able to carry out the functions of topoisomerase II when it is down regulated and therefore lead to resistance in the cells.

Although alterations in topoisomerase II activity are usually associated with resistance to the epipodophylotoxins and anthracyclines, recent reports have also demonstrated an increase in the activity of the topoisomerase II enzyme in cisplatin resistant cells. Although cisplatin is not a classical MDR drug, Barret *et al.* (1994) found that the cisplatin resistant murine leukaemia cell line, L1210/10, was approximately 2.5-fold more resistant than the parental cells to the cytotoxic effects of topoisomerase II inhibitors. Nuclear extracts of the resistant cells demonstrated a 3-fold increase in topoisomerase II activity, compared with the parental cells, which contributed to the resistant phenotype of the cells. Mestdagh *et al.* (1994) also reported an increase in topoisomerase II activity in the L1210/10 cell line and demonstrated that the activity of the topoisomerase was greater in the cisplatin resistant variant than in an adriamycin resistant variant, relative to the parental cells. Recent studies have also reported an increase in topoisomerase I levels in cisplatin resistant cell lines (Kotoh *et al.*, 1994).

### 1.7.1.2 Clinical implications of topoisomerase inhibitors

Work is ongoing to elucidate the clinical significance of altered topoisomerase II in tumour samples and cell lines and to identify possible methods of circumventing "atypical" MDR. Studies undertaken thus far, on reversal of "at-MDR", have been based on two principles. The first of these states that the cytotoxicity of the topoisomerase II inhibitors is directly related to the number of functional topoisomerase II enzymes in the cell and that increasing the number of active topoisomerases could circumvent the resistance. Phosphorylation of the topoisomerase II enzyme results in an increase in its activity; agonists whose activity is related to phosphorylation may therefore be capable of increasing the activity of the topoisomerase enzymes. Nambi *et al.* (1989) illustrated that both vasopressin and thrombin stimulated the activity of the topoisomerase I enzyme and in later studies demonstrated that the increase in activity observed in the topoisomerase I and topoisomerase II enzymes was regulated by cyclic nucleotide- and phospholipid- dependent protein kinases (Mattern *et al.*, 1991). Other agonists that have been found to increase the enzyme activity include tumour necrosis factor and leukotreine $D_4$ (Utsudi *et al.*, 1990; Mattern *et al.*, 1990). Cotreatment with a combination of topoisomerase inhibitors and these agonists could therefore play a role in the circumvention of "at-MDR", since it was previously shown that an increase in the cytotoxicity of the topoisomerase inhibitors, teniposide and amascrine, was observed when cotreated with tumour necrosis factor in the murine L929 fibrosarcoma cell line (Utsudi *et al.*, 1990).

The second principle states that the activity of topoisomerases I and II is regulated to maintain cellular homeostasis. Consequently, if the level of functional topoisomerase II enzyme is decreased in the cell then a compensatory upregulation of topoisomerase I will occur. Combination treatment with topoisomerase I and topoisomerase II inhibitors, therefore, has the potential to prevent "at-MDR". Lefevre *et al.* (1991) have shown that the human breast cell line, CALc18/AMSA, which was resistant to the topoisomerase II inhibitor amsacarine, exhibited a three-fold decrease in topoisomerase II levels when compared with the parental cells. In contrast, the cell line exhibited a three-fold increase in topoisomerase I levels and also expressed hypersensitivity to the topoisomerase I inhibitor camptothecin. These findings suggested an upregulation of the topoisomerase I gene to compensate for the reduced topoisomerase II expression. Further studies by Woessner *et al.* (1993) demonstrated an increase in resistance to the topoisomerase II inhibitor etoposide

in the P388 cell line that had been made hyperresistant to the topoisomerase I inhibitor camptothecin, thus showing an upregulation of the topoisomerase II enzyme, compensating for the decrease in the topoisomerase I. Another possible mechanism for circumventing topoisomerase resistance could involve molecular design. This theory has been studied in amsacrine resistant human leukaemia cell sublines that expressed MDR and also altered transport of the topoisomerase II inhibitor. Findlay and coworkers (1990) demonstrated that by altering substituents on the aniline acridine nucleus of the amsacrine molecule, resistance to both mechanisms was overcome.

### 1.7.2 Glutathione and Glutathione-S Transferase

The glutathione-S-transferases (GST) are a family of multifunctional cellular enzymes first discovered in the early 1960s (Booth *et al.*, 1961). The function of these enzymes was originally believed to involve the intracellular transport of various proteins and endogenous ligands such as bilirubin, haem and bile acids. However, it has long been known that the GST enzymes are an integral part of the Phase I (oxidation)/Phase II (conjugation) systems that metabolize many lipophilic drugs and other foreign compounds. The overall result of metabolism by this system is the conversion of these lipophilic compounds to more polar derivatives in a manner that can facilitate their inactivation and elimination. GSTs catalyze the conjugation of glutathione (glutamylcysteinglycine), through the sulphur atom of its cysteine residue, to various electrophiles, forming stable secretory metabolites and preventing oxidative damage through intrinsic peroxidase activity (Jokoby, 1978). Glutathione is the principal non-protein thiol in mammalian cells and functions as a scavenger of electrophilic toxins. It is a tripeptide, synthesised in two successive ATP-requiring steps. The first step involves the formation of an amide linkage between cysteine and glutamate, catalyzed by the enzyme glutathione synthetase and in the second step the enzyme mediates the reaction of glycine to the carboxyl terminal of cysteine to form glutathione. There is growing evidence that, in addition to protecting normal cells and tissues from chemical injury, glutathione and the glutathione-S-transferases may play an important role in the MDR phenotype by protecting malignant tissue from chemotherapeutic agents (Arrick and Nathan, 1984; Green *et al.*, 1984; Evans *et al.*, 1987; Hosper *et al.*, 1988).

In human, GSTs exist in cytosolic or microsomal forms and may constitute up to ten percent of total cellular protein of the liver. GSTs have also been localised in human kidney, lung, brain, intestine, skeletal muscle and adrenal glands (Tsuchida and Sato, 1992). There are three main classes of cytosolic GST: $\pi$, $\alpha$ and $\mu$, the classification being based on the enzymes N-terminal amino acid sequence, the substrate specificity, sensitivity to inhibitors and immunological techniques. Each of the GST enzymes is composed of subunits arranged as hetero- or homo-dimers of 25-29 kilodaltons. The exact structure of each subunit is unknown, but it is believed that each subunit contains two domains; the COOH terminus which has been designated the H-site and to which the electrophilic substrate binds and the glutathione binding site or G-site. The G-site is common to each subunit, while the H-site is specific for each subunit (Picket and Lu, 1989; Waxman, 1990).

#### 1.7.2.1 Glutathione and GST in classic MDR

Altered expression of GST, in particular the $\pi$ isoform, has been reported in a number of human cancers. Increases in the RNA and protein levels have been detected in tumours of the colon, bladder, oesophagus, stomach, pancreas and ovary relative to normal tissue (Kodate *et al.*, 1986; Singh *et al.*, 1990; Hayes *et al.*, 1991). Increased expression of GST has also been demonstrated in lymphoblastic leukaemia, where samples from 52 patients out of a total of 104 stained positive for GST$\pi$ (Sauerberg *et al.*, 1994). Alterations in the activity and levels of glutathione and glutathione-S-transferases have been studied in several cell lines associated with resistance to chemotherapeutic drugs. At least 30 cell lines that were selected for resistance to MDR drugs (adriamycin, etoposide, vincristine, vinblastine and colchicine) have been described in which GST activity has been characterised (reviewed by Moscow and Dixon, 1993). The majority of the cell lines studied were selected to adriamycin. However, of the 25 resistant lines studied only 10 demonstrated increased GST activity with respect to the parental cell line and only 5 of these had increases of 2-fold or greater. Two resistant variants of the human breast cell line MCF-7 that exhibited 192- and 2.6-fold resistance to adriamycin were found to exhibit 44.7- and 2.6- fold increases in GST activity respectively (Batist *et el.*, 1986; Whelan *et al.*, 1989). Cole *et al.* (1990) reported an increase in GST activity in MDR variants of two human lung cell lines, H69 and SW1573, that were also selected for resistance to adriamycin. Chao and coworkers (1992) reported a 2.5 fold increase in GST activity in the human colon cell line SW620 which

exhibited 75-fold resistance to adriamycin. The $\pi$ isoform of the GST enzyme was found to be increased in four of the cell lines that demonstrated an increase in GST activity.

When cell lines selected with other MDR chemotherapeutic agents were studied, it was observed that three resistant variants of the human breast cell line MCF-7 demonstrated an increase in GST activity. The cell lines that were 5.1-fold resistant to etoposide, 11.1-fold resistant to vincristine and 24-fold resistant to colchicine showed an increase of 6.7-fold, 6.7-fold and 70-fold in GST activity respectively. Contrary to the results obtained for the MCF-7 cell line, there was no evidence to suggest that alterations in GST activity were associated with particular cell lines, since a number of other MCF-7 resistant lines studied did not show increased levels of GST activity. There was also no direct relationship between GST activity and resistance in the lines studied since GST activity was unaltered in some of the most resistant cell lines.

The involvement of GST in the cell lines studied is, therefore, difficult to clarify, and while results obtained would indicate that increased GST activity is not necessary for MDR, they do not indicate whether increased GST activity contributes to resistance in the cell line that expressed elevated levels. A number of groups have studied GST activity in resistant cells that had reverted back to the sensitive wild type when grown in the absence of the selecting agent, to determine if the cell line retained the elevated level of GST. In one such study by Yusa *et al.* (1988), a revertant of a colchicine resistant MCF-7 cell line that had lost resistance to colchicine and adriamycin, was found to still express the 70-fold increase in GST activity that was noted in the resistant variant. In contrast, Bastist and coworkers (1986) found decreased levels of GST $\pi$ in a drug sensitive line that had reverted from one of the drug resistant MCF-7 cells that expressed increased levels of the GST enzyme. Transfection and expression of cDNA clones of the different GST isoforms in drug sensitive cell lines has been performed in various laboratories to determine if the transfectants display the resistant phenotype. A number of studies were carried out using the MCF-7 cell line, although the increased level of GST did not confer significant resistance to any of the MDR agents tested (Moscow *et al.*, 1989; Fairchild *et al.*, 1990; Leyland-Jones *et al.*, 1991). The co-transfection of GST and MDR genes showed no significant additive effect, in that no change in the sensitivity of the cells to drugs associated with the MDR phenotype was observed (Fairchild *et al.*, 1990).

### 1.7.2.2 Glutathione and GST in cisplatin resistance

Alteration in the cellular concentration of glutathione and levels of GST has been identified in several cell lines associated with resistance to alkylating agents, in particular to the alkylating agent, cisplatin. Cisplatin is sufficiently electrophilic to react directly with glutathione (Eastman, 1987), resulting in a decrease in the level of drug metabolites that can react with cisplatin intracellular targets such as DNA. Increases in GST activity and/or glutathione levels could, therefore, protect cells from exposure to cisplatin. Several groups have demonstrated increases in both glutathione levels and GST activity in cisplatin resistant cells, although its role in contributing to resistance remains controversial. Meijer *et al.* (1990) reported a 2.6-fold increase in glutathione levels in the cisplatin resistant human small cell lung cancer cell line, $GLC_4$-CDDP, with respect to the parental $GLC_4$ cells. When the cells were continuously exposed to cisplatin, a 2.6-fold increase in the glutathione level was observed in the parental cells within 5 hours. However, no further increase was noted in the $GLC_4$-CDDP cell line. Glutathione levels in the BE human colon cancer cell line were also studied. The BE cells were 5-fold resistant to cisplatin and showed a 3-fold increase in glutathione levels. When the cells were grown in the absence of drug for six weeks the revertant cells showed significantly lower levels of glutathione than the resistant cells. These results would suggest that glutathione plays a major role in the emergence of cisplatin resistance in this cell line (Fram *et al.*, 1990). Oldenburg and coworkers (1994) detected an increase in glutathione in two cisplatin resistant variants of the rat colon adenocarcinoma cell line. The two relatively stable cell lines, RL2 and RL4, were 6- and 20-fold resistant to cisplatin and showed an increase of approximately 2.5-fold and 4.8-fold in glutathione levels respectively. When the cells were cultured in the absence of drug for 12 weeks there was a decrease in resistance, although no responding decrease in glutathione levels was observed. Therefore, the results implied that cisplatin resistance was mediated by some alternative mechanism in the resistant variants.

Several investigators have also reported a relationship between cisplatin resistance and glutathione-S-transferase activity (Teicher *et al.*, 1987; Saburi *et al.*, 1989; Sharma *et al.*, 1993). However, the relation between GST and cisplatin resistance has not been clarified. In one study, GST activity and glutathione content were determined in two cisplatin resistant variants of the human small cell lung cancer cell line, H69. The two variants, H69/CDDP

and H69/CDDP$_{0.2}$ were 6- and 11-fold resistant to cisplatin compared with the parental cells. While GST activity was unaltered in the lower resistant variant, a 6.5-fold increase in activity was noted in the higher resistant line. Inhibition of GST activity resulted in sensitisation of both variants although the relative resistance of the two variants remained unchanged. This suggested that while GST may contribute to cisplatin resistance, other factors must be responsible for the observed initial difference in resistant levels (Kasahara *et al.*, 1991). Transfection and expression of GST isoforms in drug sensitive and low level cisplatin resistant lines has been associated with increased resistance to cisplatin. Miyazaki *et al.* (1990) showed a decrease in cisplatin sensitivity in the chinese hamster ovary cell line, CHO, transfected with the GST$\pi$ gene and Teicher *et al.* (1987) reported an increase in cisplatin resistance in the transfected head and neck squamous cell carcinoma cell line, SCC-25/CP. Clinical studies have also shown increased GST levels in tumours resistant to cisplatin. In one such study, Kodera *et al.* (1994) demonstrated increased GST$\pi$ expression in 30 gastric cancer specimens that were inherently resistant to cisplatin when compared with drug sensitive controls. However, no inverse relation between expression and cisplatin resistance was noted.

#### 1.7.2.3 Inhibitors of glutathione and glutathione-S-transferase

Glutathione and related enzymes have been shown to be susceptible to inhibition by a number of non-cytotoxic drugs (Van Bladeren and Van Ommen, 1991) and consequently the effect of various agents that target these enzymes has been studied. Inhibitors of glutathione and related enzymes include ethacrynic acid, a non-cytotoxic diuretic drug, buthionine sulphoxamine (BSO) and sulphasalazine, a drug used in the treatment of inflammatory bowel disorders. It is probable that these compounds act by one or more of the following mechanisms:

- competition with the alkylating agent for metabolism at the GST active site
- depletion of intracellular GSH
- inactivation of cellular GSTs by covalent modification of the enzyme

Ethacrynic acid is a specific inhibitor for glutathione-S-transferase and has been shown to enhance the cytotoxic effects of certain alkylating agents. However, a number of groups

have also reported that inhibition of GST by ethacrynic acid does not alter the cytotoxicity of cisplatin. Tew *et al.* (1988) demonstrated a reduction in resistance to the alkylating agent chlorambucil in two human colon carcinoma cell lines, HT29 and BE, following treatment with ethacrynic acid. Following this treatment both GST activity and cellular glutathione levels were decreased in both cell lines. However, when Kasahara and coworkers (1991) investigated the role of GST in the cisplatin resistant human small cell lung cell line, H69/CDDP, using ethacrynic acid they found that although GST activity was markedly decreased, the relative resistance of the cell line to cisplatin had not changed. Ethacrynic acid has also recently been shown to inhibit the activity of the GSH reductase enzyme. However, ethacrynic acid appears to have approximately 10 times greater potency in inhibiting glutathione-S-transferase relative to GST reductase activity (Hoffman *et al.*, 1995). Recently, the drug sulphasalazine has been reported to act as an effective inhibitor of the glutathione-S-transferase enzyme. Awasthi *et al.* (1994) used sulphasalazine to evaluate the relative role of GSTs in mediating cisplatin resistance in two human small cell lung cancer cell lines, NCI H-69 and H-2496. Both cell lines were found to have elevated levels of GST$\pi$ by Western blot and Northern blot analyses. Sulphasalazine inhibited the purified GSTs from both cell lines and cytotoxicity studies revealed that sulphasalazine also increased the cytotoxic effects of cisplatin in the two cell lines.

The effect of buthionine sulfoximine has been studied in a number of cell lines resistant to cisplatin and other alkylating agents. BSO is an effective and specific irreversible inhibitor of glutathione formulation, which acts by inhibiting the glutathione synthetase enzyme and thus inhibits the biosynthesis of glutathione (Griffith and Meister, 1979). Modulation of cellular glutathione levels in this way can lead to substantial reversal of alkylating agent resistance in drug resistant tumour cells. BSO was found to enhance the susceptibility of two human colon carcinoma cisplatin resistant cell lines, CP2.0 and RT, to cisplatin cytotoxicity following pretreatment for 24 hours (Yang *et al.*, 1993). Inhibition of glutathione biosynthesis by BSO also resulted in an increase in cisplatin toxicity in the human small cell lung cancer cisplatin resistant cell line, $GLC_4$-CDDP (Meijer *et al.*, 1990). A number of studies, however, have also shown that BSO did not significantly alter resistance in other platinum resistant cell lines. Osmak and Eljuga (1993) found that inhibition of glutathione by BSO did not alter the sensitivity of two human cisplatin resistant cervical carcinoma HeLa sublines to cisplatin.

### 1.7.3 Metallothioneins

Metallothioneins (MT) were first discovered in 1957, by Margoshes and Vallee, in the adrenal cortex of horses while searching for a tissue component responsible for the natural accumulation of cadmium in mammalian kidney. They are sulfhydryl-rich metalloproteins which bind to heavy metals, including zinc, copper, cadmium and platinum. Mammalian MTs have a molecular weight of 6000-7000 daltons, usually containing 61 to 62 amino acid residues, of which 20 are cysteines which bind a total of 7 equivalents of bivalent ions. All the cysteine residues occur in the reduced form and are coordinated to the metal ions through mercaptide bonds. Two major isoforms of metallothioneins have been identified in vertebrates, MT-I and MT-II (Hamer, 1986; Kobayashi and Sayto-Suzuki, 1988), which differ at neutral pH by a single negative charge. A third class of MTs, MT-III, has also been identified, however this class of MT has only been found in certain plants and microorganisms. There are at least five different human metallothionein I subtype proteins: MT-$I_A$, MT-$I_B$, MT-$I_E$, MT-$I_F$ and MT-$I_G$ and one human metallothionein II, MT-$II_A$, which have been separated by chromatographic techniques (Kagi and Schaffer, 1988; Kelley *et al.*, 1988). All the isoforms exhibit extremely high amino acid homology. The physiological role of each subtype, however, remains to be identified. Metallothioneins are found in all organs but are most abundant in the liver, kidney and intestine (Danielson *et al.*, 1982). They appear to be located primarily in the cytoplasm of cells, however they have also been located in the nuclei of hepatic and renal cells (Banerjee *et al.*, 1982). The subcellular distribution of metallothioneins has been reported to change during development and also with prolonged exposure to metals (Banerjee *et al.*, 1982; Nartey *et al.*, 1987). Although the functional significance of this altered distribution is as yet unknown, it is suspected that nuclear accumulation may occur with increased cellular metallothionein load or when cells are stimulated to undergo rapid proliferation.

The physiological function of metallothioneins remains unknown, although a number of specific functions have been postulated and include heavy metal detoxification, regulation of intracellular Cu and Zn levels, free-radical scavenging, protection from ionizing radiation and control of growth and differentiation. The most conclusive evidence supports the role of metallothioneins in heavy metal detoxification. Several laboratories have shown that cells exposed to heavy metals overexpress MT and are less sensitive to cytotoxicity of other heavy metals (Durnam *et al.*, 1984). It has also been demonstrated that cell lines which

express low levels of metallothioneins are extremely sensitive to cadmium toxicity (Compere and Palmiter, 1981). These results would suggest a prominent role for metallothioneins in heavy metal detoxification, although it does not appear to be their only biological role. Metallothioneins have been proposed to have a regulatory role in Zn and Cu homeostasis (Cousins, 1985), since the level of intestinal MT is inversely related to the absorption of Zn and Cu. This has been exploited in the treatment of Wilson's disease (an autosomal, recessively inherent disease involving abnormal copper handling by the liver) where intestinal MT expression can be increased by oral Zn therapy, with a concomitant decrease in Cu absorption (Brewer *et al.*, 1983). Metallothioneins may also have a regulatory role in growth development, this concept being supported by the findings of programmed regulation of MT mRNA and protein levels in the course of embryogenesis (Nemer *et al.*, 1984; Andrews *et al.*, 1991) and fetal development (Andrews *et al.*, 1984). It has also been reported that increased expression of metallothioneins is observed in proliferating cells and consequently metallothioneins may also serve as proliferation markers (Nagel *et al.*, 1995). There is also evidence that metallothioneins can scavenge free radicals that are produced during acute-phase response, reperfusion of hypoxic tissue and ionizing radiation (Thornally and Vasek, 1985).

Metallothionein expression can be induced by various factors, such as heavy metals, hormones, cathecolamines, corticosteroids and alkylating agents. The MT content of organs, most notably kidney and liver, as well as cultured cells can be increased by exposure to these agents. The induction of MT following exposure to heavy metals was first noticed in the liver of rabbits in 1964 following exposure to cadmium (Piscator, 1964). However, since then the number of agents that have been found to increase metallothionein expression has increased dramatically. Table 1.7.3 lists many of the factors that have been shown to induce metallothionein synthesis. The induction of metallothioneins by heavy metals such as cadmium, zinc and lead acetate has been extensively studied and has been found to result from transcriptional activation of the MT genes (Durnam and Palmiter, 1981). Cellular metallothionein levels usually reach their maximum within 24 hours following exposure to the metal, however peak levels have been observed after shorter intervals. In cultured HeLa cells, maximum levels were noted after a 6 - 8 hour exposure to cadmium. The half-life of induced metallothioneins is 1 - 4 days, although this can vary depending on the metal state of the metallothionein (Hamer 1986). In human tissue and cells, expression of the metallothionein isoforms appears to be tissue specific and induction

appears to be regulated differentially. Sadhu and Gedamu (1988) analyzed the pattern of expression of three human metallothionein genes (MT-$I_F$, MT-$I_G$ and MT-$II_A$) in the human hepatoblastoma cell line, HepG2, in response to the metal ion inducers cadmium, copper and zinc. The absolute number of transcripts of each of the three genes was measured and the results obtained indicated differential regulation of the genes by the different inducers both in terms of rate and extent of transcript accumulation. They also found that these three MT genes responded differently to induction by the metal ion inducers. The lowest level of transcript accumulation was observed for the MT-$I_F$ gene and copper was shown to be its weakest inducer. Cadmium was shown to be the weakest inducer of MT-$I_G$ even though transcript of this gene accumulated at comparatively higher level than those of MT-$I_F$. Cadmium was also found to be the weakest inducer of the MT-$II_A$ gene.

Metallothioneins are also induced by stressful conditions such as heat, cold and starvation. These conditions raise circulating steroid hormone levels which act as inducers of metallothioneins. A number of groups have studied the effect of glucocorticoids on metallothionein induction. Glucocorticoids were first shown to regulate MT levels in cultured rat liver parenchymal cells (Failla and Cousins, 1978) and were subsequently found to stimulate MT synthesis in intact rodents, primarily in the liver but also in the kidney, spleen and skeletal muscle (Hager and Palmiter, 1981). In human cells grown in culture, glucocorticoids strongly induced the MT-$I_E$ and MT-$II_A$ isoforms and slightly induced the MT-$I_A$ isoform although it did not appear to have an effect on the other isoforms (Schmidt and Hammer, 1986). In addition to steroid hormones, metallothionein synthesis is also stimulated by glucagon, angiotensin II and by $\alpha$- and ß- adrenergic agonists (Cousins, 1985). Increased expression of metallothioneins has also been demonstrated following X-irradiation, hypoxia and inflammation. Shiraishi *et al.* (1986b) reported an increase in metallothionein content in rat liver and kidney following radiation treatment, with the degree of expression comparable to levels observed following induction with heavy metals. Northern analysis and Western blotting showed an increase in MT-$II_A$ mRNA and total MT protein levels in the human squamous carcinoma cell line, A431, following hypoxia and during reoxygenation. The level of MT-$II_A$ increased as a function of time and the highest level was observed after 14 hours of hypoxia and the message was sustained for at least 24 hours of hypoxia (Murphy *et al.*, 1994). Increased synthesis of MT has also been noted in response to inflammation (Durnam *et al.*, 1984). The cytokines interferon and interleukin-1 have also been shown to increase intracellular MT concentrations (Friedman

*et al.*, 1984; Karin *et al.*, 1985).

| | |
|---|---|
| Heavy metals | Alkylating agents |
| Glucocorticoids | Phorbol esters |
| Estrogens | X-irradiation |
| Progesterone | Carbon tetrachloride |
| Glucagon | Starvation |
| Catecholamines | Infection |
| Interleukin 1 | Stress |
| Interferon | High $O_2$ tension |

**Table 1.7.3** Factors that induce metallothionein synthesis (Bahnson *et al.*, 1991)

### 1.7.3.1 Metallothionein in human tumours

The presence of metallothioneins in a number of human tumours has been demonstrated by immunohistochemical studies. Nartey and coworkers (1987) reported the detection of metallothioneins in 31 of 34 human thyroid tumours in archival paraffin embedded tissue samples while less than 20 percent of normal thyroid glands showed diffused distribution of metallothioneins. The positive staining was present in both nucleus and cytoplasm of thyroid adenomas and carcinomas. A number of studies also reported the presence of metallothioneins in testicular tumours (Chin *et al.*, 1993). There was a distinct difference between MT staining in seminomas and non-seminomas (embryonal carcinoma, teratocarcinoma and choricocarcinoma) in human testicular tumours. Pure seminomas showed little or no staining for MT, irrespective of the clinical stage while non-seminomas stained heavily, especially in the more advanced clinical stages (Chin *et al.*, 1993). Metallothionein staining in pleomorphic adenoma and adenoid cystic carcinoma of salivary gland has also been studied and the staining was found to be present, mainly in myoepithelial cells with intense staining in the proliferating edge (Chauvin *et al.*, 1992). Studies by Ofner *et al.* (1994) in 109 colorectal adenocarcinomas demonstrated strong

expression of metallothioneins in 34 cases and positivity in a further 24 cases. The remaining cases were negative for MT expression. The differences in MT expression were found to be significant and associated with the tumour stage and the lymph node involvement at the time of the operation. Expression of metallothionein has also been demonstrated in initial and relapsed childhood acute lymphoblastic leukaemia. Sauerbrey *et al.* (1994) found that in the group of patients studied, MT expression was detected in approximately 33% of patients with acute initial lymphoblastic leukaemia and in approximately 33% of patients in the relapsed state. There was no difference observed in the intensity and proportion of positively stained cells between initial and relapsed patients. Studies on nonmelanoma skin cancer have shown that metallothionein levels in a variety of tumours mirror the progressive potential of the tumours. In one example, well differentiated squamous cell carcinomas only showed MT labelling in peripheral cell layers, while in poorly differentiated squamous carcinomas, irregular clusters of tumour cells above the level of the peripheral cell layers showed MT labelling (Zelger *et al.*, 1994). In a study of samples from patients with ductal carcinoma of the breast, metallothionein expression was shown to be associated with a more aggressive behaviour. In general, myoepithelial cells surrounding benign and malignant structures stained uniformly strongly for metallothioneins (Douglas-Jones *et al.*, 1995). The immunohistochemical studies show that metallothioneins are expressed in a wide variety of human tumours, however the staining for MT is not universal to all tumour growth. The results demonstrate that expression of metallothioneins may depend on various factors, which may be related to the type of tumour, its cellular origin, morphological heterogeneity or its stage of growth.

#### 1.7.3.2 Metallothionein and cisplatin resistance

The observation that metallothioneins play a role in heavy metal detoxification led to investigations exploring the relationship between cellular metallothionein levels and sensitivity to cisplatin. This concept was first demonstrated by Bakka and coworkers (1981) where they found that cadmium resistant human and mouse cultured cells, which contained increased levels of metallothioneins, were cross-resistant to cisplatin. Further studies showed that the platinum content was higher in the resistant cells than in the non-resistant cells and therefore excluded the possibility of decreased uptake or increased efflux as a mechanism for the observed differences in cisplatin resistance. These studies also showed

that the major portion of the platinum in the resistant cells was associated with metallothionein. Numerous reports have since been published implicating metallothioneins as a cause of cellular resistance to cisplatin and other alkylating agents. In summary the reports have shown that:

- tumour cells with increased MT content are resistant to both alkylating agents and cisplatin
- cells with acquired resistance to cisplatin frequently have increased MT levels and overexpress MT mRNA
- reversal of the cisplatin resistant phenotype is accompanied by a decrease in MT content
- introduction of a eukaryotic expression vector encoding human MT-$II_A$ into murine cells confers the drug resistant phenotype.

Teicher *et al.* (1987) isolated a cisplatin resistant variant of the human head and neck squamous cell carcinoma, SCC-25. No difference in non-protein sulfhydryl content was noted, although an increase of approximately 2-fold was observed in the protein sulfhydryl content in the resistant line compared to the parental cell line. Further studies by Kelley *et al.* (1988) demonstrated that the increase in protein sulfhydryl content was due to an increase in MT expression. Kelley *et al.* (1988) generated a panel of drug resistant human cell lines by repeated exposure to increasing concentrations of cisplatin and included the breast cell line, MCF-7/CP, the small cell lung carcinoma cell line, SW2/CP, the large cell lung carcinoma cell line, SL6/CP, and the melanoma cell line G336/CP. With the exception of the MCF-7/CP cell line, all the tumour cell lines expressed elevated levels of metallothioneins compared to the corresponding parental cell line.

Andrews and Howell (1990) reported increases in MT levels in the cadmium chloride resistant human ovarian carcinoma cell line, 2008, that was cross resistant to cisplatin. Elevated levels of MT have also been demonstrated in two cisplatin resistant variants of the human small cell lung carcinoma cell line H69. The two variants were 6-fold and 11-fold resistant to cisplatin and displayed cross resistance to cadmium chloride. The expression of MT mRNA was higher in the resistant variants and the expression was related to the sensitivity of the cells to cisplatin (Kasahara *et al.*, 1991). Kondo *et al.* (1995) investigated the cellular content and subcellular distribution of metallothioneins in four human hormone-independent prostatic carcinoma cell lines. Different levels of MT expression were observed in the four cell lines and it was also demonstrated that the cell lines exhibited different patterns in the subcellular distribution of MT. The cells which had primarily

nuclear MT were more resistant to cisplatin compared to the lines that had more diffuse cytoplasmic, perinuclear and nuclear staining. Metallothionein expression has also been studied in murine cell lines. In one study, a cisplatin variant of the mouse leukaemia cell line, L1210, was shown to have elevated levels of MT when compared to the parental cells and the degree of resistance was found to correspond with MT content in the resistant cells (Kelley *et al.*, 1988). It was also demonstrated that the cisplatin revertant cell line exhibited decreased levels of metallothionein, thus suggesting that MT was involved in the resistant phenotype.

The above evidence suggests that increases in metallothionein expression occur with acquired resistance to cisplatin, although not all cell lines with acquired cisplatin resistance overexpress MT. A cisplatin variant of the MCF-7 human breast cancer cell line was shown to be approximately 3-fold resistant to cisplatin, although no alteration in metallothionein levels were noted in this cell line when compared to the parental cells (Kelley *et al.*, 1988). Andrews *et al.* (1987) demonstrated that the human ovarian cell line, 2008, which was 3-fold resistant to cadmium chloride had unaltered levels of MT. Finally Mellish *et al.* (1993) studied MT levels in a panel of five human cervical squamous carcinoma cell lines that exhibited acquired resistance to cisplatin and found that there was no significant difference in cellular MT levels.

A number of studies have been carried out modulating the toxicity and resistance of cisplatin by induction and inhibition of metallothionein synthesis. Cisplatin, when administered has various side effects including nephrotoxicity, gastrointestinal toxicity and pulmonary toxicity. The objective in a number of studies was to develop treatments to prevent toxicity of cisplatin by induction of metallothionein synthesis without compromising its antitumour activity. Naganuma *et al.* (1987) showed that preinduction of MT in the kidney by administration of bismuth nitrate decreased the renal and gastrointestinal toxicity in mice caused by a single injection of cisplatin administered 24 hours later. The renal MT content of the mice was significantly increased in comparison to the control mice and a distinctive relation was found between the protective effect of bismuth nitrate and the preinduced MT levels in the kidney. When the effect of pretreatment of bismuth nitrate on the antitumour activity of cisplatin was examined it was found that this pretreatment had no effect on the antitumour activity of cisplatin against several transplantable tumours. Preinduction of pulmonary MT by bismuth nitrate and zinc compounds has also been demonstrated in mice.

Cisplatin is known to induce lung tumours, however Satoh and coworkers (1994a) showed that pretreatment with zinc or bismuth nitrate resulted in a decrease in the occurrence of pulmonary adenomas following cisplatin treatment. An increase in pulmonary MT was noted which appeared to protect the mice from secondary carcinogenesis. Studies have also been reported on compounds which inhibit metallothionein synthesis and enhance the sensitivity of cells to cisplatin. An example of such a compound is proparglycine, an inhibitor of cystathionase (an enzyme involved in the conversion of methionine of cysteine) which has been shown to decrease MT induction by zinc in human bladder tumour cells (NMB-1) that were inoculated in nude mice. Pretreatment of the tumour bearing mice with zinc salts resulted in an increase in MT content and a marked reduction of the antitumour activity of cisplatin. When proparglycine was then administered a decrease in MT levels and diminished resistance to cisplatin was observed (Satoh *et al.*, 1994b).

## 1.8 Antibodies in the study of MDR

Antibodies to multidrug resistant-associated antigens are now widely used in the characterisation and analysis of MDR in cell lines and clinical samples. Over the last decade a number of monoclonal and polyclonal antibodies useful in the study of MDR have been generated, including antibodies to P-glycoprotein, topoisomerase, glutathione-S-transferase enzymes and metallothionein. The technique for producing virtually unlimited quantities of a single antibody, specific for a particular antigenic determinant has been a major breakthrough in MDR research. Biological production involves manipulation of the hybridization technique first developed by Cotton and Milstein (1973) and Kohler and Milstein (1975). The technique involves cell fusion or somatic cell hybridization between a normal antibody-producing B cell obtained from the spleen of mice immunized with a specific antigen and a myeloma cell line. This is followed by the selection of fused cells that secrete antibody of the desired specificity, derived from the normal B cells (figure 1.8.1).

The success of this technique depended on the development of cultured myeloma lines that would grow in normal culture medium but would not survive in defined selection medium lacking functional genes required for DNA synthesis. Normal cells synthesize nucleotides *de novo* from phosphoribosyl pyrophosphate and uridylate in several steps, one of which involves the transfer of a methyl or formyl group from activated tetrahydrofolate. Antifolate drugs, such as aminopterin, block the reactivation of tetrahydrofolate, thereby inhibiting the synthesis of purine and thymidlylate. Since these are necessary components of DNA, aminopterin blocks DNA synthesis through the *de novo* pathway. However, purine synthesis can occur through a salvage pathway from exogenously supplied hypoxanthine using the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidylate can be synthesised from thymidine using the enzyme thymidine kinase. Therefore, cells grown in aminopterin can synthesize DNA if supplemented with hypoxanthine and thymidine (figure 1.8.2). Antibody production, therefore involves selecting for fused cells that grow in medium containing hypoxanthine, aminopterin and thymidine (HAT). The principle behind this approach is that the parent myeloma cell line is HAT sensitive lacking the enzymes, HGPRT and thymidine kinase (TK) and therefore can not chemically synthesise DNA and RNA when grown in the selecting HAT medium.

While the parent lymphocytes contain these enzymes, they can not grow indefinitely. The myeloma cells confer immortality on the antibody producing B lymphocyte fused cells, and allows them to grow in culture. This technology has allowed the production of essentially unlimited quantities of pure homogenous antibodies against a large variety of antigens including enzymes, hormones, lipids, carbohydrates, bacteria, synthetic peptides and cellular extracts. This technology has been applied in MDR research with the production of both polyclonal and monoclonal antibodies to a number of multidrug resistant cell lines and synthetic peptides to MDR proteins.

### 1.8.1 Anti-P-glycoprotein monoclonal antibodies

A number of antibodies to P-glycoprotein have been reported including the mABs C219, MRK16, MRK17, JSB-1, 265/F4 and UIC2. Ling and coworkers established a number of monoclonal antibodies by immunization of BALB/c mice with a mixture of purified plasma membrane fractions from a colchicine resistant chinese hamster ovary cell line, $CH^{R}B30$ and a vinblastine resistant variant of a human leukaemia cell line, $CEM/VBL_{500}$ (Kartner *et al.*,1985). Included in this series of antibodies is the C219 mAB which recognizes a cytoplasmic epitope of the P-glycoprotein molecule six residues away from the consensus sequence that is believed to be the ATP-binding domain of the protein. This epitope is highly conserved in all P-glycoprotein isoforms, thus the C219 antibody recognizes all classes of the *mdr* gene in both human and murine cell lines (Georges *et al.*, 1990; Barrand *et al.*, 1992). Also included in this group of P-glycoprotein specific antibodies is the C494 mAB which is gene specific and binds to a sequence present only in the class I isoform of hamster and human P-glycoprotein and the mAB C32 which recognizes a sequence in hamster class 1 and II isoforms (Georges *et al.*, 1990). The C219 antibody has been used successfully for the detection of P-glycoprotein for immunohistochemistry, immunoblotting and flow cytometry.

The monoclonal antibodies, MRK16 and MRK17, were raised against the adriamycin resistant human myelogenous leukaemia cell line, K562/ADM. They recognise an extracellular epitope of the P-glycoprotein molecule and are specific for the human *mdr-1* (Hamada and Tsuruo, 1986). MRK16 has been shown to increase intracellular accumulation of vincristine and actinomycin-D and enhance vincristine cytotoxicity in the

K562 resistant cells. However, the MRK17 antibody was found to inhibit the growth of resistant cells only. Later studies demonstrated that the MRK16 antibody effectively prevented tumour development in athymic mice innoculated with drug resistant human ovarian cancer cells (Tsuruo *et al.*, 1989). The MRK16 antibody can also be used successfully for immunohistochemistry and immunoblotting. The JSB-1 antibody was established by immunization of BALB/c mice with whole intact colchicine resistant chinese hamster ovary cells (Scheper *et al.*, 1988). This antibody is not species specific and recognizes a cytoplasmic epitope closely linked to that recognized by C219. The UIC2 antibody was established by immunization of BALB/c mice with vinblastine resistant BALB/c 3T3-1000 cells. Similar to the MRK16 antibody, the UIC2 mAB recognizes an external epitope of the human P-glycoprotein. UIC2 has been shown to inhibit the efflux of P-glycoprotein substrates from MDR cells and also to significantly increase the cytotoxicity of various MDR drugs (Mechetner and Roninson, 1992).

### 1.8.2 Antibodies against non-P-glycoproteins

Monoclonal antibodies to a number of other MDR-associated antigens have also been described which have proved to be useful tools in the study of MDR. Polyclonal antibodies have frequently been used to detect levels of topoisomerase II isoforms (Drake *et al.*, 1987; Drake *et al.*, 1989; Hwang *et al.*, 1989). However, more recent studies have utilized a series of topoisomerase II specific polyclonal and monoclonal antibodies to enable detection of the topoisomerase II isoforms. A number of polyclonal antibodies were raised against two synthetic peptides of human topoisomerase II cDNA. Antibodies raised against one peptide (SPI) specifically recognized the 170 kDa $\alpha$ form of the topoisomerase II, while antibodies raised against the second peptide (SPII) selectively recognized the 180 kDa ß isoform (Chung *et al.*, 1989). Robinison *et al.* (1993) established two murine monoclonal antibodies directed against a 70 kDa polypeptide which responded to the carboxyl-terminal of human topoisomerase II$\alpha$. The antibodies were found to be specific for human topoisomerase II, although only recognizing the topoisomerase II$\alpha$ isoform. Monoclonal antibodies specific for human topoisomerase isoforms have also been established by Negri *et al.* (1992). The two antibodies, 6G2 and 8F8, specifically recognize the $\alpha$ and ß isoforms respectively.

Polyclonal and monoclonal antibodies have also been generated against multidrug resistance-associated protein (MRP), the 180 - 195 kDa membrane glycoprotein involved in non-P-glycoprotein multidrug resistance of human tumour cells. Rabbit antisera against three synthetic peptides responding to the deduced sequence of MRP was prepared. Western blot analysis with the antiserum, ASPKE, raised against one of the peptides demonstrated the presence of a 190 kDa protein contained in membranes of HL60/ADR resistant cells but absence in membranes prepared from the parental HL60 cells (Krishnamachary and Center, 1993). Rat and murine monoclonal antibodies were also raised by Flens *et al.* (1994), against two different peptide sequences of MRP, FPI and FPIII. Hybridomas secreting antibodies that reacted with MRP were selected on the basis of strong immunocytochemical staining with GLC4/ADR cells, the non-P-glycoprotein MDR subline of the small cell lung cancer cell line, GLC4. The antibodies recognized a protein of 195 kDa protein in isolates of the GLC4/ADR cells, indicating that they reacted with MRP. One rat monoclonal antibody, MRPr1, and one mouse monoclonal antibody, MRPm6, were subcloned and further characterized. Cole *et al.* (1992) established rabbit polyclonal antibodies against a synthetic peptide, the sequence of which was predicted from that of the clones MRP cDNA. The purified anti-MRP antibody recognized a glycosylated, integral membrane protein with an apparent molecular weight of 190 kDa. Five murine monoclonal antibodies were raised against extracts of the resistant H69/AR cell line. The antibodies reacted specifically with membrane proteins of the MRP positive H69/AR cells, and three of the antibodies also selectively immunoprecipitated a 190 kDa protein from $^{35}$S-labelled H69/AR cells (Hipfner *et al.*, 1994).

A monoclonal antibody has also been raised to the 110 kDa lung resistance protein (LRP) by imunization of BALB/c mice with cellular extracts of the P-glycoprotein-negative cell line SW1573/2R120 (Scheper *et al.*, 1993). The hybridomas secreting antibodies were selected by enzyme-linked immunosorbent assays with the resistant SW1573/2R120 and the parental SW1573 cells. One of the hybridomas, LRP-56, was subcloned by limiting dilution and the LRP-56 immunoglobulin purified from ascites. A number of antibodies to glutathione-S-transferase have been established, in particular to the GST$\pi$ isoform. Mannervik *et al.* (1985) developed a polyclonal anti-human glutathione-S-transferase $\pi$ antibody that recognized a 47 kDa protein, consistent with the reported molecular weight of GST$\pi$. Polyclonal antibodies to GST$\pi$ have also been established by Kodate *et al.* (1986) and Batist *et al.* (1986).

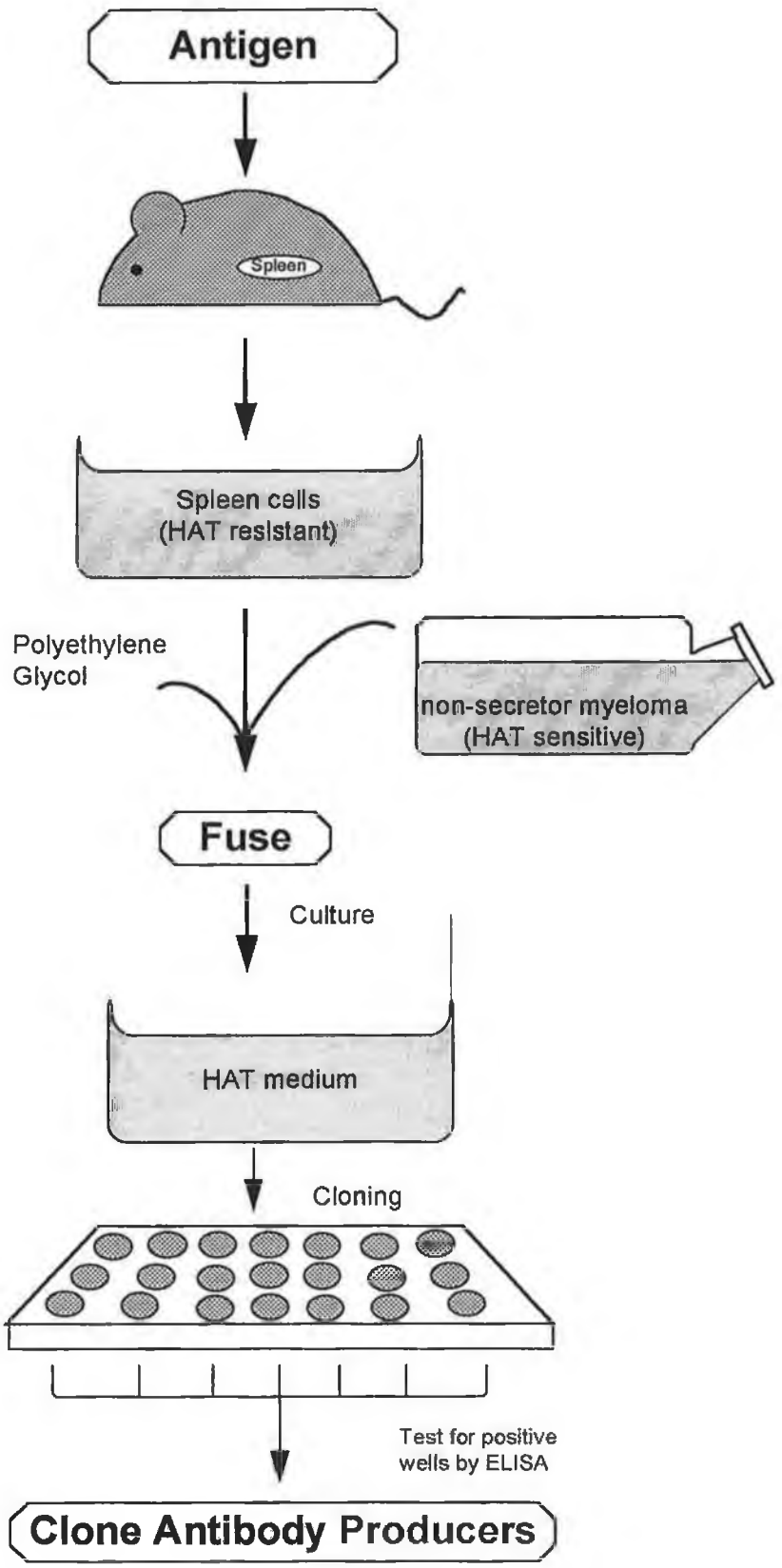



**Figure 1.8.1** Stages of hybridoma production

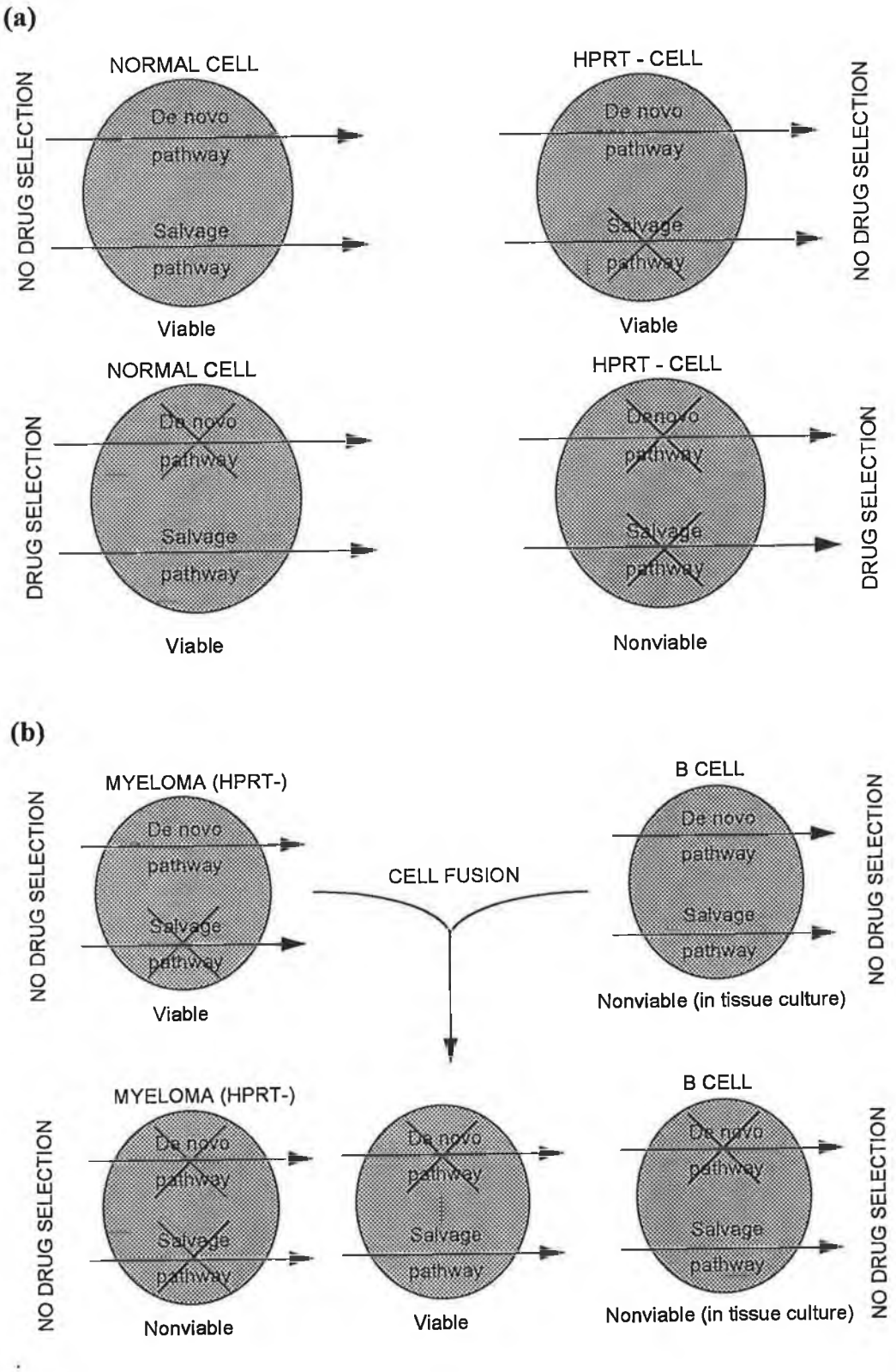



**Figure 1.8.2** Pathways of nucleotide synthesis (a) and drug selection for viable hybridomas (b)

## 1.9 Aims of thesis

The aim of the work performed in the early part of the studies described in this thesis was to establish and characterise novel, platinum resistant, variants of the human lung carcinoma cell line, DLKP. The establishment of the resistant variants was effected through continuous exposure to increasing concentrations of the the drug, carboplatin, chosen because of its lower toxicity and its widespread clinical application, particularly in the treatment of lung and ovarian carcinomas. The characterisation work involved biochemical, immunological and toxicological studies on the established variants.

Given that reduced drug accumulation is a characteristic of many MDR cell lines, the objective of the work done in the next phase was to investigate the accumulation of the anticancer agents, adriamycin and vincristine in a number of human MDR cell lines and also to determine the effect of a range of compounds on drug retention in the resistant cells. In addition, since many MDR cell lines have also been shown to display altered subcellular distribution, studies were carried out to investigate the effect of a number of agents on the subcellular distribution of adriamycin. These agents had previously been included in the studies carried out on the accumulation of anticancer agents. Since adriamycin is inherently fluorescent under ultraviolet illumination, it was considered to be an ideal drug for use in these subcellular distribution studies

The results obtained from the studies carried out on the investigation of the accumulation of anticancer agents and on the subcellular distribution of adriamycin, indicated the existance of alternative mechanisms involved in the decreased drug accumulation in MDR cell lines. Consequently, the objective of the work carried out in the final phase was to raise antibodies which may be associated with this reduced drug accumulation in MDR cell lines. These antibodies were raised against an adriamycin resistant variant of the human lung carcinoma cell line, DLKPA10, which displayed a reduction in drug accumulation and an altered subcellular drug distribution pattern relative to the parental cells.

# 2. *Materials and Methods*

## 2.1 Preparation of cell culture media

### 2.1.1 Water quality

All water used in the preparation of cell culture media was purified by a Milli-Pore ultrapure water system or an ELGA UHP system. The water was first prefiltered, followed by a double distillation step (ELGA) or passage through a reverse osmosis system (Milli-Q). It was then passaged through two ion-exchange filters and a carbon filter to remove organic solutes. Finally the water was passed through a 0.22$\mu$m cellulose acetate filter resulting in ultrapure water which was continuously monitored by an on-line conductivity meter.

### 2.1.2 Glassware treatment

All items of glassware used in cell culture (media bottles, roller bottles, waste bottles *etc.*) were soaked in a warm 2% solution (v/v) of the non-ionic detergent RBS (Chemical products; Belgium) for 1-2 hours. They were then individually scrubbed with bottle brushes and rinsed thoroughly with tap water to remove all RBS residue. This was followed by three separate washes in deionised water before a final rinse in ultra pure water.

### 2.1.3 Sterilization

All glassware used in cell culture, such as media bottles, roller bottles *etc.* were sterilized by autoclaving. All items were autoclaved before use at 121$^0$C and 15 psi pressure for 20 minutes. Phosphate buffered saline (PBS), water and the relevant solutions for media preparation (HEPES, HCl, NaOH and $NaHCO_3$) were sterilized using the above conditions. Filter sterilization was employed for unstable reagents, including cell culture media and protein solutions. Sterile disposable 0.22$\mu$m filters (Millex-GV, SLGV025BS) were used for small volumes. Larger volumes, such as culture media, were filter sterilized through a micro-culture bell filter (Gelman 12158) which has a filtering capacity of 10 litres.

### 2.1.4 Basal medium preparation

The preparation of basal medium was carried out under sterile conditions in a class ll laminar flow cabinet. All media were prepared from a 10X stock of liquid or powder concentrates and made up in batches of 5 litres with sterile ultrapure water. Hams F12 was made up exclusively from a powdered batch to 5 litres (Gibco 21700-109). The powder was dissolved in 4.7 litres of ultrapure water and supplemented with 1M HEPES (Sigma 7365-45-9), and 7.5% $NaHCO_3$ (BDH 30151). The pH was adjusted with 5M NaOH (Merck 1.06482.1000) and 1.5M HCL (BDH 10158) to give the desired pH of 7.45 - 7.55. The liquid concentrates were prepared by the addition of 500 mls of the 10X stock to 4.3 litres of ultrapure water and were supplemented with 1M HEPES and 7.5% $NaHCO_3$. The pH was again adjusted with 5M NaOH and 1.5M HCl. Table 2.1.4 illustrates the components required for the preparation of 5 litres of the three basal media used in these studies. The medium was then filter sterilized through a micro culture capsule bell filter into 500 ml sterile bottles and aliquots from each bottle were removed for sterility tests. The bottles were labelled and stored at $4^0C$ and used only after the sterility tests proved negative.

| Medium | Catalogue no. Gibco | Volume $H_2O$ | Volume 10X concentrate | Volume 1M HEPES | Volume 7.5% $NaHCO_3$ |
|---|---|---|---|---|---|
| DMEM | 12501-029 | 4.3L | 500mls | 100mls | 45mls |
| MEM | 21430-020 | 4.3L | 500mls | 100mls | 45mls |
| Hams F12 | 21700-109 | 4.7L | 5L concentrate | 100mls | 45mls |

**Table 2.1.4** Preparation of basal medium from 10X stock.

### 2.1.5 Preparation of HEPES and $NaHCO_3$

The HEPES buffer was prepared by dissolving 23.8g of HEPES in 80 mls of ultrapure water and the solution was then autoclaved. Following autoclaving, 5 mls of sterile 5M NaOH was added to give an approximate volume of 100 mls. $NaHCO_3$ was prepared by dissolving 7.5g in 100 mls of ultrapure water and autoclaving the resulting solution.

### 2.1.6 Media supplements

A 200 mM solution of L-glutamine (Gibco 28030-024) was added to the basal media to give a final dilution of 1:100 L-glutamine. The L-glutamine is a relatively unstable component of cell culture medium and was therefore only added to the media three days before use. 100 mls of L-glutamine was aliquoted and stored at -20$^0$C until required.
100 mM sodium pyruvate (Gibco 11360-039) and also a 1:100 dilution of 100X stock of non essential amino acids (NEAA; Gibco 11140-035) were added to the MEM basal medium to supplement the growth medium for the SKMES-1 and SKMES-1/ADR cell lines.

### 2.1.7 Serum

All basal media used throughout these studies were supplemented with 5% foetal calf serum (unless otherwise stated). Due to batch to batch variability of the serum, routine batch screening was carried out to select a serum suitable for the particular cell line being used. 10-15 samples of serum were screened on a number of cell lines and suitable foetal calf serum was chosen for adherent cells (Seromed S0115) and hybridoma cells (Advanced protein products AS-302-50). The serum screen was carried out using miniaturised assays similar to the toxicity assays described in section 2.6. The serum was purchased in bulk and stored longterm at -20$^0$C until required.

### 2.1.8 Sterility tests

Sterility checks were carried out on all 1X bottles of basal media prior to the preparation of complete growth medium. Aliquots of medium were removed and treated in a number of ways. A 3 ml sample of medium was incubated to test for bacterial contamination. 1ml samples were inoculated in sabauraud broth (Oxoid CM147) which supports the growth of fungus and thioglycollate broth (Oxoid CM173) which supports the growth of anaerobic bacteria. A further sample was streaked on blood agar plates (Columbian base agar: Oxoid CM331) to test for aerobic bacterial and yeast contamination. All sterility tests were incubated at 37$^0$C and left for 3-5 days to test for bacterial contamination and a minimum of 14 days to test for fungal or yeast contamination. The media was only used if all tests were negative after this time. After the basal medium was supplemented with FCS, L-glutamine and other supplements required for the particular cell line, aliquots of medium were removed and sterility checked, as above, prior to use.

## 2.2 Routine maintenance of cell lines

### 2.2.1 Cell lines used in studies

All cell culture work was performed aseptically in class II vertical down-flow recirculating laminar flow cabinets (Nuair or Holten cytoguard). The cell lines used throughout this work are outlined in table 2.2. The lines used were all human cell lines and anchorage dependent, with the exception of the SP2 cell line (murine myeloma cell line) that attaches only loosely. The human cell lines were grown in 25cm$^2$ (Costar 3055) and 75cm$^2$ (Costar 3375) tissue culture treated unvented flasks and the SP2 cells were grown in 25cm$^2$ (Costar 3056) and 75cm$^2$ (Costar 3276) vented flasks. All cell lines were incubated at 37$^0$C in an atmosphere of 5% $CO_2$ and were fed every two to three days.

| Cell line | Source | Description | Basal medium |
|---|---|---|---|
| DLKP | G. Grant NCTCC[a] | Human lung squamous carcinoma | 50% DMEM:50% Hams F12 |
| DLKP-A | A. Redmond NCTCC | Adriamycin resistant variant of DLKP | 50% DMEM:50% Hams F12 |
| SKMES-1 | ATCC[b] | Human lung squamous carcinoma | MEM |
| SKMES-1A | A. Redmond NCTCC | Adriamycin resistant variant of SKMES-1 | MEM |
| T24 | ATCC | Human bladder carcinoma | 50% DMEM:50% Hams F12 |
| T24A | G. Grant NCTCC | Adriamycin resistant variant of T24 | 50% DMEM:50% Hams F12 |
| T24V | G. Grant NCTCC | VP16 resistant variant of T24 | 50% DMEM:50% Hams F12 |
| OAW42 | ATCC | Human ovarian carcinoma | 50% DMEM:50% Hams F12 |
| OAW42-A | NCTCC | Adriamycin resistant variant of OAW42S | 50% DMEM:50% Hams F12 |
| SP2 | Immune Systems | Murine myeloma | 1X DMEM |

[a] National Cell & Tissue Culture Centre, Dublin City University.
[b] American Tissue Culture Collection, Rockville, MD, USA.

**Table 2.2** Cell lines used in this work

### 2.2.2 Subculturing of cell lines

Adherent cells grown as monolayers in tissue culture flasks were generally subcultured before confluency was reached. The cells were enzymatically detached from the flask using a 0.25% (w/v) trypsin (Gibco 25090-028)/0.01% (w/v) EDTA (BDH 10093) solution in PBS (Oxoid BR14A). The waste medium was decanted from the flasks and the cells rinsed with 2ml of prewarmed trypsin/EDTA solution. The cells were then incubated at $37^0$C with a further 2ml of trypsin/EDTA (5 ml for a $75cm^2$ flask) for 5-15 minutes (depending on the cell line) until a single cell suspension was obtained, as determined microscopically. An equal volume of complete medium was added to inhibit the action of the trypsin/EDTA and the resulting cell suspension was pelleted by centrifugation at 1000 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in 5ml of fresh complete medium. An aliquot of cell suspension was removed for cell counting and the cells were reseeded at the desired concentration in tissue culture flasks. Complete medium was added to the flasks to give approximately 8ml of medium in a $25cm^2$ flask and 15ml in a $75cm^2$ flask.

### 2.2.3 Cell counting

An aliquot of the single cell suspension was removed for counting and mixed in a ratio of 4:1 with trypan blue dye (Gibco 043-05250H). The suspension was left for approximately 1 minute to ensure equal mixing of the trypan blue with the cells before a small quantity was applied to a haemocytometer. The suspension moved into the 0.01mm deep depression of the haemocytometer by capillary action. Cells in the 16 squares of the four outer corner grids were counted (both stained and unstained cells) and averaged. The average number of cells was multiplied by $1.2 \times 10^4$ to give the number of cells per ml (1.2 being the dilution factor of the trypan blue). Non viable cells stained blue with the trypan blue dye while the viable cells remained unstained.

### 2.2.4 Large scale cell culture

In order to gain sufficient stocks of the cell lines used throughout this work the cells were grown in either glass or plastic disposable roller bottles (Falcon 3027) to acquire large numbers of cells. The roller bottles were first rinsed with 15ml of complete medium, after

which approximately $5x10^6$ cells were added (number dependent on cell line used). The roller bottle was then incubated at a rotor speed of 0.25 rpm for approximately 24 hours, after which time the rotor speed was increased to 0.5 rpm. The cells were fed every 3-4 days until 80% confluency was reached. The cells were then frozen for stocks.

### 2.2.5 Cell freezing

All the cell lines used throughout this work were stored long term at $-196^0$ C in a liquid nitrogen refrigerator. Cells in subconfluent exponential phase were most suitable for long term storage. The cells for freezing were trypsinised and a single cell suspension was obtained as described in section 2.2.2. The pellet was resuspended in 100% FCS. An equal volume of a 10% (v/v) DMSO (dimethylsulphoxide, Sigma D-5879) in FCS was added dropwise with continuous mixing over a number of minutes. After the DMSO was added the suspension was transferred to sterile cryovials (Greiner 122 278). The vials were labelled with the name of the cell line, the passage number, the date and finally the operators initials before being placed in the vapour phase of the liquid nitrogen container for 3 hours. After this time the cells were transferred to the liquid phase and stored until required. A protective face visor and protective gloves were worn at all times during handling of the liquid nitrogen.

### 2.2.6 Cell thawing

The vial of cells to be thawed was removed from the liquid phase and placed in the vapour phase for 2-3 minutes to allow any liquid nitrogen present in the vial to evaporate. The cells were then thawed rapidly in a water bath at $37^0$C and transferred to a sterile universal container containing 9ml of complete medium. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The medium was decanted and the pellet resuspended in 5 ml of complete medium. The cell viability was determined and the cell suspension was then transferred to tissue culture flasks and incubated for 24 hours. After this time the cells were refed with fresh complete medium.

## 2.3 Mycoplasma detection

The cell lines used during this project were routinely screened for the presence of mycoplasma infection. Unlike other microbial contamination, mycoplasma are not visible microscopically and infected cells may continue to grow and divide. However, infection can result in decreased proliferation rates, changes in the morphology and granulation of the cells. To detect the presence of mycoplasma, the cells were examined by a direct culture method and an indirect staining method (Hoechst 33258). The cells to be screened were grown for 3 days in drug free and antibiotic free medium and after this time the medium was collected and used for the screening process.

### 2.3.1 Hoechst DNA staining method

In this method, since cell integrity is well maintained during the fixation procedure, an indicator cell line NRK (normal rat kidney fibroblast cell line) was used. The NRK cells ($2x10^3$) were grown on sterile coverslips overnight in 1ml of DMEM supplemented with 5% FCS and 1% L-glutamine. The cells were incubated at $37^0C$ in 5% $CO_2$. 1ml of the culture medium was added to duplicate coverslips which were then incubated for 4-5 days. After this time the cells were approximately 50% confluent. The medium was removed and the cells were rinsed twice in PBS followed by one rinse in a 1:1 cold solution of PBS:Carnoys reagent (1:3 Glacial acetic acid (BDH 27013) and methanol (BDH 10158)). The cells were then fixed in 2ml of Carnoys reagent for 10 minutes. The samples were allowed to air dry before 2 ml of Hoechst (Sigma B-2883) working stock (50ng/ml in PBS) was added for 10 minutes. The Hoechst is light sensitive so all staining was carried out in darkness. The coverslips were then rinsed 3 times with ultrapure water and mounted on a slide in a drop of glycerol mounting medium (50% glycerol (BDH 10118) in 0.1M citric acid ; 0.2M disodium sulphate at pH 5.5). The slides were then examined using a mercury fluorescence microscope. The Hoechst stain binds specifically to DNA, staining the nucleus of the cells and any extranuclear DNA that may be present. If mycoplasma contamination was present, small fluorescent bodies were visible in the cytoplasma of the cells. Mycoplasma contaminated medium and medium not exposed to cells were used as positive and negative controls respectively.

### 2.3.2 Mycoplasma culture method

100 mls of mycoplasma agar (Oxoid CM401) and mycoplasma broth (Oxoid CM403) were prepared and 90 mls of each were supplemented with 20 ml FCS, 1 ml fungizone (Gibco 042-05290), 250$\mu$l penicillin (Sigma Pen-3), 1.3 ml of a 0.2% stock of DNA (BDH 42026) and 10 mls of a 25% (w/v) stock of yeast extract (which was boiled for 5 minutes before being filtered and aliquoted). 0.5 ml of the growth medium from the cell lines was incubated in 3 ml of mycoplasma broth for 48 hours at $37^0$C and then a streak from the broth was inoculated onto 10 mls of the solidified agar. The agar plates were incubated in a $CO_2$ gaspak system for 3-4 weeks during which time the plates were examined for colony formation. If mycoplasma contamination was present, typical "fried egg" type colonies, consisting of a dense centre and a less dense surround would have been visible.

## 2.4 Safe handling of cytotoxic drugs

Cytotoxic drugs were treated with extreme caution in the laboratory at all times due to the potential risks in handling these drugs. Generally two pairs of latex disposable gloves (Medical Supplies Company Ltd) and a face mask were worn when dealing with the concentrated stocks of the drugs and all work was carried out in cytotoxic cabinets (Gelman cytoguard or Holten Laminar Air cytotoxic cabinet). All drugs were stored in a safety cabinet at room temperature or in designated areas at $4^0$C or at $-20^0$C. Table 2.4 outlines the storage and means of disposal of the cytotoxic drugs used in this work. All liquid drug waste was disposed of in the same way as the drug.

| Cytotoxic agent | Supplier | Storage | Disposal |
|---|---|---|---|
| Adriamycin (doxorubicin) 2mg/ml | Farmitalia | $4^0$C in darkness | Inactivated with 1% hyperchloride solution and disposed with excess water |
| Vincristine 1mg/ml | David Bull Laboratories | $4^0$C in darkness | Inactivated by autoclaving and disposed with excess water |
| VP16 (etoposide) 20mg/ml | Bristol-Myers Pharmaceuticals | Room temperature | Incineration |
| 5-Fluorouracil 25mg/ml | Farmitalia | Room temperature in darkness | Inactivated with 5M NaOH and disposed with excess water |
| Cisplatin 1mg/ml | Lederle Laboratories | $4^0$C in darkness | Incineration |
| Carboplatin 1mg/ml | Bristol-Myers Pharmaceuticals | Room temperature in darkness | Incineration |

**Table 2.4** Storage and disposal of cytotoxic drugs

## 2.5 Adaptation of MDR variants

A toxicity assay was initially carried out (section 2.6.1) on the parental cell lines in order to determine the concentration of the selective agent that resulted in 90% cell kill. The selective agent was then added to cells in the exponential phase of growth at the relavant concentration. The cells were grown at this concentration of drug until they appeared healthly and had reached approximately 70% confluency, after which time the concentration of drug to which the cells were exposed was increased. This process was continued over a number of months until the desired concentration was reached. The selection process for the DLKP cell line with the cytotoxic agent carboplatin was commenced at 1$\mu$g/ml carboplatin and increased to a final concentration of 25$\mu$g/ml carboplatin (DLKPC 25).

## 2.6 Miniaturized *in vitro* toxicity assays

The toxicity profile of the cell lines used throughout this work was determined using miniaturized toxicity assays. Two days prior to the assay, the cells were subcultured and reseeded in a 75cm$^2$ flask at a concentration of 5x10$^5$ cells per ml. The cells were allowed to attach and the following day the cells were refed in complete medium and incubated overnight at 37$^0$C. The cells were trypsinised and a single cell suspension was prepared as described in section 2.2.2. The cell count and cell viability was then determined using trypan blue.

### 2.6.1 Toxicity assay - 96 well plates

The cells were plated in 96 well plates (Costar 3596) at a concentration of 1x10$^3$ cells/well in 100$\mu$l of complete medium. The first lane of each plate was used as a cell blank to which 100$\mu$l of complete medium was added. The plates were gently rotated to ensure equal distribution of the cells within the wells. The cells were then allowed to attach overnight at 37$^0$C in 5% $CO_2$. The following day a series of 2X drug dilutions were prepared ranging from a non toxic concentration to a concentration that resulted in 100% cell kill. 100$\mu$l of the drug dilution was added to each well in replicates of eight and 100$\mu$l of complete medium was added to lanes one and two which served as a blank and a 100%

cell survival control respectively. The plates were then incubated for 5-6 days until cells in the control wells had reached approximately 80% confluency. The medium was decanted from the wells and the cells were rinsed twice in PBS. 100$\mu$l of the assay substrate (10mM p-nitrophenyl phosphate (Sigma 104) in 0.1M sodium acetate (Sigma S-2889), 0.1% Triton X-100 (Sigma X100)) was added to each well (Martin and Clynes, 1991). The assay substrate was prepared just before use and the pH was adjusted to pH 5.5. The plates were incubated for two hours at 37$^0$C in 5% $CO_2$ and then read in a dual beam ELISA plate reader at a wavelength of 405nm (reference wavelength 620nm). The first lane of each plate was used to blank the ELISA plate reader. If the colour obtained was slight it was enhanced by the addition of 50$\mu$l of 1N NaOH which also stopped the enzymatic reaction.

### 2.6.2 Toxicity assays - 24 well plates

Cells were plated in 24 well plates (Costar 3424) at a concentration of 2x10$^3$ cells per well in 500$\mu$l of complete medium. The first lane of each plate was used as a blank to which 500$\mu$l of medium was added. The cells were allowed to attach overnight at 37$^0$C in 5% $CO_2$. A series of 2X drug dilutions were prepared and 500$\mu$l of each dilution was added to four replica wells. The plates were incubated for a further 5-6 days, until the control cells had reached approximately 80% confluency. The medium was then decanted from the plates and the cells rinsed twice in PBS. The cells were then fixed in 10% formalin (Riedel de Haen D-3016) for 10 minutes, after which time the formalin was removed and the plates air dried. The cells were stained with 0.25% crystal violet (Sigma C-3886) for 10 minutes and then washed thoroughly to remove the excess stain. The plates were allowed to air dry and the stain eluted by the addition of 33% glacial acetic acid (BDH 27013). 200$\mu$l of the resulting solution was transferred to 96 well plates and read on a dual beam plate reader at a wavelength of 570nm (reference wavelength 620).

### 2.6.3 Circumvention assay - 96 well plates

The cells were plated in 96 well plates at a concentration of 1x10$^3$ cells per well in 100$\mu$l of medium. 100$\mu$l of medium was added to the first lane of each plate which served as a blank. The cells were incubated for 24 hours at 37$^0$C in 5% $CO_2$, to allow the cells to attach. A series of 4X drug dilutions were prepared and 50$\mu$l of the drug was added to the

wells in replicas of eight. A 4X stock of the circumvention agent was also prepared (the concentration that resulted in 95% cell survival, as determined from its toxicity profile, was used in the assays) and 50$\mu$l was added to each of the appropriate wells. Control wells containing only the drug or circumvention agent were also set up. The plates were then incubated at 37$^0$C in 5% $CO_2$ for 5-6 days until the control wells had reached approximately 80% confluency. The medium was decanted and the cells rinsed twice with PBS before the toxicity was determined using the acid phosphatase method, as described in section 2.6.1.

### 2.6.4 Transport circumvention assays - 96 well plates

The effect of the circumvention agents cyclosporin A (Sandoz), verapamil (Sigma V-4629) and monensin (Sigma M-5273) on the toxicity of adriamycin was studied under conditions similar to those used in the adriamycin accumulation assays *ie* similar concentrations and time length exposures (section 2.10). The cells were plated in 96 well plates at a concentration of 1x10$^3$ cells per well in 100$\mu$l of medium. 100$\mu$l of medium was added to the wells in the first lane which served as a blank. The cells were allowed to attach for 24 hours at 37$^0$C in 5% $CO_2$. The following day the appropriate drug dilutions were prepared and 100$\mu$l of medium was added to the first three lanes, 100$\mu$l of a 2X stock of adriamycin (10$\mu$M) was added to lanes 4,5 and 6 and 100$\mu$l of a 2X stock of the circumvention agent was added to lanes 7,8 and 9. To the remaining three lanes 50$\mu$l of a 4X stock of adriamycin and 50$\mu$l of a 4X stock of the circumvention agent were added. The plates were then incubated at 37$^0$C in 5% $CO_2$. After 2 hour and 4 hour incubation time periods, the medium was decanted and the cells rinsed twice with fresh medium. 200$\mu$l of medium was then added to each well and the plates incubated at 37$^0$C in 5% $CO_2$ for 5-6 days until the cells in the control wells had reached approximately 80% confluency. The medium was then decanted from the wells and the cells rinsed twice in PBS. The toxicity of adriamycin and the circumvention agent alone or in combination was determined by the acid phosphatase method, as described in section 2.6.1.

## 2.7 Protein analysis by Western blotting

Protein analysis was carried out by Western blotting using whole cell extracts, purified cell membrane extracts and purified nuclear extracts. The extracts were prepared as follows:

### 2.7.1 Whole cell extract preparation

The cells used in the preparation of whole cell extracts were grown in $75cm^2$ flasks until approximately $1x10^7$ cells were obtained. The cells were then trypsinised and pelleted by centrifugation at 1000 rpm for 5 minutes. The cell pellet was washed three times in PBS by centrifugation. Once the cell pellet was obtained the procedure was carried out on ice. Cell pellets were resuspended in 5ml of a lysis buffer containing 10mM KCl (BDH 10198), 1.5mM $MgCl_2$ (Sigma M-1250), 2mM PMSF (Sigma P-7626) and 10mM Tris-HCl (Sigma T-8404) pH 7.4. PMSF was added to the lysis buffer just before use and the buffer was stored on ice. The cells were then sonicated (Labsonic U) until lysis occurred, as determined microscopically. The cellular extracts were used immediately.

### 2.7.2 Cell membrane preparation

The cells used for membrane preparations were grown in $75cm^2$ flasks until approximately $2x10^7$ cells were obtained. Cells were trypsinised in the exponential phase of growth and pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was washed three times by centrifugation at 2000 rpm for 5 minutes and then placed on ice. The cells were resuspended in 5ml of lysis buffer (10mM KCl, 1.5mM $MgCl_2$, 2mM PMSF and 10mM Tris-HCl pH 7.4). The cells was then sonicated until cell lysis was observed microscopically. The lysed cells were centrifuged at 7,800 rpm in an ultracentrifuge at $4^0C$ for 10 minutes. The supernatant was collected and centrifuged at 38,000 rpm at $4^0C$ for 1 hour. The resulting pellet was resuspended in 0.5 - 1ml of lysis buffer and a $50\mu l$ aliquot of the suspension was removed for total protein analysis. The remaining supernatant was aliquoted in $100\mu l$ lots in eppendorf tubes and lyophilized overnight. The lyophilized samples were stored at $-20^0C$ until required.

### 2.7.3 Nuclear extract preparation

The cells used for nuclear extracts were grown in $75cm^2$ flasks until approximately $1x10^7$ cells were obtained. Cells were then trypsinised and pelleted by centrifugation. The cell pellet was washed three times after which the cells were permeabilized by incubation for 10 minutes in 3 ml of a hypotonic buffer (5mM $KH_2PO_4$, 2mM $MgCl_2$, 4mM DTT, 0.1mM $Na_2EDTA$ and 1mM PMSF, pH 7.0). The cells were then pelleted at 1500 rpm for 5 minutes. The resulting pellet was resuspended in 300$\mu$l of a 0.1% SDS (Riedel-de Haen 62862) solution. An aliquot was removed for protein determination and the remaining supernatant was used immediately.

### 2.7.4 Quantification of protein

The protein levels in the cellular extracts were determined using the BCA protein assay (Pierce). The Bicinchoninic acid protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration. It is based on the reaction of the protein with $Cu^{2+}$ in an alkaline medium containing bicinchoninic acid which is a detection reagent for $Cu^{+}$ (Smith *et al.*, 1985). The BCA protein assay working reagent was prepared by mixing 50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2N NaOH) with one part of reagent B (4% copper sulfate solution). A set of protein standards of known concentrations were prepared by diluting the BSA standard (2mg/ml stock). 0.1ml of each standard or unknown protein sample was pipetted into a test tube and 2 ml of the working reagent was added to each tube. 0.1 ml of diluent was used as a blank. The test tubes were then incubated at $60^0C$ for 30 minutes and the absorbance of each sample measured at 562nm. A standard curve was plotted of the known protein standards and the protein concentration of the unknown samples was determined by extrapolation from the standard curve.

### 2.7.5 Separation of proteins by SDS - Polyacrylamide gel electrophoresis (PAGE)

7.5% and 10% polyacrylamide gels were used in this work to separate proteins present in the cellular extracts. The gels were prepared as shown in table 2.7.5.

| Gel component | Resolving Gel | | Stacking gel |
|---|---|---|---|
| | 7.5% | 10% | 5% |
| 30% Acrylamide Stock | 3.8 ml | 5.0 ml | 0.8 ml |
| Ultrapure $H_2O$ | 8.0 ml | 6.8 ml | 3.6 ml |
| 1.875 M Tris-HCl pH 8.8 | 3.0 ml | 3.0 ml | - |
| 1.25 M Tris-HCl pH 6.8 | - | - | 0.5 ml |
| 10% SDS (Sigma L-4509) | 150 $\mu$l | 150 $\mu$l | 50 $\mu$l |
| 10% Ammonium Persulphate (Sigma A-1433) | 60 $\mu$l | 50 $\mu$l | 17 $\mu$l |
| TEMED (Sigma T-8133) | 9 $\mu$l | 7.7 $\mu$l | 5 $\mu$l |

**Table 2.7.5** Preparation of resolving and stacking gels for electrophoresis

The 30% acrylamide stock was prepared by dissolving 29.1g of acrylamide (Sigma A-8887) and 0.9g of NN-Methylene bis-acrylamide (Sigma M-7279) in 100 mls of ultrapure water. The resolving gels were immediately cast into two gel cassettes which comprised of a glass plate and an aluminium plate separated by two 0.75cm$^2$ plastic spacers. The gels were allowed to polymerise and then the stacking gel was carefully poured on top of the resolving gel. Combs of suitable thickness were then inserted into the stacking gel. When the stacking gel had set and the wells had been formed, the gels were transferred to an LKB mini-gel system to which electrode buffer was added (1.9M glycine (Sigma G-6761), 0.25M Tris (Sigma T-8404) and 0.1% SDS - pH 8.3). The samples to be separated were adjusted to equal protein concentration by the addition of an appropriate volume of loading buffer (1.25M Tris-HCl, 1% SDS, 5% glycerol (BDH 10118), 5% 2-mercaptoethanol (Sigma M-6250) and 0.1% bromophenol blue (BDH 44305)). 10$\mu$g/10$\mu$l of each sample was loaded onto the gel. The gels were run for approximately 50 minutes at a voltage of 250V and at a current of 45mA.

### 2.7.6 Western blotting

Western blotting was preformed by the method of Towbin *et al.* (1979). Following SDS-polyacrylamide gel electrophoresis (PAGE), the gels were equilibrated for 15 minutes in transfer buffer (25mM Tris, 192mM glycine (Sigma G-8898) - pH 8.5 without adjustment). The gels were then aligned onto nitrocellulose sheets (Amersham, Hybond-C) which had been equilibrated in the same transfer buffer. A number of sheets of Whatman 3mm filter paper were soaked in transfer buffer and then placed underneath the nitrocellulose paper and also on top of the gel. The transfer of protein from the acrylamide gel to the nitrocellulose filter was carried out using a Bio-Rad semi-dry blotting system for approximately 20 minutes at 15 volts and a maximum of 345 mA. Following protein transfer, the nitrocellulose sheets were placed in blocking buffer which consisted of 5% non-fat dried milk (Marvel skimmed milk) in TBS (20mM Tris, 500mM NaCl (BDH 1.060400.1000), pH 7.5) for 90 - 120 minutes. The nitrocellulose blots were then rinsed with Tris buffered saline (TBS, 0.05M Tris/HCl, 0.15M NaCl) prior to the addition of the primary antibody.

#### 2.7.6.1 P-glycoprotein

The primary antibody used to detect the presence of P-glycoprotein was the anti-P-glycoprotein monoclonal antibody, C219 (Centicor Diagnostics). 0.25$\mu$g/ml of the C219 in TBS containing 0.1% Tween-20 (Sigma P-1379) was added to the nitrocellulose and incubated overnight at 4$^0$C. The blots were then washed three times with TBS containing 0.5% Tween-20 and then exposed to the secondary antibody, at a 1:10,000 dilution of the alkaline phosphatase conjugated anti-mouse IgG and IgM antibody (Pierce), for 90 minutes. The blots were washed three times and developed in an alkaline phosphatase substrate.

#### 2.7.6.2 Topoisomerase II

The antibody used to detect the presence of topoisomerase II was a mixture of topoisomerase II $\alpha$ and ß monoclonal antibodies (gifts from Dr. G. Astaldi-Ricotti). A 1:700 dilution of the serum in TBS containing 0.1% Tween-20 was prepared and incubated with the nitrocellulose blots overnight at 4$^0$C. Following this incubation period, the blots were washed three times with TBS containing 0.5% Tween-20 and incubated with the secondary antibody (1:500 dilution of anti-mouse immunoglobulin (DAKO) in TBS

containing 0.1% Tween-20) for 90 minutes at room temperature. The blots were then washed three times before development in the alkaline phosphatase substrate.

#### 2.7.6.3 D8-8 antibody

When the D8-8 antibody, raised against the DLKPA10 cell line, was characterized using western blotting, a 1:100 dilution of the ascitic fluid in TBS containing 0.1% Tween-20 was added to the nitrocellulose blots and incubated overnight at $4^0$C. The blots were then washed three times and incubated with 1:500 dilution of the anti-mouse immunoglobulin secondary antibody (DAKO) for 90 minutes at room temperature. The blots were washed three times with TBS containing 0.5% Tween and then developed using the alkaline phosphatase substrate.

### 2.7.7 Development of Western blots

All nitrocellulose blots were developed using an alkaline phosphatase substrate. A 0.1% stock (w/v) of nitroblue tetrazolium (Sigma H-5514) in 10mM Tris-HCl, pH 8.9 and a 5mg/ml stock of 5-bromo-4-chloro-3-indoxyl phosphate (Sigma B-0274) in N,N-dimethylformamide (Sigma D-8654) were prepared. 5ml of the nitroblue tetrazolium stock and 2 ml of the 5-bromo-4-chloro-3-indoxyl phosphate stock were added to 44 ml of a 0.5M Tris-HCl buffer, pH 8.9 containing 0.2 ml of 1M $MgCl_2$. The blots were developed by incubating the nitrocellulose with the developing substrate until bands of suitable colour development were visible. The developing reaction was stopped by decanting the developing substrate and rinsing the blots thoroughly in water. The blots were then carefully dried and stored protected from the light to prevent the bands from fading.

## 2.8 Determination of Glutathione-S-transferase activity

Glutathione-S-transferase activity was determined using cytosolic extracts of the DLKP cells and the DLKPC resistant variants. The activity was determined spectrophotometrically and the assay was based on the conjugation of glutathione to 2,4-dinitrochlorobenezene forming 2,4-dinitrophenyl-glutathione which absorbs light at 340nm and has an extinction co-efficient of 9.6 mM/cm (Gibson *et al.*, 1987).

### 2.8.1 Cytosolic extract preparation

Approximately $2x10^7$ cells at the exponential phase of growth were trypsinised and pelleted by centrifugation. The pellet was washed three times with PBS and then resuspended in 2 ml of lysis buffer (10mM KCl, 1.5mM $MgCl_2$, 2mM PMSF and 10mM Tris-HCl, pH 7.4). The cells were sonicated until cell lysis was observed microscopically. The lysed cells were centrifuged at 7,800 rpm for 10 minutes at $4^0$C. The supernatant was collected and centrifuged at 38,000 rpm for 1 hour at $4^0$C. An aliquot of the supernatant was retained for protein determination by the BCA assay and the remaining supernatant was used directly in the glutathione-S-transferase activity assay.

### 2.8.2 Assay for glutathione-S-transferase

A 30 mM stock of glutathione (Sigma G-4251), a 30 mM stock of dinitrochlorobenezine (BDH 10076) in ethanol and a 100 mM potassium phosphate buffer ($KH_2PO_4$ and $HK_2PO_4$) pH 6.5 were prepared. 2 cuvettes containing 0.1 ml of the glutathione stock, 0.1 ml of the dinitrochlorobenzene stock and 2.2 ml of potassium phosphate buffer were placed in the constant temperature chamber of a dual beam spectrophotometer (Shimadzu UV 160A). 0.6 ml of the cytosolic extract was added to the sample cuvette and 0.6 ml of water was added to the reference cuvette. The reaction was followed at 340nm over a time period of three minutes. The change in absorbance per minute was calculated and this was related to the conjugation of glutathione by the extinction co-efficient. The rate of glutathione conjugation was calculated from the following formula:

glutathione conjugated $min^{-1}$ mg $protein^{-1}$ = OD $min^{-1}$ / extinction coefficient x [protein]

## 2.9 Immunological studies

Throughout the course of this work immunological studies were carried out on both live and fixed cells. Immunofluorescence was performed on live cells to detect the presence of cell surface antigens and immunocytochemistry was performed on fixed cells to detect the presence of both intracellular and membrane associated antigens.

### 2.9.1 Live cell immunofluorescence

The cells used for immunofluorescence were grown in $75cm^2$ flasks until they had reached approximately 80% confluency. The cells were then trypsinised and pelleted by centrifugation. The cell pellet was washed three times and resuspended in PBS. An aliquot of the cell suspension was removed and the cells counted using a haemocytometer. A single cell suspension of $1x10^6$ cells per ml was prepared and 100$\mu$l of this suspension was added to two eppendorf tubes. 100$\mu$l of the test antibody was added to one eppendorf (1:100 dilution of the D8-8 ascitic fluid in PBS) and 100$\mu$l of PBS was added to the second eppendorf tube as a negative control. As a positive control for the study, 100$\mu$l of a cell suspension of the SCC-9 cell line (human squamous carcinoma of the tongue) was incubated with 100$\mu$l of the monoclonal antibody, EP-16. The cells were incubated with the primary antibody for 30 minutes at $4^0$C and then washed three times with PBS by centrifugation. The cell pellet obtained was resuspended in 100$\mu$l of PBS. 100$\mu$l of the secondary antibody (FITC-labelled anti-mouse IgM (Sigma F-9259) diluted to 1:50 in PBS for the EP-16 antibody and FITC-labelled antimouse IgG (Boehringer Mannheim) for the D8-8 antibody) was added and the cells were incubated for a further 30 minutes at $4^0$C. The FITC-labelled antibodies were light sensitive, consequently the reactions were carried out in darkness. After the 30 minute incubation period the cells were washed three times with PBS. The supernatant was aspirated and the cell pellet was resuspended in one drop of mounting medium (Vectra shield H1000). Approximately 20$\mu$l of the cell suspension was then mounted on a glass slide and the cells were viewed for fluorescence under ultra violet illumination using a Nikon microscope equipped with a mercury lamp. If the antibody recognised a cell surface antigen a ring of fluorescence was visible on the surface of the cells.

### 2.9.2 Immunocytochemistry of fixed cells

#### 2.9.2.1 Preparation of cytospins

The glass micro slides (Chance propper LTD) used for the cytospins were washed thoroughly in hot water containing 0.5% Tween-20 and then rinsed in running water for approximately 15 minutes. Following this washing step, the slides were soaked in alcohol for 15 minutes and then dried thoroughly. The slides were then coated with poly-l-lysine (Sigma P-1274) by adding approximately 10$\mu$l of poly-l-lysine (1mg/ml in ultrapure water) to one end and spreading it evenly over the slide to give an equal coating of the poly-l-lysine. The slides were air dried overnight and stored at -20$^0$C until required.

The cells required for the cytospins were grown in 75cm$^2$ flask until they had reached approximately 80% confluency. The trypsinisation procedure was carried out and a single cell suspension of 1x10$^6$ cells per ml was prepared. Two 100$\mu$l aliquots of the cell suspension was cytofuged (Heraeus Labofuge 400) onto the glass slide at 400 rpm for 4 minutes. A number of cytospins were prepared and allowed to air dry overnight. The cytospins were stored at -20$^0$C until required.

#### 2.9.2.2 Immunocytochemistry

The cytospins were removed from -20$^0$C and allowed to stand at room temperature for approximately 20 minutes prior to starting the assay. Fixation of the cells was carried out by incubating the cells in acetone (BDH 10003) for 10 minutes at -20$^0$C. The slides were then allowed to air dry for approximately 15 minutes. The cells were incubated in 0.6% hydrogen peroxide (BDH 10128) in methanol (BDH 10158) for 5 minutes to quench any endogenous peroxidase activity. The slides were rinsed with water and then washed for 5 minutes in TBS (Tris buffered saline; 0.05M Tris/HCl, 0.15M NaCl, pH 7.6) containing 0.1% Tween-20. Non-specific background in the cells was blocked by the addition of a 1:5 dilution of normal rabbit serum (DAKO X0902) for 20 minutes. The primary antibody was added and the cells were incubated either at room temperature for 1 - 2 hours or overnight at 4$^0$C (depending on the antibody used) after which time the slides were washed

three times with TBS/0.1% Tween-20. The cells were incubated with 50$\mu$l of a biotinylated secondary antibody for 1 hour at room temperature and then washed thoroughly three times with TBS/0.1% Tween-20. 50$\mu$l of a freshly prepared stock of Streptavidin-binding complex (DAKO K377) was added to each of the sections and incubated for 30 minutes at room temperature. The slides were washed thoroughly and the cells incubated with 50$\mu$l of the peroxidase substrate, 3,3-diaminobenzidine tetrahydrochloride (DAB, DAKO S3000). When suitable colour had developed (red-brown reacting product) the cells were rinsed in water and counterstained with hematoxylin (Bayers). A series of dehydration steps in alcohol (70%, 90% and 100% methanol) were carried out before the cells were mounted in DPX (BDH 36029) and viewed under a Nikon microscope when dry.

#### 2.9.2.3 Metallothionein

The presence of metallothionein in a number of cell lines was investigated using a monoclonal mouse anti-metallothionein antibody. The cells were incubated at $4^0$C for 16 hours with a 1:20 dilution of the metallothionein antibody (DAKO-MT M0639). After this incubation period, the slides were washed thoroughly with TBS/0.1% Tween-20 and the secondary antibody added for 1 hour at room temperature. A 1:300 dilution of the biotinylated rabbit anti-mouse IgG antibody (DAKO E354) was used as the secondary antibody for the detection of metallothioneins.

#### 2.9.2.4 Lung resistance protein (LRP)

The presence of LRP in a number of DLKP variants was detected using a mouse monoclonal antibody to LRP (LRP-56) which was a generous gift from Prof. Rik Scheper's laboratory (Dept. of Pathology and Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam). The cells were incubated with a 1:500 dilution of the primary antibody, LRP-56, for 2 hours at room temperature and then washed three times over a period of 15 minutes. The cells were then incubated with the secondary antibody (1:300 dilution of the biotinylated rabbit anti-mouse IgG antibody) for 1 hour at room temperature.

#### 2.9.2.5 Multidrug resistance related protein (MRP)

The primary antibody used for the detection of MRP was a rat monoclonal antibody MRPr1 which was also a generous gift from Prof. Rik Scheper's laboratory. The cells were incubated with a 1:40 dilution of the antibody for 2 hours at room temperature and then washed three times in TBS/0.1% Tween-20. The binding of the MRPr1 antibody was detected using a 1:100 dilution of a biotinylated rabbit anti rat IgG antibody (DAKO E0468) with which the cells were incubated for 1 hour at room temperature.

#### 2.9.2.6 D8-8 antibody

For immunocytochemical studies, the cells were incubated with a 1:100 dilution of the primary D8-8 antibody at $4^0$C overnight. The cells were then washed thoroughly and incubated with the secondary antibody, 1:300 dilution of the biotinylated rabbit anti-mouse IgG antibody, at room temperature for 1 hour.

## 2.10 Drug transport studies

### 2.10.1 Adriamycin accumulation studies

The accumulation of adriamycin in a number of cell lines was measured by a modification of the method of Ganapathi and Grabowski (1983). The cells used in the studies were grown in $75cm^2$ flasks until approximately 80% confluency was reached. The cells were then trypsinised and a single cell suspension prepared. 1ml of the cell suspension was added to each well of 6 well cluster plates (Costar 3516) at a concentration of $1x10^5$ - $5x10^5$ cells per ml (concentration dependent on the cell line). A further 3 ml of complete medium was added to each well and the cells incubated at $37^0C$ in 5% $CO_2$ for approximately 48 hours. After this time the medium was decanted and 4 ml of complete medium, containing 10$\mu$M adriamycin, was added to each of the wells except the control wells, to which 4 ml of complete medium was added. These plates were incubated at $37^0C$ in 5% $CO_2$. After specified time intervals the drug containing medium was decanted and the cells were rinsed twice in ice-cold PBS. 2 ml of ice-cold ultrapure water was added to the wells and left for approximately 5 minutes to facilitate lysis of the cells. The adriamycin was then directly extracted from the cells by the addition of 2 ml of 0.6N HCl-Methanol solution for approximately 5 minutes. The resulting solutions were transferred to universal containers and centrifuged at 4000 rpm for 10 minutes at $4^0C$. The supernatants were collected and the fluorescence of each solution determined using a Perkin Elmer LC50 luminescence spectrometer with an excitation wavelength of 470nm and an emission wavelength of 585nm. The slit widths for excitation and emission were 10nm and 15nm respectively. The concentration of adriamycin present in each sample was quantitated from a linear standard curve prepared from the fluorescence of known adriamycin concentrations.

### 2.10.2 Adriamycin efflux studies

The efflux of adriamycin was studied following preloading of the cells with the drug. The cells were plated in 6 well plates at a concentration of $1x10^5$ - $5x10^5$ cells per well (concentration dependent on cell line) and fed with 3 ml of complete medium. Following an incubation period of approximately 48 hours at $37^0C$, the medium was decanted and 4 ml of glucose free medium (DMEM: Gibco 11963-022) containing 10$\mu$M adriamycin and either 10$\mu$M antimycin A or 10mM sodium azide was added to each of the wells with the

the exception of the control wells, to which complete medium was added. After 3 hours the drug containing medium was removed and the cells were rinsed twice with medium. 4 ml of medium containing glucose was added to each of the wells and the cells were further incubated at 37$^0$C in 5% $CO_2$. After specified time intervals the medium was decanted and the cellular adriamycin was extracted. The resulting solution was centrifuged and the supernatants collected. The fluorescence of the supernatants was determined and the concentration of adriamycin determined from the standard linear curve.

### 2.10.3 Vincristine accumulation studies

The cellular uptake of vincristine was determined by measuring the quantity of radiolabelled vincristine (Amersham, TRK.478) accumulated in the cells. Cells at the exponential growth phase were trypsinized and a single cell suspension prepared. These cells were plated in 6 well plates at a concentration of $1x10^5$ - $5x10^5$ cells per well (concentration dependent on cell line) and incubated at 37$^0$C for approximately 48 hours. Following this incubation period, the medium was decanted and medium containing $^3$H-vincristine was added to the cells. The plates were incubated at 37$^0$C in 5% $CO_2$. After specified time intervals the medium was quickly aspirated, and the cells washed twice in ice-cold PBS. The plates were blotted dry and the cells solubilized by overnight incubation in 0.2N NaOH at room temperature. The cell lysates were neutralized by the addition of 0.2N HCl and the radioactivity determined by liquid scintillation counting (Beckman LS 6500). The concentration of vincristine accumulated was quantitated from a standard curve prepared from known concentrations of vincristine.

### 2.10.4 Vincristine efflux studies

The efflux of vincristine was investigated following preloading of the cells with vincristine. The cells were plated in 6 well plates and incubated at 37$^0$C in 5% $CO_2$. Approximately 48 hours after plating, the medium was removed and the cells incubated in glucose free medium containing radiolabelled vincristine and 10$\mu$M antimycin A for two hours. At the end of the uptake period, the cells were rinsed thouroghly and incubated in complete medium at 37$^0$C in 5% $CO_2$. After specified time intervals the medium was decanted and the vincristine extracted as described in section 2.10.3

## 2.11 Subcellular distribution of adriamycin

The subcellular localisation of adriamycin in a number of cell lines was investigated by fluorescence microscopy and confocal laser microscopy. The cells studied were grown on glass coverslips and then viewed under ultraviolet illumination.

### 2.11.1 Fluorescence microscopy

Microscope glass coverslips (Chance propper LTD) were washed with 70% alcohol and flamed with a bunsen burner. The sterile cover slips were placed in 35mm petri dishes (Greiner 627160). The cells used in the studies were grown in $25cm^2$ flasks until approximately 80% confluent and then trypsinised at the exponential phase of growth. The cells were pelleted by centrifugation and a single cell suspension of $1x10^5$ cells per ml was prepared. 1 ml of the cell suspension was added to each of the petri dishes which were then incubated overnight at $37^0C$ in 5% $CO_2$ to allow attachment of the cells to the sterile coverslips. The following day the medium was carefully removed from the petri dishes and the cells incubated with 1 ml of medium containing 10$\mu$M adriamycin for 2 hours. After this time the drug containing medium was decanted and the cells were washed twice with ice-cold PBS. The coverslips were then inverted onto clean glass slides and the edges sealed with silicon grease to protect the cells against dehydration. The cells were viewed for fluorescence under ultraviolet illumination using a Nikon microscope equipped with a mercury lamp. The ultraviolet illumination induced an orange fluorescence at the site of adriamycin accumulation and as a result the localisation of adriamycin within the cells could be studied. The cells were viewed immediately after mounting since fading of the fluorescence occured very rapidily.

### 2.11.2 Confocal laser microscopy

Cells were grown on sterile coverslips and exposed to adriamycin (10$\mu$M) as described in section 2.11.1. After specified incubation time periods the medium was decanted and the cells rinsed with ice-cold PBS. The coverslips were inverted on to glass slides and sealed with silicon grease and viewed using an Odyssey XL digital video confocal laser scanning imaging system.

## 2.12 Effect of a number of compounds on adriamycin accumulation and distribution

A number of compounds were tested to determine if they were effective at altering the accumulation and/or subcellular distribution of adriamycin in various cell lines. Table 2.12.1 and table 2.12.2 illustrates the solubility and concentration of each of the compounds used for the study of adriamycin accumulation and adriamycin distribution respectively.

| Compound | Supplier | Solubility | Concentration |
|---|---|---|---|
| Verapamil | Sigma V-4629 | Soluble in methanol | 30 - 100 $\mu$g/ml |
| Cyclosporin A | Sandoz | Soluble in medium | 10 - 100 $\mu$g/ml |
| Sodium azide | Sigma S-8032 | Soluble in medium | 500 - 2500 $\mu$g/ml |
| 2-Deoxy-D-Glucose | Sigma D-6134 | Soluble in medium | 10 - 50 mg/ml |
| Antimycin A | Sigma A-8674 | Soluble in ethanol | 10 $\mu$M |
| Brefeldin A | Sigma B-7651 | Soluble in methanol | 10 - 50 $\mu$M |
| Bafilomycin A1 | Sigma B-8281 | Soluble in DMSO | 10 - 50 $\mu$g/ml |
| Genestein | Sigma G-6649 | Soluble in DMSO | 100 - 1000 $\mu$g/ml |
| Monensin | Sigma M-5273 | Soluble in ethanol | 5 - 100 $\mu$g/ml |
| Stearic Acid | Sigma S-4751 | Soluble in medium | 50 - 500 $\mu$M |
| Cholestryl hemisuccinate | Sigma C-6512 | Soluble in medium | 50 - 500 $\mu$M |

**Table 2.12.1** Compounds used to alter adriamycin accumulation

| Compound | Supplier | Solubility | Concentration |
|---|---|---|---|
| Verapamil | Sigma V-4629 | Soluble in methanol | 10 - 30 μg/ml |
| Cyclosporin A | Sandoz | Soluble in medium | 10 - 50 μg/ml |
| Sodium azide | Sigma S-8032 | Soluble in medium | 10 - 100 mM |
| 2-Deoxy-D-glucose | Sigma D-6134 | Soluble in medium | 10 - 25 mg/ml |
| Antimycin A | Sigma A-8674 | Soluble in ethanol | 10 μM |
| Brefeldin A | Sigma B-7651 | Soluble in methanol | 10 - 50 μM |
| Bafilomycin A1 | Sigma B-8281 | Soluble in DMSO | 10 - 50 μg/ml |
| Chloroquine | Sigma C-6628 | Soluble in medium | 10 - 100 μM |
| Methylamine | Sigma M-0505 | Soluble in ethanol | 1 - 10 mM |
| Monensin | Sigma M-5273 | Soluble in ethanol | 10 μg/ml |
| Nigericin | Sigma N-7143 | Soluble in chloroform | 5 μg/ml |
| Genestein | Sigma G-6649 | Soluble in DMSO | 300 μg/ml |

**Table 2.12.2** Compounds used to alter the subcellular distribution of adriamycin

## 2.13 Hybridoma production

### 2.13.1 Immunization procedure

The immunogen used for the production of hybridomas was a whole cell preparation of the human lung tumour cell line, DLKPA10. The cells were grown in 75cm$^2$ flasks until they had reached approximately 80% confluency. Trypsinisation procedure was carried out and the cells were pelleted by centrifugation. The cell pellet was washed three times in PBS and a single cell suspension of 1x10$^7$ cells per 0.5 ml was prepared. Intraperitoneal injection of the immunogen (0.5ml) was performed on day one, on day 14 and on day 28 on male Balb/c mice. The fusion was carried out three days after the final injection.

### 2.13.2 Fusion protocol

The murine myeloma cell line, SP2, was grown in 75cm$^2$ vented flasks until approximately 80% confluency was reached. The cells were then harvested and at least 1x10$^7$ cells were resuspended in serum free DMEM (Hybri-Max 1X DMEM; Sigma D-5660) containing L-glutamine (1%) and penicillin/streptomycin (Gibco 15140-023).

The immunized mouse was sacrificed and a splenectomy was performed. The spleen was filtered through a gauze and suspended in 10 ml of serum free medium. The debris was allowed to pellet and the supernatant was then decanted and centrifuged at 1000 rpm for 5 minutes. The spleen cells were resuspended in serum free medium and a cell count was performed. The myeloma cells and spleen cells were mixed at a ratio of 1:10 in a final volume of 10 mls and then centrifuged at 2000 rpm for 5 minutes. The resulting cell pellet was resuspended in 10 mls of serum free medium and centrifuged at 2000 rpm for a further 5 minutes. After the second centrifugation the supernatant was removed and 1 ml of prewarmed polyethylene glycol (PEG 1500, Boehringer) was slowly added to the cell pellet over 1 minute. This was followed by the addition of 10 mls of medium containing 10% foetal calf serum over a time period of 7 minutes during which time the cells were gently rotated. 1 ml was added over the first minute, a second ml was added during the second minute and a third ml was added over the third minute. The final 7 mls was slowly added over 4 minutes. The cells were then centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in approximately 25 ml of DMEM complete medium containing 5% FCS, 1%

L-glutamine, 1% pen/strep, 5% Briclone (Bioresearch Ireland) and 1% HAT ($5x10^{-3}$M hypoxanthine, $2x10^{-5}$M aminopterin and $8x10^{-4}$M thymidine; Sigma H-0262). The fused cells were incubated at room temperature for 15 minutes and plated in 8 x 48 well tissue culture plates (Costar 3548). Complete medium was added to give a final volume of 0.5 ml per well. The plates were incubated at 37$^0$C in 5% $CO_2$ for 7 - 10 days. After this incubation time, the plates were examined for hybridoma formation and any clones present were fed with complete medium. The cells were refed with HAT medium at least twice. After this time the cells were fed twice with medium containing 1% HT (Sigma H-0137; $5x10^{-3}$M hypoxanthine and $8x10^{-4}$M thymidine) in which the aminopterin was omitted.

### 2.13.3 Screening of hybridomas for antibody production

All hybridomas, resulting from the fusion, were screened against the immunogen (DLKPA10 cell line) and the parental cell line DLKP for antibody production to establish if antibodies produced were raised to an antigen that was overexpressed or downregulated in either the parental or resistant cell line. When the hybridoma cells had reached approximately 50% confluency the supernatants were removed and screened by the ELISA method. The DLKP and DLKPA10 cells were plated in 96 well plates at a concentration of $2x10^4$ cells per well and incubated overnight at 37$^0$C in 5% $CO_2$ to allow the cells to attach. The following day the medium was decanted and the plates were washed twice in TBS prior to fixation in 70% acetone for 5 minutes at room temperature. The cells were blocked with a 0.1% gelatin (Sigma G-9382) solution for approximately 90 minutes at room temperature. Following the blocking step, the cells were washed with TBS and incubated with 100 $\mu$l of the supernatants that were removed from the cells. 100 $\mu$l of diluted serum from the immunized mouse and 100 $\mu$l of irrelevant supernatant were included as a positive and negative control respectively. The cells were incubated with the primary antibody for 90 minutes at 37$^0$C after which time the primary antibody was removed and the cells were washed thoroughly three times with TBS containing 0.1% Tween-20. The cells were incubated with the secondary antibody (1:10,000 dilution of Pierce IgG/IgM antibody) for 1 hour at 37$^0$C. The plates were washed three times with TBS/0.1% Tween-20 and then 100 $\mu$l of a 1mg/ml solution of p-nitrophenyl phosphate (PNPP, Sigma N-9389) substrate was added to each well. The substrate was prepared by dissolving the PNPP in a buffer consisting of 100mM glycine, 1mM $MgCl_2$ and 1mM ZnCl (Merck 8816). The cells were incubated at 37$^0$C until a bright yellow colour had developed in positive wells. The absorbance of each well was read at 405nm on a dual beam ELISA plate reader.

## 2.14 Determination of antibody class

The class and subclass of the antibodies produced were determined using a commercial isotyping kit from Serotec (MMT RC1). The kit was used specifically for tissue culture supernatants and was based on red cell agglutination. A positive agglutination result was obtained when specific antibodies recognised and bound to the particular isotype to which it was directed. The following rat monoclonal antibodies were used to isotype the antibodies that were screened; IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. Negative control cells that were coupled to irrelevant antibodies and positive control cells coupled to a polyclonal antibody directed against mouse immunoglobulins were also included in the assay. 30 $\mu$l of the supernatant to be tested was added to 30 $\mu$l of each of the specific isotyping reagent across a microtitre plate (Greiner 650160). The solutions were mixed gently and then incubated at room temperature for 1 hour. The plate was then examined for agglutination, a carpet of agglutination indicated a positive result and a small red circle represented a negative result.

## 2.15 Production of ascitic fluid

In order to produce large quantities of antibodies the hybridomas were injected into mice (Balb/c) and grown as ascites tumours. The mice were initially given an intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetra-methylpenta-decane) to stimulate the immune system. 7 days later the mice were injected intraperitoneally with the hybridomas ($1x10^7$ cell/0.5 ml PBS) and then observed for tumour growth over 1 - 2 weeks. When tumour growth was evident the mice were sacrificed and the ascitic fluid was collected from the peritoneal cavity using a sterile needle. The ascitic fluid was centrifuged at 2000 rpm for 5 minutes and the supernatant was removed, aliquoted and stored at $-20^0$C until required. The cell pellet was resuspended in complete medium (containing FCS, L-glutamine, Briclone and pen/strep) and reseeded in tissue culture flasks. The cells were cultured and frozen in liquid nitrogen for stock.

# *3. Results*

## 3.1 Establishment of novel platinum resistant cell lines

### 3.1.1 Establishment of DLKPC 6.2, DLKPC 14 and DLKPC 25

Three new carboplatin resistant variants of the human lung squamous carcinoma cell line DLKP were established, as described in section 2.5. The parental DLKP cells readily adapted to growth in carboplatin. The cells were initially exposed to 1$\mu$g/ml carboplatin and adjusted to growth in 6.2 $\mu$g/ml after approximately four months, to 14$\mu$g/ml after a further six months and to a final concentration of 25$\mu$g/ml within another three months. The resistant cell lines selected to and maintained in 6.2$\mu$g/ml, 14$\mu$g/ml and 25$\mu$g/ml were designated DLKPC 6.2, DLKPC 14 and DLKPC 25 respectively.

### 3.1.2 Cross resistance profile of the DLKPC variants

The sensitivities of the three resistant variants to a range of chemotherapeutic agents was determined, in order to establish their cross resistance profile. The chemotherapeutic agents included the selective drug carboplatin, the alkylating platinum agent cisplatin, the anthracycline adriamycin, the vinca alkaloid vincristine, the semisynthetic derivative of podophyllotoxin VP16 and the antimetabolite agent, 5-fluorouracil. Table 3.1.2.1 represents the $IC_{50}$ values of each of the chemotherapeutic agents for the parental DLKP cell line and the three DLKPC selected variants, ascertained from the toxicity profile as described in section 2.6. Table 3.1.2.2 represents the fold resistance of the DLKPC variants relative to the control DLKP cell line. All three DLKPC variants showed similar cross resistance profiles. They were found to be resistant to the selective agent, carboplatin and cross resistant to cisplatin. There was no significant cross resistance to the chemotherapeutic agents, adriamycin, vincristine and 5-fluorouracil observed in the DLKPC cells. The resistant variants showed slight cross resistance to VP16. The fold resistant values illustrated that the three variants were most resistant, not to the selecting agent carboplatin but to cisplatin (approximately 5-, 12- and 25-fold respectively), then to the selecting agent carboplatin (approximately 4-, 8.3- and 15.8- fold respectively) and finally to VP16 (approximately 2 fold for DLKP 14 and 2.5 fold for the DLKPC 25 variant).

| $IC_{50}$ (nM) | DLKP | DLKPC 6.2 | DLKPC 14 | DLKPC 25 |
|---|---|---|---|---|
| Carboplatin | 1400 ± 61.9 | 5678 ± 369 | 11894 ± 892 | 22708 ± 817 |
| Cisplatin | 1500 ± 90 | 7650 ± 712 | 18300 ± 774 | 37650 ± 817 |
| Adriamycin | 12.9 ± 0.9 | 20.6 ± 1.3 | 21.5 ± 1.5 | 20.5 ± 1.44 |
| Vincristine | 1.3 ± 0.2 | 1.35 ± 0.17 | 1.49 ± 0.2 | 1.29 ± 0.13 |
| VP16 | 76 ± 6.3 | 100 ± 5.2 | 144.6 ± 9.8 | 190 ± 10.2 |
| 5-Fluorouracil | 5000 ± 210 | 4600 ± 358 | 3850 ± 192 | 4550 ± 223 |

**Table 3.1.2.1** $IC_{50}$ values for DLKP and the DLKPC variants

| Drug | DLKPC 6.2 | DLKPC 14 | DLKPC 25 |
|---|---|---|---|
| Carboplatin | 4.04 | 8.26 | 15.77 |
| Cisplatin | 5.1 | 12.2 | 25.1 |
| Adriamycin | 1.6 | 1.67 | 1.59 |
| Vincristine | 1.04 | 1.12 | 0.9 |
| VP16 | 1.34 | 1.9 | 2.5 |
| 5-Fluorouracil | 0.92 | 0.77 | 0.91 |

**Table 3.1.2.2** Fold resistance of the DLKPC variants relative to the parental DLKP cell

### 3.1.3 Stability of the DLKPC variants

The stability of the DLKPC variants, when maintained in the absence of carboplatin was studied over a number of months. Up to three months out of drug, all three cell lines maintained their resistance to carboplatin. After this time, however, the cells began to lose their resistance to the drug. Figure 3.1.3 illustrates the toxicity profile of the DLKPC 25 after three months and six months maintained in the absence of drug. After six months maintenance in drug free medium a decrease of approximately 1.3-fold was observed in the $IC_{50}$ value.

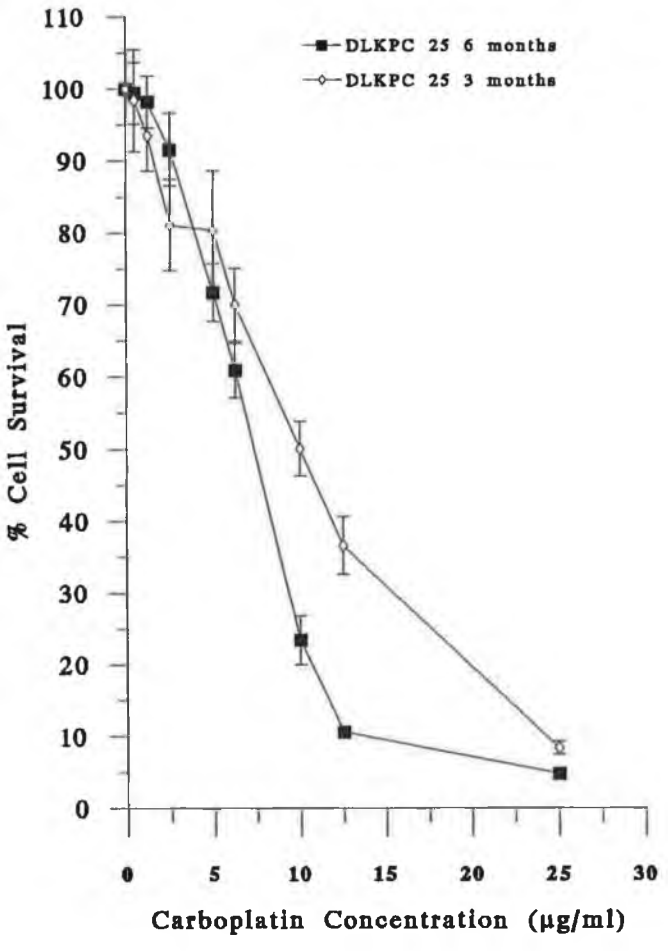



**Figure 3.1.3** The toxicity profile of carboplatin in the DLKPC 25 cell line. The cells were cultured in the absence of the selecting agent for 3 months and 6 months.

### 3.1.4 Morphology of the DLKPC variants

The morphology of the three variants was studied and compared to the DLKP cells, to determine if the resistant cells differed from the parental cells from which they were derived. Figure 3.1.4 presents the morphology of the parental DLKP cells and the three carboplatin resistant variants. The morphology of the variants were similar to the parental DLKP cell line, in that all three variants had the squamous cell type, typical of the parental DLKP cell line.

### 3.1.5 Doubling times in the DLKPC variants

The rate of cell growth, calculated during the exponential phase, was determined in the DLKP parental cells and in the DLKPC variants over a time period of 72 hours. The cells were plated in 25cm$^2$ tissue culture flasks and cell counts performed every 12 hours. The results obtained are presented in table 3.1.5. The DLKP and the DLKPC 6.2 cells were found to have similar doubling times of approximately 30.1 and 30.63 hours respectively. However, both the DLKPC 14 and DLKPC 25 resistant variants were shown to have longer doubling times than the parental cells. The doubling time observed in the DLKPC 14 was approximately 39 hours and approximately 41 hours in the DLKPC 25 variant.

| Cell line | Doubling time (hours) |
|---|---|
| DLKP | 30.1 |
| DLKPC 6.2 | 30.63 |
| DLKPC 14 | 38.92 |
| DLKPC 25 | 41.3 |

**Table 3.1.5** Doubling times in DLKP cells and DLKPC variants

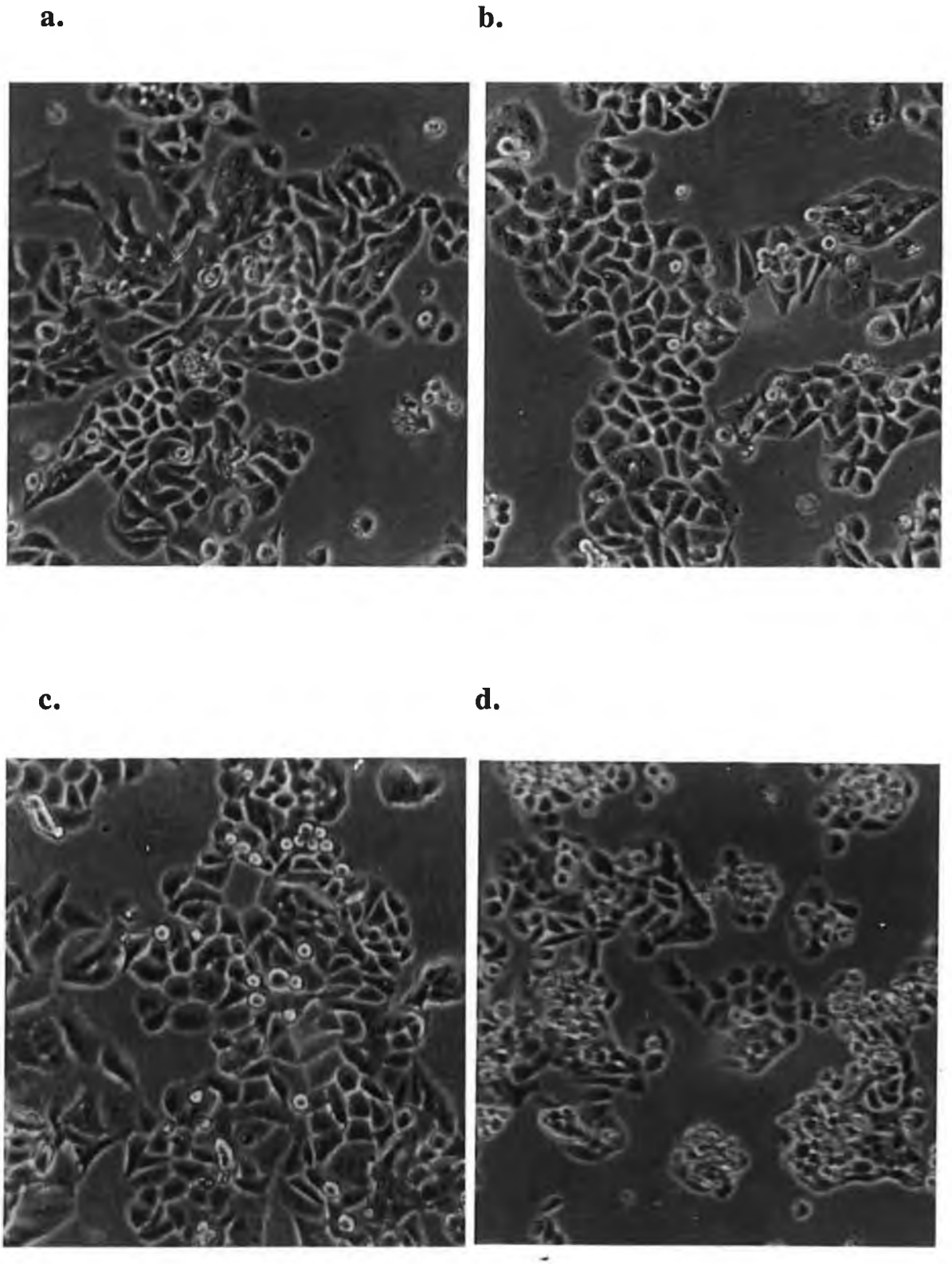



**Figure 3.1.4** Morphology of the parental DLKP and carboplatin resistant DLKP variants. DLKP (a), DLKPC 6.2 (b), DLKPC 14 (c) and DLKPC 25 (d).

### 3.1.6 DNA fingerprint analysis of the DLKPC 25 variant

DNA fingerprint analysis was carried out on the DLKPC 25 variant to establish its genetic identity to the DLKP cells from which they were established. DNA was extracted from the cell lines by Cellmark Diagnostics, Abingdon, Oxfordshire and fingerprints analysed with the multilocus probes 33.15 and 33.6. The results obtained illustrated that the DLKPC 25 variant shared almost identical bands with the DLKP cell line and therefore must have originated from the DLKP cell line. Since the DLKPC 25 variant was derived from the DLKPC 6.2 and DLKPC 14 lines, DNA fingerprint analysis was not carried out on these variants.

## 3.2 Protein Analysis in the DLKPC variants

Alterations in the expression of multidrug resistance associated proteins was studied by Western blot analysis, as described in section 2.7. The expression of the membrane associated P-glycoprotein pump was investigated in the sensitive DLKP cells, its adriamycin resistant variant, DLKPA10 and in the DLKPC resistant variants. Possible alterations in the level of the nuclear enzyme, topoisomerase II $\alpha$ and II $\beta$, were studied in the DLKP cells and also in the highest carboplatin resistant variant, DLKPC 25.

### 3.2.1 Western blot analysis of P-glycoprotein expression

The expression of P-glycoprotein in the DLKPC variants was investigated, using the anti-P-glycoprotein monoclonal antibody, C219, by Western blotting on purified membrane fractions prepared, as described in section 2.7.2. The results obtained for the each of the cell lines studied are presented in figure 3.2.1 Strong immunoreactive P-glycoprotein expression was observed in the adriamycin resistant DLKPA10 cell line at a molecular weight of approximately 170 kDa. However, no detectable level of P-glycoprotein expression was evident in the parental DLKP or in the carboplatin resistant variants.

### 3.2.2 Western blot analysis of topoisomerase II $\alpha$ and $\beta$

The expression of the enzyme topoisomerase II in the parental DLKP and the DLKPC 25 variant was determined by western blotting, using a mixture of polyclonal antibodies raised against the topoisomerase II $\alpha$ and topoisomerase II $\beta$ isoforms. Total cell lysates were prepared, as in section 2.7.1 and analysed for topoisomerase II expression. As illustrated in figure 3.2.1, both the DLKP and DLKPC cell lines possessed immunoreactive topoisomerase II, although no significant difference in the expression of the topoisomerase II isoforms was observed between the two cell lines.

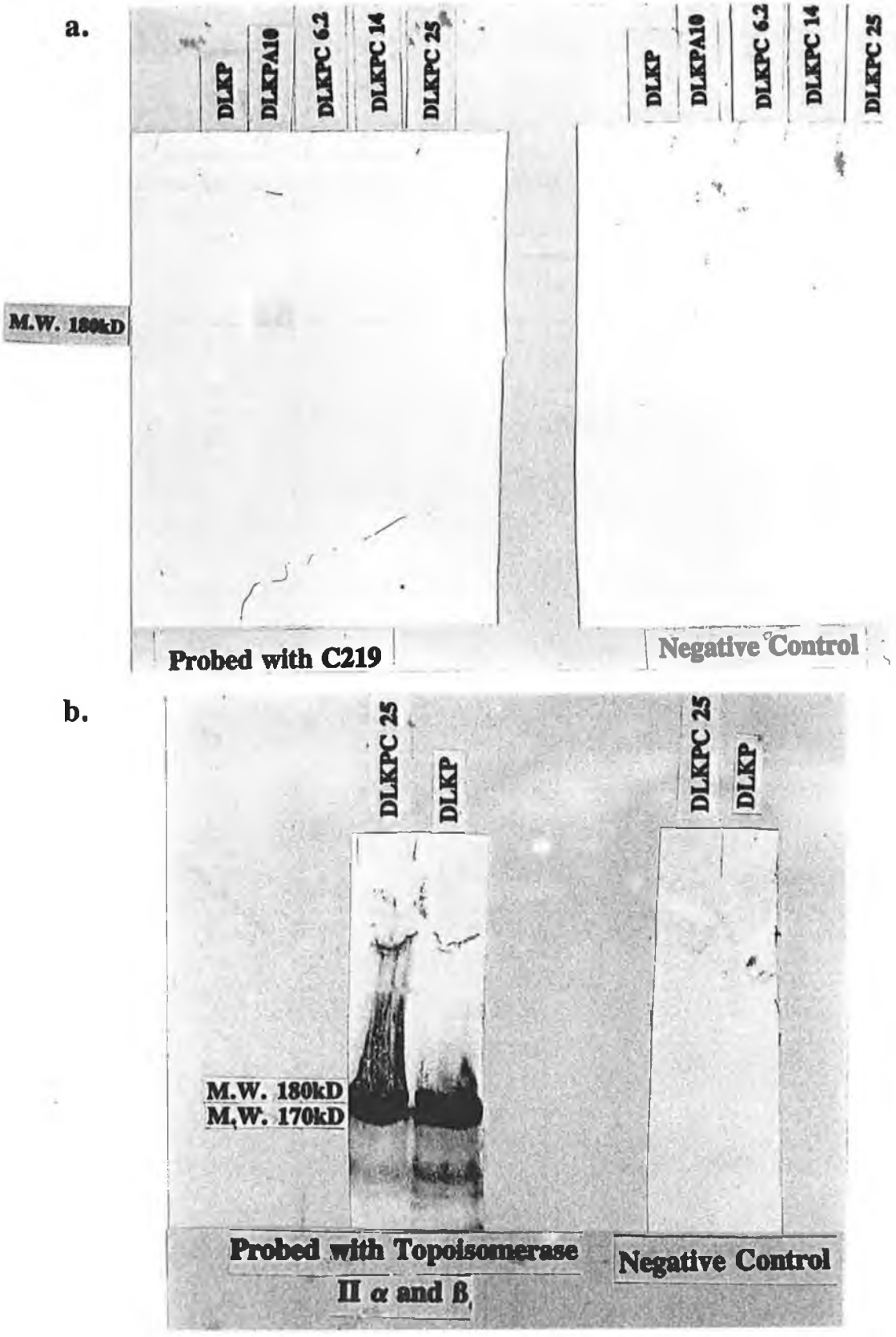



**Figure 3.2.1** Western blot detection of P-glycoprotein in cell membrane preparations of the DLKP, DLKPA10 and DLKPC resistant variants (a) and Western blot detection of topoisomerase IIα and ß in nuclear extracts of the DLKP and DLKPC 25 variants (b).

## 3.3 Glutathione studies in the DLKP variants

### 3.3.1 Determination of Glutathione-S-Transferase activity in DLKPC variants

Alterations in the activity of the metabolising enzyme, glutathione-S-transferase, was investigated in the DLKPC resistant variants using cytosolic cellular extracts, prepared as described in section 2.8.1. A decrease in GST activity was observed in the DLKPC 6.2 and DLKPC 14 variants when compared to the parental cells, although the level of activity in the DLKPC 25 variant was comparable to that noted in the parental cells (Figure 3.3.1). The range from the highest level of activity to the lowest activity level, however, was not significant, a decrease of less than two fold being noted.

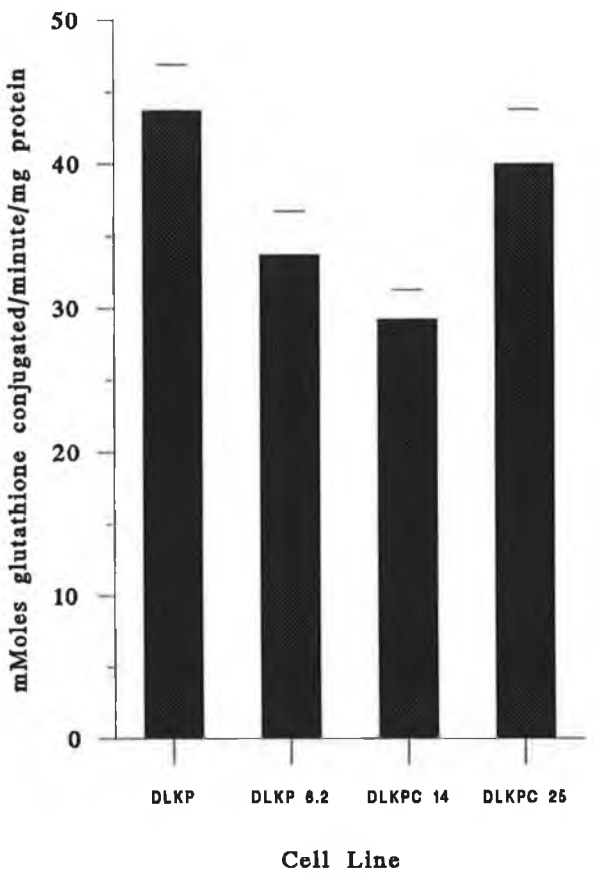


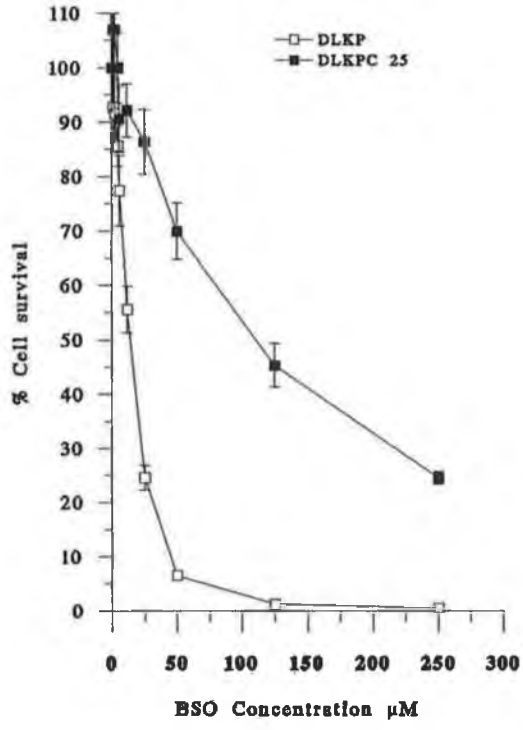

**Figure 3.3.1** The activity of the glutathione-S-transferase enzyme in the parental DLKP and DLKPC resistant variants.

### 3.3.2 Toxicity of BSO and ethacrynic acid in the DLKPC variants

The toxicity profiles of buthionine sulphoxamine (BSO), an inhibitor of glutathione synthesis and ethacrynic acid, a specific inhibitor of glutathione-S-transferase, were determined in the DLKP cells and DLKPC variants. The toxicity of BSO, over a concentration range of 0 - 250$\mu$M, was investigated in both the sensitive DLKP and DLKPC 25 cell lines. Figure 3.3.2.1 illustrates that the DLKP parental cells exhibited far greater susceptibility to BSO toxicity than the DLKPC 25 variant. The calculated $IC_{50}$ values for BSO in the DLKP and DLKPC 25 cells were approximately 20$\mu$M and 120$\mu$M respectively. The effect of BSO on the toxicity profile of carboplatin was investigated in the DLKPC variants. The concentration of BSO resulting in more than 95% cell survival ($IC_{95}$) was chosen for the assay, in which the cells were exposed to both carboplatin and BSO. The results obtained illustrated that the non-toxic concentration of BSO had no significant effect on the toxicity profile of carboplatin. Figure 3.3.2.1 presents the results obtained for the DLKPC 25 cell variant.

The toxicity profile of ethacrynic acid, over a concentration range of 0 - 5mg/ml, was also studied in the parental DLKP cells and DLKPC resistant variants. Figure 3.3.2.2 illustrates that all four cell lines studied exhibited slightly different toxicity profiles. Although the DLKPC cells were found to be more susceptible to ethacrynic acid than the parental cells, only a maximum of a two fold difference in the $IC_{50}$ values was observed. The effect of ethacrynic acid ($IC_{95}$) on the toxicity of carboplatin was studied in the three resistant variants and the results obtained illustrated that ethacrynic acid did not significantly alter the toxicity profile in any of the cell lines studied. Figure 3.3.2.2 illustrates the results obtained for the DLKPC 25 variant.

a.




b.

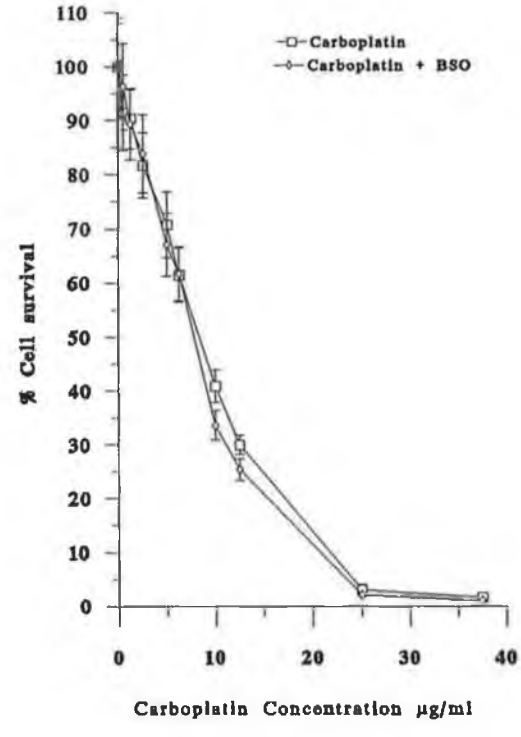
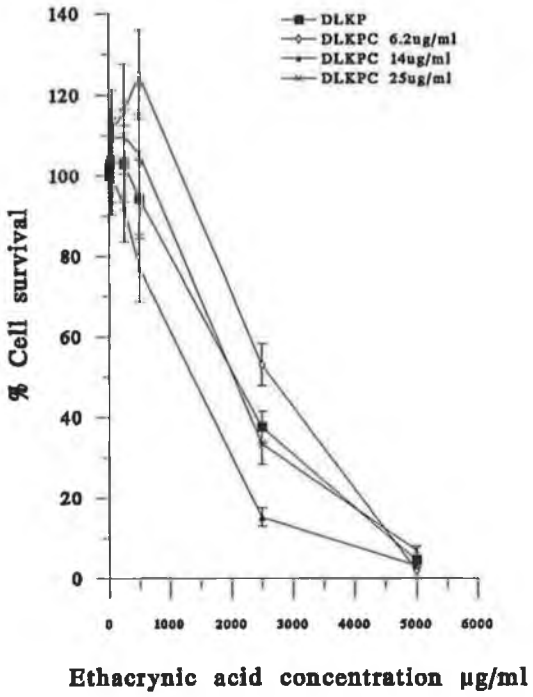



**Figure 3.3.2.1** The toxicity profile of the glutathione inhibitor, BSO in the parental DLKP and the carboplatin resistant DLKPC 25 variants (a) and the effect of BSO on the toxicity profile of carboplatin in the DLKPC 25 variant (b).

a.




b.

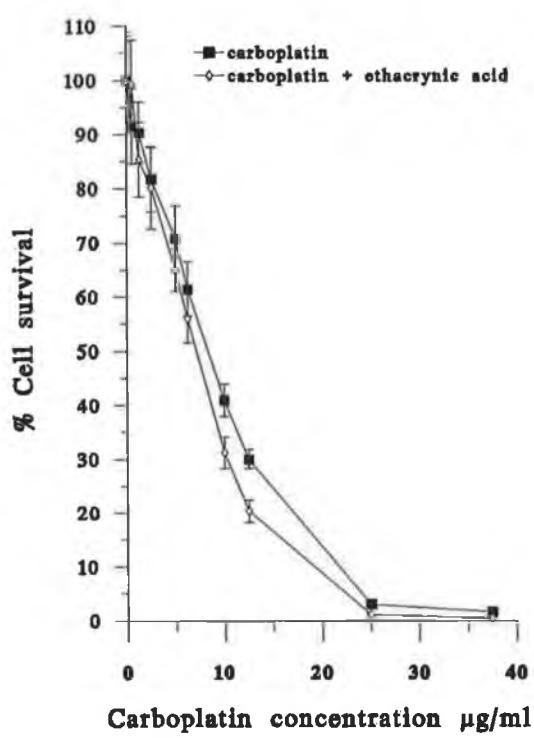



**Figure 3.3.2.2** The toxicity profile of ethacrynic acid in the parental DLKP and in the carboplatin resistant DLKPC variants (a) and the effect of ethacrynic acid on the toxicity profile of carboplatin in the DLKPC 25 variant (b).

### 3.4.1 Camptothecin toxicity in the DLKPC variants

The toxicity of the topoisomerase I inhibitor, camptothecin, in DLKP and DLKPC variants was investigated to determine if the DLKPC variants were susceptible to topoisomerase I inhibition. The toxicity profile of camptothecin, over a concentration range of 0 - 2500 ng/ml (0 - 2.5$\mu$g/ml), was determined in the parental DLKP and DLKPC cells. Figure 3.4.1 illustrates that the camptothecin exhibited slightly lower toxicity in all three variants, relative to the parental cell line. However, there was only a maximum of a two fold decrease between the DLKP cells, where camptothecin proved to be the most toxic and DLKPC 14 variant, which were the least susceptible to camptothecin toxicity.

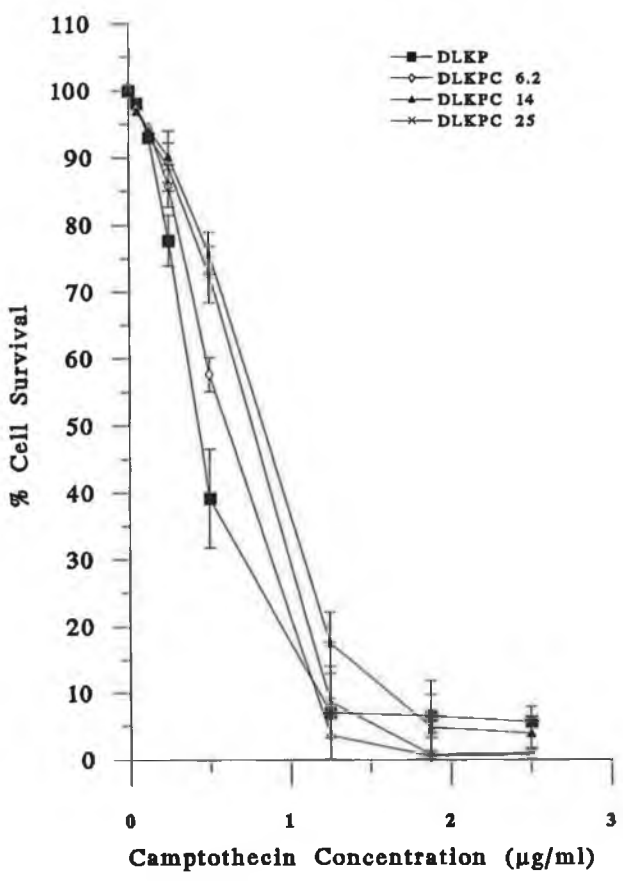



**Figure 3.4.1** The toxicity profile of the topoisomerase I inhibitor, camptothecin in the parental DLKP cells and in the DLKPC resistant variants.

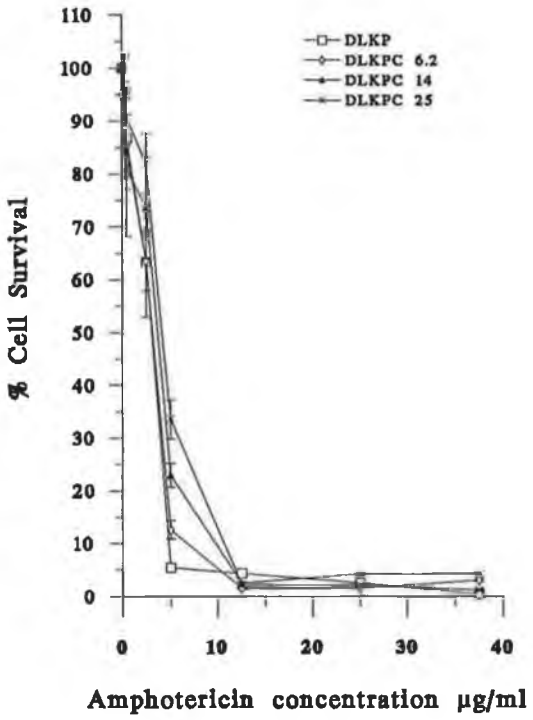
### 3.4.2 Amphotericin B toxicity in the DLKPC variants

The effect of the polyene antibiotic, amphotericin B, on carboplatin resistance was investigated in the DLKPC resistant variants. Amptothericin B alters membrane permeability by binding to cholesterol and forming pores in the membranes. The aim of this work was to determine if alterations in membrane permeability affected the toxicity of carboplatin in the DLKPC resistant variants. An initial toxicity assay for amphotericin was carried out, over a concentration range of 0 - 40$\mu$g/ml, in the DLKP and DLKPC cell lines. Figure 3.4.2 illustrates that a similar toxicity profile was observed in the four cell lines studied, with $IC_{50}$ values of approximately 3.5 - 5$\mu$g/ml amphotericin B. Figure 3.4.2 also illustrates the effect of a non-toxic concentration of amphotericin B (0.2$\mu$g/ml) on carboplatin toxicity in DLKPC 25 cells. Although this agent has been shown to reverse platinum resistance in a number of cell lines, the results obtained showed that amphotericin B did not significantly alter the toxicity profile of carboplatin in the DLKPC 25 cells. The effect of amphotericin B was also studied in the DLKPC 6.2 and DLKPC 14 variants. However, no significant effect was observed.

### 3.4.3 Ouabain toxicity in the DLKPC variants

Since the $Na^+$ $K^+$-ATPase inhibitor, ouabain, has been demonstrated to decrease platinum accumulation in a number of cell lines, the effect of the compound on carboplatin toxicity was studied in the DLKPC variants. The aim of this work was to investigate the role of $Na^+K^+$-ATPases in drug accumulation in the DLKPC variants. The toxicity profile of ouabain, over a concentration range of 0 - 50ng/ml, was initially determined in the parental DLKP and the three DLKPC resistant variants. The results obtained illustrated that the DLKP cells were slightly more susceptible to ouabain toxicty than the resistant lines, having an $IC_{50}$ value of 8.5ng/ml. The DLKPC 14 and DLKPC 25 cells exhibited similar toxicity profiles with $IC_{50}$ values of approximately 16ng/ml while the DLKPC 6.2 cells had an $IC_{50}$ value of 10.5ng/ml (figure 3.4.3). Studies on the effect of ouabain (0.2ng/ml) on carboplatin toxicity in the DLKPC variants revealed that ouabain did not significantly alter the toxicity profile in any of the variants studied. Figure 3.4.3 illustrates the results obtained for the DLKPC 25 cell line.

a.




b.

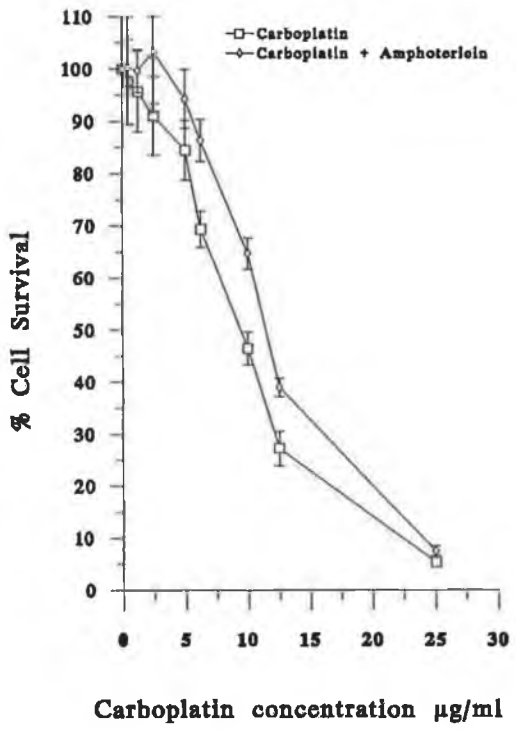
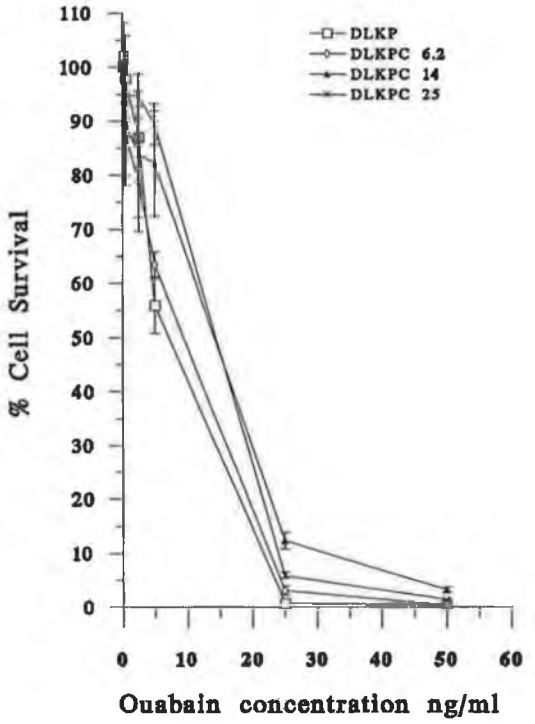



**Figure 3.4.2** The toxicity profile of amphotericin B in the parental DLKP and carboplatin resistant DLKPC variants (a) and the toxicity profile of amphotericin B (0.2$\mu$g/ml) on the toxicity profile of carboplatin in the DLKPC 25 variant (b).

a.




b.

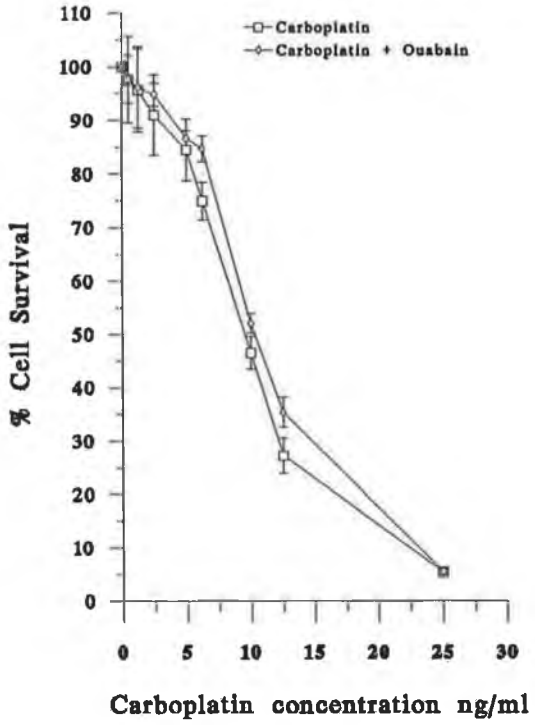



**Figure 3.4.3** The toxicity profile of ouabain in the parental DLKP and carboplatin resistant DLKPC variants (a) and the effect of ouabain (0.2$\mu$g/ml) on the toxicity profile of carboplatin in the DLKPC 25 variant (b).

### 3.4.4 Cadmium chloride toxicity in the DLKPC variants

Since resistance to the heavy metal, cadmium chloride is associated with increased levels of metallothionein expression the toxicity of cadmium chloride in the DLKP parental cells and in the DLKPC resistant variants was studied. This work was carried out to establish if there was any alteration in the level of the cytoplasmic protein, metallothionein, in the carboplatin resistant lines. Cadmium chloride was shown to exhibit a notably different toxicity profile in all four DLKP variants studied. It proved to be the most toxic to the DLKP parental cells, having an $IC_{50}$ value of approximately 0.22 $\mu$g/ml and least toxic to the DLKPC 25 resistant variant, with an $IC_{50}$ of approximately 2.2 $\mu$g/ml (Figure 3.4.4). There was approximately a ten fold decrease in cadmium chloride toxicity from the DLKP to the DLKPC 25 variant. The DLKPC 14 cells exhibited approximately a nine fold resistance to cadmium and the DLKPC 6.2 a 2.7 fold resistance to cadmium.

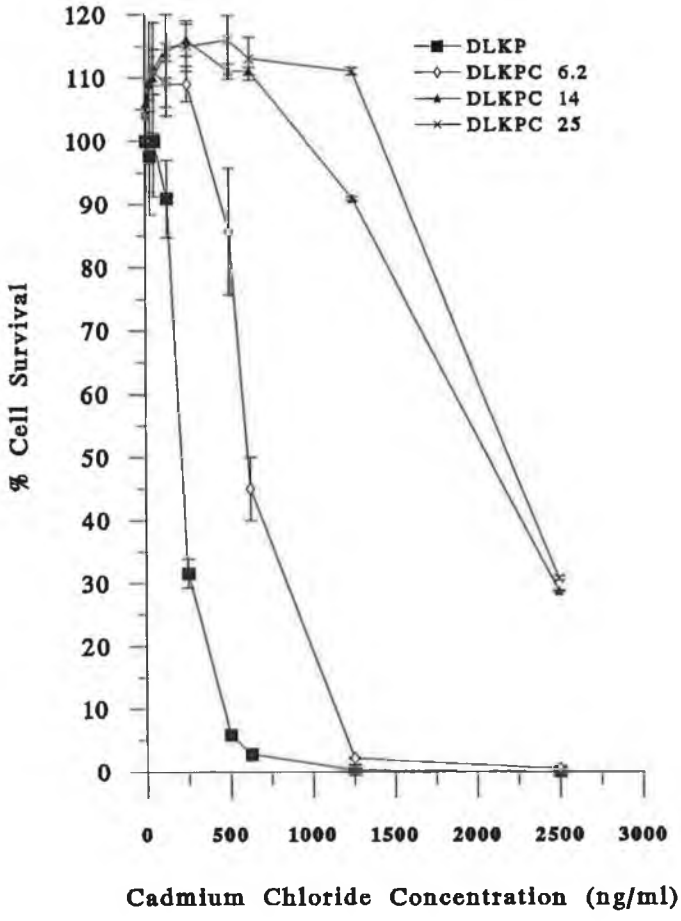



**Figure 3.4.4** The toxicity profile of cadmium chloride in the parental DLKP and DLKPC resistant cell lines.

## 3.5 Immunological Studies in the DLKPC variants

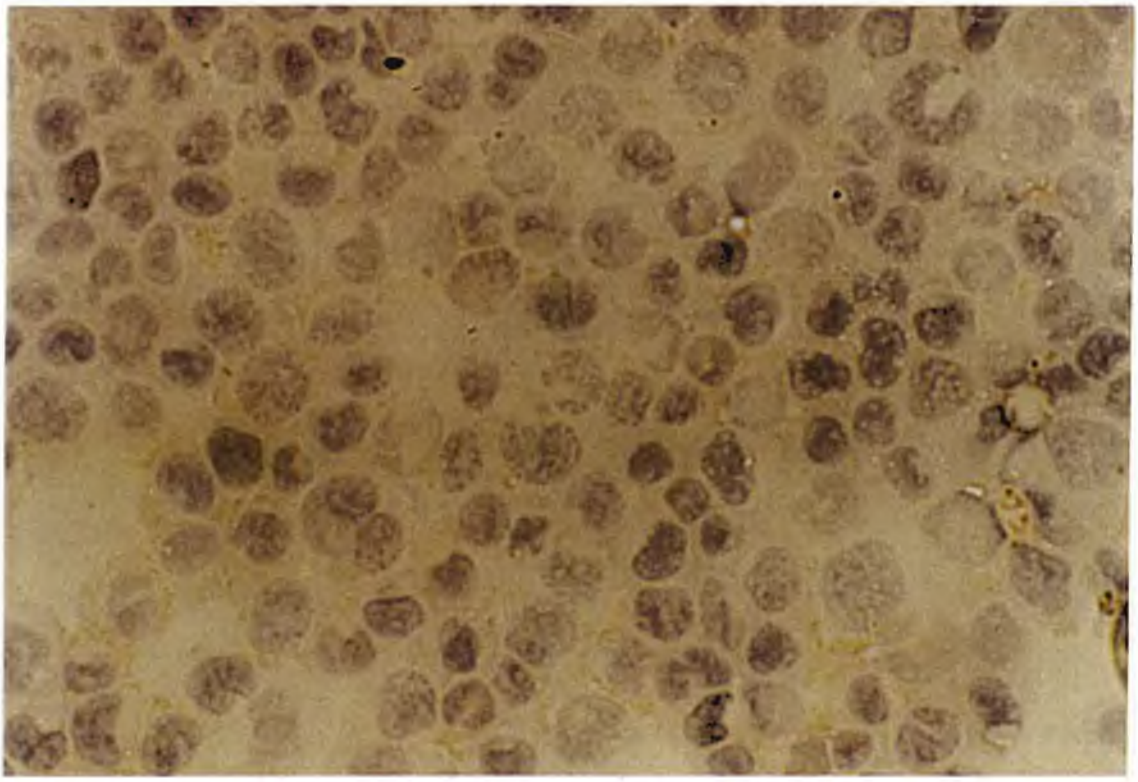
### 3.5.1 Immunological detection of metallothionein in the DLKPC variants

The presence of metallothionein in the parental DLKP cells and the DLKPC variants was investigated, using a monoclonal mouse anti-metallothionein antibody as outlined in section 2.9.2.3. Metallothionein expression was predominantly observed in the cytoplasm of the cells, with the intensity of staining observed varying, depending on the DLKP variant studied. Faint cytoplasmic staining was visible in approximately 60% of the DLKP parental cells. All three DLKPC variants stained positive for metallothionein expression. However, the staining intensity increased with increasing level of resistance. The DLKPC 6.2 variant was only slightly more positive than the DLKP cells as shown in figure 3.5.1.1. However, both the DLKPC 14 and DLKPC 25 variant proved to be substantially more immunoreactive to the anti metallothionein antibody than the parental cells (figure 3.5.1.2). Intense cytoplasmic staining was noted in approximately 85% of the DLKPC 25 cells. Cells that were incubated in the absence of the primary metallothionein antibody were negative for metallothionein expression.

### 3.5.2 Immunological detection of LRP and MRP in the DLKPC variants

The presence of the 110 kilodalton protein, LRP, was investigated in the parental DLKP and the resistant DLKPC cells, using the LRP-56 antibody, as described in section 2.9.2.4. A typical staining pattern for LRP in the DLKP and DLKPC 25 cells is shown in figure 3.5.2. LRP expression was localized predominantly in the cytoplasmic regions of the DLKP and DLKPC cells. Approximately 60% of the DLKP cells were positive for LRP, although the intensity of staining varied within a cell population, from faint staining in some cells to very intense staining in others. The three DLKPC variants showed similar LRP staining patterns to the parental cells. Cytoplasmic staining was observed in the cells and the intensity of staining again varied within the cell populations. There was no significant difference in LRP staining and intensity observed in the DLKPC 6.2, DLKPC 14 and DLKPC 25 cells. The presence of MRP was investigated using the rat monoclonal antibody MRPr1 in the DLKP and DLKPC cell variants. No detectable levels of MRP was observed in any of the four variants studied. Very faint staining was observed, however, the staining pattern was similar to the negative control cells.

a.



b.

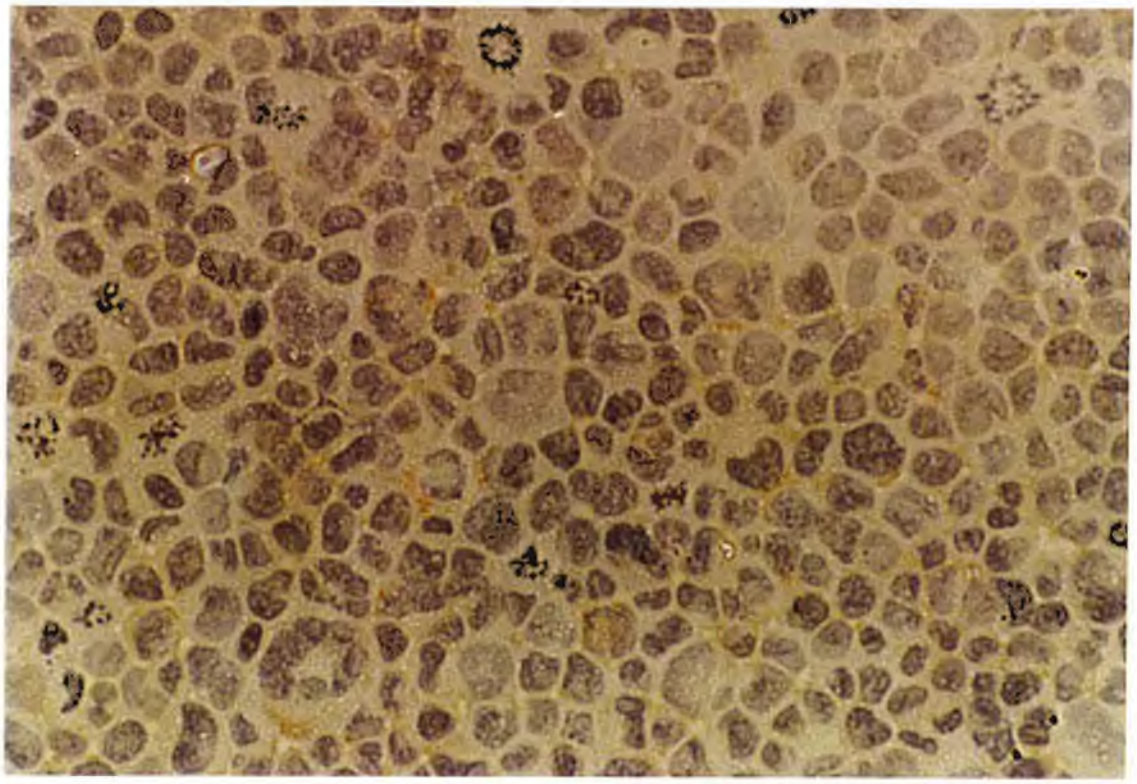
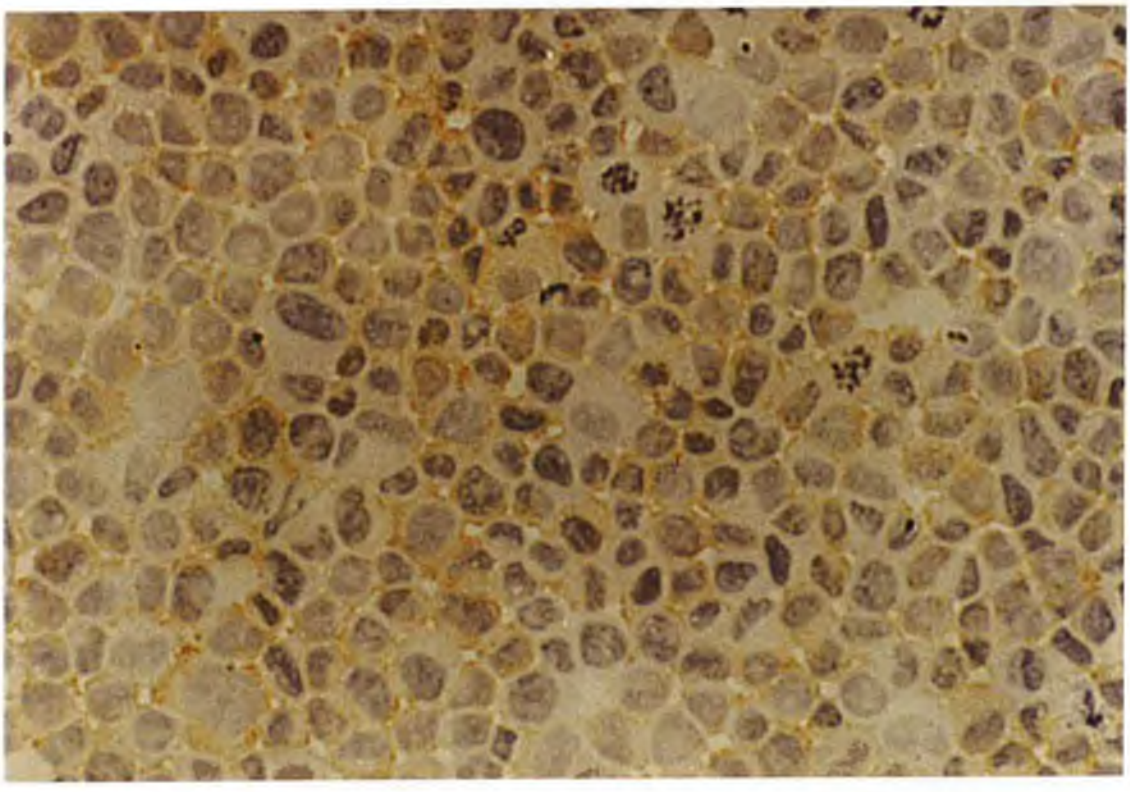


**Figure 3.5.1.1** Immunocytochemical detection of metallothionein in the parental DLKP (a) and DLKPC 6.2 resistant variant (b) with a mouse monoclonal anti-metallothionein antibody.

a.



b.

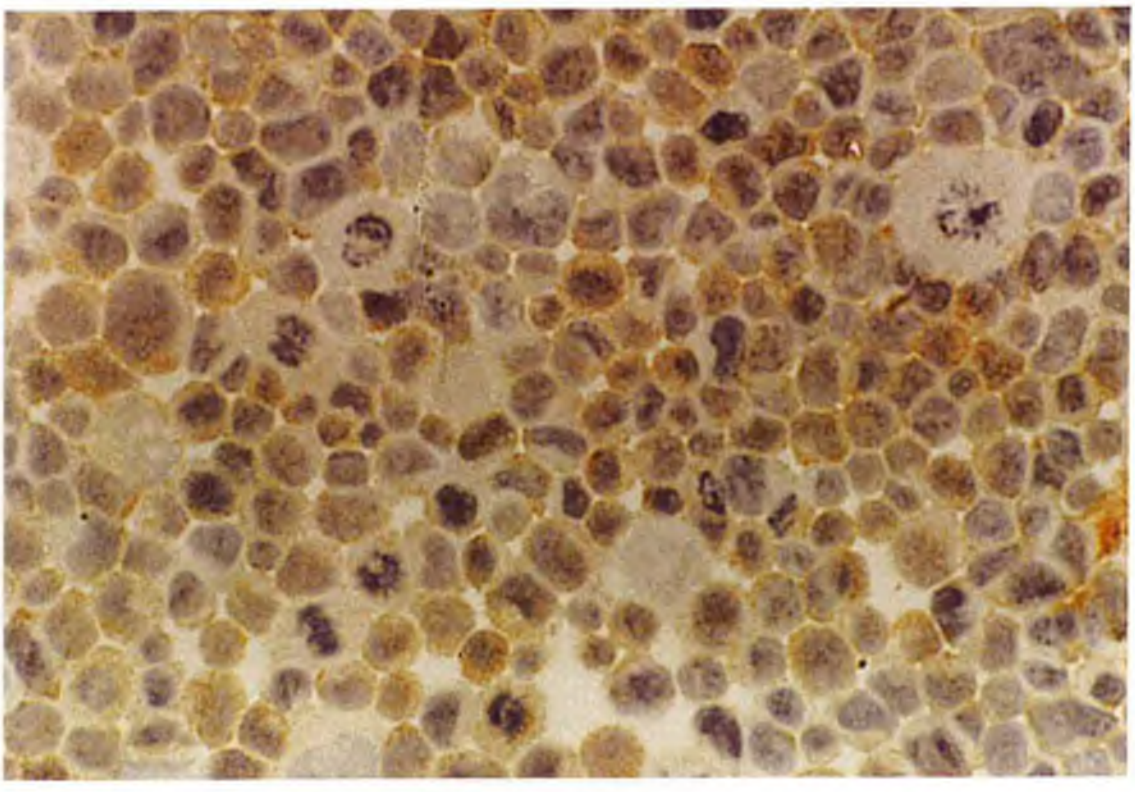
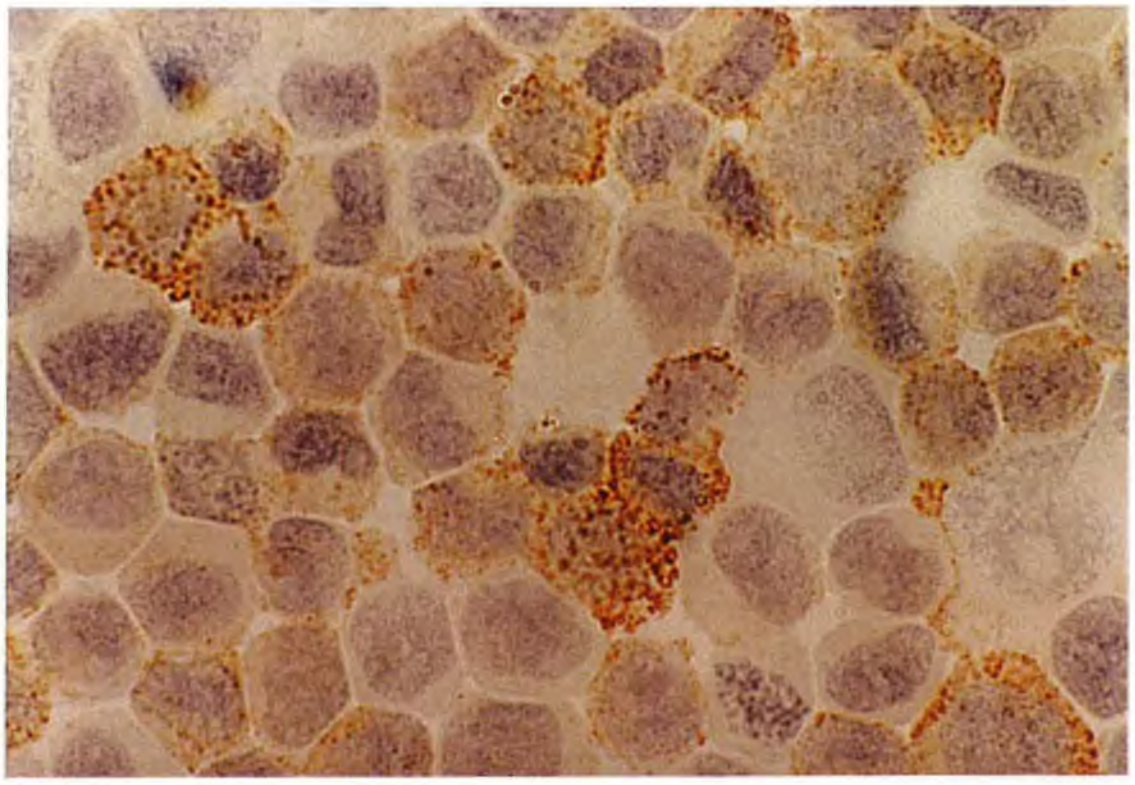


**Figure 3.5.1.2** Immunocytochemical detection of metallothionein in the DLKP 14 (a) and DLKPC 25 resistant variants (b) with a mouse monoclonal anti-metallothionein antibody.

a.



b.

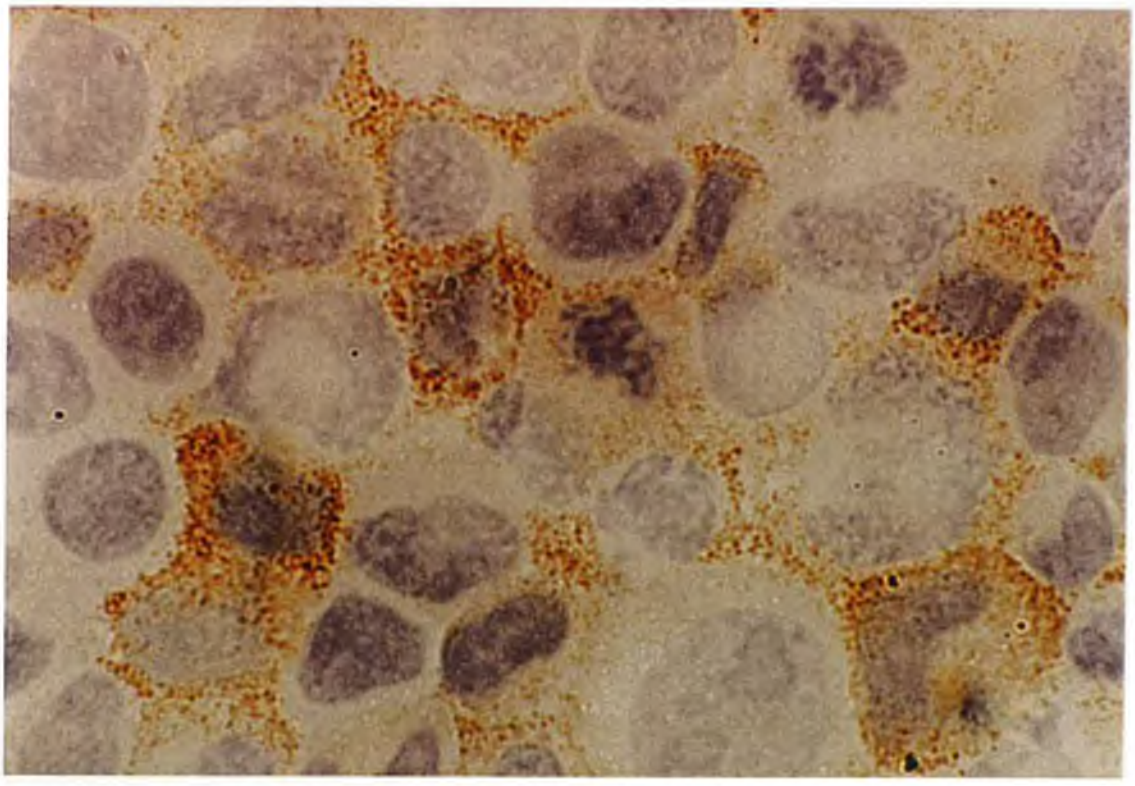


**Figure 3.5.2** Immunocytochemical detection of LRP (p110) in the parental DLKP (a) and DLKPC 25 resistant variant (b) with the mouse monoclonal anti-LRP 56 antibody.

## 3.6 Establishment of MDR cell lines

### 3.6.1 Establishment of DLKPA10 and SKMES-1/ADR cell lines

The DLKPA10 variant of the human lung carcinoma cell line DLKP was established by further exposing the adriamycin resistant variant, DLKP-A (selected to 2.1$\mu$g/ml adriamycin; Clynes *et al.*, 1992) to increasing concentrations of adriamycin over a time period of approximately three months. The cells readily adapted to growth in the higher drug concentrations. The cells were exposed to a final concentration of 10$\mu$g/ml adriamycin and were designated DLKPA10. The SKMES-1/ADR variant of the human lung cell line, SKMES-1, was established by exposing the low level adriamycin resistant variant, SKMES-1A, to increasing concentrations of adriamycin over a time period of four months. The $IC_{50}$ value for adriamycin in the SKMES-1A cell was found to be approximately 350nM before the selection process was started. The cells adapted slowely to increasing concentration of the drug over the first two months, although after this time they readily adapted to growth. The cells were exposed to a final concentration of 0.25$\mu$g/ml adriamycin. The cells were routinely maintained at this concentration and were designated SKMES-1/ADR.

### 3.6.2 Cross resistance profile of the DLKPA10 cell line

The sensitivity of the DLKPA10 variant to a number of chemotherapeutic drugs was determined in order to establish its cross resistance profile. The chemotherapeutic agents included the selecting agent, adriamycin, the vinca alkaloid, vincristine, the antimetabolite agent, 5-fluorouracil, the semisynethic derivative of podophyllotoxin, VP16 and the alkylating agent, carboplatin. Table 3.6.2.1 represents the $IC_{50}$ values obtained for each of the chemotherapeutic agents for the parental DLKP cells and the DLKPA10 resistant variant. Table 3.6.2.2 represents the fold resistance of the DLKPA10 and DLKP-A cell lines relative to the control DLKP parental cells. The DLKPA10 cells were found to be resistant, not only to the selecting agent adriamycin but also to vincristine and VP16. The cells exhibited greatest cross resistance to vincristine (3000-fold approximately), then to the selecting agent adriamycin (765-fold approximately), showing the least resistance to VP16 (approximately 63-fold). The DLKPA10 cells were found to exhibit slight sensitivity to the chemotherapeutic agents carboplatin and 5-fluorouracil. However, a significance difference was not noted.

| $IC_{50}$ (nM) | DLKP | DLKPA10 |
|---|---|---|
| Adriamycin | 8.75 ± 0.77 | 6696 ± 453 |
| Vincristine | 0.728 ± 0.062 | 2184 ± 132 |
| VP16 | 233 ± 18.5 | 1448 ± 98.2 |
| Carboplatin | 3243 ± 278 | 2676 ± 198 |
| 5-Fluorouracil | 7923 ± 57.7 | 7308 ± 522 |

**Table 3.6.2.1** $IC_{50}$ values for the DLKP and DLKPA10 cell lines

| Drug | DLKP-A | DLKPA10 |
|---|---|---|
| Adriamycin | 322.2 | 765.3 |
| Vincristine | 79.5 | 3000 |
| VP16 | 36.2 | 63.3 |
| Carboplatin | ND | 0.825 |
| 5-Fluorouracil | ND | 0.92 |

ND - not determined

**Table 3.6.2.2** Fold resistance of the DLKP-A and DLKPA10 cells relative to the parental DLKP cells

### 3.6.3 Cross resistance profile of the SKMES-1/ADR cell line

To determine if the SKMES-1/ADR cell line exhibited the multidrug resistant phenotype, the sensitivity of the resistant cells to a number of chemotherapeutic agents was investigated. Table 3.6.3.1 represents the $IC_{50}$ values obtained for the SKMES-1 parental cells and the SKMES-1/ADR cell line with the selecting agent adriamycin, the vinca

alkaloid vincristine, the alkylating agent carboplatin and the podophyllotoxin VP16. Table 3.6.3.2 presents the fold resistant values obtained for the SKMES-1/ADR cells, relative to the control SKMES-1 parental cells. The SKMES-1/ADR cells were found to be most resistant, not to the selecting agent adriamycin but to vincristine. The cells exhibited approximately a 50-fold resistance to vincristine and a 45-fold resistance to adriamycin. The cells also showed cross resistance to VP16, although only a three fold resistant level was noted. A 2-fold resistance level was observed with carboplatin in the SKMES-1/ADR cells.

| $IC_{50}$ (nM) | SKMES-1 | SKMES-1/ADR |
|---|---|---|
| Adriamycin | 26.8 ± 3.1 | 1205 ± 98.9 |
| Vincristine | 9.1 ± 0.86 | 455 ± 32.2 |
| VP16 | 114.3 ± 10.6 | 343 ± 27.8 |
| Carboplatin | 337.8 ± 25.7 | 729.9 ± 55.4 |

**Table 3.6.3.1** $IC_{50}$ values for SKMES-1 and SKMES-1/ADR cell lines

| Drug | SKMES-1/ADR |
|---|---|
| Adriamycin | 45 |
| Vincristine | 50 |
| VP16 | 3 |
| Carboplatin | 2.16 |

**Table 3.6.3.2** Fold resistance values for SKMES-1/ADR with respect to the SKMES-1 cells

### 3.7 Stability of the DLKPA10 and SKMES-1/ADR cell lines

The stability of the DLKPA10 cells when maintained, in the absence of adriamycin, was studied over a number of months. Over a time period of six months the cells maintained their resistance to adriamycin and, therefore, was characterised as a stable cell line. The stability of the SKMES-1/ADR cell line was also investigated over a time period of four to six months. Although the cells maintained their resistance to adriamycin over a four month time period, a slight decrease in the resistance levels was observed following six months maintenance in drug free medium. The SKMES-1/ADR cells were, therefore, exposed to adriamycin every four months to maintain their resistance level.

### 3.8 DNA fingerprint analysis of the DLKPA10 and SKMES-1/ADR cell lines

DNA fingerprint analysis was carried out on the DLKPA10 cells and SKMES-1/ADR cells to establish its genetic identity to the DLKP and SKMES-1 parental cells, from which they were established. The DLKPA10 cell line was found to share almost identical bands with the DLKP cells and, therefore, must have originated from the DLKP cells. The SKMES-1/ADR cells were found to share almost identical bands with the SKMES-1 cells and consequently must have originated from this cell line.

## 3.9 Protein analysis in the DLKP and SKMES-1 variants

The expression of the MDR associated membrane protein, P-glycoprotein, was studied in the DLKPA10 and SKMES-1/ADR resistant cell lines by western blot analysis. The results obtained were compared to the levels of protein detected in the parental DLKP and SKMES-1 cells respectively.

### 3.9.1 Western blot analysis of P-glycoprotein expression

Alterations in the levels of P-glycoprotein expression was investigated in the parental DLKP and SKMES-1 cells and in the MDR variants, DLKPA10 and SKMES-1/ADR. Purified membrane fractions of each of the cell lines were prepared, as described in section 2.7.2 and and stored lyophilized until required. The extracts were reconstituted and the protein separated by gel electrophoresis. The expression of P-glycoprotein was detected, using the anti-P-glycoprotein monoclonal antibody, C219. Figure 3.9.1. illustrates the results obtained for the four cell lines. The results show that the C219 antibody reacted with a low level of P-glycoprotein in the DLKP and SKMES-1 parental cells. In contrast strong immunoreactivity was observed in both the DLKPA10 and SKMES-1/ADR cell lines, indicating high expression of P-glycoprotein. The most significant increase in P-glycoprotein expression was noted in the DLKPA10 cells.

To determine if P-glycoprotein localisation was confined to cellular membrane extracts, the expression of P-glycoprotein was also studied in nuclear extracts of the DLKP and DLKA10 cell lines. Nuclear extracts were prepared, as outlined in section 2.7.3 and western blot analysis carried out using the anti-P-glycoprotein monoclonal antibody, C219. The results obtained are presented in figure 3.9.2. The overexpression of P-glycoprotein in the nuclear extracts was comparable to the level detected in the membrane preparations. Strong immunoreactivity was observed in the nuclear extracts of the DLKPA10 cells, while only a very low level was detected in the parental DLKP cells. Western blot analysis was also carried out on membrane preparations of both cell line to compare the expression of P-glycoprotein in the different cellular extracts. The results show that the level of P-glycoprotein overexpression in the nuclear extracts was comparable to that detected in the DLKPA10 membrane preparations.

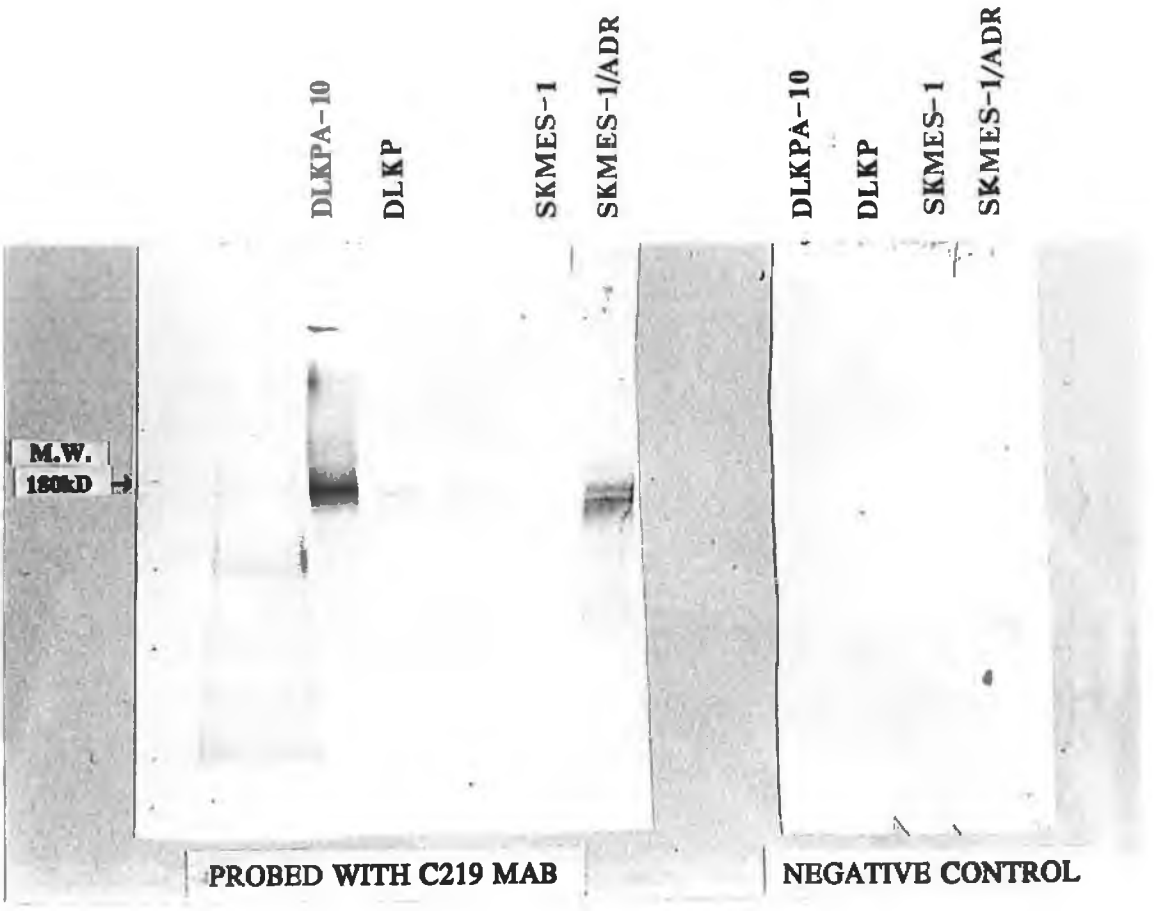



**Figure 3.9.1** Western blot detection of P-glycoprotein in cell membrane preparations of the DLKP, DLKPA10, SKMES-1 and SKMES-1/ADR cell lines with the C219 monoclonal antibody.

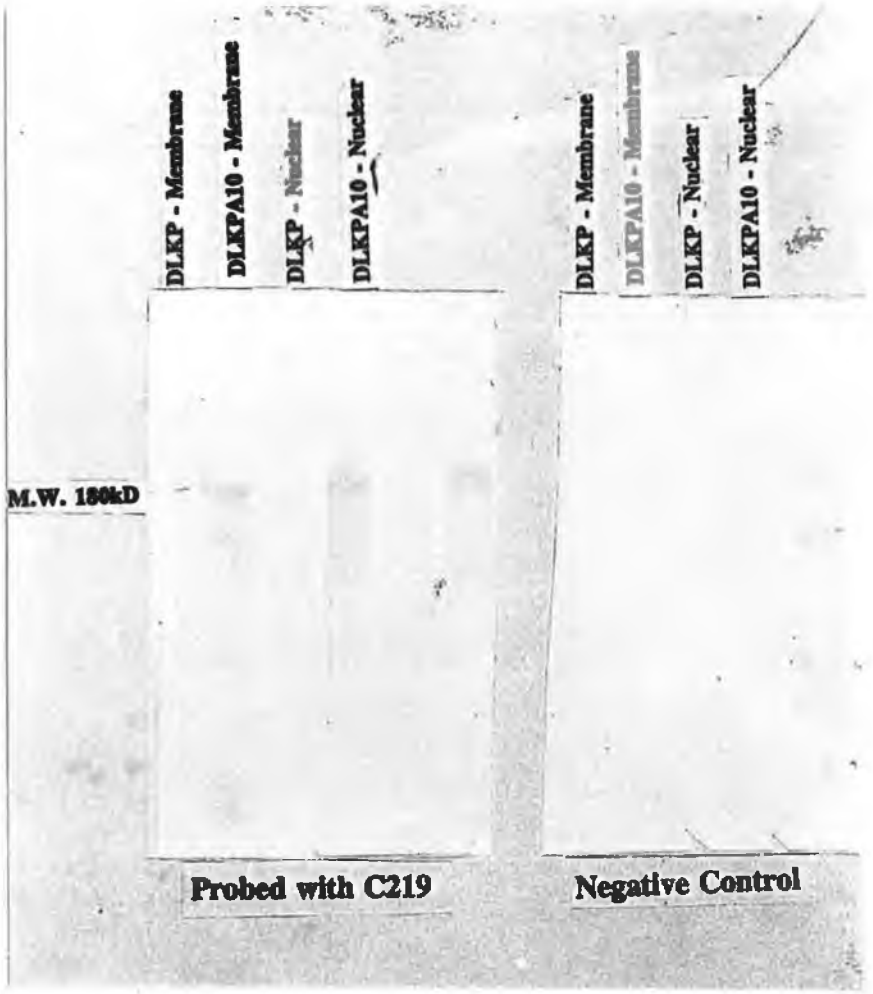



**Figure 3.9.2** Western blot detection of P-glycoprotein in cell membrane and nuclear extract preparations of the DLKP and DLKPA10 cell lines with the C219 monoclonal antibody.

### 3.10 Adriamycin accumulation in SKMES-1 and SKMES-1/ADR cell lines

The cellular concentration of adriamycin in the SKMES-1 and SKMES-1/ADR cells was determined after various incubation time periods, as described in section 2.10.1. The effect of a number of compounds on adriamycin accumulation in both cell lines was also investigated.

Figure 3.10.1 illustrates the time course of adriamycin accumulation in the parental SKMES-1 and the multidrug resistant SKMES-1/ADR cell lines. The results show that over a four hour time period an increase in the accumulation of adriamycin in the SKMES-1 cells was observed up to three hours incubation, after which time a steady state of arimaycin uptake was reached. Adriamycin accumulation was significantly reduced in the SKMES-1/ADR cells relative to the parental cell line. The SKMES-1 cells accumulated approximately 525 pmoles of adriamycin per $10^6$ cells within four hours. However, only 135 pmoles of adriamycin accumulated per $10^6$ cells was noted in the SKMES-1/ADR cells within the same time period. The rate of accumulation was greatest within the first hour in the SKMES-1/ADR cells following the addition of drug, after which time a steady rate was observed. No further increase in adriamycin accumulation was noted in the SKMES-1/ADR cells following exposure to the drug for longer incubation periods.

#### 3.10.1 Effect of verapamil on adriamycin accumulation

The effect of the calcium channel antagonist, verapamil, on adriamycin accumulation was investigated in the SKMES-1 and the SKMES-1/ADR cell lines. An initial concentration response assay was carried out, over a range of 0 - 100$\mu$g/ml, and a suitable working concentration of verapamil was determined. When the cells were coincubated with verapamil (30$\mu$g/ml) a slight increase in the cellular concentration of adriamycin was observed in the parental cell line within a four hour time period, although verapamil did not significantly alter the rate of accumulation. The addition of verapamil to the SKMES-1/ADR cells resulted in an marked increase in the rate of adriamycin accumulation and also in the cellular concentration of the drug. Verapamil reversed the accumulation defect in this cell line and restored the maximum level of adriamycin accumulated to that observed in the parental SKMES-1 cell line (Figure 3.10.1).

### 3.10.2 Effect of cyclosporin A on adriamycin accumulation

The effect of the immunosupressive agent, cyclosporin A (10$\mu$g/ml), on adriamycin accumulation was also studied in the SKMES-1 and SKMES-1/ADR cells. A concentration response curve was established, over the range of 0 - 100$\mu$g/ml and a suitable working concentration was determined. Cotreatment with cyclosporin A resulted in an increase in the cellular concentration of adrimaycin over the four hour time period studied. Cyclosporin A was found to be more effective than verapamil at reversing the accumulation defect in the SKMES-1/ADR cells. The presence of cyclosporin A enhanced adriamycin accumulation in the SKMES-1/ADR cells to a level greater than that observed in the SKMES-1 cell line (figure 3.10.1). The addition of cyclosporin A also caused a slight increase in the cellular concentration of adriamycin in the parental cells .

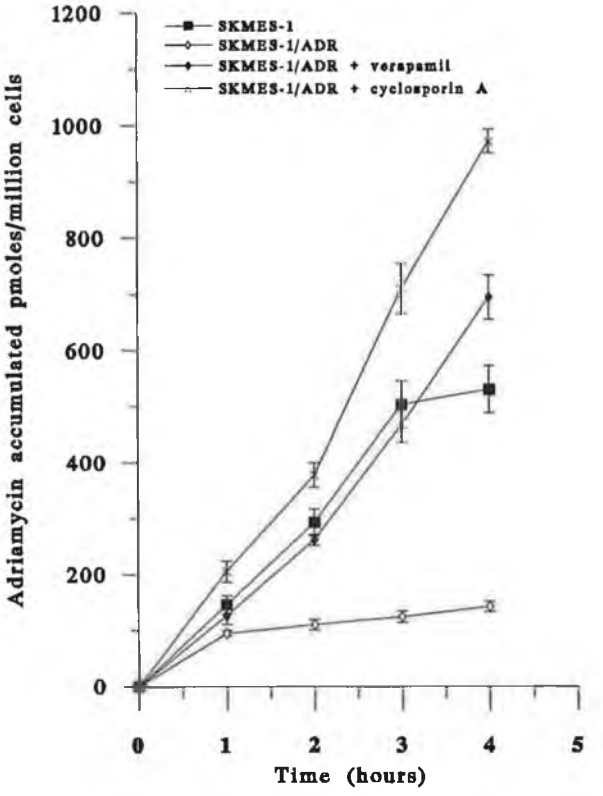



**Figure 3.10.1** The time course of adriamycin accumulation in the SKMES-1 and SKMES-1/ADR cell lines. The effect of verapamil (30$\mu$g/ml) and cyclosporin A (10$\mu$g/ml) on adriamycin accumulation in SKMES-1/ADR cells.

### 3.10.3 Effect of energy inhibition on adriamycin accumulation

The effect of sodium azide (an inhibitor of oxidative phosphorylation) and 2-deoxy-D-glucose (an inhibitor of glycolysis) on adriamycin accumulation in the SKMES-1 and SKMES-1/ADR cell lines was studied, to determine if the accumulation defect observed in the SKMES-1/ADR cells could be restored by the inhibition of ATP production. Adriamycin accumulation was first determined in the presence of glucose free medium, containing sodium azide (10mM), over a time course of three hours, as described in section 2.10.1. An increase in adriamycin accumulation was observed in the SKMES-1 cell line in the presence of sodium azide when compared to treatment with adriamycin alone. When incubated with sodium azide, the maximum cellular concentration of adriamycin was 1 nmole per $10^6$ cells after three hour incubation. The SKMES-1 cells accumulated approximately 600 pmoles adriamycin per $10^6$ cells when incubated with adriamycin for the same time period. A marked increase in adriamycin accumulation was observed in the SKMES-1/ADR cells following treatment with sodium azide. Figure 3.10.3 illustrates that the accumulation defect was restored in the resistant cells and that the cellular concentration of drug was comparable to the level observed in the SKMES-1 parental cell.

The addition of 2-deoxy-D-glucose (25mM) resulted in a slight decrease in adriamyicn accumulation in the SKMES-1 parental cells. However, when the SKMES-1/ADR cells were exposed to 2-deoxy-D-glucose, an increase in the uptake of adriamycin to a level comparable to the parental cell line was again observed.

### 3.10.4 Adriamycin efflux in SKMES-1 and SKMES-1/ADR cell lines

Adriamycin efflux was studied in the SKMES-1 and SKMES-1/ADR cells after the cells were preloaded with adriamycin in the presence of sodium azide (10mM) for three hours, as described in section 2.10.2. Figure 3.10.3 illustrates adriamycin efflux in the SKMES-1 and SKMES-1/ADR cell lines over a period of four hours. The results obtained demonstrated that when the cells were reintroduced to drug free complete medium, a marked decrease in the cellular concentration of adriamycin was observed in the SKMES-1/ADR cells within the first hour. Over the next two hours a slight decrease was recorded, after which time the cellular concentration had reached a level of approximately 75 pmoles

per $10^6$ cells. Drug efflux was also observed within the first hour in the parental SKMES-1 cells when the cells were reintroduced into drug free medium, although the decrease in cellular concentration of adriamycin was not as marked as in the SKMES-1/ADR cell line. A further decrease in adriamycin accumulation was noted within the next hour, after which time the cellular concentration of adriamycin reached a steady level of approximately 900 pmoles per $10^6$ cells.

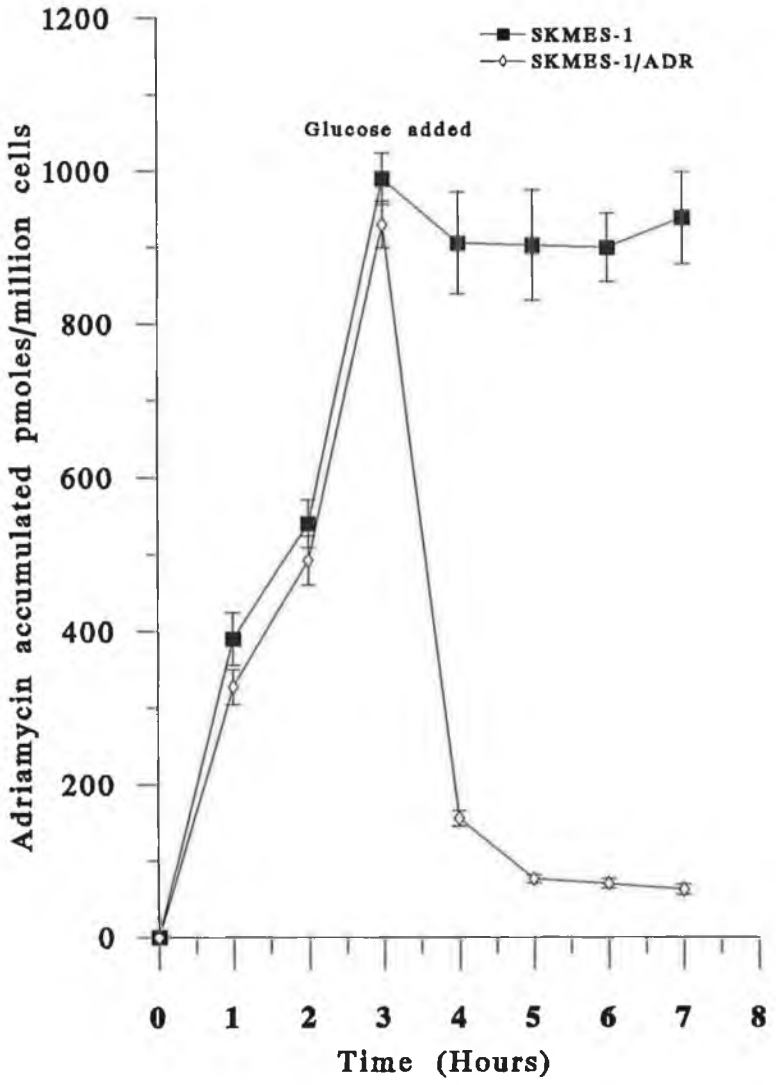



**Figure 3.10.3** Adriamycin accumulation and efflux in the SKMES-1 and SKMES-1/ADR cell lines. The cells were preloaded with adriamycin in glucose free medium, containing sodium azide (10mM), for 3 hours and then washed and incubated in drug free complete medium. Drug efflux was studied over a period of four hours.

### 3.10.5 Vincristine accumulation in SKMES-1 and SKMES-1/ADR cell lines

Vincristine accumulation was also studied in the SKMES-1 and SKMES-1/ADR cells over a period of three hours. The results obtained illustrated a decrease in the cellular concentration of vincristine in the SKMES-1/ADR, relative to the parental cell line. A linear increase in vincristine accumulation was observed in the SKMES-1 parental cells, within the three hour period studied. An increase in vincristine accumulation was also noted in the SKMES-1/ADR cells up to a thirty minute time period, although after this time a steady state of accumulation of approximately 0.3 pmoles per $10^6$ cells was reached. Accordingly, a decrease of approximately six fold was observed in the resistant cells within the time period studied. When the cells were coincubated with cyclosporin A (10$\mu$g/ml), a marked increase in vincristine accumulation was noted in the SKMES-1/ADR cells. The cellular concentration of vincristine in the resistant cells after three hour incubation with cyclosporin A was over two fold greater than the concentration in the parental cells which were exposed to vincristine for the same time period (figure 3.10.5). The effect of the carboxylic ionophore, monensin, on vincristine accumulation was also investigated in the SKMES-1/ADR cells. The results obtained illustrated that, although monensin was not as effective as cyclosporin A at enhancing vincristine accumulation in the resistant cells, the addition of monesnin completely reversed the accumulation defect observed in the cells. The maximun level of drug accumulated within the three hour period studied was greater than the level observed in the parental cells.

### 3.10.6 Effect of cell density on adriamycin accumulation in SKMES-1 and SKMES-1/ADR cell lines

The uptake of adriamycin in the SKMES-1 and SKMES-1/ADR cell lines at different cells density was investigated to determine if drug accumulation was cell density dependent. The cells were plated at two different cell densities, $7.5x10^4$ and $2x10^5$ cells per well and incubated for approximately 48 hours. The cells were exposed to adriamycin for two and four hour time periods and the cellular concentration of adriamycin was determined, as described in section 2.10.1. Both the SKMES-1 and the SKMES-1/ADR cell lines were found to be cell density dependent, when plotted as pmoles adriamycin accumulated per $10^6$ cells (figure 3.10.6). The results illustrated that when the cells were plated at a low seeding density ($7.5 \times 10^4$ cells per well) the amount of adriamycin accumulated per $10^6$ cells was

greater than that observed when the cells were plated at a higher density (2 x $10^5$ cells per well). As the cell density was increased the cellular concentration of adriamycin per $10^6$ cells decreased.

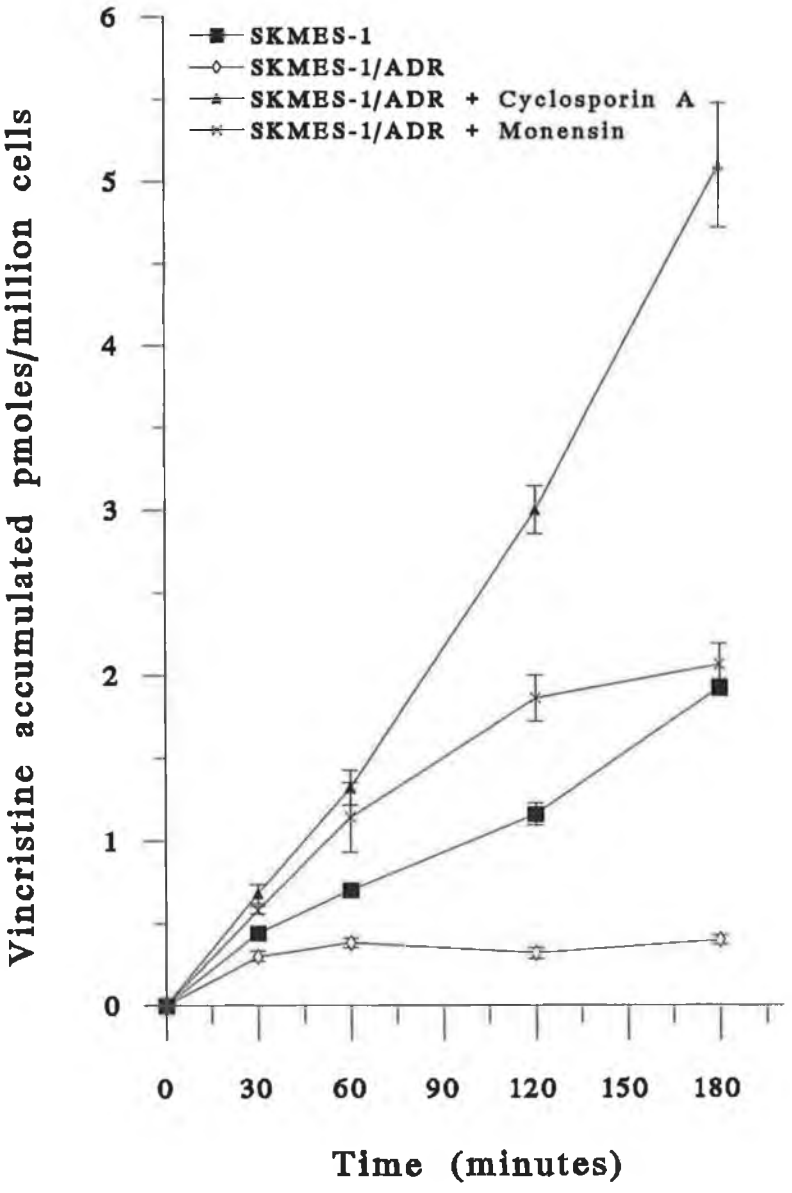
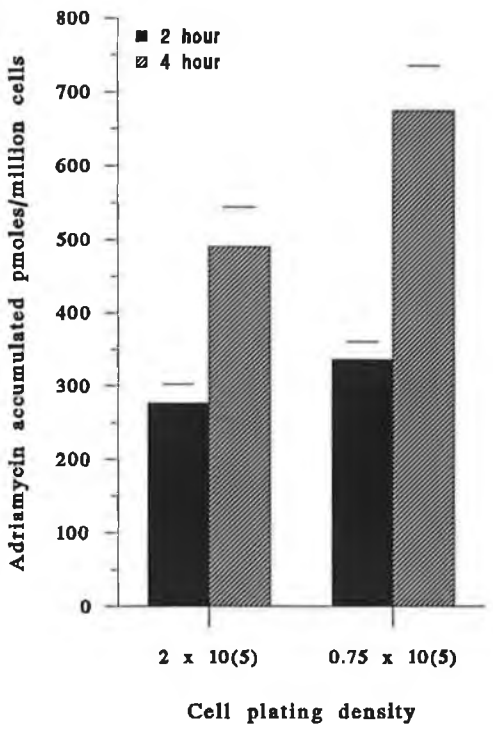



**Figure 3.10.5** The time course of vincristine accumulation in the SKMES-1 and SKMES-1/ADR cell lines. The effect of cyclosporin A (10$\mu$g/ml) and monensin (10$\mu$g/ml) on vincristine accumulation in SKMES-1/ADR cells.

a.




b.

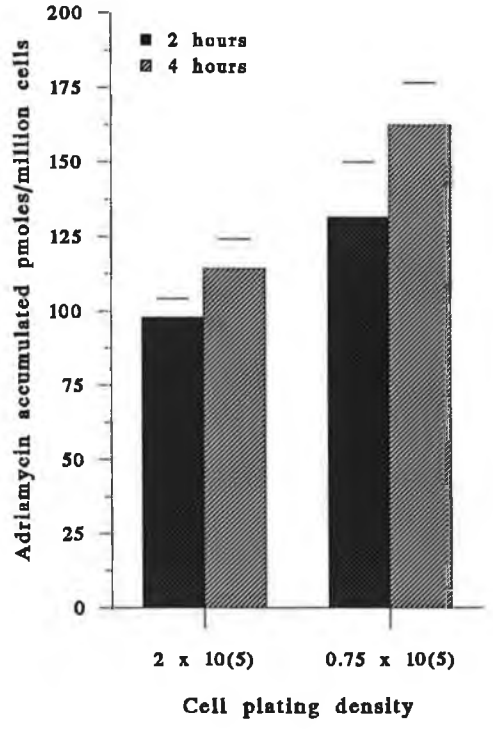



**Figure 3.10.6** The effect of cell plating density on adriamycin accumulation in the SKMES-1 (a) and SKMES-1/ADR (b) cell lines. The cells were plated at a concentration of $2x10^5$ and $0.75x10^5$ cells per well and adriamycin accumulation determined after 48 hours.

## 3.11 Drug accumulation studies in DLKP and DLKPA10 cell lines

### 3.11.1 Adriamycin accumulation in DLKP and DLKPA10 cell lines

Adriamycin accumulation was investigated in the DLKP and DLKPA10 cell lines to determine if these cell lines exhibited similar accumulation patterns to those observed in the SKMES-1 and SKMES-1/ADR cell lines. The effect of a number of compounds on adriamycin accumulation was also studied. Figure 3.11.1 represents the time course of adriamycin accumulation in the DLKP and DLKPA10 cells. An increase in accumulation was observed in the DLKP cells within the four hours studied, with maximum accumulation of approximately 950 pmoles per $10^6$ cells. A marked reduction in adriamycin accumulation was observed in the DLKPA10 cell line, with respect to the parental DLKP cells. A decrease of approximately 16-fold in the cellular concentration of adriamycin was observed in the DLKPA10 cells. A maximum accumulation level of approximately 60 pmoles per $10^6$ cells was reached after two hour exposure. No further increase in accumulation was noted in the DLKPA10 cell lines at longer exposure times. Studies were carried out on the DLKP and DLKPA10 cell lines to determine the time point at which the cellular concentration of adriamycin was equivalent in both cell lines. Adriamycin accumulation was determined in the parental cells over a time period of 0 - 30 minutes and over a time period of 0 - 4 hours in the DLKPA10 cell lines. The cellular concentration of adriamycin in the DLKPA10 cells after a 4 hour incubation period was approximately 75 pmoles per $10^6$ cells. When the DLKP cells were exposed to adriamycin, rapid uptake of the drug was observed in the cells. The cellular concentration of adriamycin in the DLKP cells after a five minute incubation period was comparable to the level observed in the DLKPA10 cells after four hours. After five minutes the concentration of adriamycin in the DLKP cells was found to be 79 pmoles per $10^6$ cells.

#### 3.11.1.1 Effect of verapamil on adriamycin accumulation

The addition of verapamil (30$\mu$g/ml) resulted in a slight increase in the maximum cellular concentration of adriamycin in the DLKP cell line within the time period studied, although no significant alteration in the rate of accumulation was observed. Verapamil also increased adriamycin accumulation in the DLKPA10 cell line but only to a level of approximately 45% of that observed in the parental cells after four hour exposure (figure 3.11.1). The

accumulation defect could not be reversed in the DLKPA10 cell lines following longer incubation periods with verapamil, or at higher concentrations of verapamil (up to 100$\mu$g/ml verapamil was studied).

### 3.11.1.2 Effect of cyclosporin A on adriamycin accumulation

When the cells were incubated with cyclosporin A (10$\mu$g/ml), the maximum adriamycin accumulation was, again, slightly enhanced in the parental DLKP cells but the rate of accumulation was unaltered within the four hour time period. However, in the DLKPA10 cell line, the addition of cyclosporin A resulted in an approximate 10-fold increase in adriamycin accumulation after four hour exposure and, therefore, was more effective than verapamil (Figure 3.11.1). No further increase in the cellular concentration of adriamycin in the DLKPA10 cells was observed following exposure to increasing concentration of cyclosporin (up to 100$\mu$g/ml).

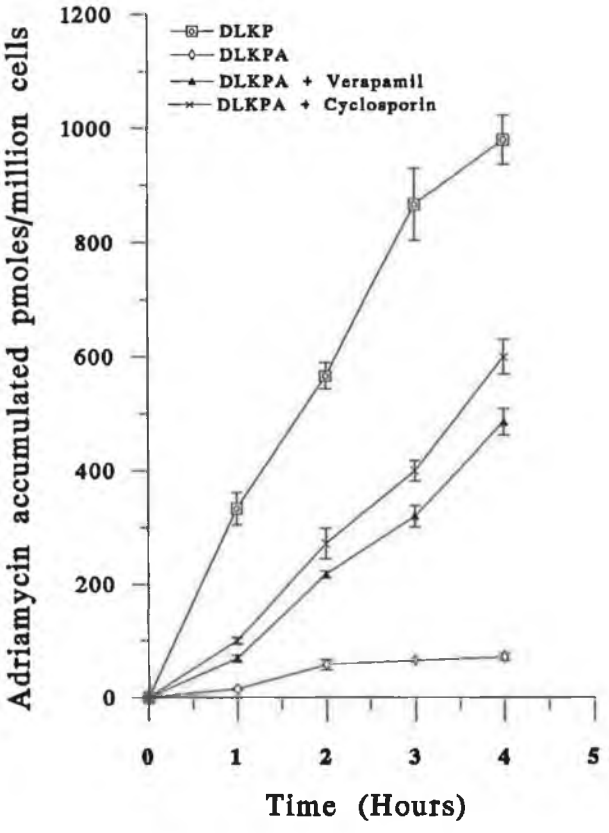



**Figure 3.11.1** The time course of adriamycin accumulation in DLKP and DLKPA10 cell lines. The effect of verapamil (30$\mu$g/ml) and cyclosporin A (10$\mu$g/ml) on adriamycin accumulation in DLKPA10 cells.

### 3.11.1.3 Effect of cyclosporin A pretreatment on adriamycin accumulation

The cellular concentration of adriamycin was determined in the DLKPA10 cells, following pretreatment with cyclosporin A (10$\mu$g/ml) for two hours. The aim of this work was to determine if pretreatment was more effective than cotreatment with cyclosporin A at restoring the accumulation defect observed in the DLKPA10 cells. Figure 3.11.1.3 illustrates the time course of adriamycin accumulation in the DLKPA10 cells following pretreatment and cotreatment with cyclosporin A. The results show that, although cyclosporin A pretreatment increased accumulation, it did not fully restore the accumulation defect in the DLKPA10 cell line. Cyclosporin A pretreatment was, however, found to be more effective at increasing the cellular concentration of drug.

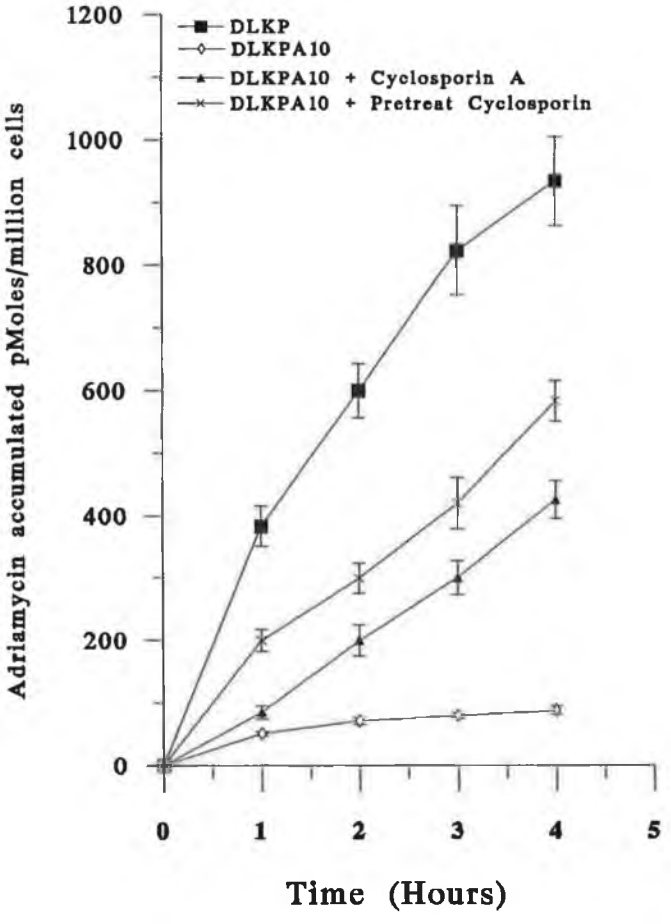



**Figure 3.11.1.3** The effect of cyclosporin A pretreatment on adriamycin accumulation in DLKPA10 cells. The cells were pretreated with cyclosporin A (10$\mu$M) for two hours prior to the addition of adriamycin (10$\mu$M).

### 3.11.1.4 Effect of energy inhibition on adriamycin accumulation

The effect of the energy inhibitors sodium azide, 2-deoxy-D-glucose and antimycin A on adriamycin accumulation in the parental DLKP and resistant DLKPA10 cells was studied to determine if the accumulation defect observed in the DLKPA10 cell line could be reversed by inhibition of ATP production. The cells were incubated in glucose free medium, containing either sodium azide (10mM), 2-deoxy-D-glucose (25$\mu$g/ml) or antimycin A (10$\mu$M). Adriamycin accumulation was then determined over a period of three hours (figure 3.11.1.4). Following exposure to sodium azide, an increase in the maximum level of adriamycin accumulated was observed in the DLKP cells, although the rate of accumulation was unaltered. The cellular concentration of adriamycin in the presence of sodium azide was found to be approximately 660 pmoles adraimycin per $10^6$ cells after three hour exposure, in comparison to 444 pmoles accumulated per $10^6$ cells following incubation in standard culture medium. Coincubation with sodium azide resulted in a 2.5-fold increase in adriamycin accumulation in the DLKPA10 cells within the same time period. Adriamycin accumulation in the DLKPA10 cells was determined over a concentration range of sodium azide (5 - 50mM). However, it was found that the accumulation defect could not be reversed at higher concentrations. When the cells were exposed to sodium azide at concentations greater than 10mM cell lysis was observed in both the DLKP and DLKPA10 cell lines.

When the effect of 2-deoxy-D-glucose on adriamycin accumulation in DLKP and DLKPA10 cells was investigated, the results were similar to those obtained with sodium azide. A slight increase in adriamycin accumulation was observed in the DLKP parental cells following three hour exposure to the drug. Coincubation with 2-deoxy-D-glucose resulted in an increase of approximately 2-fold in adriamycin accumulation in the DLKPA10 cells, thus indicating that it was not as effective as sodium azide at enhancing the cellular concentration of drug. 2-deoxy-D-glucose proved to be more toxic than sodium azide to the cells within the time period studied. The accumulation of adriamycin in the presence of antimycin A was also investigated in the DLKP and DLKPA10 cell lines. The addition of 10$\mu$M antimycin A resulted in an increase of greater than 2-fold in the cellular accumulation of adriamycin in the parental DLKP cells. The DLKP cells accumulated approximately 444 pmoles of adriamycin per $10^6$ cells within the three hour period, while the subcellular concentration was approximately 980 pmole per $10^6$ cells following incubation with antimycin A. Antimycin A was even more effective at enhancing the

cellular levels of adriamycin in the DLKPA10 cells. Following treatment with antimycin A, an 8.5-fold increase in the level of adriamycin accumulation was noted in the DLKPA10 cells. The cellular concentration of adriamycin was approximately 70 pmoles per $10^5$ cells after a three hour incubation period, while the subcellular concentration was approximately 590 pmoles following incubation of the cells in the presence of antimycin A. Accordingly, antimycin A was shown to completely restore adriamycin accumulation in the DLKPA10 cells to a level comparable to that observed in the parental DLKP cell line.

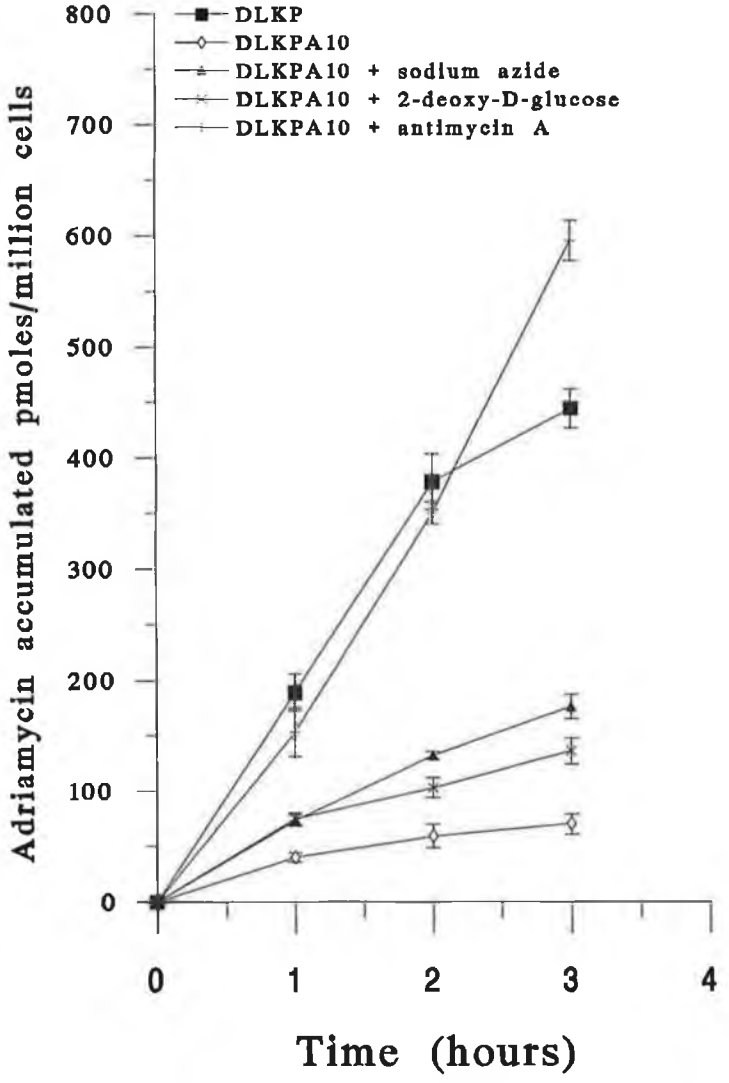



**Figure 3.11.1.4** The effect of sodium azide (10mM), 2-deoxy-D-glucose (25$\mu$g/ml) and antimycin A (10$\mu$M) on adriamycin accumulation in DLKPA10 cells.

### 3.11.1.5 Effect of cotreatment with cyclosporin A and sodium azide on adriamycin accumulation

The effect of cotreatment with cyclosporin A and sodium azide on adriamycin accumulation was studied in the DLKP and DLKPA10 cells. When the cells were incubated in glucose free medium, containing cyclosporin A (10$\mu$g/ml) and sodium azide (10mM), a decrease in adriamycin accumulation was observed in the DLKP parental cells. The cellular concentration of adriamycin, following four hour incubation, was approximately 750 pmoles per $10^6$ cells with cotreatment, as opposed to 920 pmoles adriamycin per $10^6$ cells when the cells were treated with adriamycin alone. Cotreating the DLKPA10 cells with cyclosporin A and sodium azide resulted in an increase in adriamycin accumulation from approximately 70 pmoles per $10^6$ cells to 550 pmoles per $10^6$ cells, representing an increase to approximately 60% of the adriamycin accumulated in the DLKP cells (figure 3.11.1.5).

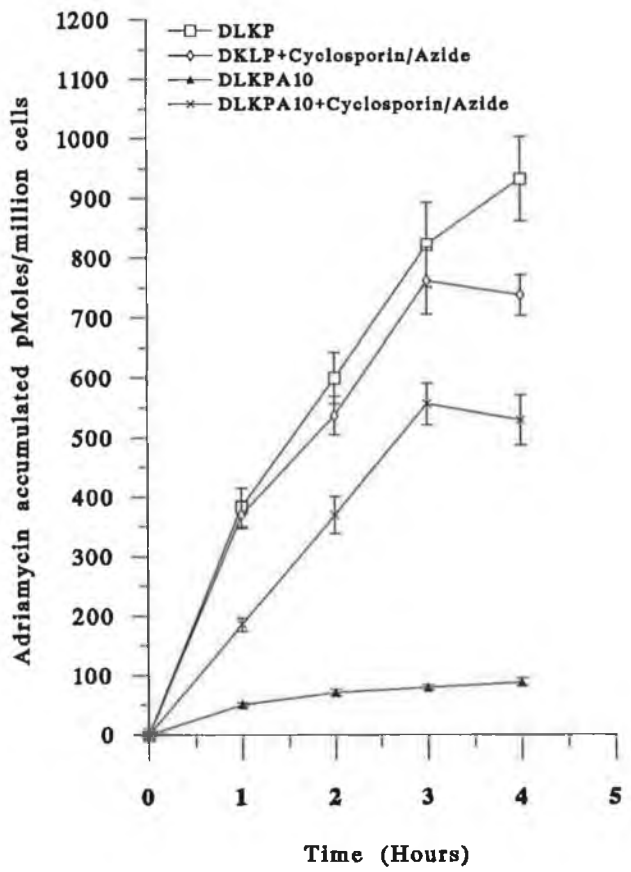


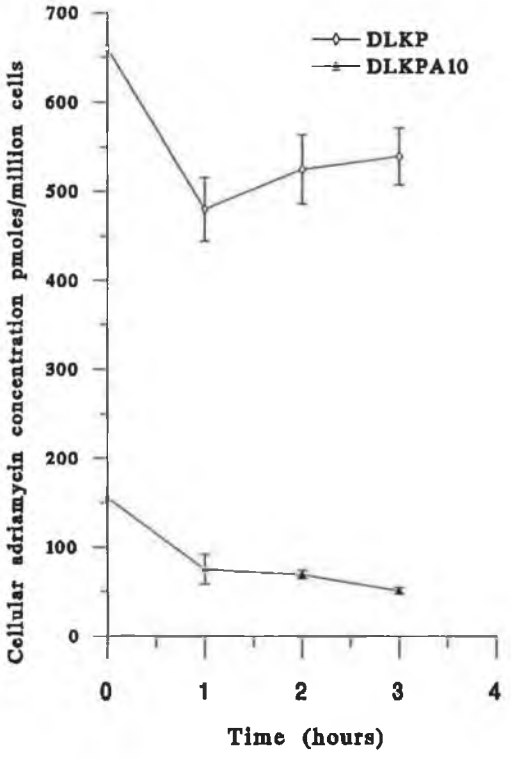

**Figure 3.11.1.5** The effect of cotreatment with cyclosporin A (10$\mu$g/ml) and sodium azide (10mM) on adriamycin accumulation in DLKP and DLKPA10 cells.

### 3.11.2 Adriamycin efflux in DLKP and DLKPA10 cell lines

The efflux of adriamycin from the DLKP and DLKPA10 cells was studied over a period of three hours, following preloading of the cells for three hours in glucose free medium, containing either sodium azide or antimycin A (Figure 3.11.6). When the cells were coincubated with sodium azide (10mM), the cellular accumulation of adriamycin in the DLKP and DLKPA10 cell lines after three hours was approximately 660 pmoles per $10^6$ cells and 150 pmoles per $10^6$ cells respectively. When the drug was removed and the cells incubated in drug free complete medium, a rapid efflux of adriamycin was observed in the DLKP cells within the first hour. After this time the cellular concentration of adriamycin increased slightly and then a steady rate was reached over the next two hours. Rapid drug efflux was also noted in the DLKPA10 variant within the first hour when the cells were reintroduced into drug free medium. Over the next two hours there was a slight decrease in the cellular concentration of adriamycin to a level comparable with that observed in the time course assay (figure 3.11.2.1).

Following coincubation with antimycin A (10$\mu$M), the cellular concentration of adriamycin in the DLKP and DLKPA10 cells was approximately 1.4 nmoles per $10^6$ cells and 1 nmole per $10^6$ cells respectively. When the drug was removed, a decrease in the cellular drug concentration was observed in both cell lines, although a greater decrease was noted in the DLKPA10 cells. Within the first 60 minutes, rapid efflux of adriamycin from the DLKPA10 cells was observed, after which time a slower rate of efflux was noted. After three hour incubation in drug free medium, the cellular concentration of adriamycin in the DLKPA10 cells was approximately 200 pmoles per $10^6$ cells. A decrease in the maximun level of cellular adriamycin was also noted in the DLKP parental cells when incubated in drug free medium, although the efflux rate was slower than that observed in the DLKPA10 cells. The cellular concentration of adriamycin was approximately 800 pmoles per $10^6$ cells following three hour incubation in drug free medium (figure 3.11.2.1).

a.




b.

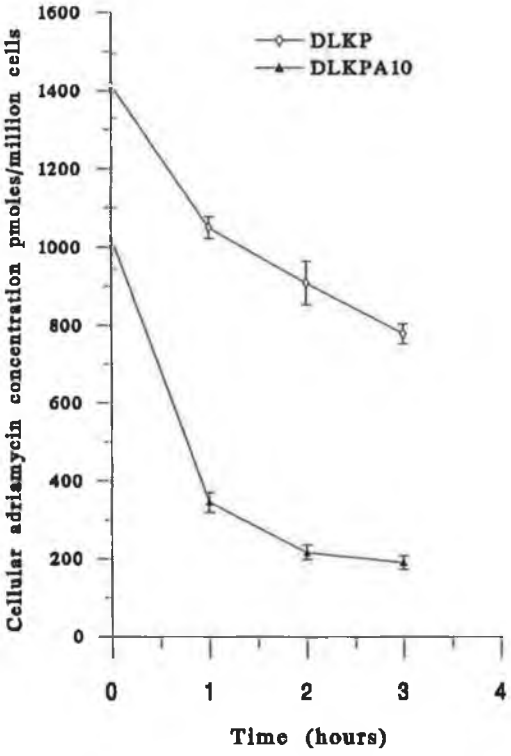



**Figure 3.11.2.1** Adriamycin efflux in DLKP and DLKPA10 cell lines following preloading with (a) sodium azide (10mM) and (b) antimycin A (10$\mu$M) for three hours.

### 3.11.2.1 The effect of cyclosporin A on adriamycin efflux in DLKP and DLKPA10 cells

The effect of cyclosporin A (10$\mu$g/ml) on adriamycin efflux was studied in the DLKP and DLKPA10 cell lines, following preloding with antimycin A (40$\mu$g/ml) for three hours. Figure 3.11.2.1 illustrates the efflux pattern observed in both the DLKP and DLKPA10 cells in the presence of cyclosporin A. The results show that cyclosporin A did not significantly alter adriamycin efflux in the parental cells within the first two hours studied. Although, after this time an increase in the cellular concentration of ariamycin was noted in the presence of cyclosporin A. A marked decrease in adriamycin efflux was observed in the DLKPA10 cells when incubated with cyclosporin A. The cellular concentration of drug after three hours was approximately 2.5-fold greater than that observed in the absence of the circumvention agent.

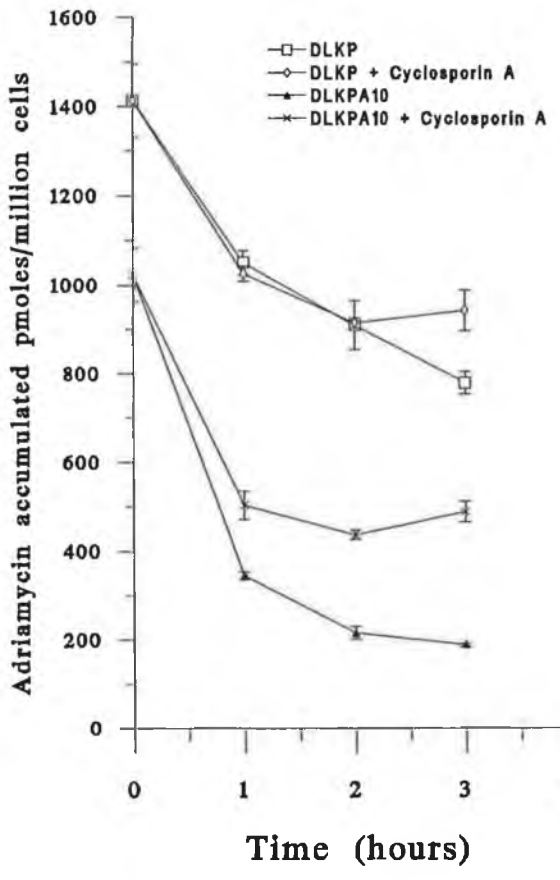



**Figure 3.11.2.1** The effect of cyclosporin A (10$\mu$g/ml) on adriamycin efflux in DLKP and DLKPA10 cells lines. The cells were preloaded in the presence of antimycin A (10$\mu$M) for three hours and efflux was studied over a further three hours

### 3.11.3 Vincristine accumulation in the DLKP and DLKPA10 cell lines

Vincristine accumulation was also studied in the DLKP and DLKPA10 cells, over a period of three hours. The results obtained illustrated a marked reduction in the cellular concentration of vincristine in the DLKPA10 cells, relative to the parental cells (figure 3.11.3). Vincristine accumulation was shown to increase with increasing incubation time in the DLKP cells within the three hours studied, with the maximum level of drug accumulation of approximately 4 pmoles per $10^6$ cells. In contrast, the maximum level of vincristine accumulated in the DLKPA10 cells after the same time exposure was only approximately 0.25 pmoles per $10^6$ cells, representing a 16-fold decrease in drug accumulation in the resistant cells. The addition of verapamil enhanced vincristine accumulation in the DLKPA10 cells, although only a 3.6-fold increase in the cellular concentration of drug was observed. An increase in vincristine accumulation was also noted in the parental cells following treatment with cyclosporin A. Cyclosporin A was found to more effective than verapamil at increasing drug accumulation, in both the DLKP and DLKPA10 cells. Although treatment with cyclosporin A resulted in a greater enhancement of vincristine accumulation, the maximum cellular concentration of drug was still substantially less than that observed in the parental cells. The cellular concentration of drug after three hours was approximately 1.25 pmoles per $10^6$ cells, representing a 5-fold increase in vincristine accumulation in the resistant cells. No further increase in accumulation was observed following longer exposure to the drug.

### 3.11.4 Vincristine efflux in the DLKP and DLKPA10 cell lines

The efflux of vincristine from the DLKP and DLKPA10 cells was studied, following preloading of the cell in the presence of antimycin (10$\mu$M) for two hours. When the cells were reintroduced to drug free complete medium, a marked decrease in drug retention was observed in both the DLKP and DLKPA10 cell lines within the first 60 minutes. After this time, a steady level of drug retention was reached in the DLKP cells. However, in the DLKPA10 cells, a further decrease was observed within the second hour, although a slower rate of drug efflux was noted (figure 3.11.4). The cellular concentration of vincristine in the DLKPA10 cells was comparable to the level observed following two hour exposure to the drug (figure 3.11.3). The addition of cyclosporin A resulted in an increase in drug retention in the DLKPA10 cells of approximately 3.6-fold after two hours. A significant

decrease in the rate of drug efflux was also observed in the DLKPA10 cells, following treatment with cyclosporin A. The addition of cyclosporin A did not significantly alter either the rate of drug efflux or the retention of drug in the DLKP cells within the first 60 minutes, although after this time a decrease in vincristine retention was observed.

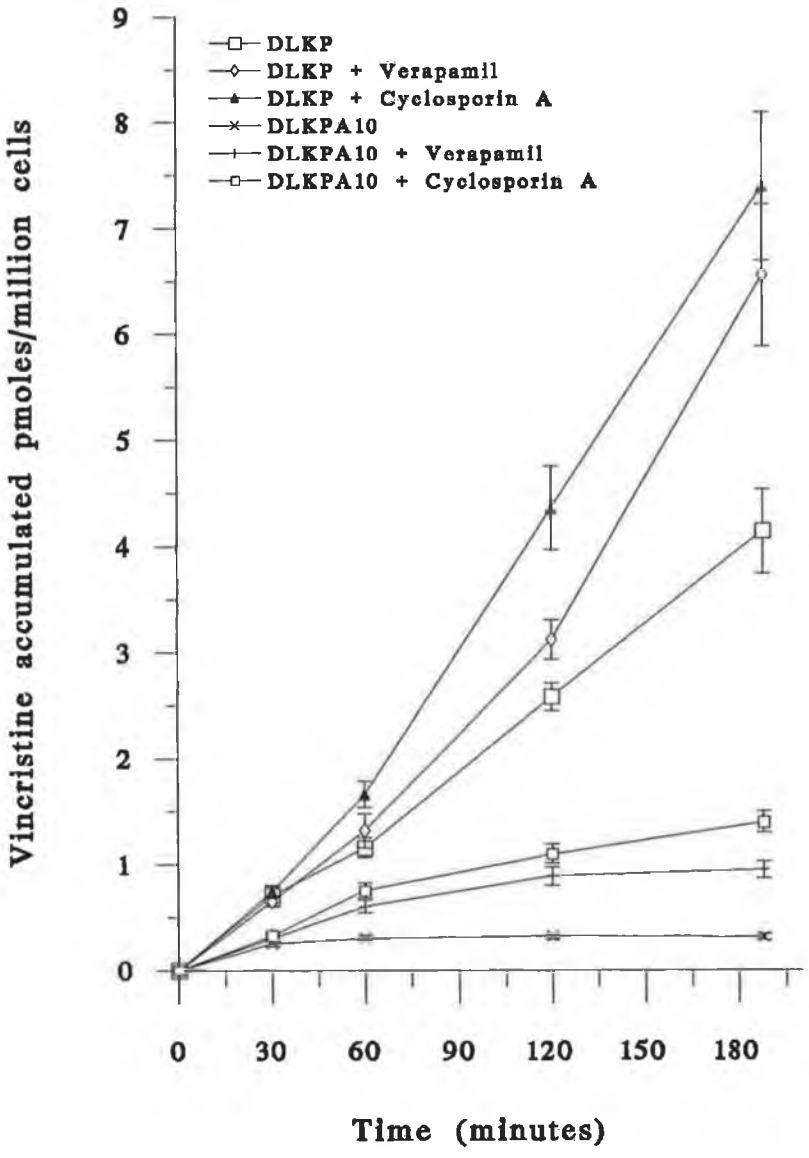



**Figure 3.11.3** The effect of verapamil (30$\mu$g/ml) and cyclosporin A (10$\mu$g/ml) on the time course of vincristine accumulation in the DLKP and DLKPA10 cell lines.

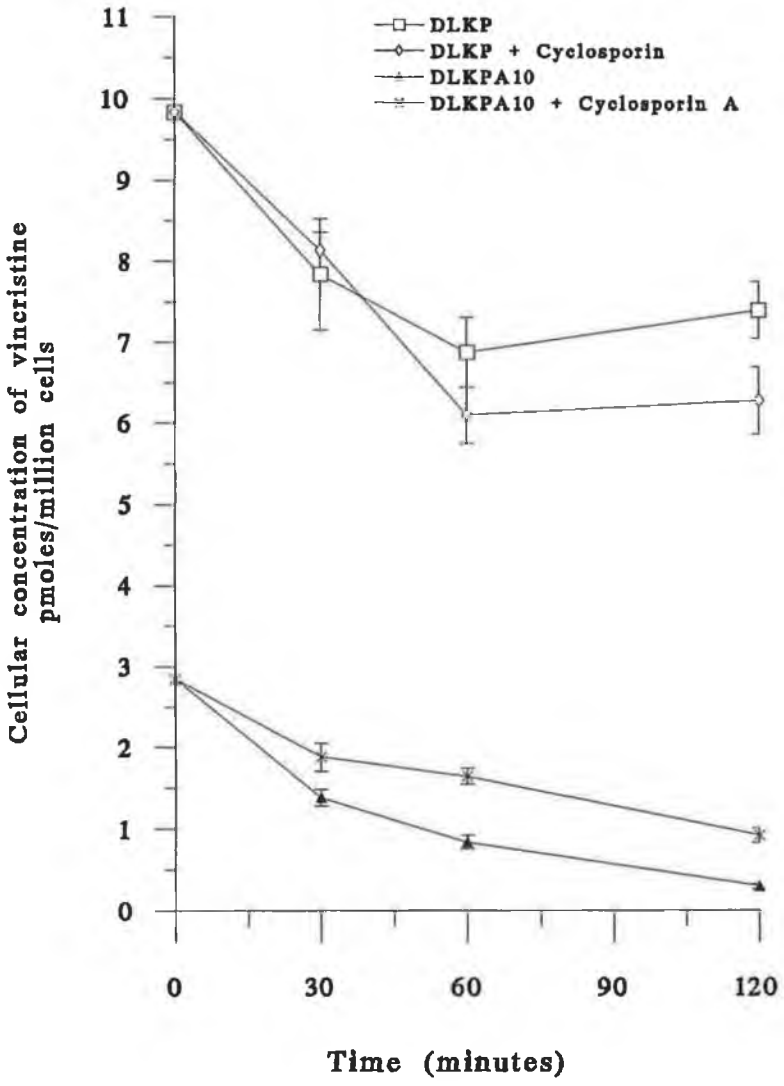
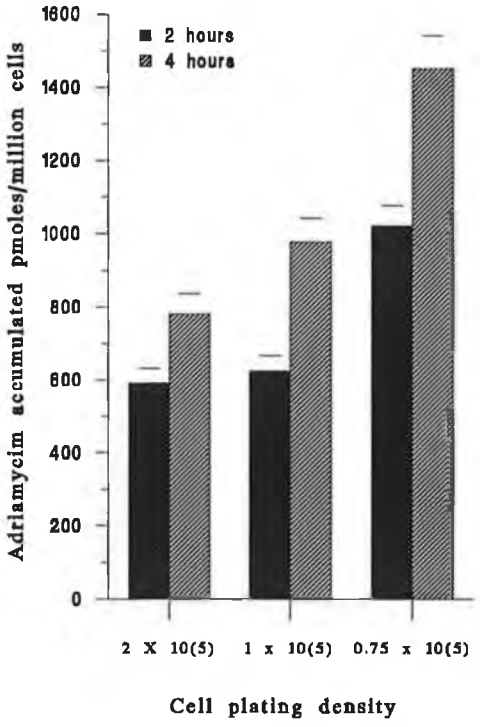



**Figure 3.11.4** The effect of cyclosporin A (10$\mu$g/ml) on vincristine efflux in the DLKP and DLKPA10 cell lines. The cells were preloaded in the presence of antimycin A (10$\mu$M) for two hours, washed and incubated in drug free medium.

### 3.11.5 Effect of cell density on adriamycin accumulation in DLKP and DLKPA10 cell lines

Adriamycin accumulation was determined in the DLKP and DLKPA10 cells at three different cell plating densities to establish if drug accumulation was cell density dependent. The cellular concentration of adriamycin was deterined following two and four hour incubation periods. The results obtained are presented in figure 3.11.5. Drug uptake in both DLKP and DLKPA10 cell lines was found to be cell density dependent, within the time course studied. When the DLKP cells were plated at a low cell density ($7.5 \times 10^4$ cells per well) approximately 1.5 nmoles adriamycin was accumulated per $10^6$ cells within four hours as opposed to 780 pmoles accumulated per $10^6$ cells when plated at a high density ($2 \times 10^5$ cells per well). Therefore, a two fold increase was observed in adriamycin accumulation in the low cell plating density, relative to the high cell plating density. Figure 3.11.5 also illustrates that, as the cell density increased, the level of adriamycin accumulated per $10^6$ cells decreased. Similar results were noted in the DLKPA10 cell when plated at different cell densities. Following four hours incubation, approximately 240 pmoles adriamycin was accumulated per $10^6$ cells when the cell were plated at a low density ($7.5 \times 10^4$ cells per well). However, when the cells were plated at a higher density ($2 \times 10^5$ cells per well) only 96 pmoles adriamycin was accumulated per $10^6$ cells, within the same time period.

a.

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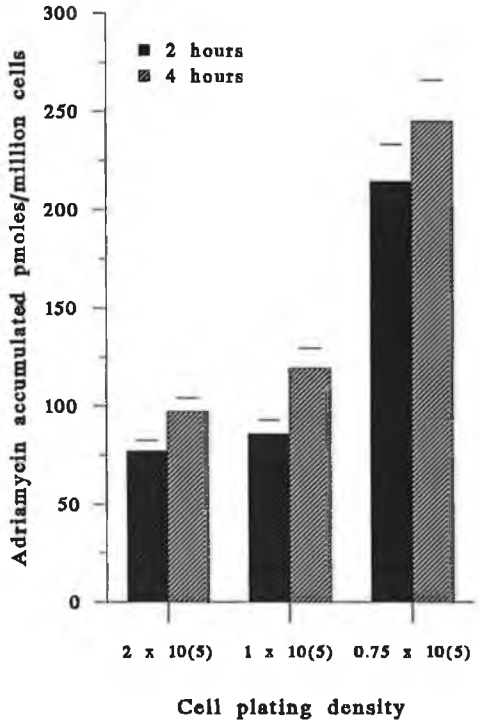



**Figure 3.11.5** The effect of cell plating density on adriamycin accumulation in the DLKP (a) and DLKPA10 (b) cell lines. The cells were plated at a concentration of $2x10^5$, $1x10^5$ and $0.75x10^5$ cells per well and adriamycin accumulation determined after 48 hours.

### 3.12 Adriamycin accumulation in the DLKPA clones

Adriamycin accumulation was studied in four adriamycin resistant clones previously isolated from the resistant cell line, DLKP-A. The cellular concentration of adriamycin in each of these clones was determined and the maximum level of accumulation compared with the levels observed in the parental DLKP and the DLKPA10 resistant cell line. Figure 3.12.1 illustrates the level of adriamycin accumulated in the six cell lines following two hour and four hour exposure to the drug. A significant difference was noted in the cellular concentration of adriamycin in each cell line. The DLKP parental cells accumulated approximately 620 pmoles adriamycin per $10^6$ cells, which represented the highest level of adriamycin uptake. The DLKPA10 cells were found to accumulate the least amount of drug within the same time period (approximately 120 pmoles per $10^6$ cells). Of the four clones studied, the highest cellular concentration of adriamycin was observed in the DLKPA 2B clone, followed by the DLKPA 6B clone and then the DLKPA 11B clone. The cellular concentration of adriamycin was found to be the lowest in the DLKPA 5F clone.

#### 3.12.1 Effect of verapamil on adriamycin accumulation

The effect of verapamil on adriamycin accumulation was studied to determine if the accumulation defect observed in the DLKPA clones could be reversed. The addition of verapamil (30$\mu$g/ml) resulted in an increase in adriamycin accumulation in all four clones, although a significant difference in the cellular concentration of adriamycin was observed (Figure 3.12.2). Following four hour exposure, verapamil fully reversed the accumulation defect in the DLKPA 5F and DLKPA 2B clones, causing an increase in adriamycin accumulation to a level greater than that seen in the parental DLKP cells. Although verapamil also enhanced adriamycin accumulation in the DLKPA 6B and DLKPA 11B clones, the maximum cellular concentration of the drug was only 70% of that observed in the parental DLKP cells.

#### 3.12.2 Effect of cyclosporin A on adriamycin accumulation

The effect of cyclosporin A on adriamycin accumulation was also investigated in the DLKPA clones. The addition of cyclosporin A was found to enhance adriamycin

accumulation in all of the clones, although as was the case with verapamil, a notable difference in the cellular concentration of the drug was observed in each clone (Figure 3.12.2). Cyclosporin A appeared to be most effective at reversing the accumulation defect in the DLKPA 5F clone, enhancing adriamycin accumulation to a level significantly greater than the parental DLKP cells (approximately 100 pmoles per $10^6$ cells more adriamycin was accumulated within the same time period). Cyclosporin A restored drug accumulation in the DLKPA 2B clone to a level comparable with the DLKP cell line. However, the presence of cyclosporin A only restored the accumulation to approximately 50% in the DLKPA 6B clone and 70% in the DLKPA 11B clones. The results, therefore, illustrated that the accumulation defect observed in these two clones could not be fully reversed by cyclosporin A.

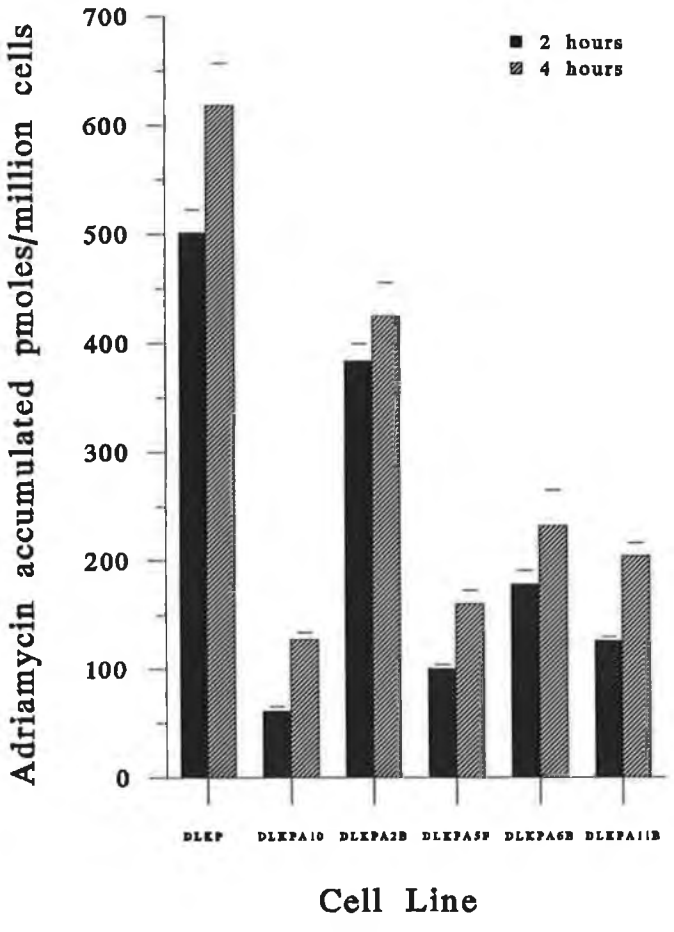
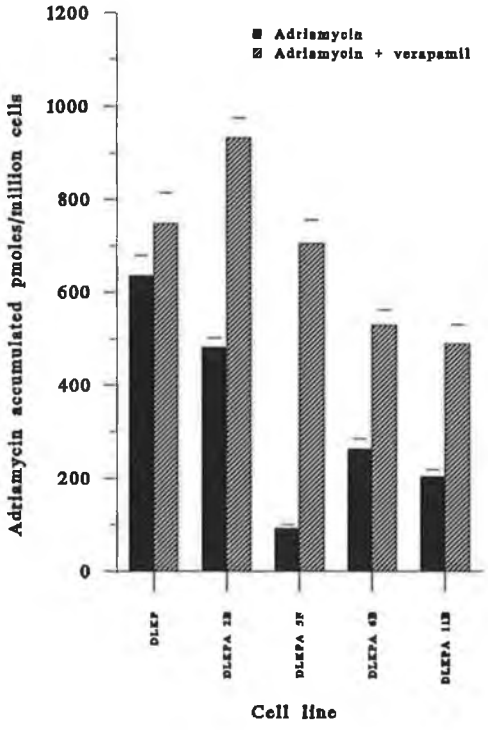



**Figure 3.12.1** Adriamycin accumulation in DLKP, DLKPA10 and DLKPA clones following two hour and four hour exposure to the drug.

a.




b.

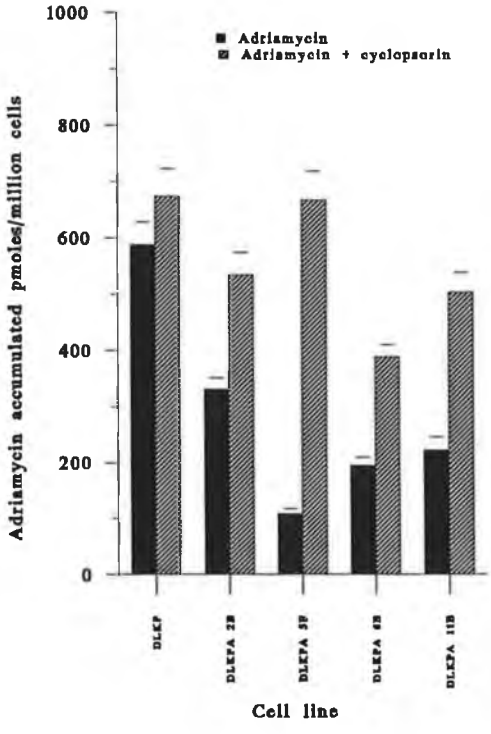



**Figure 3.12.1** The effect of (a) verapamil (30$\mu$g/ml) and (b) cyclosporin A (10$\mu$g/ml) on the cellular concentration of adriamycin in the parental DLKP cells and DLKP resistant clones. The cells were exposed to the drugs for four hours.

### 3.13 Adriamycin accumulation in T24, T24A and T24V cell lines

Adriamycin accumulation was studied in the human bladder carcinoma cell line, T24 and its MDR resistant variants, T24A and T24V. The T24A and T24V resistant lines were previously derived in the laboratory by continuous exposure to adriamycin and VP16 respectively. The T24A cells were exposed to a maximum concentration of 2.2$\mu$g/ml adriamycin and the T24V cells to a maximum concentration of 20$\mu$g/ml VP16. Figure 3.13.1 presents the time course of adriamycin accumulation in the parental and resistant cell lines. The cellular concentration of adriamycin was found to increase with drug exposure time in the T24 cells over a period of three hours. However, after this time the level of accumulation decreased, probably due to the toxicity exerted by the high concentration of adriamycin on the cells. The accumulation of adriamycin in the T24A and T24V cells was significantly reduced relative to the level observed in the parental cells. The T24A cells accumulated the least amount of intracellular drug within the time period studied. Following four hour adriamycin exposure, only approximately 30% of the level accumulated in the T24 cells was observed in the T24A cells.

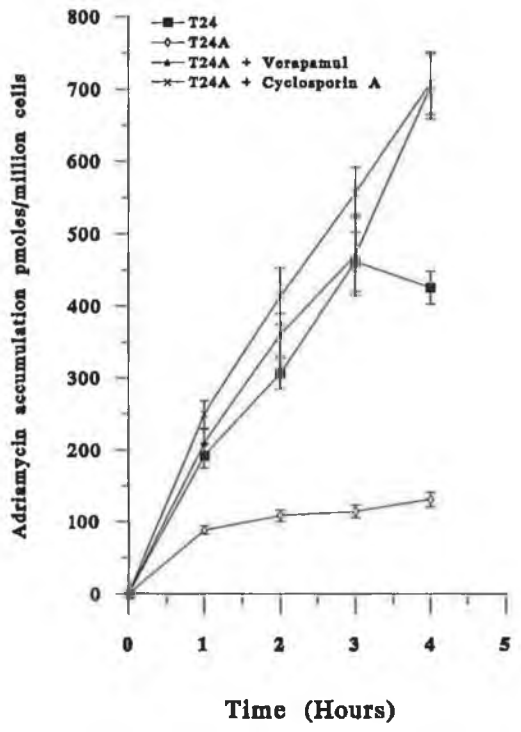
#### 3.13.1 Effect of verapamil on adriamycin accumulation

The addition of verapamil (30$\mu$g/ml) resulted in a slight increase in the cellular concentration of adriamycin in the parental T24 cells, although it did not significantly alter the rate of accumulation. When adriamycin accumulation in the presence of verapamil was studied in the T24A and T24V cells, both the rate of accumulation and the maximum cellular level of adriamycin accumulated were significantly altered. In the presence of verapamil the cellular level of adriamycin in both resistant variants reverted to a level comparable with the parental T24 cells (figure 3.13.1).

#### 3.13.2 Effect of cyclosporin A on adriamycin accumulation

The addition of cyclosporin A (10$\mu$g/ml) also enhanced the accumulation of adriamycin in the T24A and T24V cell lines. Cyclosporin A proved to be more effective than verapamil causing a marked increase in the uptake of adriamycin to levels greater than that observed in the T24 cells (figure 3.13.1). Under similar experimental, conditions cyclosporin A did not significantly alter the rate or maximum level of adriamycin accumulation in the T24 cells.

a.




b.

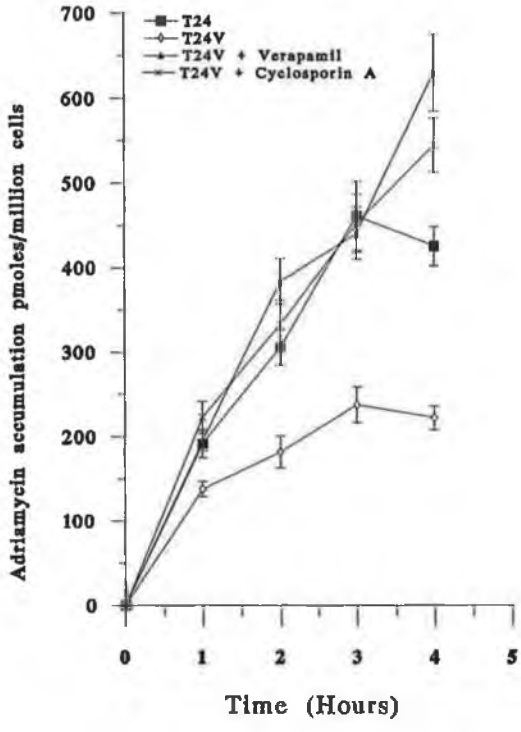



**Figure 3.13.1** The effect of verapamil (30$\mu$g/ml) and cyclosporin (10$\mu$g/ml) on the time course of adriamycin accumulation in the T24A (a) and T24V (b) resistant variants.

## 3.14 Adriamycin Studies in the OAW42 variants

### 3.14.1 Cross resistance profile of the OAW42 variants

Adriamycin accumulation was studied in a number of variants of the human ovarian cell line, OAW42. The OAW42 parental cell line, originally derived from the ascites of a patient with cystadenocarcinoma of the ovary, was obtained from the ECACC (European collection of animal cell cultures). One of the variants studied, OAW42-SR, was a spontaneous MDR resistant strain of the parental cell line. Two resistant variants, OAW42-A1 and OAW42-A, were established by exposing the OAW42-SR cells to increasing concentrations of adriamycin. An adriamycin sensitive variant, OAW42-S, was also included in the study. The OAW42-S variant was a clonal subpopulation derived from the OAW42-SR cell line. The cross resistance profiles of a number of chemotherapeutic agents in the OAW42 variants were determined and table 3.14.1 presents the $IC_{50}$ values obtained for each of the chemotherapeutic agents studied. Table 3.14.2 illustrates the fold resistance of the resistant variants relative to the sensitive OAW42-S line.

| Drug $IC_{50}$ (nM) | OAW42-S | OAW42-SR | OAW42-A1 | OAW42-A |
|---|---|---|---|---|
| **Adriamycin** | 53.5 ± 6.2 | 444.1 ± 33. | 749 ± 54.2 | 1380.5 ± 429 |
| **Vincristine** | 15.2 ± 1.3 | 304 ± 16.7 | 212.8 ± 19.7 | 608 ± 51 |
| **VP16** | 914.9 ± 77 | 1921.3 ± 128 | 1829 ± 102 | 6861 ± 421 |
| **Cisplatin** | 1667 ± 127 | 1100 ± 94.6 | 1533 ± 102 | 2517 ± 177 |
| **5-Fluorouracil** | 9231 ± 654 | 4808 ± 319 | 6923 ± 234 | 8864 ± 906 |

**Table 3.14.1** $IC_{50}$ values for the OAW42 variants

| Drug | OAW42-SR | OAW42-A1 | OAW42-A |
|---|---|---|---|
| **Adriamycin** | 8.3 | 14 | 25.8 |
| **Vincristine** | 20 | 14 | 40 |
| **VP16** | 2.1 | 2 | 7.5 |
| **Cisplatin** | 0.66 | 0.92 | 1.55 |
| **5-Fluorouracil** | 0.52 | 0.75 | 0.99 |

**Table 3.14.2** Fold resistance of the OAW42 resistant variants relative to OAW42-S cells

### 3.14.2 Adriamycin accumulation in the OAW42 variants

The time course of intracellular adriamycin accumulation was studied in the OAW42 variants. Figure 3.14.1 illustrates the cellular concentration of adriamycin in each of the four variants over a period of three hours. The results demonstrate a linear increase in the cellular concentration of adriamycin in the OAW42-S cells within the time period studied. After three hours, the OAW42-S cells had accumulated approximately 600 pmoles adriamycin per $10^6$ cells. Adriamycin accumulation was significantly reduced in both the OAW42-SR and OAW42-A1 variants with respect to the OAW42-S cell line, with a maximum cellular concentration of approximately 390 pmoles per $10^6$ cells. The uptake rate and cellular drug concentrations were similar in these variants to the OAW42-S cells within the first hour studied. However, after this time a steady rate was observed in both the OAW42-SR and OAW42-A1 cells. The lowest level of drug accumulated was observed in the OAW42-A resistant variant. The cellular concentration of adriamycin after three hours was approximately 200 pmoles per $10^6$ cells representing only 25 % of the level observed in the sensitive OAW42-S cells after the same time period.

### 3.14.3 Effect of verapamil on adriamycin accumulation

The effect of verapamil on adriamycin accumulation was investigated in the four OAW42 variants. When the cells were coincubated with the circumventing agent (30$\mu$g/ml), a significant increase in adriamycin accumulation was observed in the OAW42-SR, OAW42-A1 and OAW42-A cells (figure 3.14.2). The addition of verapamil reversed the accumulation defect in the three resistant cell lines and restored the cellular concentration of adriamycin to a level observed in the OAW42-S cell line. Verapamil also caused a slight increase in the cellular concentration of adriamycin in the OAW42-S variant.

### 3.14.4 Effect of cyclosporin A on adriamycin accumulation

The addition of cyclosporin A (10$\mu$g/ml) also enhanced adriamycin accumulation in the OAW42-SR, OAW42-A1 and OAW42-A cell lines (figure 3.14.2). A marked increase in adriamycin uptake was observed in each of the cells lines. Cyclosporin A again proved to be more effective than verapamil, causing an increase in the cellular concentration of the

drug to levels greater that observed in the OAW42-S cells. The addition of cyclosporin A also resulted in a slight increase in adriamycin accumulation in the OAW42-S cells. The results illustrate that both cyclosporin A and verapamil effectively restore the accumulation defects seen in the resistant variants, indicating that the mechanism of resistant in the variants appears to be predominately P-glycoprotein mediated.

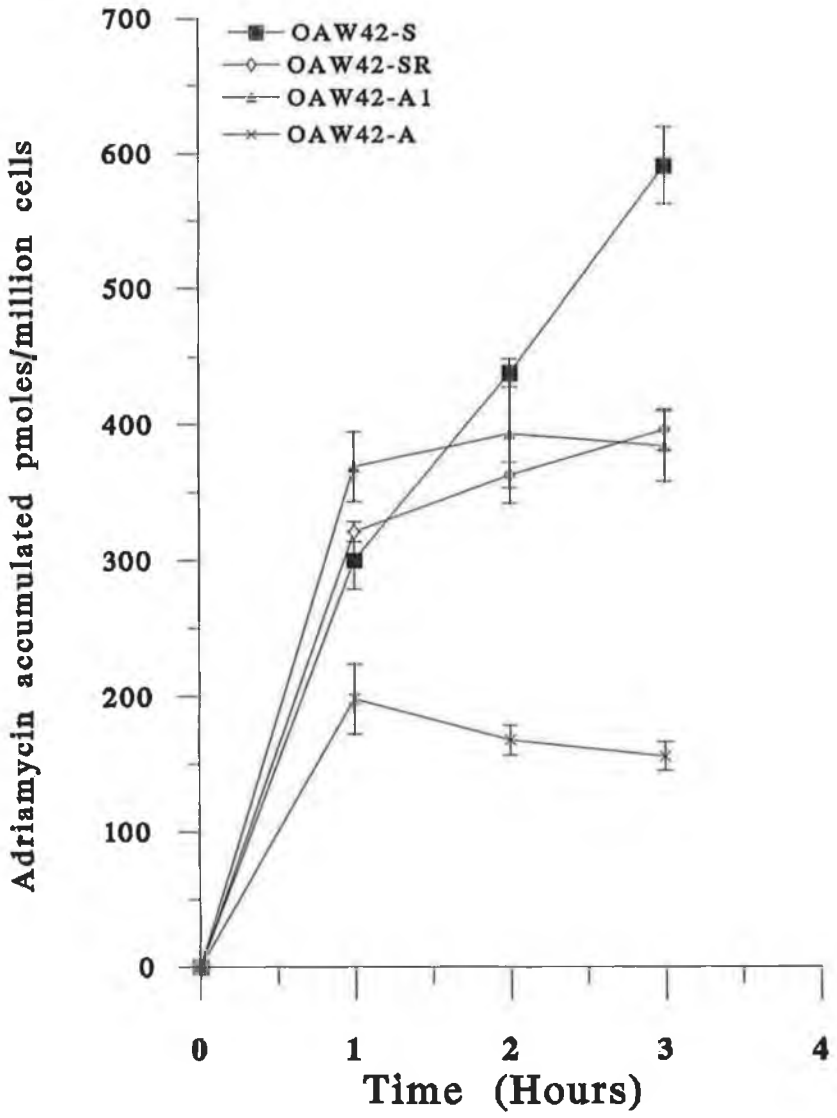
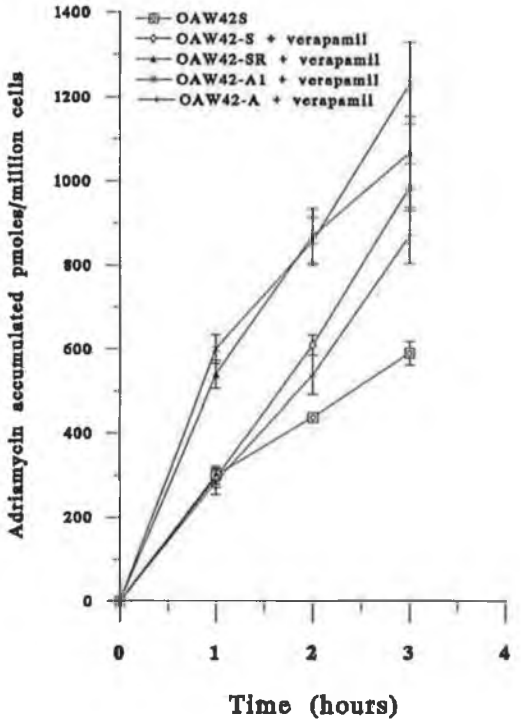



**Figure 3.14.1** The time course of adriamycin accumulation in OAW42-S, OAW42-SR, OAW42-A1 and OAW42-A variants

**a.**




**b.**

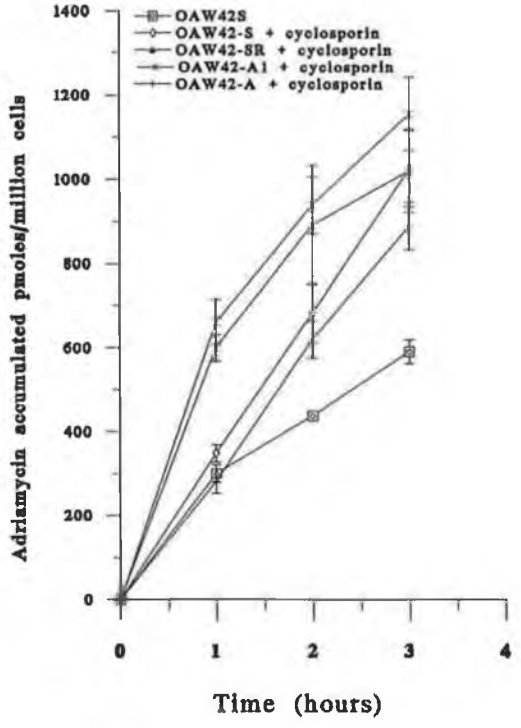



**Figure 2.14.2** The effect of (a) verapamil (30$\mu$g/ml) and (b) cyclosporin A (10$\mu$g/ml) on the time course of adriamycin accumulation in the OAW42-S, OAW42-SR, OAW42-A1 and OAW42-A variants.

## 3.15 Adriamycin subcellular localisation studies

### 3.15.1 Subcellular distribution of adriamycin in SKMES-1 and SKMES-1/ADR cell lines

The subcellular localisation of adriamycin in the SKMES-1 and SKMES-1/ADR cell lines was studied by fluorescent microscopy, as described in section 2.11. The effect of a number of compounds on adriamycin distribution was also investigated. The compounds tested included verapamil, cyclosporin, sodium azide and 2-deoxy-D-glucose.

Figure 3.15.1.1 illustrates the fluorescence pattern observed in the parental SKMES-1 cells and the resistant SKMES-1/ADR cells following two hour exposure to adriamycin (10$\mu$M). Intense nuclear fluorescence was observed in the SKMES-1 cells with distinct areas of more intense fluorescence visible within the nuclei. Faint cytoplasmic fluorescence was also visible, consistent with the localisation of adriamycin in cytoplasmic vesicles. When the cells were exposed to adriamycin for longer time periods, an increase in the intensity of nuclear fluorescence and a decrease in cytoplasmic fluorescence was noted. When the SKMES-1/ADR cells were exposed to adriamycin under the same experimental conditions, the fluorescence pattern was notably different from that observed in the SKMES-1 parental cell line. While cytoplasmic fluorescence was visible in the majority of cells viewed, only faint fluorescence was visible within the nuclei of the cells. An increase in adriamycin cytoplasmic fluorescence was observed following longer exposure times. However, no corresponding increase in the intensity of nuclear fluorescence was noted.

#### 3.15.1.1 Effect of verapamil on adriamycin distribution

When the subcellular localisation of adriamycin was studied in the presence of verapamil (30$\mu$g/ml) only a slight increase in the intensity of nuclear fluorescence was observed in the SKMES-1 cell line. However, a decrease in the intensity of cytoplasmic fluorescence was noted, in particular with longer time exposure to the drug. In contrast, when the SKMES-1/ADR cells were coincubated with verapamil, a marked increase in nuclear fluorescence was observed after two hours in the majority of cells. Adriamycin nuclear fluorescence was enhanced in the SKMES-1/ADR with the intensity similar to that observed in the SKMES-1 cells (Figure 3.15.1.1). A decrease in the cytoplasmic fluorescence was also noted.

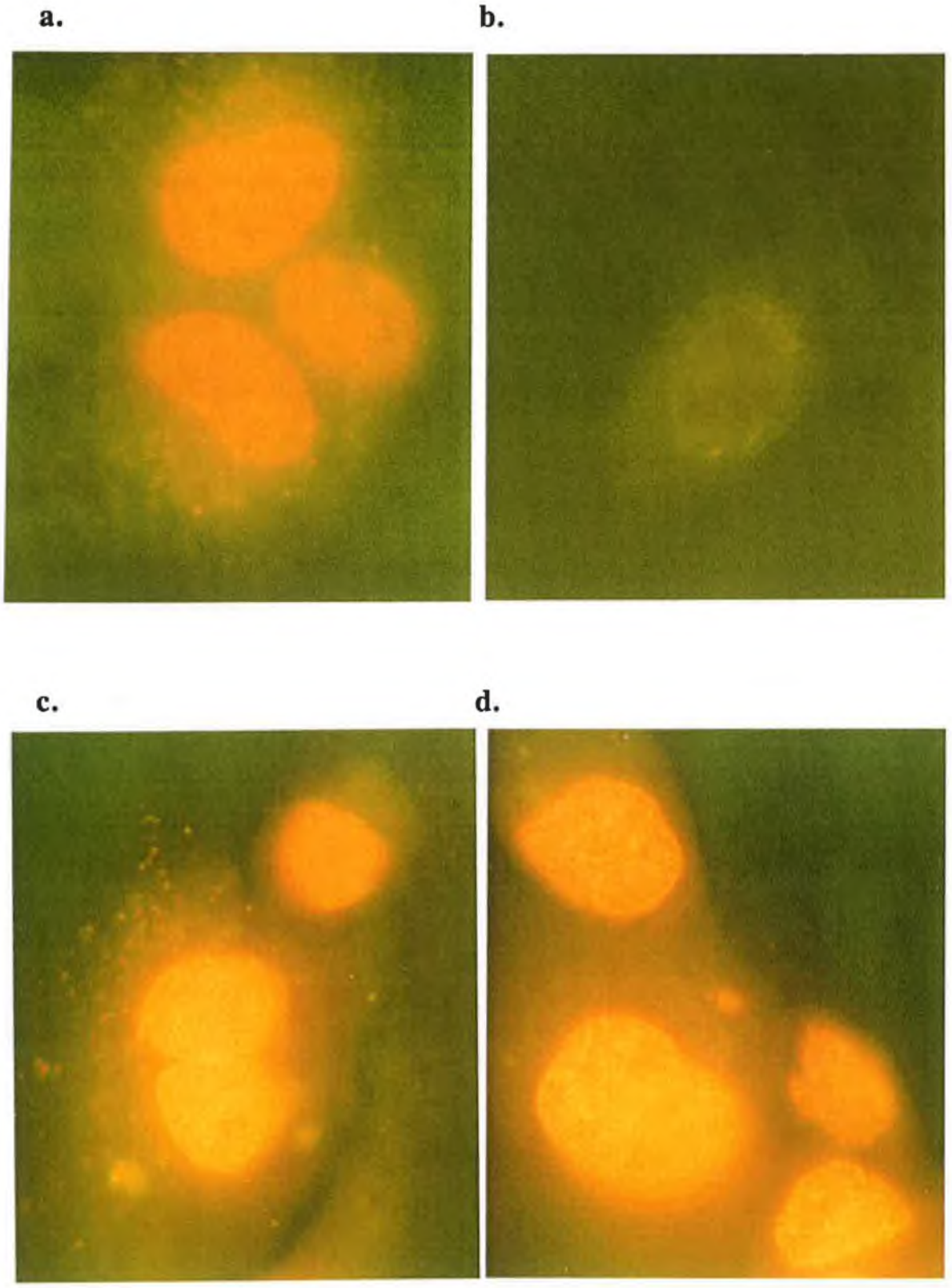



**Figure 3.15.1.1** The subcellular adriamycin (10$\mu$M) distribution pattern in the SKMES-1 and SKMES-1/ADR cell lines following two hour exposure to the drug; (a) SKMES-1, (b) SKMES-1/ADR, (c) SKMES-1 in the presence of verapamil (30$\mu$g/ml) and (d) SKMES-1/ADR in the presence of verapamil.

### 3.15.1.2 Effect of cyclosporin A on adriamycin distribution

Cyclosporin A (10$\mu$g/ml) was also found to enhance adriamycin nuclear fluorescence in the SKMES-1/ADR cells. The intensity of nuclear fluorescence observed appeared to be greater than that seen in the parental SKMES-1 cells, within the same time period (Figure 3.15.1.2). Cytoplasmic fluorescence was still visible within the cells, although an overall cytoplasmic fluorescence pattern was observed, in contrast to the fluorescence pattern observed when the cell were exposed to adriamycin alone.

### 3.15.1.3 Effect of energy inhibition on adriamycin distribution

The effect of the metabolic inhibitors, sodium azide and 2-deoxy-D-glucose, in glucose free medium on the localisation of adriamycin in the SKMES-1 and the SKMES-1/ADR cell lines was also investigated. The aim of this work was to determine if depletion of ATP levels resulted in an increase in nuclear fluorescence in the resistant cells. When the SKMES-1 cells were exposed to sodium azide (10mM) for two hours, cell lysis was observed in approximately 75% of the cells. The viable cells, however, displayed nuclear fluorescence, with the intensity similar to that observed when the parental cells were exposed to adriamycin alone. Reducing the concentration to 1mM sodium azide resulted in an increase in cell viability but no significant alteration in the intensity of nuclear fluorescence. The addition of 10mM sodium azide did not appear to have a toxic effect on the SKMES-1/ADR cells within the time period studied. Treatment with sodium azide was found to effectively enhance adriamycin nuclear fluorescence in the SKMES-1/ADR cells. The intensity of fluorescence observed was comparable to the SKMES-1 parental cells (Figure 3.15.1.2). A decrease in cytoplasmic fluorescence was also noted in the SKMES-1/ADR cells following exposure to sodium azide.

When the subcellular distribution of adriamycin was studied in the presence of 2-deoxy-D-glucose (25$\mu$g/ml), cell lysis was observed in both the SKMES-1 cells (100%) and the SKMES-1/ADR cells, (approximately 50%). The viable SKMES-1/ADR cells displayed intense nuclear fluorescence, similar to that noted with sodium azide. When the concentration of 2-deoxy-D-glucose was reduced to 2.5$\mu$g/ml, although nuclear fluorescence was observed in the SKMES-1/ADR cells, the intensity of fluorescence was low, with respect to the parental SKMES-1 cell line.

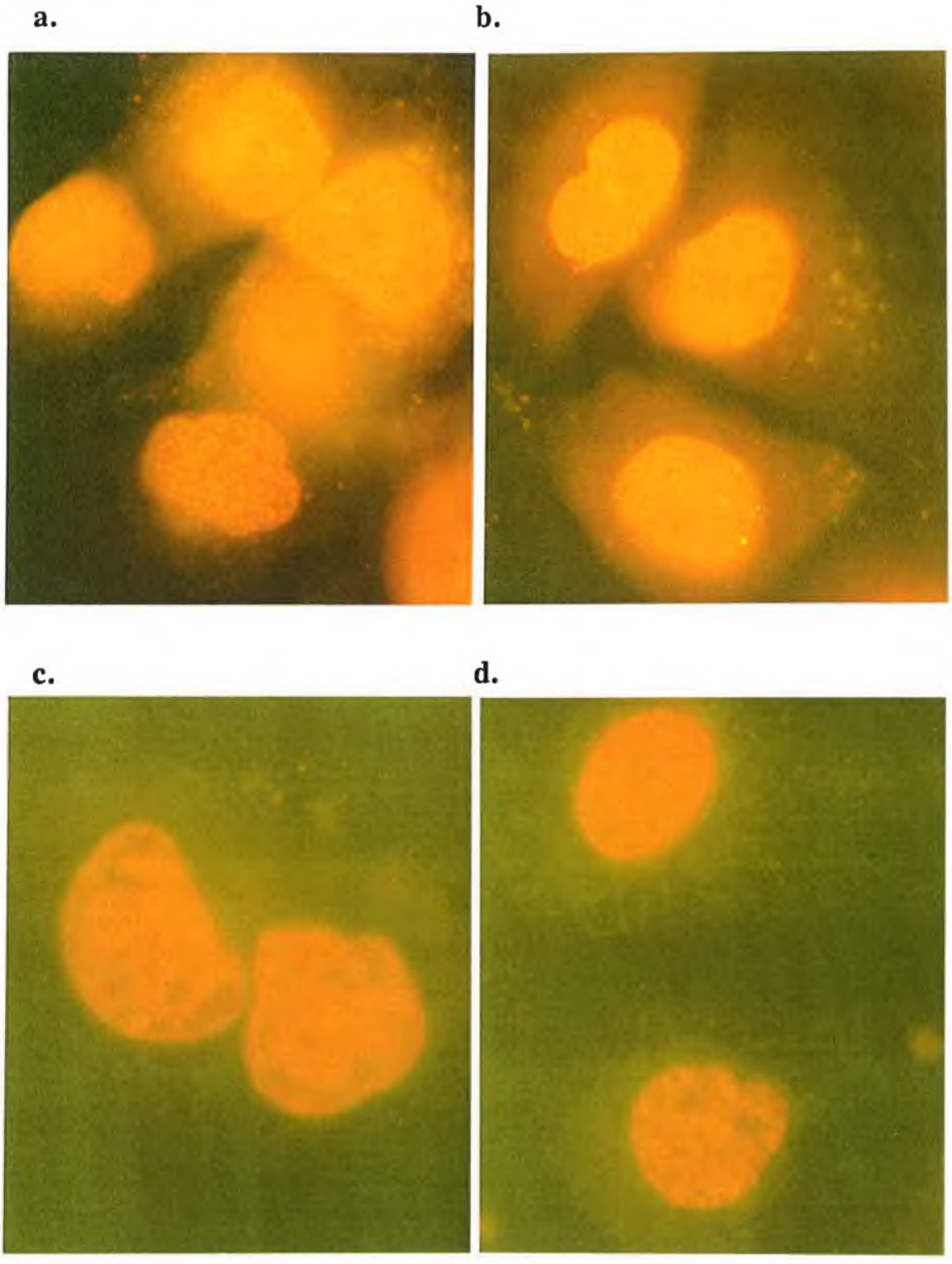



**Figure 3.15.1.2** The subcellular adriamycin (10$\mu$M) distribution pattern in the SKMES-1 and SKMES-1/ADR cell lines following two hour exposure to the drug in the presence of cyclosporin A (10$\mu$g/ml) or sodium azide (10mM); (a) SKMES-1 in the presence of cyclosporin A, (b) SKMES-1/ADR in the presence of cyclosporin A, (c) SKMES-1 in the presence of sodium azide and (d) SKMES-1/ADR in the presence of sodium azide.

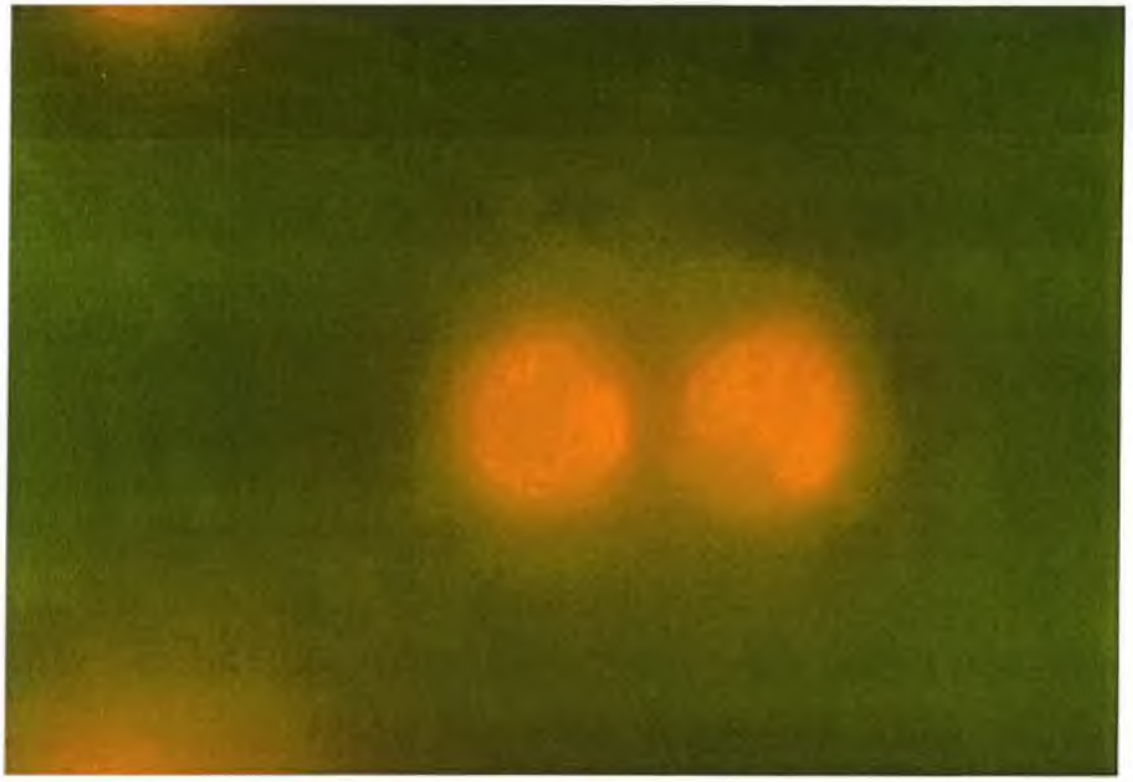
### 3.15.2 Adriamycin distribution studies in DLKP and DLKPA10 cell lines

#### 3.15.2.1 Subcellular distribution of adriamycin in DLKP and DLKPA10 cell lines

The localisation of adriamycin in the DLKP and DLKPA10 cells was also investigated by fluorescent microscopy. Figure 3.15.2.1 illustrates the fluorescence pattern observed in the parental DLKP and resistant DLKPA10 cells following two hour incubation with the drug (10$\mu$M). The results obtained showed intense nuclear fluorescence in the DLKP cells, with more distinct regions of intense fluorescence visible within the nuclei. Faint cytoplasmic fluorescence was also observed in a small proportion of cells viewed. When the DLKPA10 cells were incubated with adriamycin for the same time period, the majority of cells displayed only faint nuclear fluorescence, although speckles of more intense fluorescence were visible throughout the cytoplasmic region. Increasing the concentration of adriamycin to a maximum level of 50$\mu$M resulted in an increase in the intensity of cytoplasmic fluorescence in the DLKPA10 cells, although no corresponding increase in nuclear fluorescence was observed (figure 3.15.2.2). When the subcellular distribution of adriamycin was studied in the DLKPA10 cells, following longer exposure time periods to 10$\mu$M adriamycin (up to 48 hours), an increase in the intensity and quantity of fluorescent cytoplasmic speckles was again noted. However, no significant alteration in the intensity of nuclear fluorescence was observed (Figure 3.15.2.3).

The subcellular distribution of adriamycin was investigated in the DLKP and DLKPA10 cell lines following 5 minute and 4 hour exposure times respectively. These time periods represented the time at which equivalent amounts of drug were present in the cells, as determined from quantitative accumulation assays (section 3.11.1). After 4 hour exposure to adriamycin, cytoplasmic fluorescence was clearly visible in the DLKPA10 cells, although no nuclear fluorescence was observed. However, after 5 minutes exposure to the drug, faint nuclear fluorescence was observed in the DLKP cells, thus showing the rapid localisation of adriamycin within the nucleus (figure 3.15.2.4). These results demonstrate the different subcellular distribution of adriamycin in the sensitive and resistant cell lines. The results also indicate that intracellular mechanisms, in addition to bulk uptake/efflux rates may play a role in intracellular drug distribution in multidrug resistant cell lines.

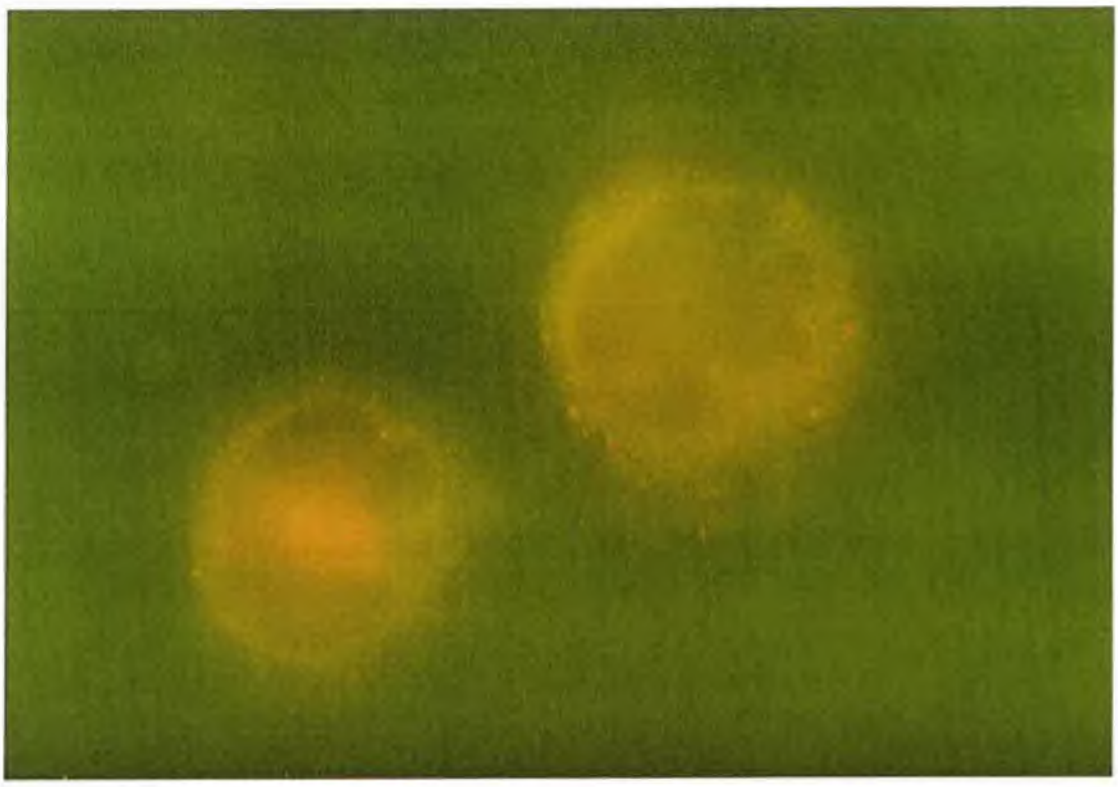
a.



b.

**Figure 3.15.2.1a** The subcellular adriamycin (10$\mu$M) distribution pattern in the DLKP cell line following two hour exposure to the drug (a). Light microscopy image of the DLKP cells illustrating the localisation of adriamycin in the nuclear region (b).

a.



b.

**Figure 3.15.2.1b** The subcellular adriamycin (10$\mu$M) distribution pattern in the DLKPA10 cell line following two hour exposure to the drug (a). Light microscopy image of the DLKPA10 cells illustrating the localisation of adriamycin within the cytoplasm (b).

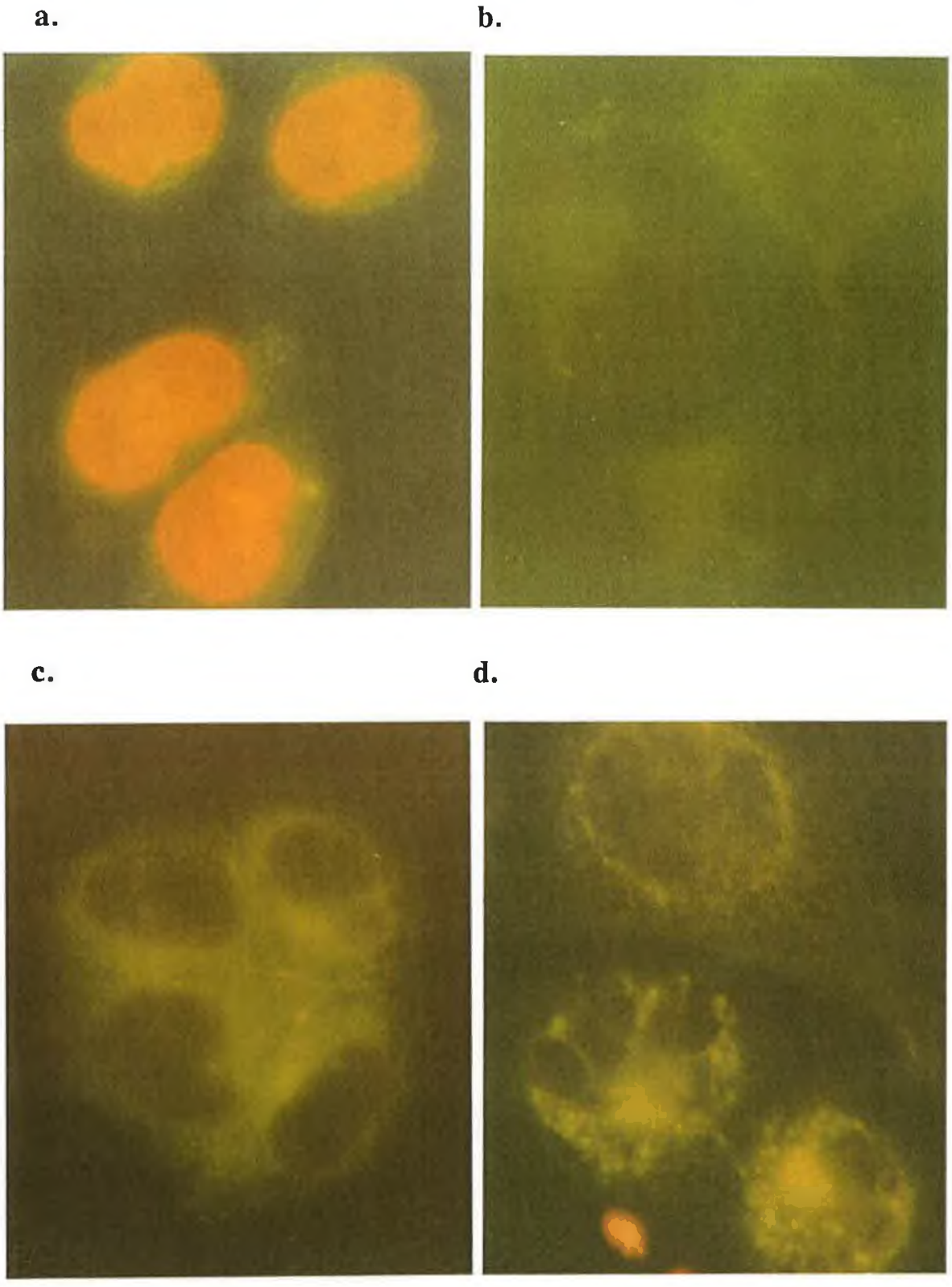



**Figure 3.15.2.2** The subcellular adriamycin distribution pattern in the DLKP and DLKPA10 cell lines. The DLKP cells were exposed to adriamycin (10$\mu$M) for 2 hours (a); the DLKPA10 cells were exposed to increasing concentrations of adriamycin; 10$\mu$M (b), 25$\mu$M (c) and 50$\mu$M (d) for 2 hours.

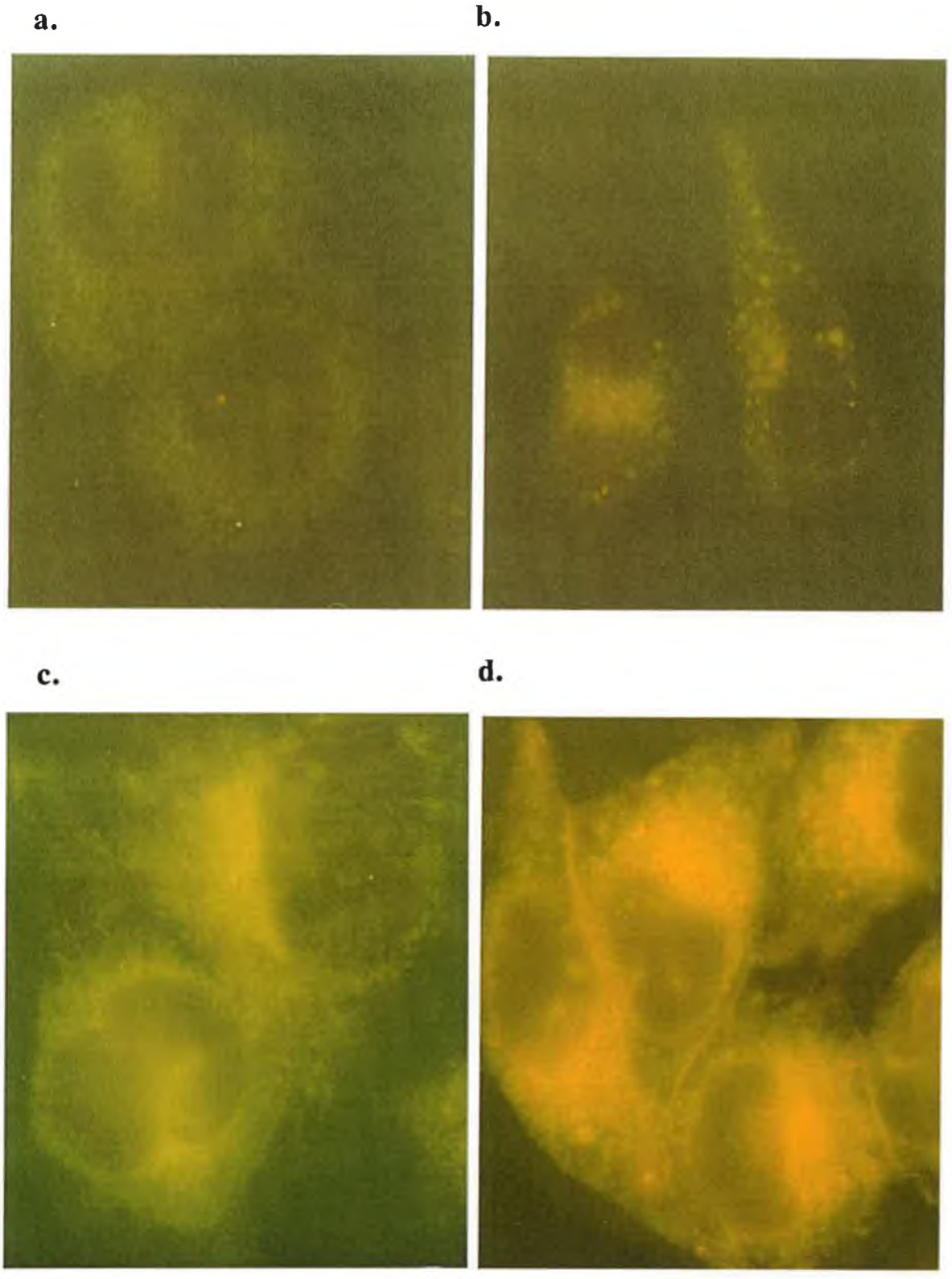
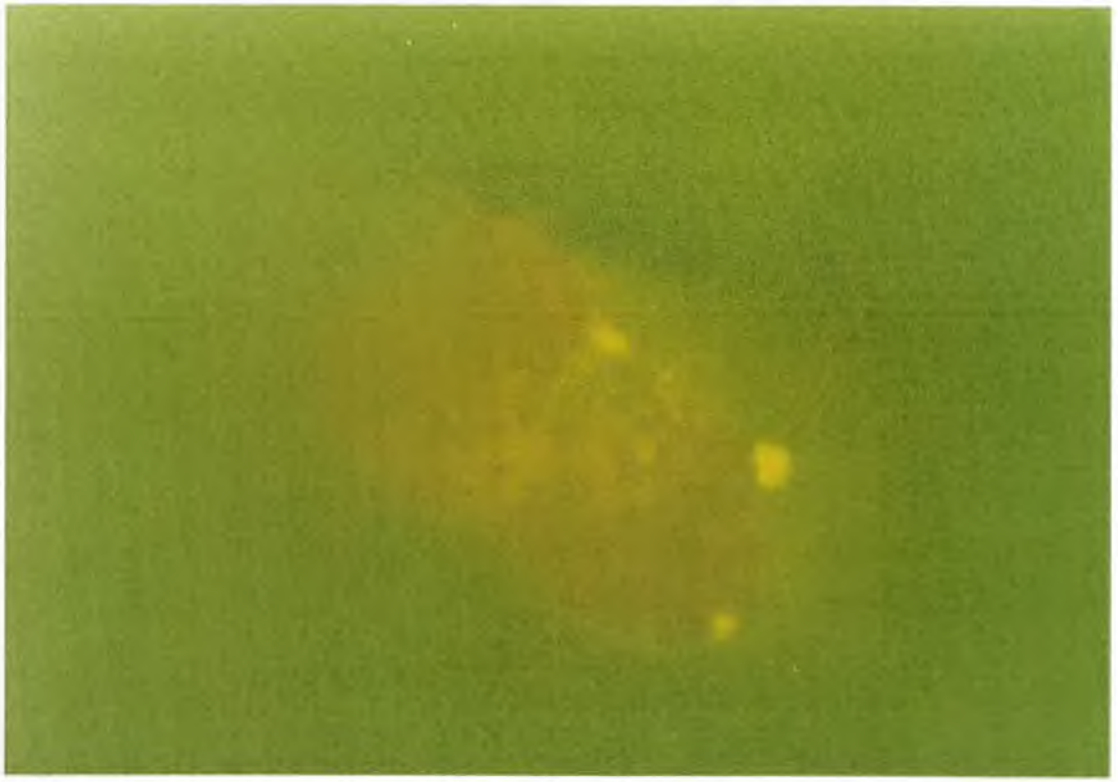



**Figure 3.15.2.3** The time course for the subcellular adriamycin distribution pattern in the DLKPA10 cell line. The cells were exposed to adriamycin (10$\mu$M) for 2 hours (a), 4 hours (b), 12 hours (c) and 24 hours (d).

a.



b.

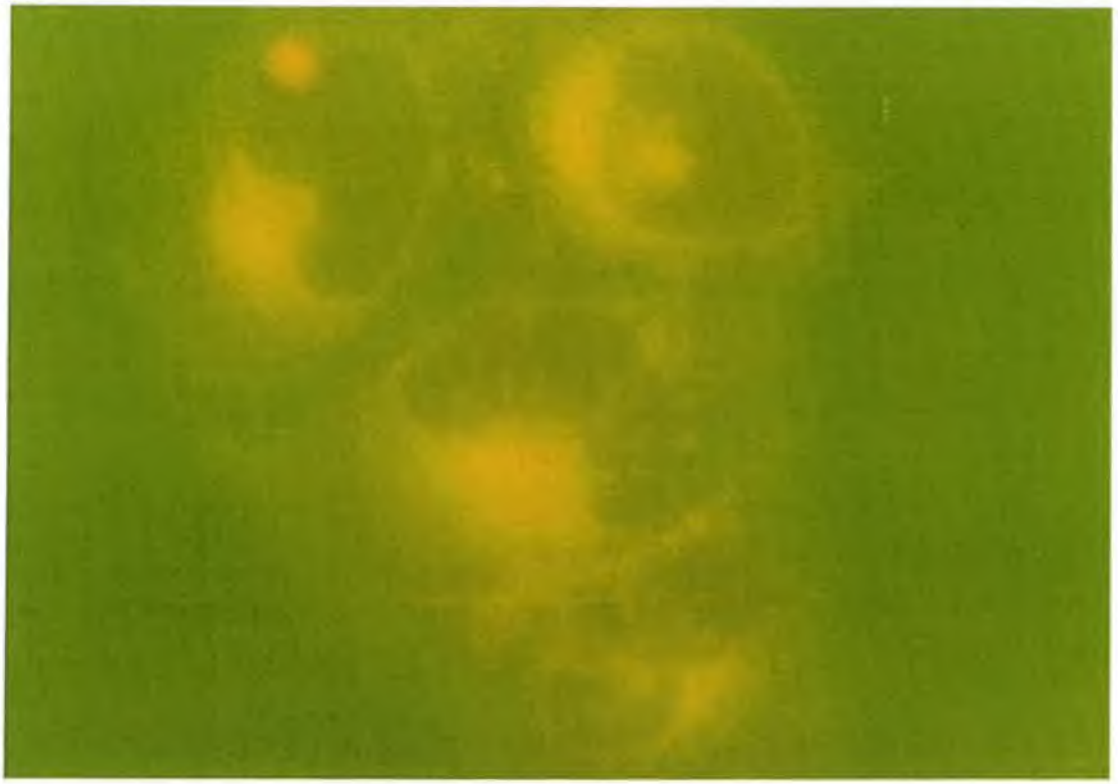


**Figure 3.15.2.4** The subcellular adriamycin (10$\mu$M) distribution pattern in the DLKP cells following 5 minute exposure to adriamycin (a) and in the DLKPA10 cells following 4 hour exposure to the drug (b).

### 3.15.2.2 Effect of verapamil on adriamycin distribution

When the cells were incubated with verapamil (30$\mu$g/ml), a slight increase in the intensity of nuclear fluorescence was observed in the DLKP cells with faint cytoplasmic fluorescence also visible in a small proportion of the cells. In contrast, the addition of verapamil to the DLKPA10 cells resulted in a marked increase in nuclear fluorescence in all of the cells viewed (Figure 3.15.2.5). However, the intensity of fluorescence was substantially less than that observed in the parental cells. Pretreatment with verapamil for two hours prior to the addition of adriamycin did not result in any further increase in the intensity of nuclear fluorescence in the DLKPA10 cells. When the concentration of verapamil was increased (concentrations up to 100$\mu$g/ml), no significant alteration in the intensity of nuclear fluorescence was observed.

### 3.15.2.3 Effect of cyclosporin A on adriamycin distribution

The addition of cyclosporin A (10$\mu$g/ml) also enhanced adriamycin nuclear fluorescence in the DLKPA10 cell line. Although the intensity of fluorescence was greater than that observed with verapamil treatment, it was still substantially less than that observed in the parental cell line (Figure 3.15.2.5). Increasing the concentration of cyclopsorin A (concentrations up to 100$\mu$g/ml) did not significantly alter the intensity of nuclear fluorescence observed in the DLKPA10 cells. When the cells were pretreated with cyclosporin A for two hours, prior to the addition of adriamycin, no significant increase in the intensity of fluorescence was noted. The addition of cyclosporin A resulted in a slight increase in the intensity of nuclear fluorescence in the parental DLKP cells.

### 3.15.2.4 Effect of energy inhibition on adriamycin distribution

To determine if the inhibition of ATP production could alter the subcellular distribution of adriamycin in the DLKPA10 cells, the effect of sodium azide, 2-deoxy-D-glucose and antimycin A was investigated. When the cells were incubated with sodium azide (10mM) in a glucose free environment, cell lysis was observed in the majority of the DLKP parental cells. However, the viable cells displayed intense nuclear fluorescence, similar to that observed in the DLKP cells when incubated with adriamycin. When the concentration of

sodium azide was reduced to 1mM, an increase in cell viability was noted, although no significant alteration in the intensity of nuclear fluorescence was observed. The addition of sodium azide did not appear to have a toxic effect on the DLKPA10 cells within the two hour period studied. Although, with longer incubation periods, cell lysis was observed in a large percentage of the cells. When the DLKPA10 cells were incubated with sodium azide, in glucose free medium, an increase in cytoplasmic adriamycin fluorescence was observed in the cells. However, no significant alteration in nuclear fluorescence was noted. Distinct regions of fluorescence were clearly visible throughout the cytoplasm, in particular, in areas close to the nucleus where very intense fluorescence was observed (Figure 3.15.2.6). When the concentration of sodium azide was increased to 25mM, no significant enhancement of nuclear fluorescence was observed. Coincubating the cells with higher concentrations of sodium azide resulted in total cell lysis. Adriamycin distribution in the DLKPA10 cells was also studied, following pretreatment with sodium azide. When the cells were treated with sodium azide (10mM) two hour prior to the addition of adriamycin, nuclear fluorescence was observed in all of the cells viewed (figure 3.15.2.6). The intensity of nuclear fluorescence, however, was less than that noted in the parental cells.

Treatment with 2-deoxy-D-glucose (25$\mu$g/ml) was also found to be toxic to the parental DLKP cells, even at low concentrations (2.5$\mu$g/ml). Although cell lysis was not observed in the DLKPA10 cells within the two hour incubation period studied, it appeared to be toxic to the cells, following longer exposure periods. Coincubating the DLKPA10 cells with 2-deoxy-D-glucose resulted in an increase in adriamycin cytoplasmic fluorescence, to a level comparable with that observed with sodium azide treatment. No significant increase in the intensity of nuclear fluorescence was observed when the cells were coincubated with 2-deoxy-D-glucose for longer time periods. However, pretreatment with 2-deoxy-D-glucose also resulted in a marked increase in the nuclear fluorescence. The intensity of fluorescence was comparable with that observed with verapamil treatment and sodium azide pretreatment.

The subcellular distribution of adriamycin was also studied in the DLKP and DLKPA10 cell lines, in the presence of 10$\mu$M antimycin A (figure 3.15.2.7). Treatment with antimycin A resulted in an increase in the intensity of nuclear fluorescence in the parental DLKP cells. Intense nuclear fluorescence was also observed in the DLKPA10 cells, following incubation with antimycin A. The fluorescence intensity appeared greater than the intensity observed in the DLKP cells treated with adriamycin alone. Coincubation with antimycin A also resulted in a decrease in the intensity of cytoplasmic fluorescence in the DLKPA10 cells.

The effect of antimycin A on adriamycin distribution was also studied in the DLKPA10 cells following preloading with adriamycin (10$\mu$M). The cells were exposed to adriamycin for two hours, washed and incubated in adriamycin free medium, containing antimycin A (10$\mu$M) for a further two hours. When the cells were viewed after two hour exposure to the drug, the typical cytoplasmic fluorescence pattern was observed. Intense fluorescence was clearly visible in distinct regions throughout the cytoplasm. Following treatment with antimycin A, faint nuclear fluorescence was observed in the majority of cells viewed. A decrease in cytoplasmic fluorescence was also distinguishable, in particular in the number of fluorescent vesicles scattered throughout the cytoplasmic region (figure 3.15.2.8).

### 3.15.3.1 Subcellular distribution of adriamycin in the DLKPA clones

The subcellular localisation of adriamycin in the DLKPA clones was studied, to establish if the four adriamycin resistant clones displayed similar drug distribution patterns. The effect of verapamil and cyclosporin A on adriamycin distribution within the four DLKPA resistant clones was also investigated.

Figures 3.15.3.1 - 3.15.3.4 illustrates the fluorescence patterns observed in the four DLKPA clones following exposure to adriamycin (10$\mu$M) for two hours. The results obtained showed notably different adriamycin fluorescence patterns in each of the four clones. Following exposure to adriamycin, nuclear fluorescence was clearly visible in the DLKPA 2B cells, although the intensity of the fluorescence varied within the cell population. Approximately 65% of the cell population displayed intense nuclear fluorescence, while the remaining cells had faint nuclear fluorescence (figure 3.15.3.1). Faint nuclear fluorescence was observed in the majority of the DLKPA 5F cells, although quite intense distinct areas of fluorescence were noted in the cytoplasmic regions. An intense area of fluorescence was also visible in the proximity of the nucleus (figure 3.15.3.2). The DLKPA 6B and DLKPA 11B clones displayed a mixed fluorescence pattern within the population of cells viewed. The DLKPA 6B cells displayed quite intense nuclear fluorescence in approximately 50% of the cells while very faint nuclear fluorescence was observed in the remaining cells. Distinct spots of cytoplasmic fluorescence were also visible, particularly in the cells that displayed faint nuclear fluorescence (figure 3.15.3.3). When adriamycin distribution was studied in the DLKPA 11B cells approximately 35% of the population had quite intense nuclear fluorescence, while the remaining cells had faint

nuclear fluorescence, with distinct areas of fluorescence visible within the cytoplasm (figure 3.15.3.4).

### 3.15.3.2 Effect of verapamil on adriamycin distribution in the DLKPA clones

When the subcellular distribution of adriamycin in the DLKPA clones was studied in the presence of verapamil (30$\mu$g/ml), the intensity of fluorescence was notably different in each of the DLKPA clones (figures 3.15.3.1 - 3.15.3.4). Intense nuclear fluorescence was observed in the DLKPA 2B cells, with more intense regions of fluorescence visible within the nucleus. The DLKPA 5F cells displayed a similar fluorescence pattern as the DLKPA 2B cells, with the majority of cells displaying very intense nuclear fluorescence. The addition of verapamil also resulted in an increase in the intensity of nuclear fluorescence in the DLKPA 6B and DLKPA 11B clones, although the intensity of nuclear fluorescence was significantially less than that observed in the DLKPA 2B and DLKPA 5F clones. When the cells were exposed to higher concentrations of verapamil (concentration range 5 - 50$\mu$g/ml), or for longer incubation periods (time periods up to 10 hours), no further increase in nuclear fluorescence was observed.

### 3.15.3.3 Effect of cyclosporin A on adriamycin distribution in the DLKPA clones

Treatment of the DLKPA clones with cyclosporin A (10$\mu$g/ml) resulted in subcellular distribution patterns, similar to that observed with verapamil. The intensity of adriamycin nuclear fluorescence was again enhanced in both the DLKPA 2B cells and DLKPA 5F cells, to a level comparable with the parental DLKP cells. Cyclosporin A also increased the nuclear fluorescence in the DLKPA 6B cells and DLKPA 11B cells, although the intensity of fluorescence was significantly less than that observed in the DLKPA 2B and DLKPA 5F clones. Treatment with higher concentrations of cyclosporin (10-50$\mu$g/ml) did not result in any further increase in the intensity of nuclear fluorescence observed in the DLKPA 6B and DLKPA 11B clones. Although the cells were exposed to cyclosporin A for incubation time periods up to 10 hours, no further increase in fluorescence intensity was noted in any of the DLKPA clones.

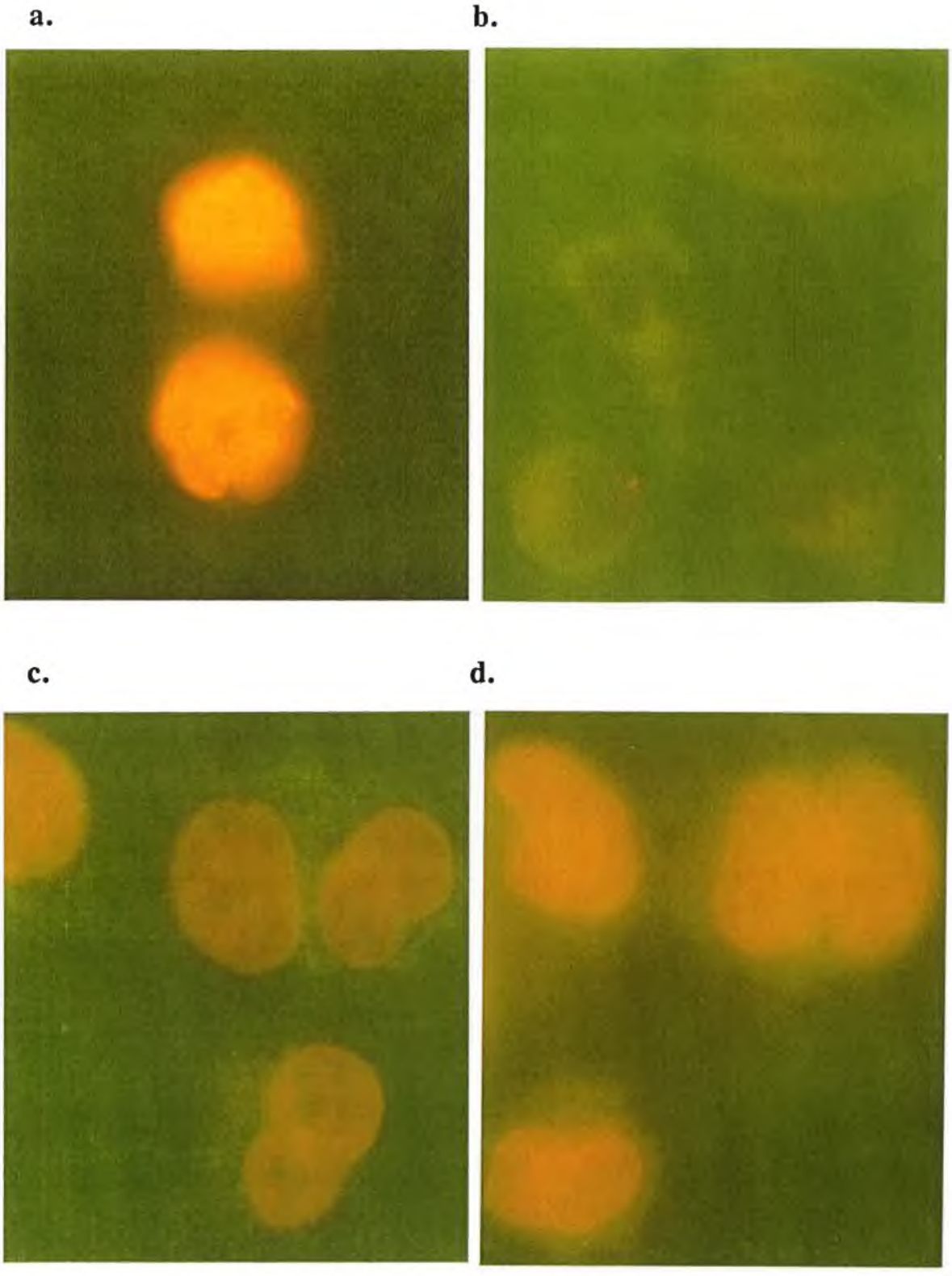



**Figure 3.15.2.5** The subcellular adriamycin (10$\mu$M) distribution pattern in the DLKPA10 cell line following two hour exposure to the drug in the presence of verapamil (30$\mu$g/ml) or cyclosporin A (10$\mu$g/ml); (a) DLKP, (b) DLKPA10 (c) DLKPA10 in the presence of verapamil and (d) DLKPA10 in the presence of cyclosporin A.

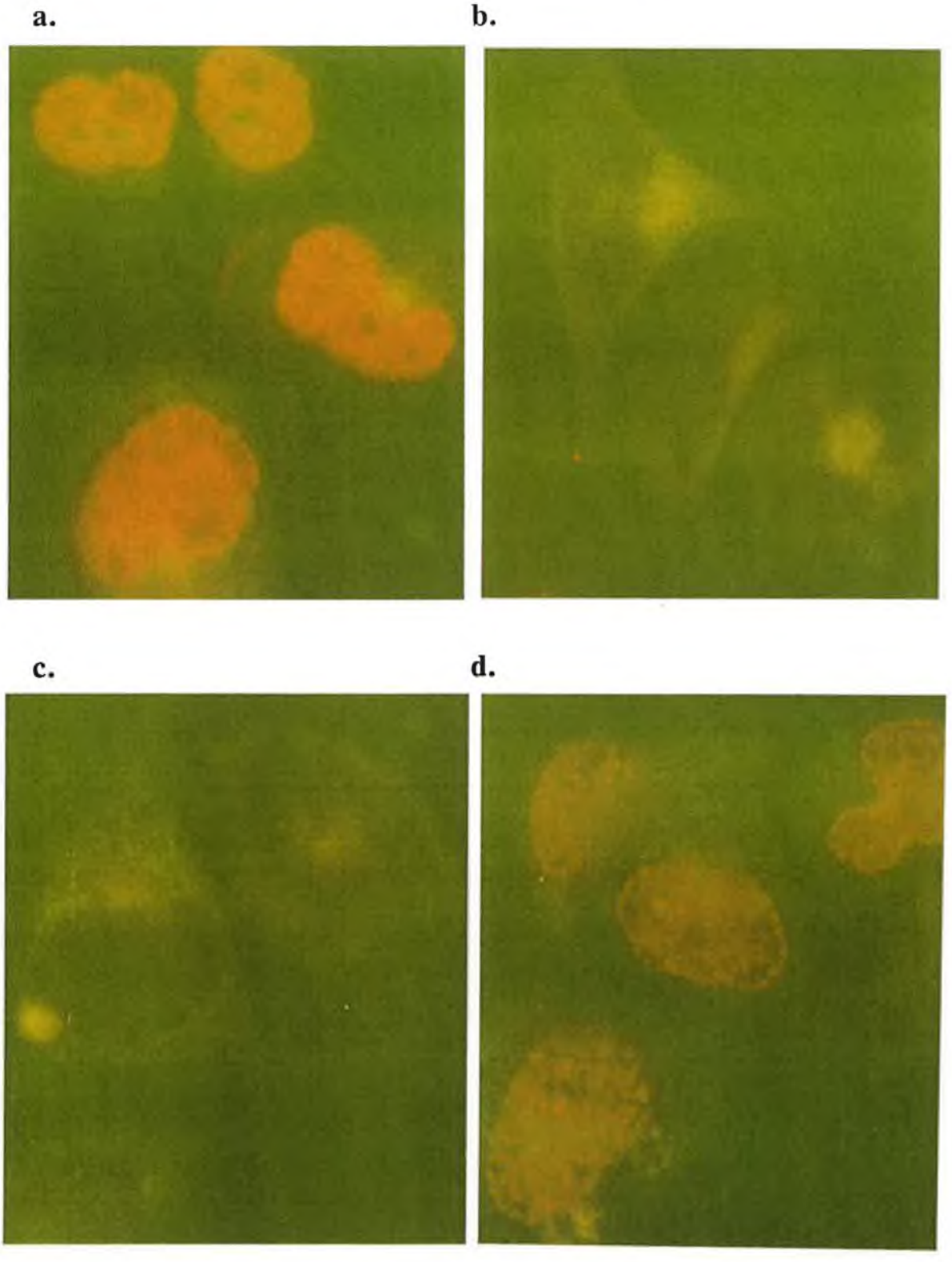
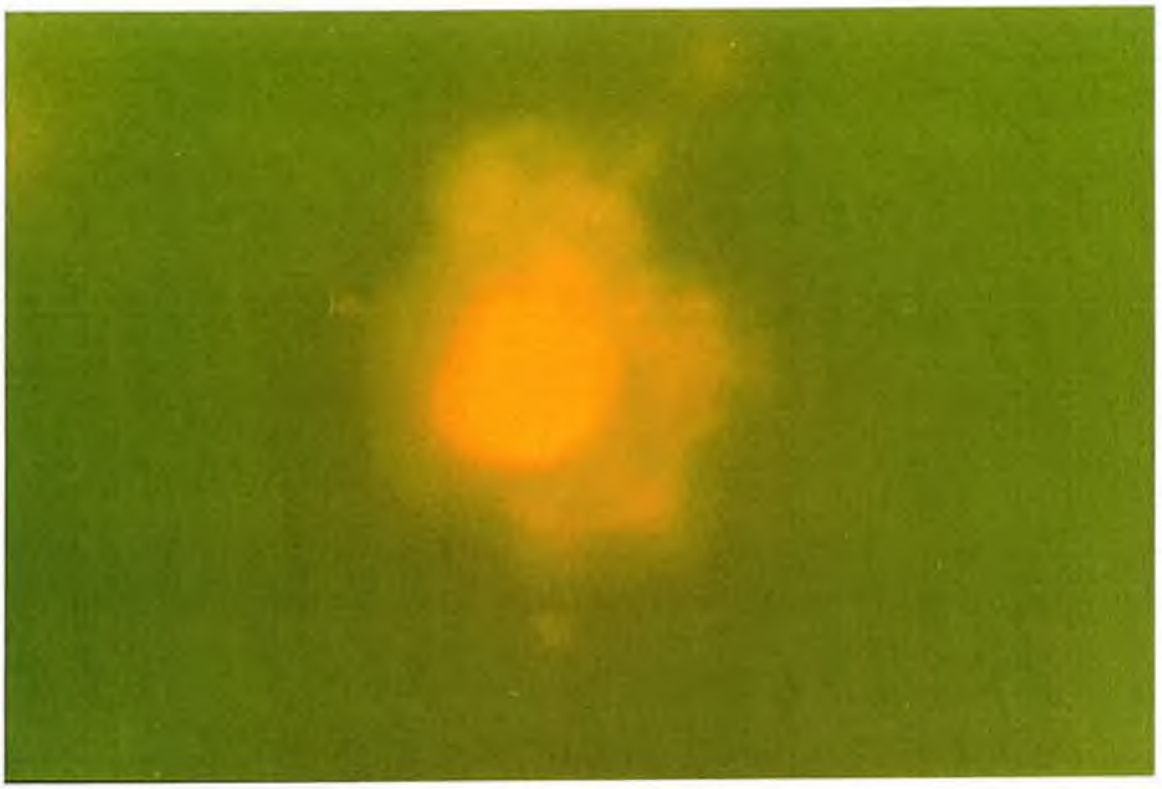



**Figure 3.15.2.6** The effect of metabolic inhibitors on the subcellular distribution of adriamycin (10$\mu$M) in the DLKP and DLKPA10 cell lines. The cells were exposed to the drug for 2 hours in the presence of sodium azide (10mM) or 2-deoxy-glucose (25$\mu$g/ml); (a) DLKP in the presence of sodium azide, (b) DLKPA10 in the presence of sodium azide, (c) DLKPA10 in the presence of 2-deoxy-glucose and (d) DLKPA10, following pretreatment with sodium azide for 2 hours.

a.



b.

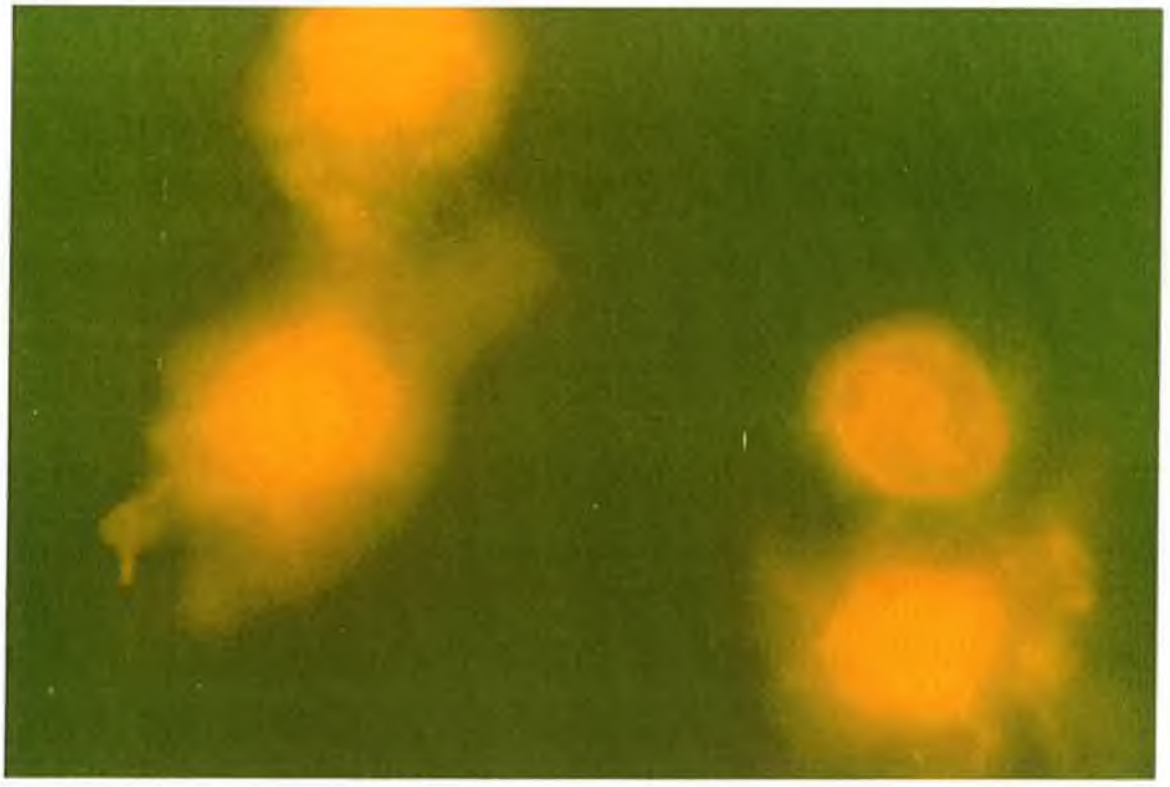


**Figure 3.15.2.7** The subcellular adriamycin distribution pattern in the DLKP (a) and DLKPA10 (b) cells following 2 hour exposure to the drug in the presence of the metabolic inhibitor, antimycin A (10$\mu$M).

a.

b.

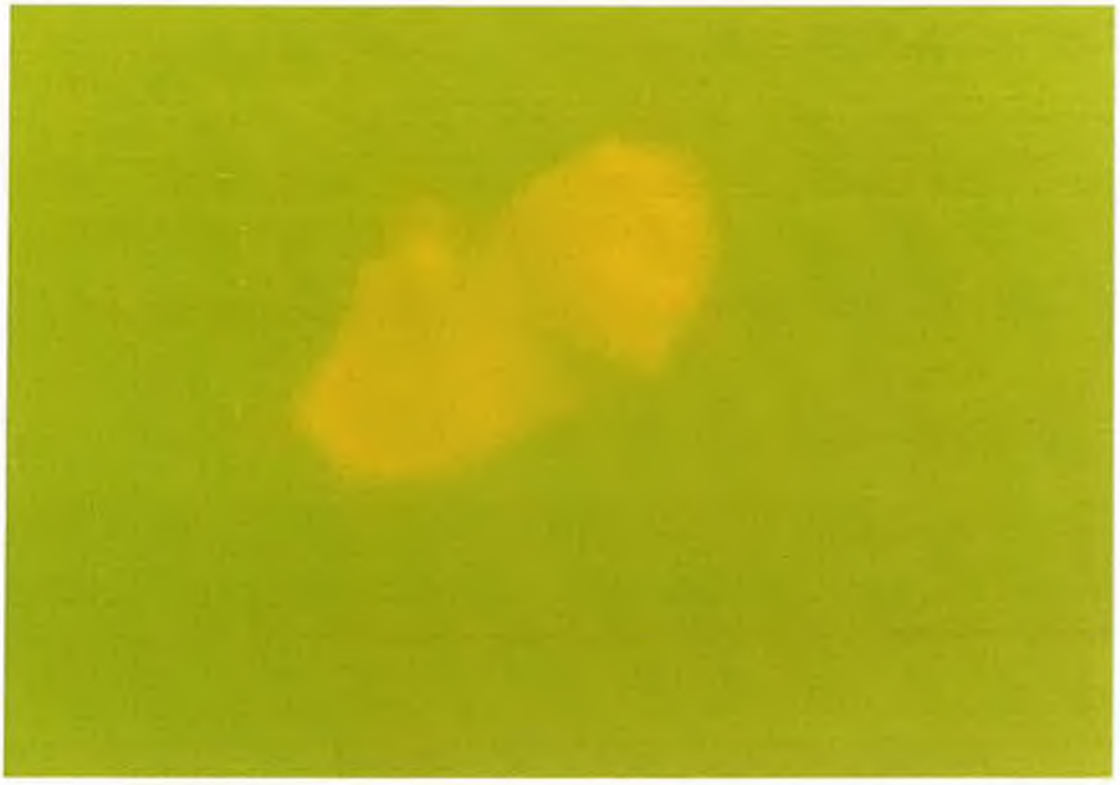
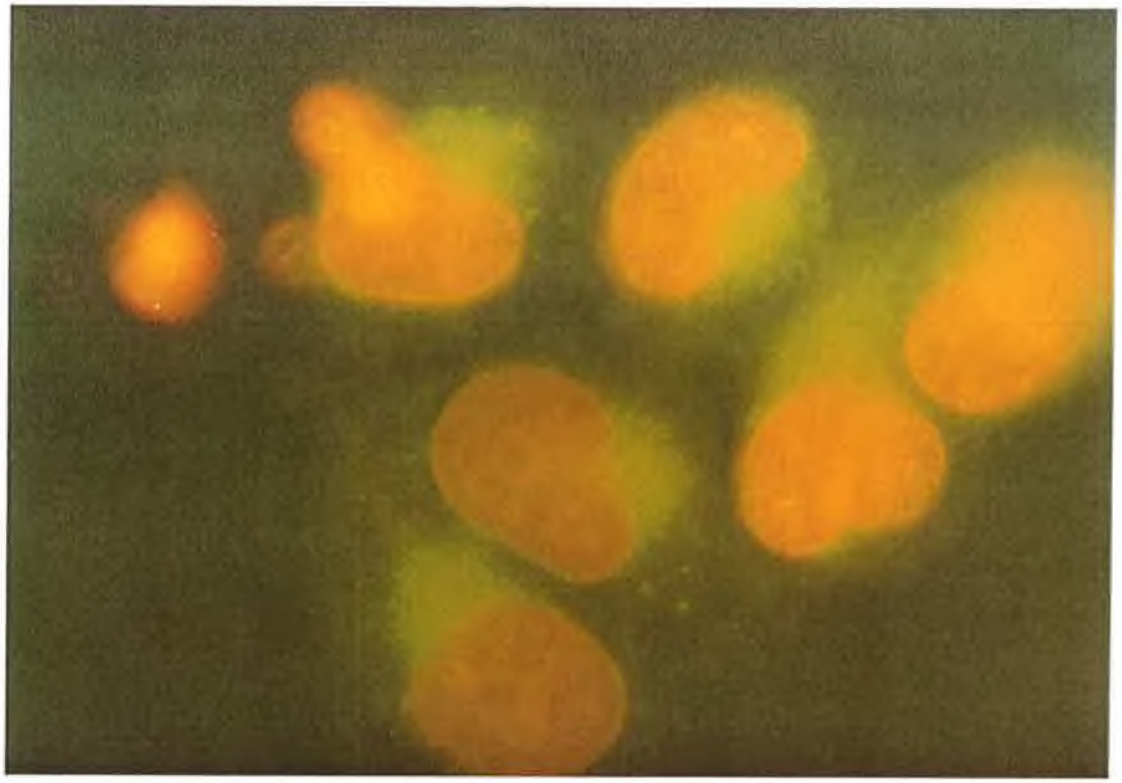


**Figure 3.15.2.8** The subcellular adriamycin distribution pattern in the DLKPA10 cells. The cells were preloaded with adriamycin (10$\mu$M) for 4 hours (a), washed and incubated with antimycin A (10$\mu$M) for 2 hours (b).

a.



b.

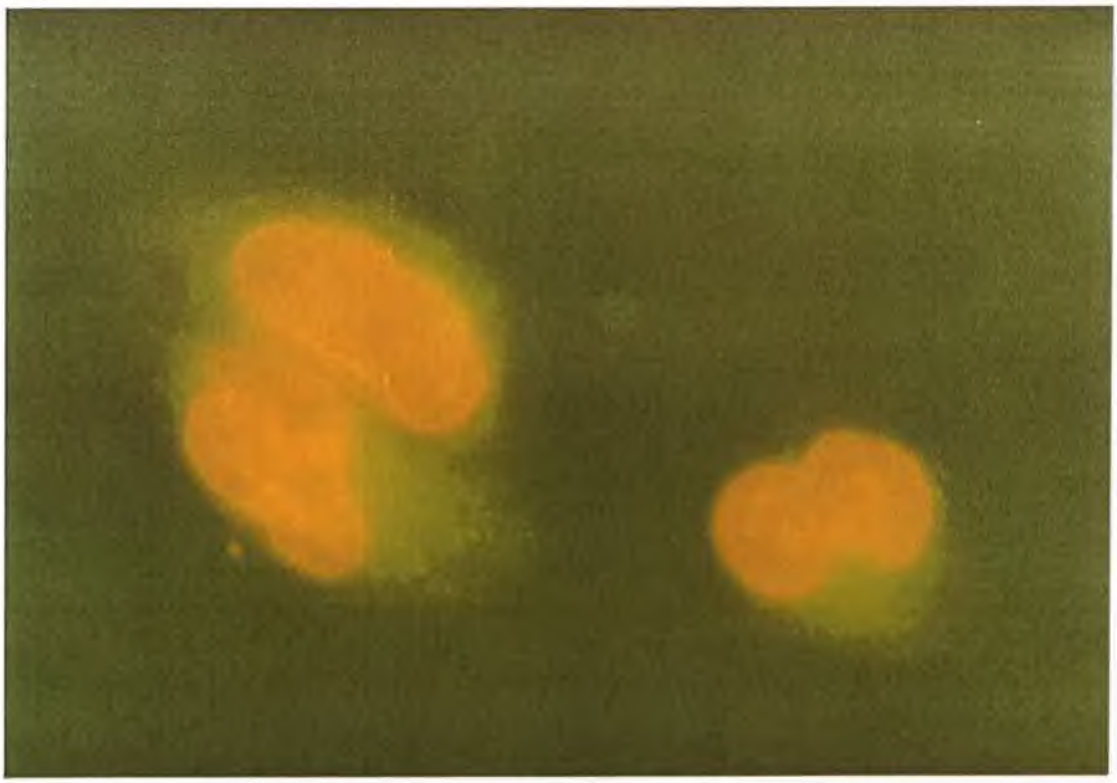
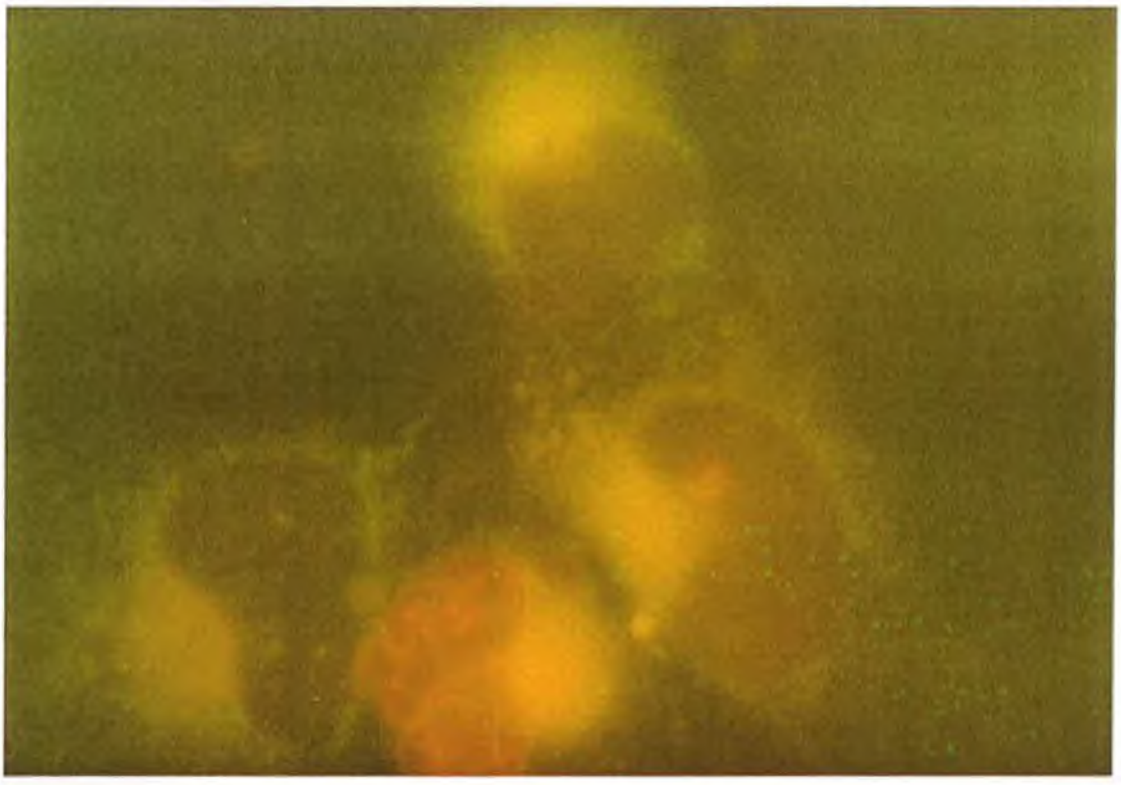


**Figure 3.15.3.1** The subcellular adriamycin distribution pattern in the DLKPA 2B cells. The cells were exposed to adriamycin (10$\mu$M) for 2 hours in the absence (a) or presence (b) of verapamil (30$\mu$g/ml).

a.



b.

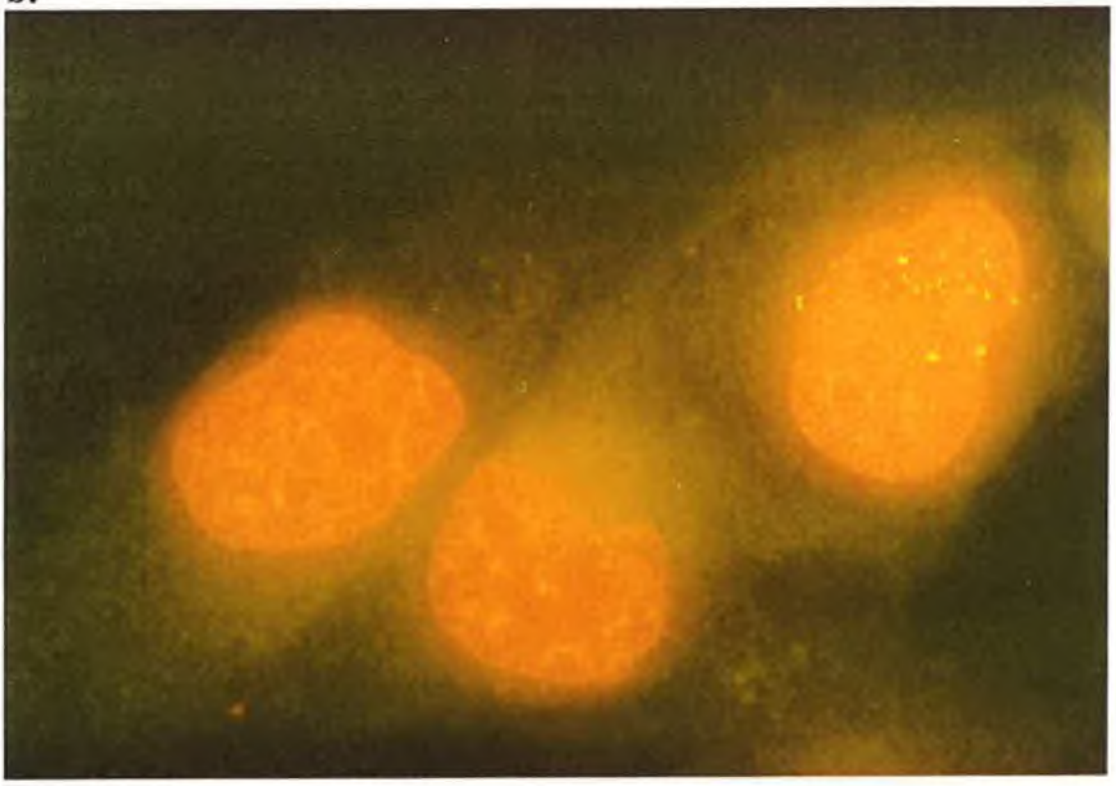
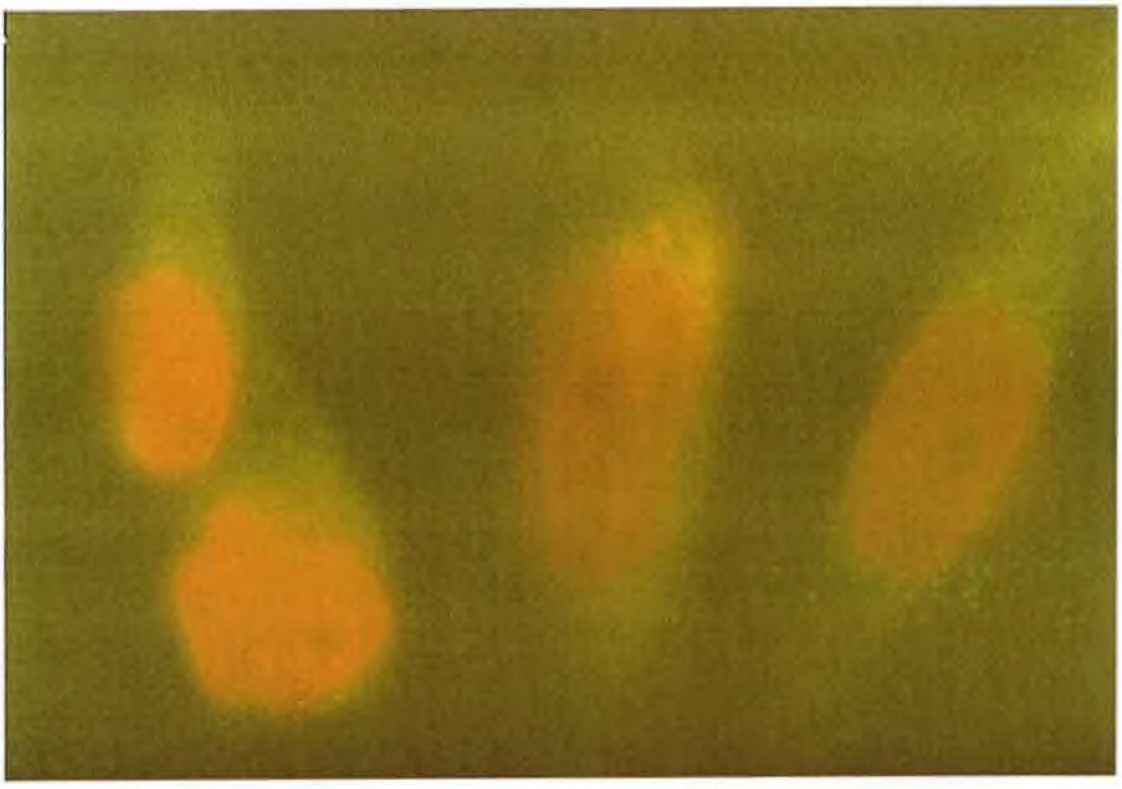


**Figure 3.15.3.2** The subcellular adriamycin distribution pattern in the DLKPA 5F cells. The cells were exposed to adriamycin (10$\mu$M) for 2 hours in the absence (a) or presence (b) of verapamil (30$\mu$g/ml).

a.



b.

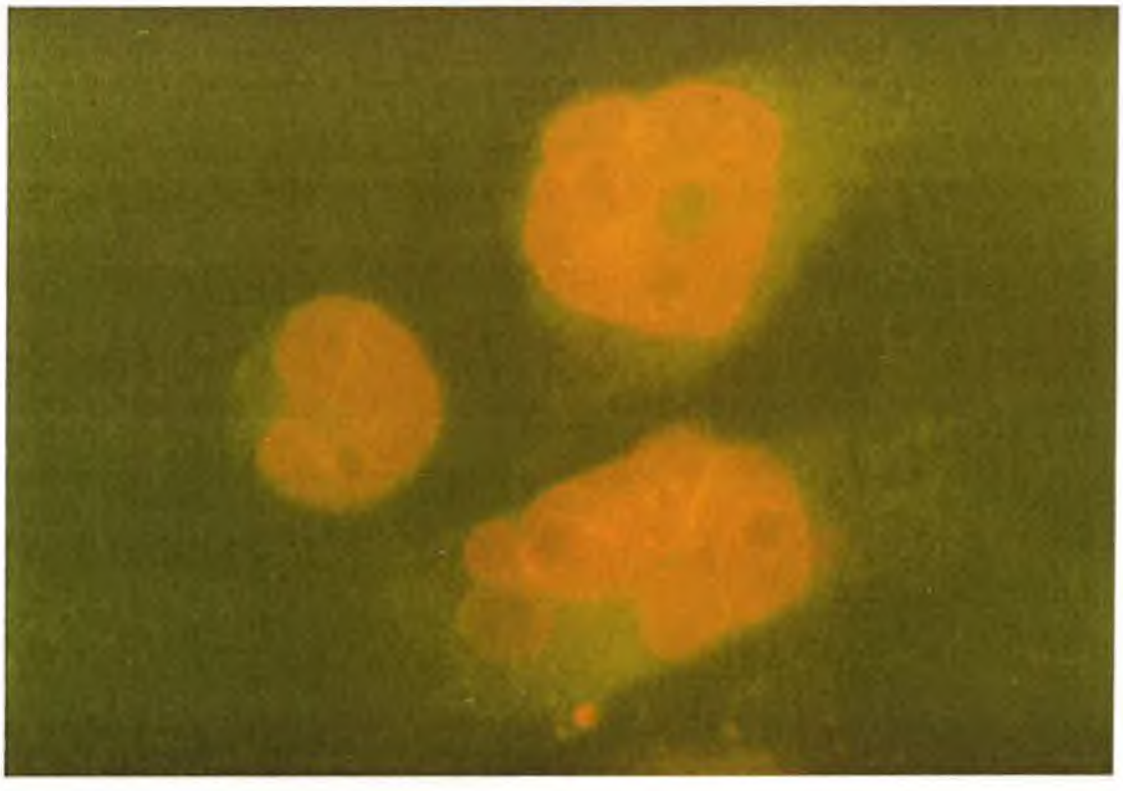
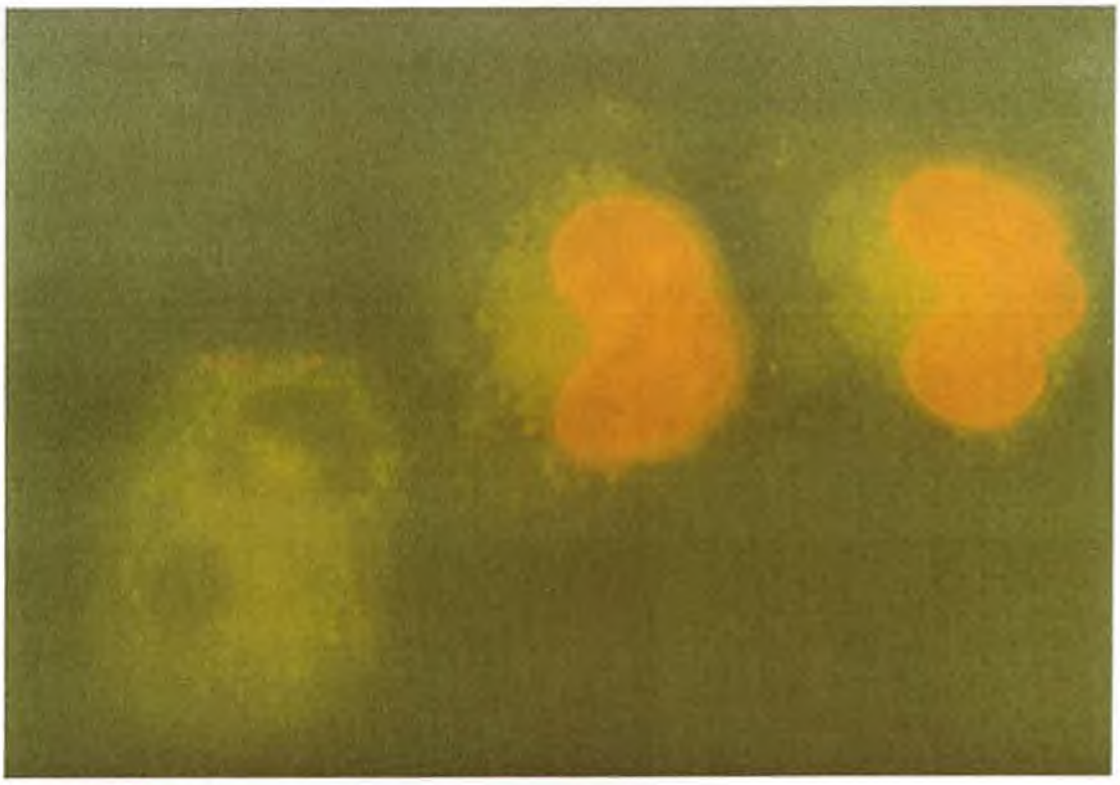


**Figure 3.15.3.3** The subcellular adriamycin distribution pattern in the DLKPA 6B cells. The cells were exposed to adriamycin (10$\mu$M) for 2 hours in the absence (a) or presence (b) of verapamil (30$\mu$g/ml).

a.



b.

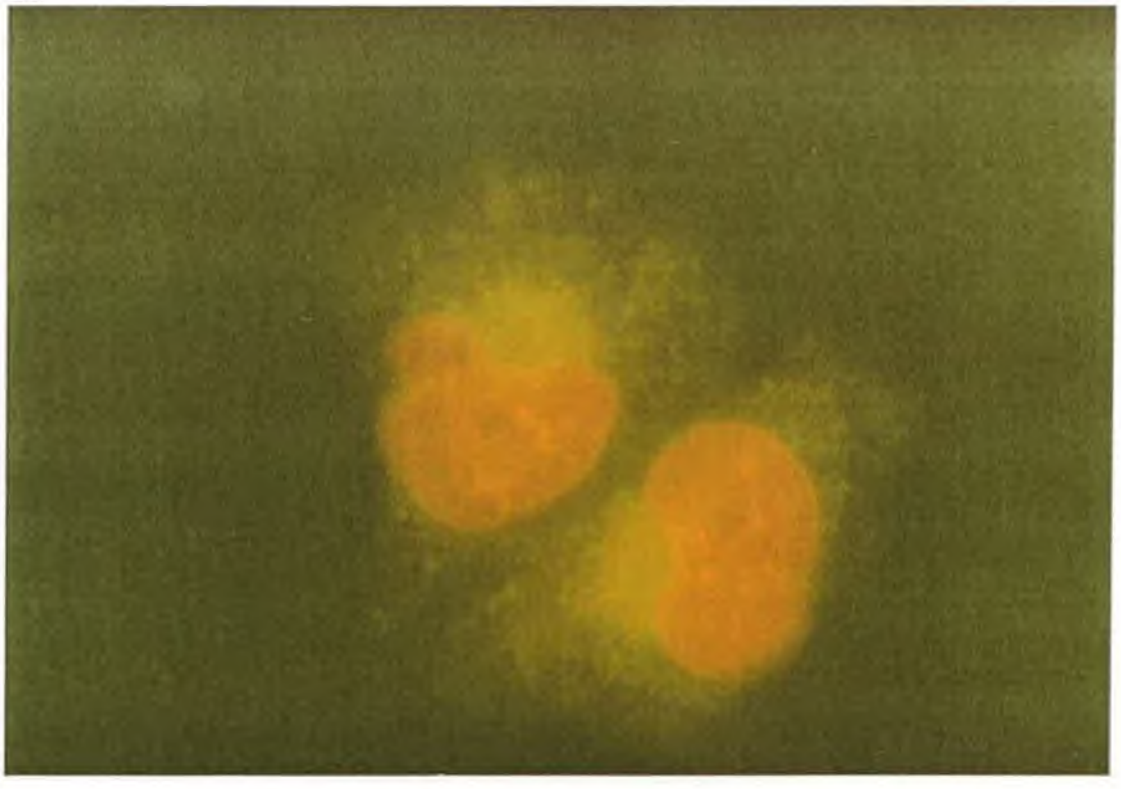


**Figure 3.15.3.4** The subcellular adriamycin distribution pattern in the DLKPA 11B cells. The cells were exposed to adriamycin (10$\mu$M) for 2 hours in the absence (a) or presence (b) of verapamil (30$\mu$g/ml).

### 3.15.4 Subcellular distribution of adriamycin in the OAW42 variants

Adriamycin distribution was also studied in the OAW42-S, OAW42-SR, OAW42-A1 and OAW42-A cells. Figure 3.15.4.1 illustrates the subcellular adriamycin distribution pattern observed in each of the four variants following two hour exposure to the drug. The results showed that adriamycin fluorescence was predominantly localised in the nucleus of the OAW42-S, OAW42-SR and OAW42-A1 cells. However, the intensity of nuclear fluorescence was found to be greatest in the sensitive OAW42-S cell line. The OAW42-SR and OAW42-A1 variants displayed similar fluorescence patterns, predominately displaying nuclear fluorescence, although regions of faint cytoplasmic fluorescence were also visible in the resistant variants. When the OAW42-A cells were exposed to adriamycin, only faint nuclear fluorescence was observed. The intensity of fluorescence was substantially less than that noted in the OAW42-S, OAW42-SR and OAW42-A1 cell lines. Cytoplasmic fluorescence was also visible in the OAW42-A cells. No significant alteration in the fluorescence pattern in the OAW42-A cells was observed following longer incubation time periods (up to 8 hours). The addition of cyclosporin A and verapamil resulted in an increase in the intensity of nuclear fluorescence in each of the variants, to a level greater than that observed in the OAW42-S sensitive cells. However, the most significant increase in fluorescence intensity was noted in the OAW42-A cell line (figure 3.15.4.2).

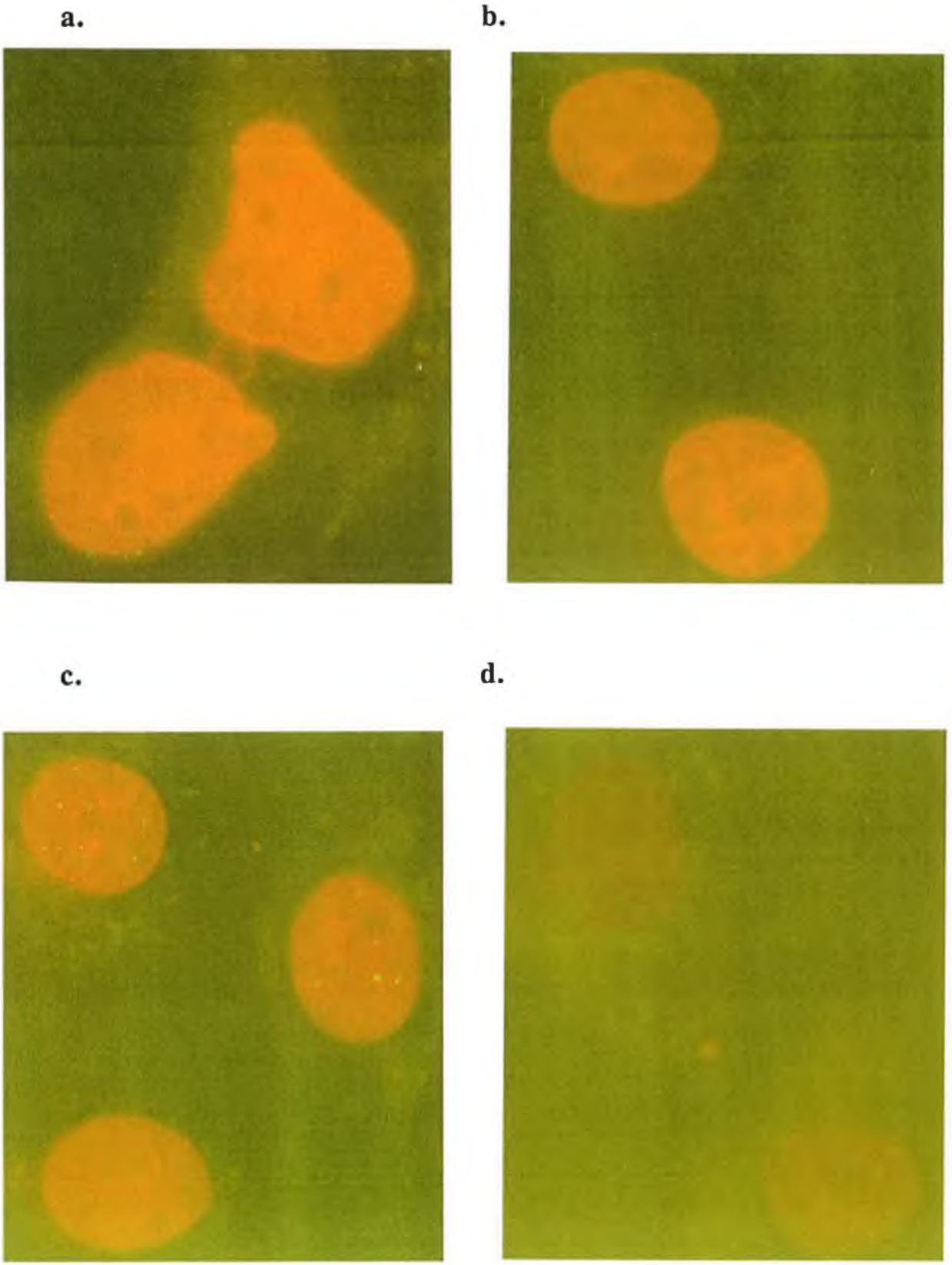
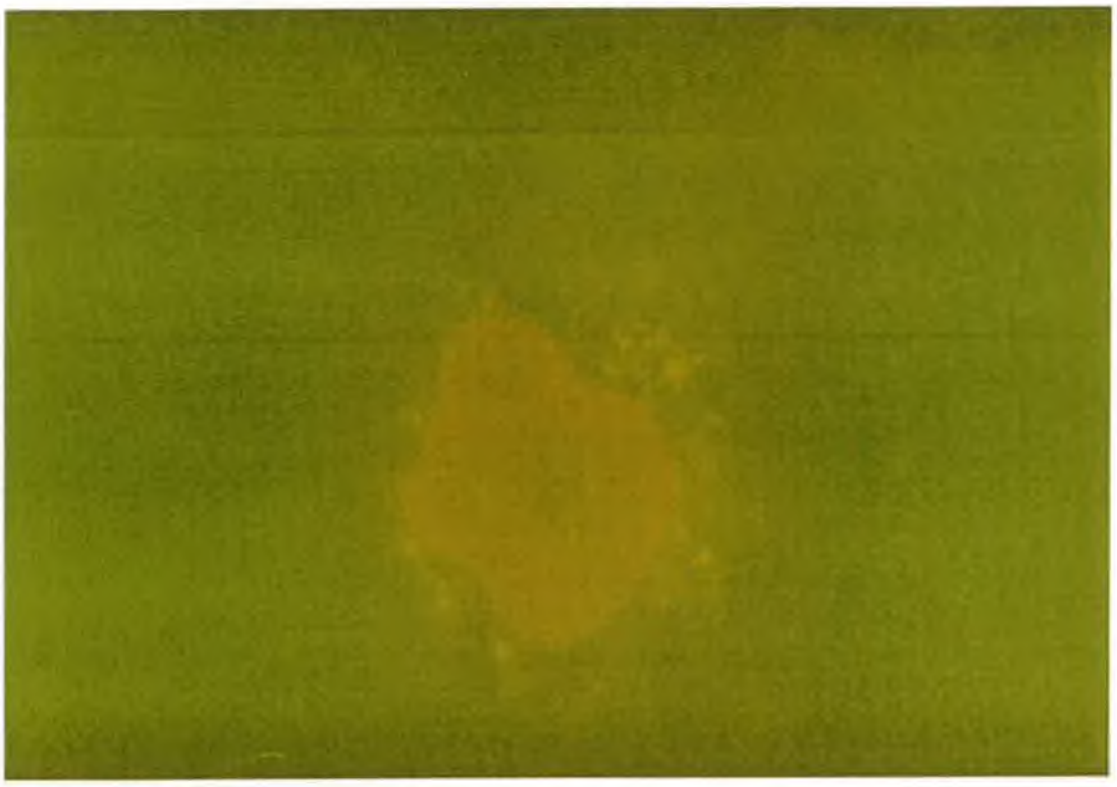



**Figure 3.15.4.1** The subcellular adriamycin (10$\mu$M) distribution in the OAW42 variants following 2 hour exposure to the drug; (a) OAW42-S, (b) OAW42-SR, (c) OAW42-A1 and (d) OAW42-A.

a.



b.

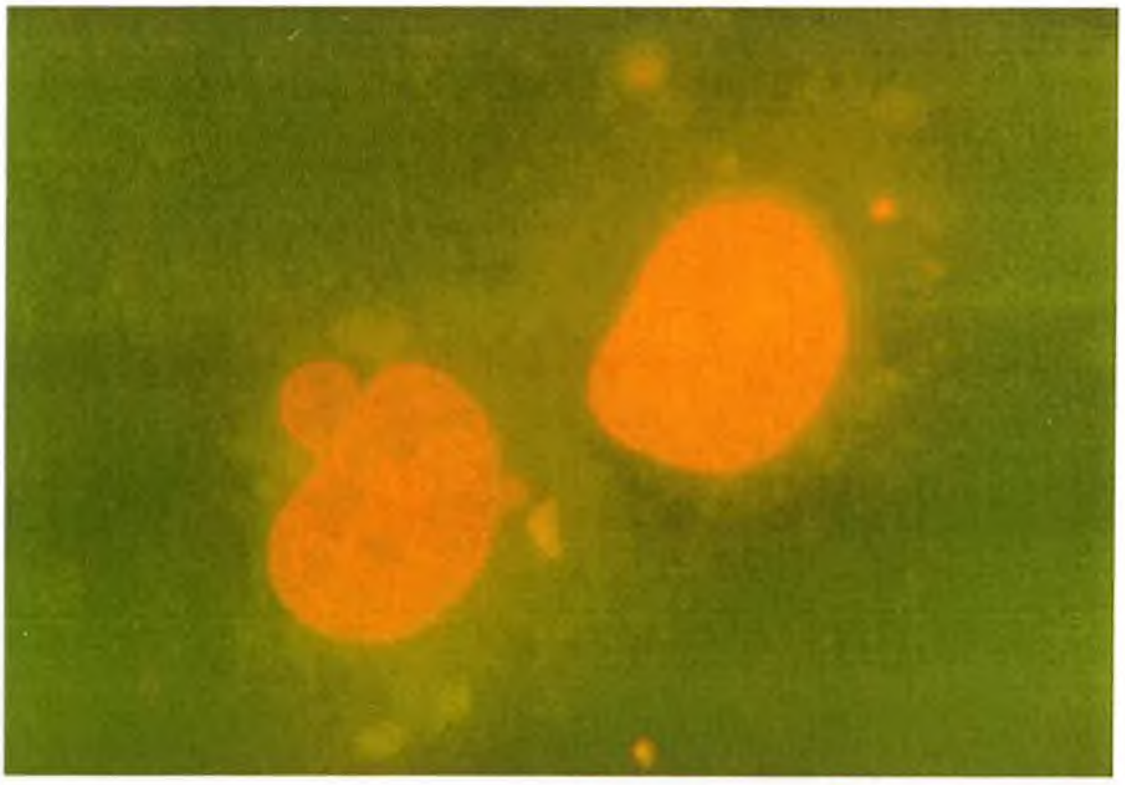


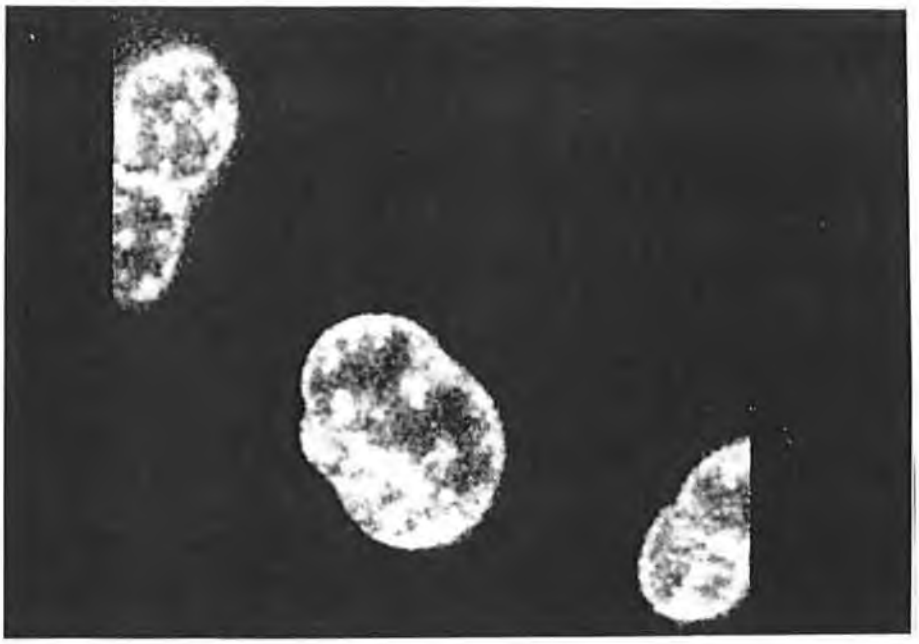
**Figure 3.15.4.2** The subcellular adriamycin distribution pattern in the OAW42-A resistant variant. The cell were exposed to the drug for 2 hours in the absence (a) or presence of cyclosporin A (b).

### 3.16 Confocal laser microscopy studies

The subcellular localisation of adriamycin in the SKMES-1 and DLKP parental cells and in their resistant variants, SKMES-1/ADR and DLKPA10, was also studied by confocal laser microscopy. When the SKMES-1 cells were incubated with adriamycin (10$\mu$M) for two hours, intense nuclear fluorescence was observed in all of the cells viewed. The nuclear envelope was quite distinct, with more intense regions of fluorescence clearly visible within the nucleus of the cell (Figure 3.16.1). The addition of verapamil (30$\mu$g/ml) and cyclosporin A (10$\mu$g/ml) resulted in an increase in the intensity of nuclear fluorescence. The DLKP parental cell line exhibited a similar fluorescence pattern when exposed to adriamycin (Figure 3.16.1). Intense nuclear fluorescence, with more intense regions within the nucleus, was again observed. Verapamil and cyclosporin A caused a slight increase in the intensity of adriamycin nuclear fluorescence in the DLKP cells.

Following exposure of the SKMES-1/ADR cells to adriamycin, faint nuclear fluorescence was observed in the majority of cells viewed, although the intensity was substantially less than that observed in the parental SKMES-1 cells. Distinct regions of fluorescence were also visible, scattered throughout the cytoplasm, particularly in areas close to the nucleus of the cells (Figure 3.16.2). The addition of verapamil or cyclosporin A enhanced the intensity of nuclear fluorescence in the SKMES-1/ADR cells to a level comparable with the SKMES-1 cell line. A decrease in cytoplasmic fluorescence was also observed in the presence of verapamil and cyclosporin A. When adriamycin distribution was studied in the DLKPA10 cells, faint nuclear fluorescence was observed in a small number of cells, although the majority of cells displayed no nuclear fluorescence. Distinct spots of intense fluorescence were clearly visible within the cytoplasm, with more intense regions of fluorescence distinguishable in the perinuclear region (Figure 3.16.3). The cytoplasmic fluorescence was quite intense following two hour exposure to adriamycin. Although exposure of the cells to adriamycin for longer incubation periods resulted in an increase in cytoplasmic fluorescence, no corresponding increase in nuclear fluorescence was observed. Verapamil and cyclosporin A enhanced the nuclear fluorescence in the DLKPA10 cells, although the intensity observed was significantly less than that observed in the DLKP parental cells. Both agents also decreased the intensity of cytoplasmic fluorescence, while reducing the quantity of fluorescent vesicles visible in the cytoplasm. No further increase in nuclear fluorescence was observed in the DLKPA10 cells following longer exposure times to either cyclosporin A or verapamil.

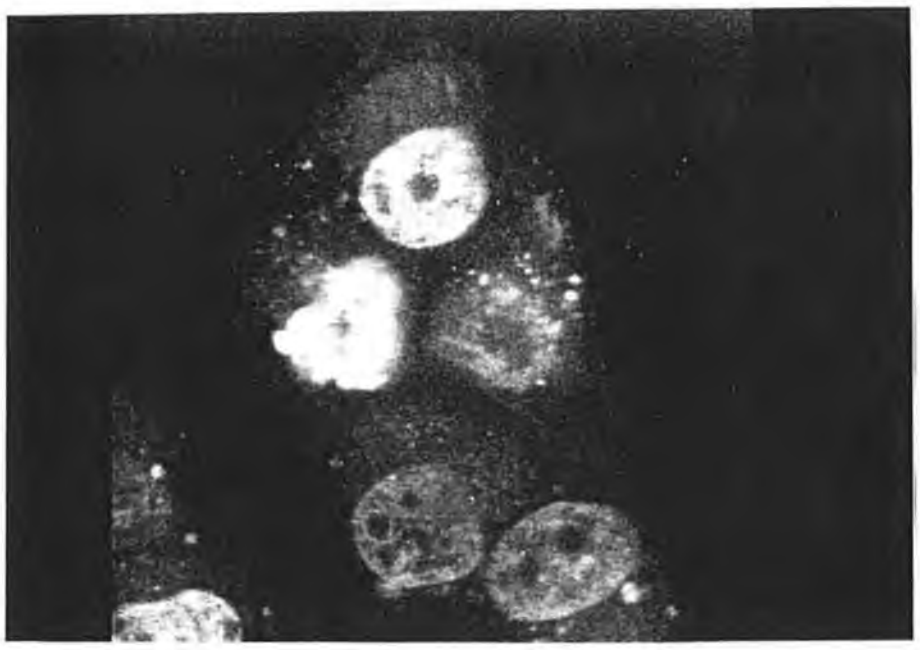
a.



b.

**Figure 3.16.1** Confocal laser microscopy illustrating the subcellular distribution of adriamycin (10$\mu$M) in the SKMES-1 (a) and the DLKP (b) sensitive cells.

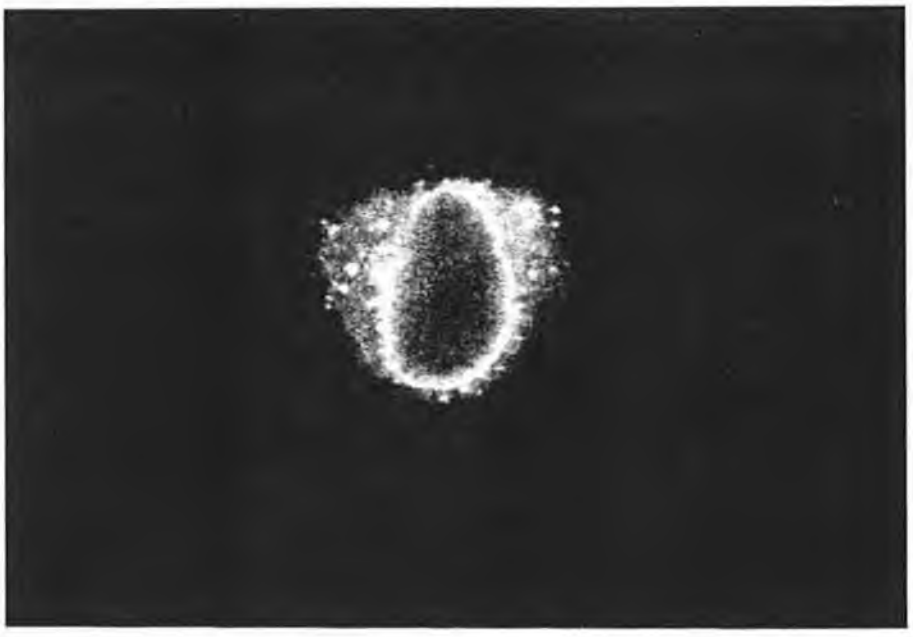
a.



b.

**Figure 3.16.2** Confocal laser microscopy illustrating the subcellular distribution of adriamycin (10$\mu$M) in the SKMES-1/ADR cells. The cells were exposed to the drug for 2 hours in the absence (a) or presence (b) of cyclosporin A (10$\mu$g/ml).

a.



b.

**Figure 3.16.3** Confocal laser microscopy illustrating the subcellular distribution of adriamycin (10$\mu$M) in the DLKPA10 cells. The cells were exposed to the drug for 2 hours in the absence (a) or presence (b) of cyclosporin A (10$\mu$g/ml).

## 3.17 Circumvention studies in DLKP and SKMES-1 cells

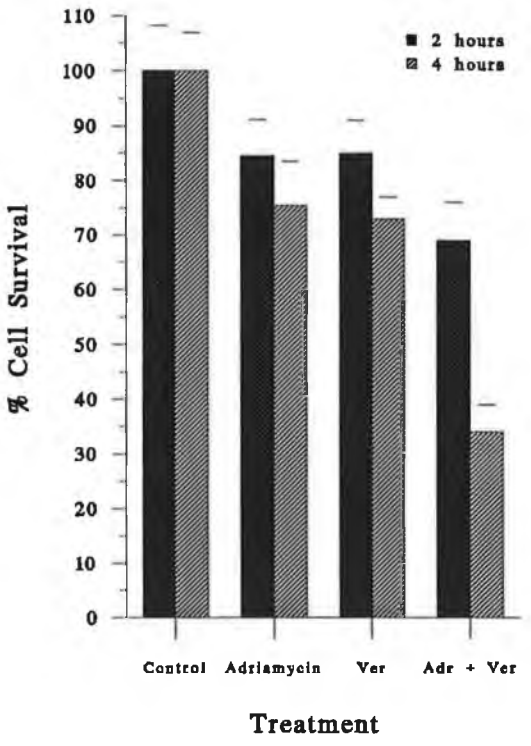
### 3.17.1 Effect of verapamil and cyclosporin A on adriamycin toxicity in SKMES-1 and SKMES-1/ADR cell lines

The effect of verapamil and cyclosporin A on the toxicity of adriamycin in the SKMES-1 and SKMES-1/ADR cell lines was investigated, under conditions similar to those employed in the accumulation studies. The toxicity was determined, as described in section 2.6. Adriamycin proved to be extremely toxic to the parental SKMES-1 cells, with total cell kill observed following two hour and four exposure to the drug (10$\mu$M). The addition of verapamil (30$\mu$g/ml) or cyclosporin A (10$\mu$g/ml), again resulted in total cell kill in the SKMES-1 cells. When the SKMES-1/ADR cells were exposed to adriamycin, approximately 15% kill was noted after two hours, with 25% cell kill after four hour exposure. The addition of verapamil resulted in a decrease in cell viability of approximately 10% and 40% following two hour and four hour exposure respectively (Figure 3.17.1). Cyclosporin A also enhanced the toxicity of adriamycin in the SKMES-1/ADR cell line. When the cells were exposed to cyclosporin A for two hours, a decrease of approximately 20% and 50% was observed following, two and four hour exposures (figure 3.17.1).

### 3.17.2 Effect of verpamil and cyclosporin A on adriamycin toxicity in DLKP and DLKPA10 cell lines

Adriamycin toxicity was also studied in the DLKP and DLKPA10 cell lines, in the presence of verapamil and cyclosporin A. Treatment of the parental DLKP cells with adriamycin (10$\mu$M) resulted in total cell kill after a two hour time period. When the cells were coincubated with verapamil (30$\mu$g/ml) or cyclosporin A (10$\mu$g/ml) total cell kill was, again, observed in the parental cells. When the DLKPA10 cells were exposed to adriamycin only slight toxicity was observed even, after four hour incubation (approximately 5% cell kill noted). However, the addition of cyclosporin enhanced adriamycin toxicity in the cells. Following two hour incubation with cyclosporin A (10$\mu$g/ml) a decrease of approximately 38% cell viability was observed, while exposure for four hours resulted in a decrease of approximately 45% (Figure 3.17.2). Verapamil also increased the toxicity of adriamycin, causing a decrease of approximately 25% and 40% cell viability after two hour and four hour time periods respectively.

a.




b.

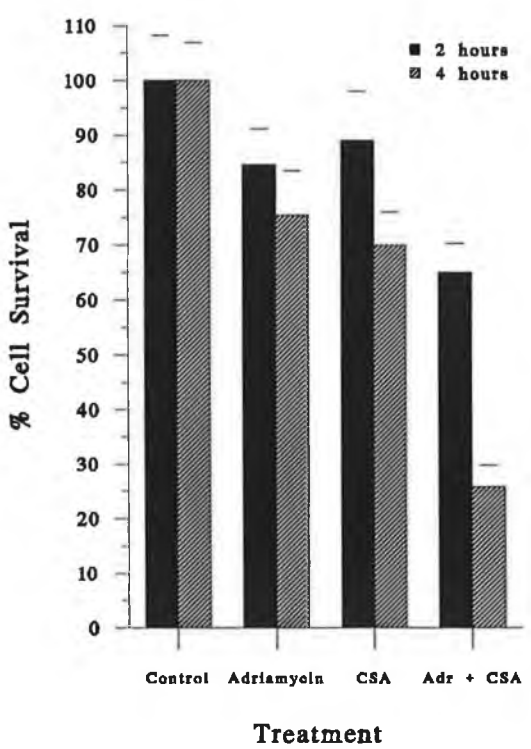
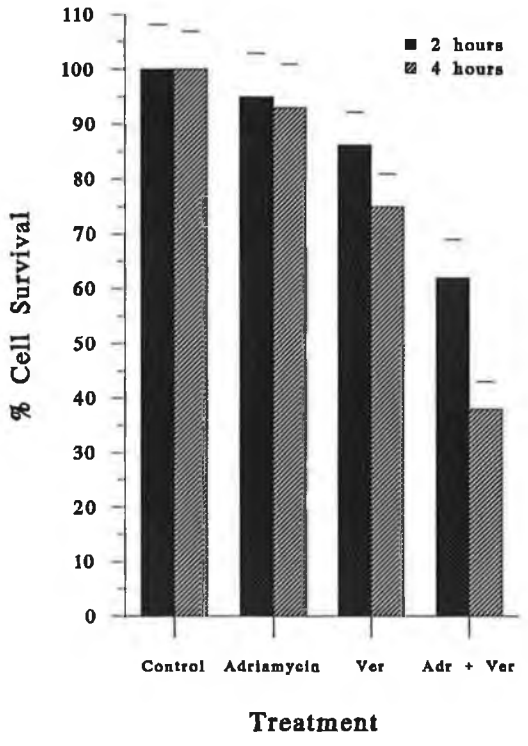



**Figure 3.17.1** The effect of verapamil (a) and cyclosporin A (b) on adriamycin toxicity in the SKMES-1/ADR cell line. The cells were exposed to adriamycin (10$\mu$M) in the presence of verapamil (30$\mu$g/ml) and cyclosporin A (10$\mu$g/ml) for two and four hour incubation periods.

a.




b.

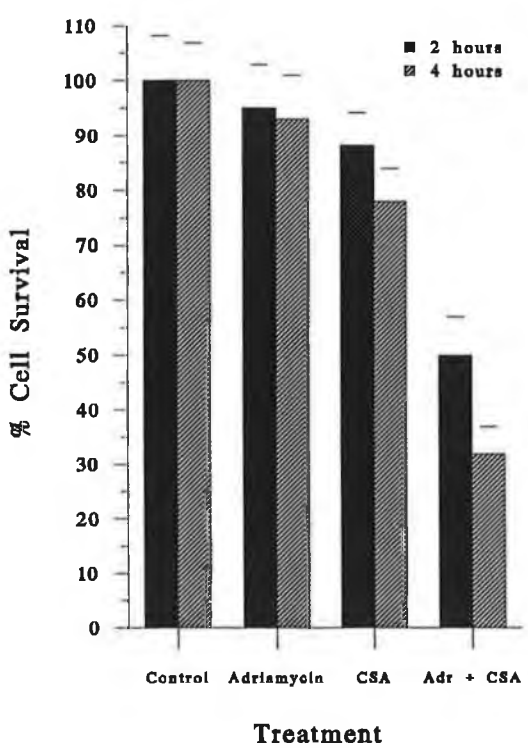



**Figure 3.17.2** The effect of verapamil (a) and cyclosporin A (b) on adriamycin toxicity in the DLKPA10 cell line. The cells were exposed to adriamycin (10$\mu$M) in the presence of verapamil (10$\mu$g/ml) and cyclosporin A (30$\mu$g/ml) for two and four hour incubation periods.

## 3.18 Investigation of a number of compounds on adriamycin accumulation and distribution in DLKP and DLKPA10 cell lines

The effect of various compounds on the accumulation and subcellular distribution of adriamycin in the DLKP and DLKPA10 cell lines was investigated. The compounds studied included the fungal antibiotic brefeldin A, the tyrosine kinase inhibitor genestein, the macrolide antibiotic bafilomycin A1, the carboxylic ionophores monensin and nigericin, the calmodulin inhibitor trifluoroperazine and the lysosomotropic agents chloroquine and methylamine. The aim of these studies was to determine if the cytoplasmic vesicles observed in the resistant cells could be eliminated, enabling the adriamycin to enter the nucleus of the cells.

### 3.18.1 The effect of brefeldin A on adriamycin accumulation and distribution

The effect of the Golgi apparatus disrupting agent, brefeldin A, on the accumulation and subcellular distribution of adriamycin was investigated in the DLKP and DLKPA10, cells to establish if the cytoplasmic fluorescent vesicles observed were associated with the Golgi apparatus. Figure 3.18.1 illustrates the time course of adriamycin accumulation in the parental DLKP and resistant DLKPA10 cell lines following incubation with brefeldin A (10$\mu$M). No significant difference in adriamycin uptake was noted in the DLKPA10 cells. However, the addition of brefeldin A resulted in a slight decrease in adriamycin accumulation in the DLKP parental cells. When the cells were pretreated with brefeldin A (10$\mu$M) for two hours prior to the addition of adriamycin, no significant alteration in adriamycin accumulation was observed in the DLKPA10 cells. Pretreatment with brefeldin A, again, resulted in a slight decrease in drug accumulation in the DLKP cells. When the subcellular localisation of adriamycin was examined following two hour exposure to brefeldin A, intense nuclear fluorescence was observed in the parental DLKP cells. The intensity of fluorescence was comparable to that observed in the control cells incubated with adriamycin alone. When the cells were exposed to brefeldin A for longer incubation periods, cell lysis was observed in the DLKP cells. The addition of brefeldin A did not significantly alter the distribution of adriamycin in the DLKPA10 cells. Faint nuclear fluorescence was observed in a small number of cells, although the majority of cells displayed only cytoplasmic fluorescence. Distinct regions of cytoplasmic fluorescence were still visible in all of the cells viewed following longer exposure to brefeldin A. Brefeldin A did not exert an observable toxic

effect on the DLKPA10 cells within the four hour time period studied.

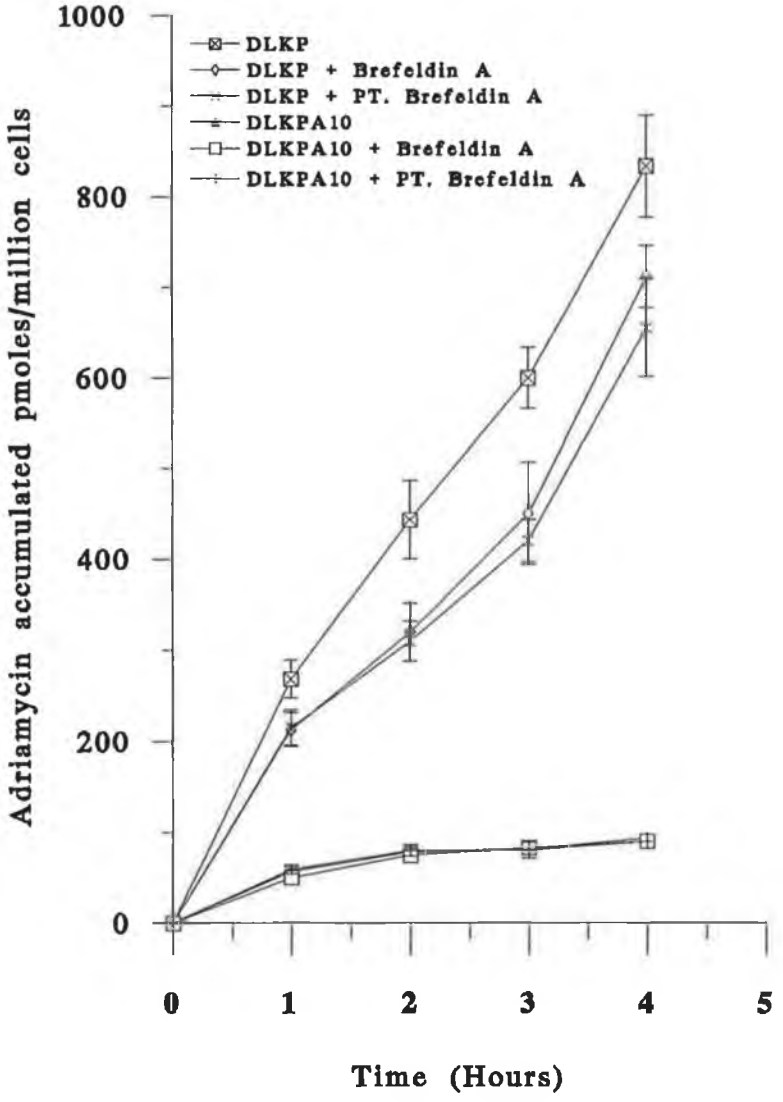



**Figure 3.18.1** The effect of pretreatment (2 hours) and cotreatment with the Golgi apparatus disrupting agent, brefeldin A (10$\mu$M) on the time course of adriamycin accumulation in the DLKP and DLKPA10 cell lines.

### 3.18.2 The effect of genistein on adriamycin accumulation and distribution

The effect of the tyrosine kinase inhibitor genistein on adriamycin accumulation was investigated in the DLKP and DLKPA10 cell lines. Genistein (300$\mu$g/ml) was coincubated with adriamycin and its effect on the accumulation of adriamycin was monitored over four hours. The results obtained showed that genistein did not significantly alter the rate or maximum level of adriamycin accumulation in either the DLKP or DLKPA10 cells within the time period studied (Figure 3.18.2). When the concentration of genistein was increased up to 1mg/ml, no further increase in the cellular concentration of adriamycin was observed. When the subcellular localisation of adriamycin was studied in the presence of genistein (300$\mu$g/ml), intense nuclear fluorescence was noted in the parental cells, while only cytoplasmic fluorescence was observed in the DLKPA10 cell line. When the concentration of genistein was increased to 500$\mu$g/ml, cell lysis was observed in the majority of the cells viewed. No significant difference in adriamycin distribution was noted in the viable cells.

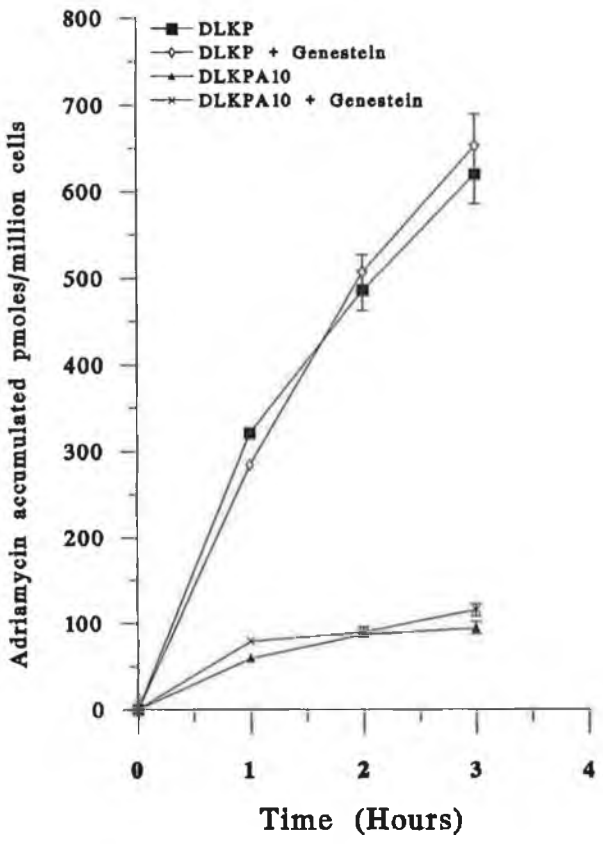



**Figure 3.18.2** The effect of genistein (300$\mu$g/ml) on the time course of adriamycin accumulation in the DLKP and DLKPA10 cells.

### 3.18.3 The effect of bafilomycin A1 on adriamycin accumulation and distribution

The effect of the vacuolar $H^+$ATPase inhibitor, bafilomycin A1, on the subcellular distribution of adriamycin was also investigated in the DLKP and DLKPA10 cell lines. This work was carried out to determine if vacuolar $H^+$ATPases were involved in the sequestration of adriamycin into the cytoplasmic vesicles, observed in the DLKPA10 cells following exposure to the drug. When the DLKP cells were exposed to bafilomycin A1 (10$\mu$g/ml), nuclear fluorescence was observed in all of the cells viewed. The intensity of fluorescence was comparable with that noted in cells exposed to adriamycin alone. Bafilomycin A1 did not appear to exert a toxic effect on the parental cells following incubation time periods of up to six hours. Coincubation with bafilomycin A1 did not significantly alter the fluorescent pattern observed in the DLKPA10 cell line. No significant increase in nuclear fluorescence was noted, with regions of intense fluorescence still visible throughout the cytoplasmic regions. When the time course of adriamycin accumulation was studied in the presence of bafilomycin A1, no significant alteration in either the rate of accumulation or maximum level of adriamycin accumulated was observed in the DLKP and DLKPA10 cells.

### 3.18.4 The effect of trifluoroperazine on adriamycin distribution

The effect of the calmodulin inhibitor, trifluoroperazine on adriamycin distribution was studied to determine if it could enhance the nuclear level of adriamycin in the DLKPA10 cells. When the DLKP cells were incubated with trifluoroperazine (10$\mu$g/ml), total cell lysis was observed after two hours. The DLKPA10 cells were not as susceptible to the toxicity of trifluoroperazine, although some cell kill was observed. The addition of trifluoroperazine did not enhance the intensity of nuclear fluorescence in the DLKPA10 cells. Speckles of cytoplasmic fluorescence were still clearly visible in the cells.

### 3.18.5 The effect of lysosomotropic agents on adriamycin distribution

The subcellular distribution of adriamycin, in the presence of the lysosomotropic agents, chloroquine and methylamine was investigated to determine if the cytoplasmic fluorescence observed in the DLKPA10 cell line was associated with the lysosomal regions of the cells.

No significant alteration in the adriamycin fluorescence pattern was observed in the DLKPA10 cells following coincubation with chloroquine (100$\mu$M) or methylamine (10mM). The speckled cytoplasmic fluorescence was still clearly distinguishable in all the cells viewed. No further increase in nuclear fluorescence was observed following longer incubation time periods. The effect of increasing concentrations of chloroquine and methylamine on adriamycin distribution was also studied. However, when the cells were exposed to higher concentrations of the drugs, lysis was observed in the majority of cells. When the parental DLKP cells were exposed to chloroquine and methylamine, no alteration in the intensity of nuclear fluorescence was observed. When the time course of adriamycin accumulation was studied in the presence of chloroquine and methylamine, no significant increase in the cellular concentration of drug was observed.

### 3.18.6 The effect of carboxylic ionophores on adriamycin distribution

The effect of the ionophores, monensin and nigericin, on the localisation of adriamycin in the DLKP and DLKPA10 cells was studied to determine if these agents could enhance drug uptake in the cells. Coincubation with monensin (10$\mu$g/ml) resulted in a slight increase in the intensity of nuclear fluorescence in the parental DLKP cells, when compared with the intensity observed in the control cells exposed to adriamycin alone. When the DLKPA10 cells were exposed to monensin, a marked increase in nuclear fluorescence was observed in all of the cells viewed. Distinct regions of intense fluorescence were still visible within the cytoplasmic regions, although the quantity and intensity of the regions of fluorescence was less than that observed in the absence of monensin. Although a marked increase in nuclear fluorescence was observed in the DLKPA10 cells in the presence of monensin, the intensity of fluorescence was significantly less that that observed in the parental cell line (figure 3.18.6).

Coincubation with nigericin (10$\mu$g/ml) also resulted in an increase in nuclear fluorescence in the DLKPA10 cells. Although, similar to the effect observed with monensin treatment, the intensity was less than that observed in the DLKP parental cell line. Cytoplasmic fluorescence was still visible in the majority of cells studied, although a decrease in the intensity of cytoplasmic fluorescence was observed in all of the cells viewed. A slight increase in the intensity of nuclear fluorescence was also observed in the DLKP cells following treatment with nigericin.

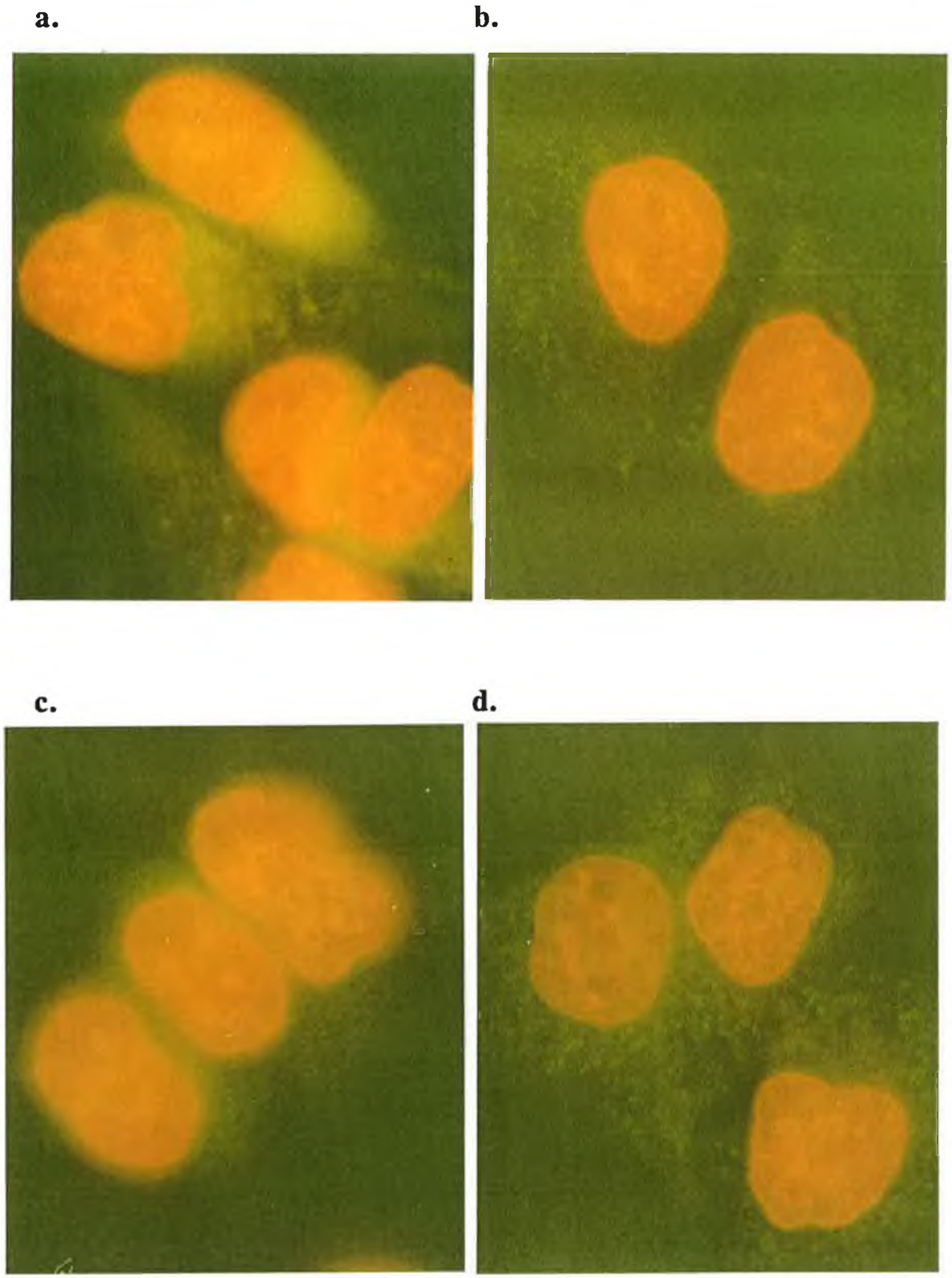



**Figure 3.18.6** The subcellular adriamycin (10$\mu$M) distribution in the DLKP and DLKPA10 cell lines. The cells were exposed to adriamycin for 2 hour in the presence of monensin (10$\mu$g/ml) or nigericin (10$\mu$g/ml); (a) DLKP in the presence of monensin, (b) DLKPA10 in the presence of monensin, (c) DLKP in the presence of nigericin and (d) DLKPA10 in the presence of nigericin.

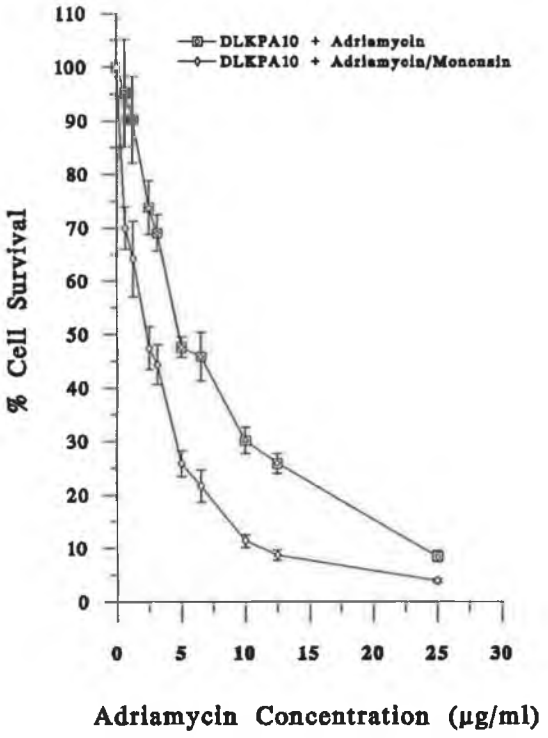
## 3.19 Monensin Studies

### 3.19.1 Monensin toxicity in DLKP and DLKPA10 cell lines

The toxicity of the carboxylic ionophore, monensin, in the DLKP and DLKPA10 cell lines was investigated within the concentration range of 0 - 5$\mu$g/ml monensin. The parental DLKP cells were found to be more susceptible to the toxicity of monensin than the resistant DLKPA10 cells. The $IC_{50}$ values obtained for the DLKP and DLKPA10 cells were approximately 0.005$\mu$g/ml and 0.05$\mu$g/ml monensin respectively, thus representing a 10-fold difference in monensin toxicity. Monensin appeared to be relatively non-toxic to the DLKPA10 cells at concentrations below 0.02$\mu$g/ml and relatively non-toxic to DLKP parental cells at concentrations less than 0.006$\mu$g/ml.

The effect of non-toxic concentrations ($IC_{95}$) of monensin on the toxicity of the chemotherapeutic agents adriamycin, vincristine and VP16 was also examined in the DLKP and DLKPA10 cell lines. The aim of this work was to determine if monensin could modulate the toxicity profile of these drugs. The addition of monensin did not significantly alter adriamycin toxicity in the DLKP parental cells. However, an increase in adriamycin toxicity was observed in the DLKPA10 cells (Figure 3.19.1.1). A 2-fold decrease was observed in the $IC_{50}$ value for adriamycin toxicity in the DLKPA10 cells when treated with monensin. The addition of monensin also enhanced the toxicity profile of VP16 in the DLKPA10 cells. Figure 3.19.1.1 represents the toxicity profile of VP16 in the presence and absence of monensin. Coincubation with monensin resulted in a decrease in the $IC_{50}$ value obtained from 9.5$\mu$g/ml to 4.8$\mu$g/ml in the DLKPA10 cell line, representing a 2-fold increase in the potency of VP16. Monensin was found to have no significant effect on VP16 toxicity in the DLKP cells. When the effect of monensin on the toxicity profile of vincristine was studied, no alteration in toxicity was noted in either the DLKP or DLKPA10 cell lines (Figure 3.19.1.2). No significant difference in the $IC_{50}$ values obtained for vincristine was observed in the presence and absence of monensin. The effect of monensin on carboplatin toxicity in the parental DLKP and the carboplatin resistant DLKPC 25 cell lines was also studied. However, no significant alteration was observed in the toxicity profile of carboplatin in either cell line following treatment with monensin (figure 3.19.1.2).

a.




b.

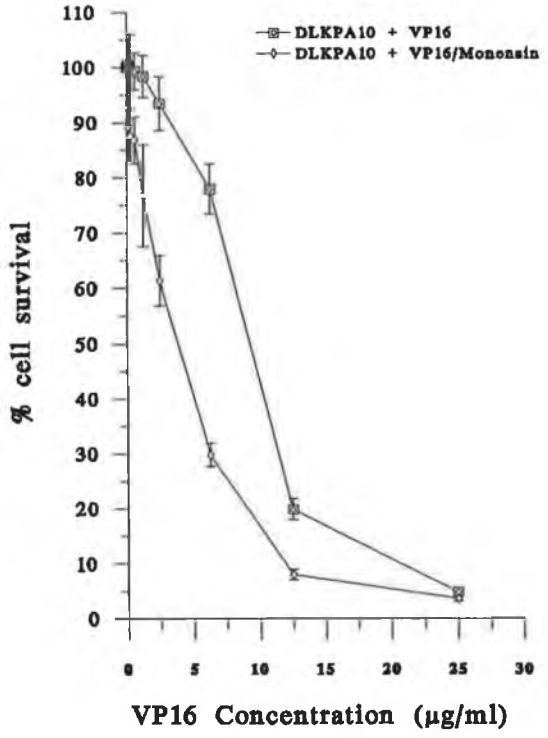
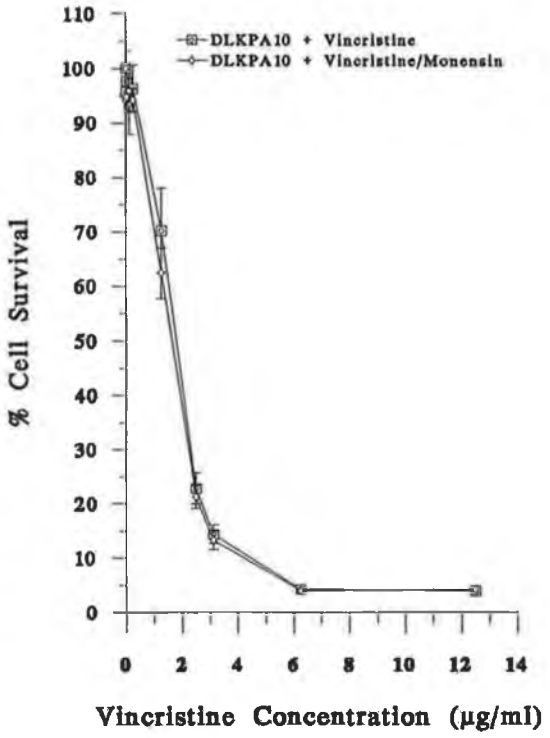



**Figure 3.19.1.1** The effect of monensin on the toxicity profile of adriamycin (a) and VP16 (b) in the DLKPA10 cell line.

a.




b.

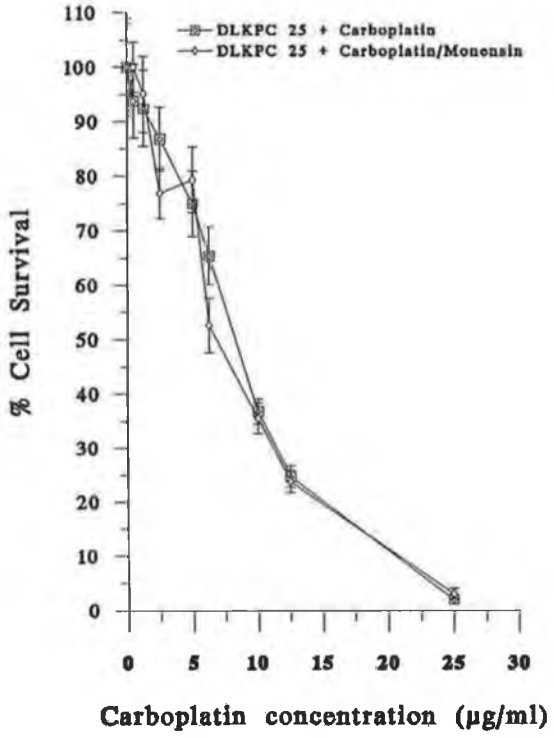



**Figure 3.19.1.2** The effect of monensin on vincristine toxicity in the DLKPA10 cell line (a) and carboplatin toxicity in the DLKPC 25 cell line(b).

### 3.19.2 The effect of monensin on drug accumulation in DLKP and DLKPA10 cells

Since monensin proved to be effective at increasing the nuclear accumulation of adriamycin in the DLKPA10 cells, the effect of monensin on the cellular concentration of adriamycin and vincristine was studied in the DLKP and DLKPA10 cell lines. An initial concentration response curve was established over a range of 0 - 100$\mu$g/ml and a suitable working concentration of monensin was determined. Figure 3.19.2.1 illustrates the time course of adriamycin accumulation in both cell lines over a period of four hours. The addition of monensin (10$\mu$g/ml) resulted in only a slight increase in adriamycin accumulation in the parental cells within the four hours studied. However, monensin proved to be effective in partially reversing the accumulation defect observed in the DLKPA10 cells within the same time period (Figure 3.19.2.1). Although coincubation with monensin resulted in a 6-fold increase in adriamycin accumulation in the DLKPA10 cells, the maximum level of adriamycin was still significantly less than that observed in the DLKP cells. Exposure of the cells for longer incubation time periods (4-10 hours) or with increasing concentration of monensin did not lead to any further increase in adriamycin accumulation in the DLKPA10 cells. Figure 3.19.2.2 illustrates the time course of vincristine accumulation in the DLKP and DLKPA10 cells. No significant increase in the cellular concentration of vincristine was observed in the parental cells following treatment with monensin. However, monensin was found to enhance drug accumulation in the DLKPA10 resistant cells. An increase in the cellular concentration of vincristine of approximately 3.2 fold was observed in the cells.

### 3.19.3 Effect of monensin pretreatment on adriamycin accumulation

Adriamycin accumulation was also investigated in the DLKP and DLKPA10 cells following pretreatment with monensin, to establish if adriamycin accumulation could be completely restored in the resistant cells, to a level comparable with the parental DLKP cells. The cells were pretreated with monensin (10$\mu$g/ml) for two hours prior to the addition of adriamycin. The results obtained showed that monensin pretreatment was more effective than cotreatment at enhancing adriamycin accumulation in both the DLKP and DLKPA10 cells. The cellular concentration of adriamycin following pretreatment was approximately 900 pmoles per $10^6$ cells in the DLKP cells, as opposed to 830 pmoles per $10^6$ cells in the absence of monensin. A significant increase in adriamycin accumulation was observed in the DLKPA10 cells

(figure 3.19.2.1). After a four hour incubation period, approximately 750 pmoles adriamycin per $10^6$ cells was observed in the DLKPA10 cells, representing a 7.5-fold increase in drug accumulation.

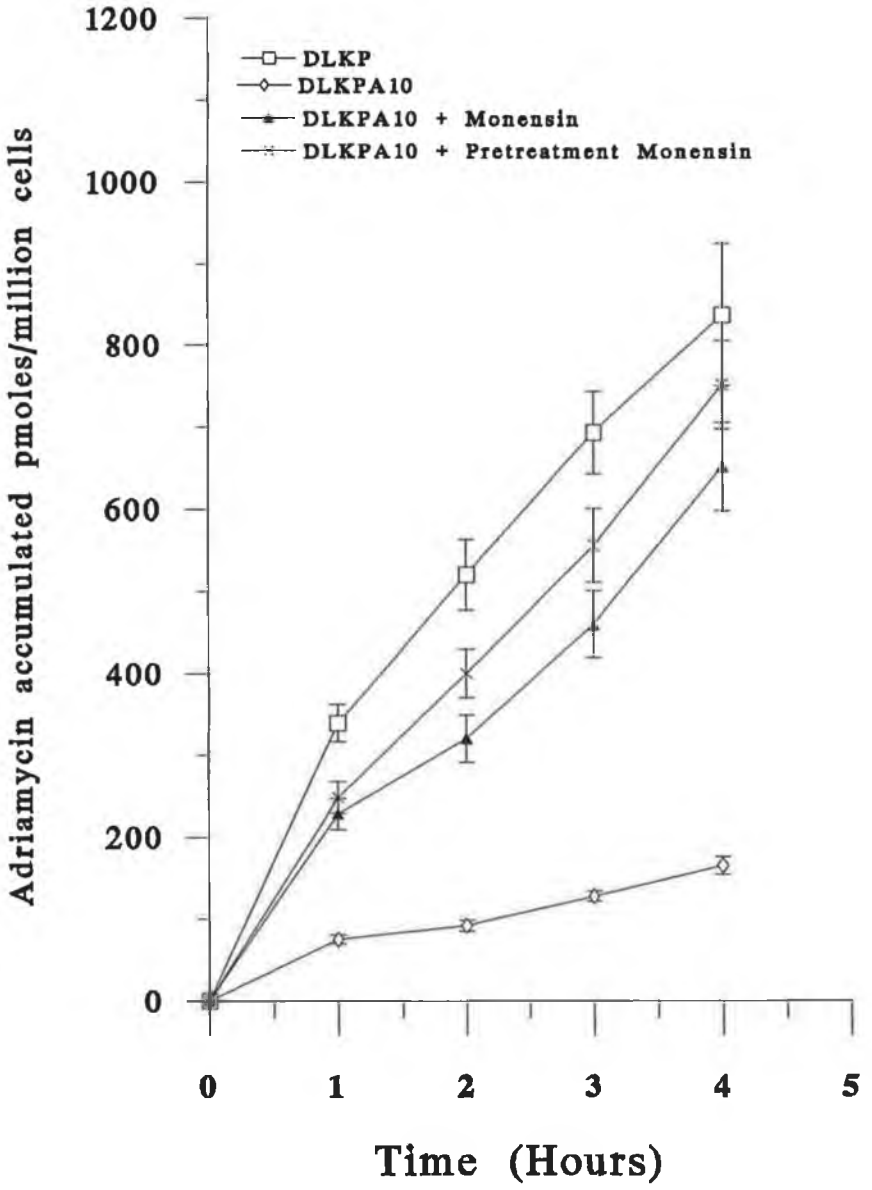



**Figure 3.19.2.1** The effect of monensin pretreatment (2 hours) and cotreatment on the time course of adriamycin accumulation in the DLKPA10 cells.

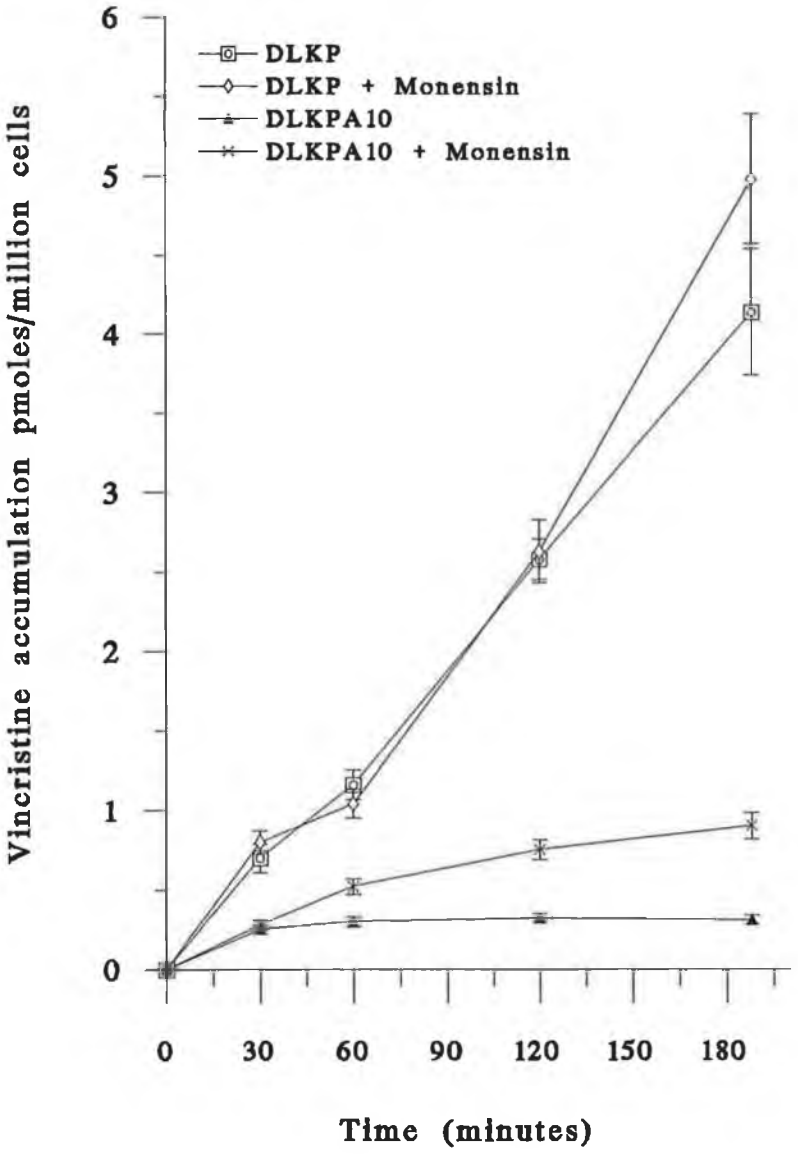



**Figure 3.19.2.2** The effect of monensin (10$\mu$g/ml) on the time course of vincristine accumulation in DLKP and DLKPA10 cell lines

### 3.19.4 Effect of cotreatment with monensin and cyclosporin A on adriamycin accumulation

The effect of monensin and cyclosporin A cotreatment on adriamycin accumulation was studied, to determine if cotreatment could effectively reverse the adriamycin accumulation defect in the DLKPA10 cells. The addition of monensin and cyclosporin A resulted in a marked increase in adriamycin accumulation in the DLKPA10 cells, to a level comparable with that observed with monensin pretreatment (Figure 3.19.4). Although an increase in adriamycin accumulation was also noted in the DLKP cells, the maximum level of adriamycin accumulation was lower than that observed with monensin pretreatment.

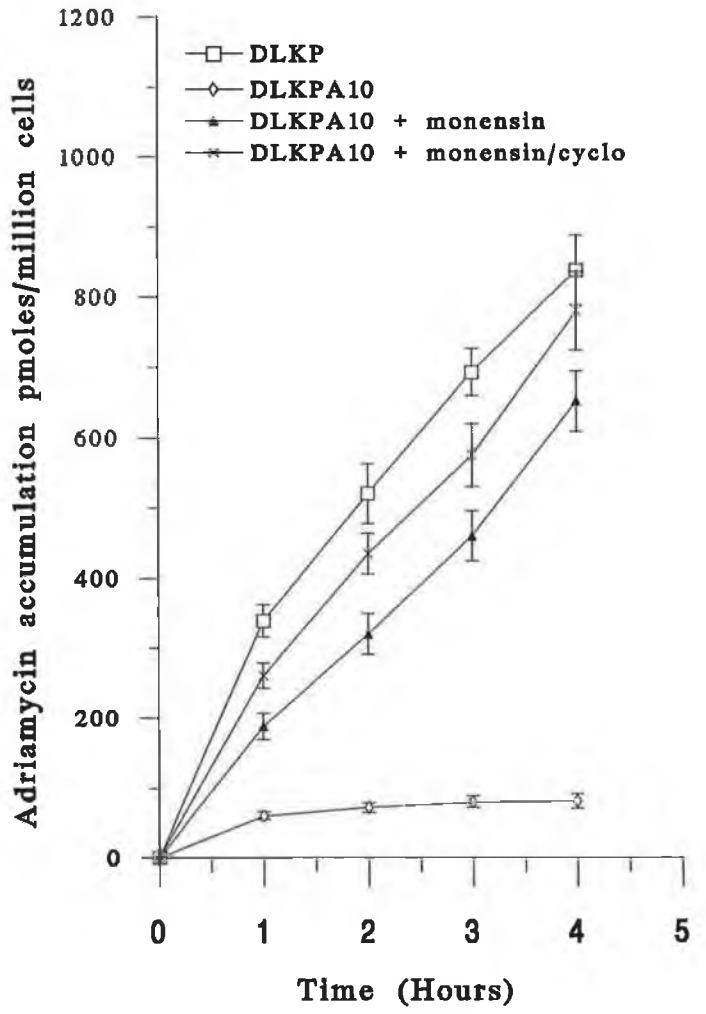



**Figure 3.19.4** The effect of monensin (10$\mu$g/ml) and cyclopsorin A (10$\mu$g/ml) cotreatment on the time course of adriamycin accumulation in DLKPA10 cells.

### 3.19.5 Effect of monensin on adriamycin efflux in DLKP and DLKPA10 cell lines

The effect of monensin on adriamycin efflux in the DLKP and DLKPA10 cell lines was investigated, to determine if monensin could enhance drug retention in the cells. The cells were preloaded with adriamycin for three hours, by incubation in glucose free medium, containing antimycin A (10$\mu$M). The cells were then incubated in drug free medium and the adriamycin efflux studied over a period of three hours. A rapid efflux was observed in both cell lines within the first hour, after which time the rate of efflux decreased, as illustrated in figure 3.19.5. When adriamycin efflux was studied in the presence of monensin, no significant alteration in the rate of efflux was observed in the DLKP parental cells. However, treatment with monensin resulted in a decrease in the rate of adriamycin efflux in the DLKPA10 cells and a significant increase in the cellular retention of drug.

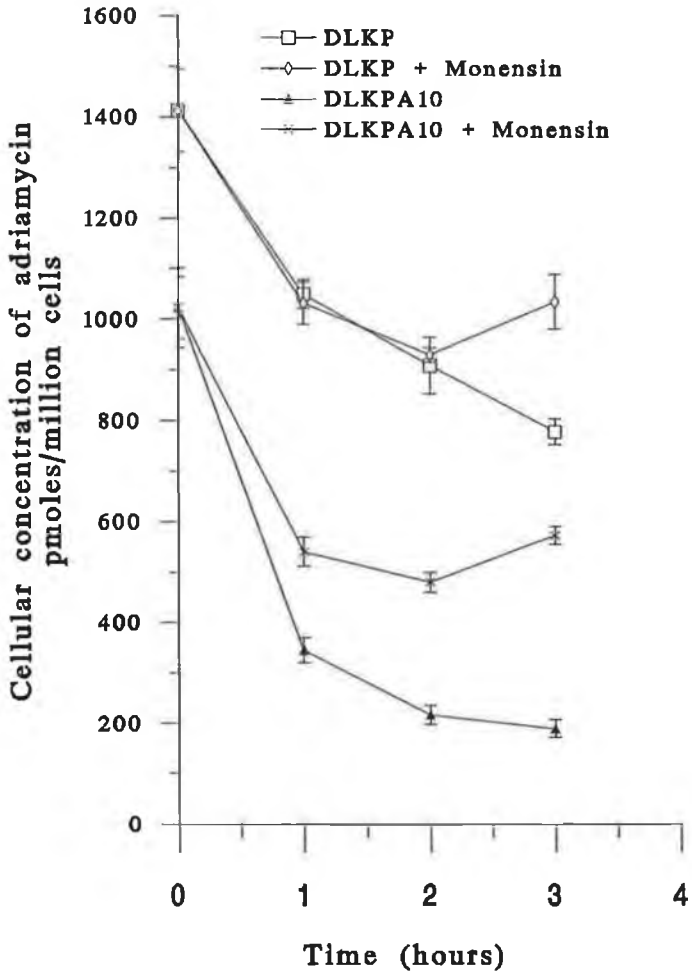



**Figure 3.19.5** The effect of monensin (10$\mu$g/ml) on adriamycin efflux in DLKP and DLKPA10 cell lines. The cells were preloaded with adriamycin for three hours in glucose free medium containing antimycin A (10$\mu$M) and efflux was studied over a further 3 hours.

### 3.19.6 Effect of monensin on vincristine efflux in DLKP and DLKPA10 cells

The effect of monensin on vincristine retention in the DLKP and DLKPA10 cells was also investigated. The cells were preloaded with vincristine for two hours, in the presence of antimycin A. A rapid efflux was observed in both cell lines within the first hour when incubated in drug free medium (figure 3.19.5). After this time, a decrease in the rate of efflux was observed in the DLKPA10 cells, while a steady state of drug retention was noted in the parental cells. Treatment with monensin resulted in approximately a 4-fold decrease in drug efflux in the resistant cells. However, an increase in drug efflux was observed in the parental DLKP cells, following treatment with monensin.

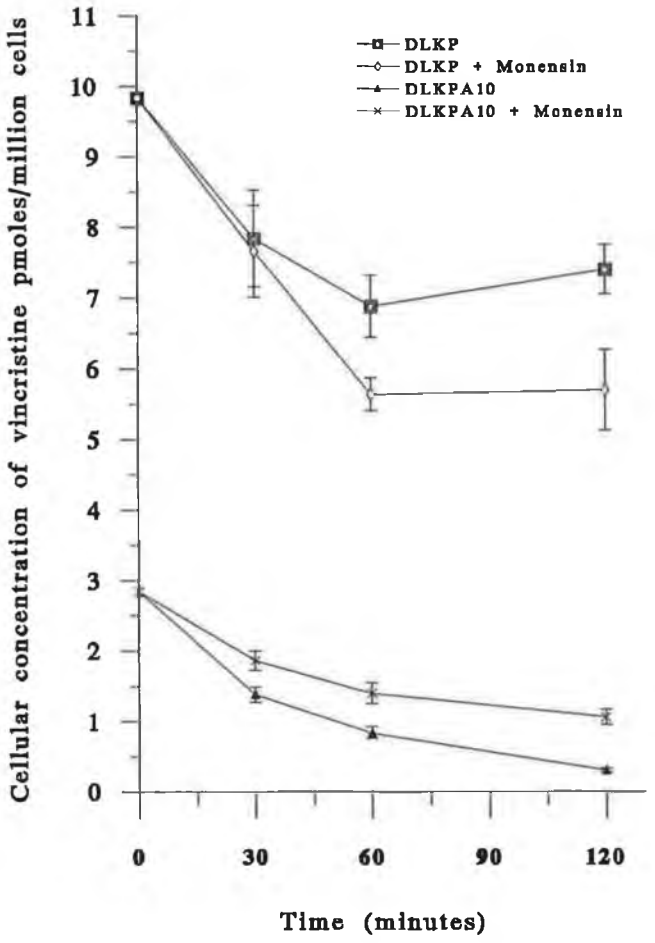



**Figure 3.19.5** The effect of monensin (10$\mu$g/ml) on vincristine efflux in the DLKP and DLKPA10 cell lines. The cells were preloaded with vincristine in the presence of antimycin A (10$\mu$M) for two hours and efflux was studied over a further 2 hours.

### 3.19.7 Effect of monensin on adriamycin toxicity in the DLKP and DLKPA10 cell lines

The effect of monensin on the toxicity of adriamycin in the DLKP and DLKPA10 cells was investigated under conditions similar to those employed in the accumulation assay (section 2.6). The parental DLKP cells were found to be extremely susceptible to adriamycin toxicity. When the cells were treated with the drug for two hour and four hour incubation periods, total cell kill was observed in the cells, as determined by the acid phosphatase assay (section 2.6.1). When the cells were incubated with monensin, total cell kill was again observed in the DLKP cells.

Figure 3.19.7 illustrates the results obtained for the DLKPA10 cells. The DLKPA10 cells were much less susceptible to adriamycin toxicity. Following exposure to adriamycin, approximately 95% cell survival was observed after two and four hour incubations. When the cells were treated with monensin (10$\mu$g/ml), approximately 45% cell kill was observed after two hours and 50% after four hour exposure. Coincubation with adriamycin and monensin resulted in a further decrease in the percentage of cell survival in the DLKPA10 cells. After two hours, the cell viability was reduced to approximately 40%, while after four hours, only 25% cell viability was observed.

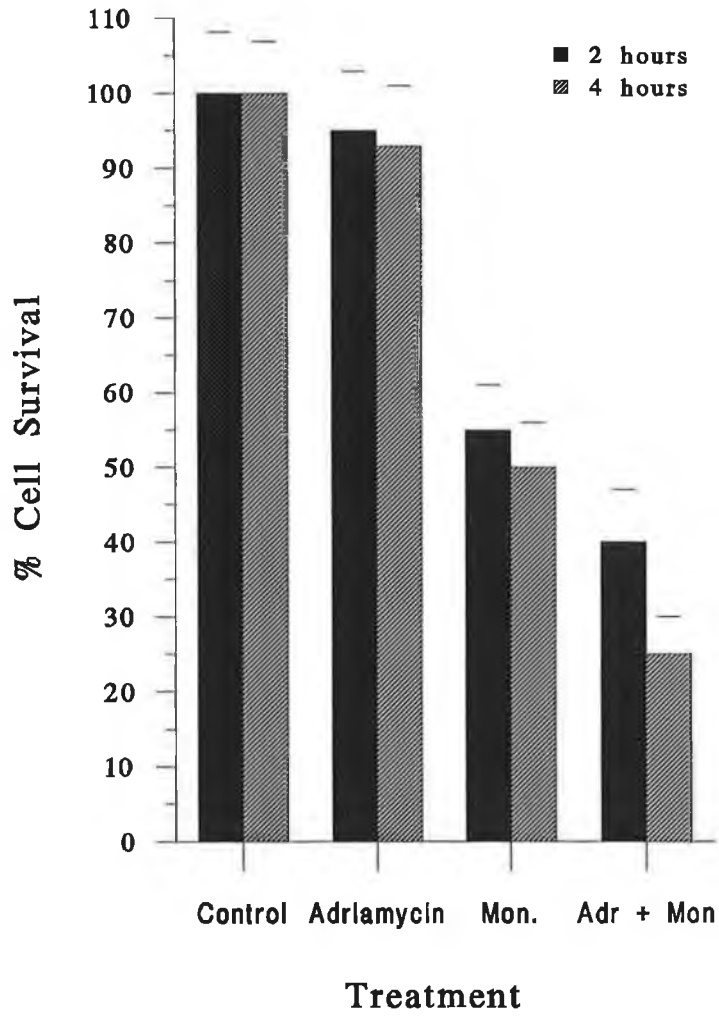



**Figure 3.19.7** The effect of monensin on adriamycin toxicity in the DLKPA10 cell line. The cells were exposed to adriamycin (10$\mu$M) in the presence of monensin (10$\mu$g/ml) for two and four hour incubation periods.

## 3.20 Effect of membrane altering agents in DLKP and DLKPA10 cell lines

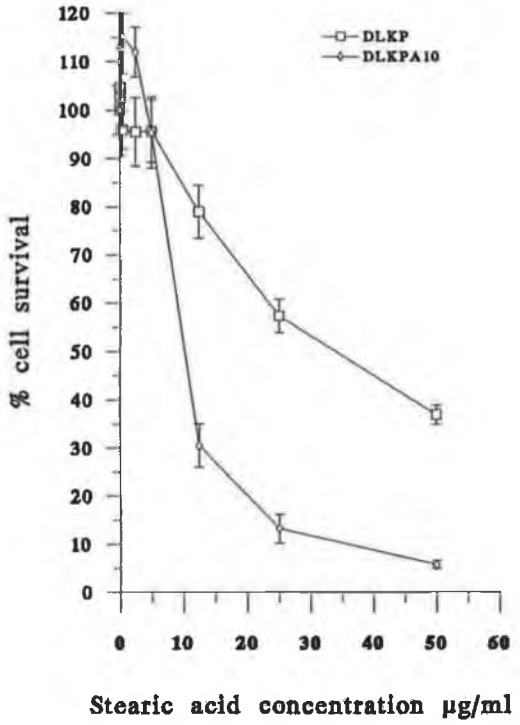
### 3.20.1 Toxicity of membrane rigidifying agents in DLKP and DLKPA10 cells

The toxicity of the fatty acids, stearic acid and cholesteryl hemisuccinate, was studied in the DLKP and DLKPA10 cells. The resistant DLKPA10 cells were found to be more susceptible than the parental cell to the toxicity of both compounds. The $IC_{50}$ values obtained for the DLKP and DLKPA10 cells with stearic acid were approximately 36$\mu$g/ml and 10$\mu$g/ml respectively, representing a 3.6-fold difference in stearic acid toxicity in the cell lines. Cholestryl hemisuccinate proved to be more toxic than stearic acid to the parental and resistant cell lines. The $IC_{50}$ values obtained for the DLKP and DLKPA10 cells were approximately 6.2$\mu$g/ml and 3.3$\mu$g/ml respectively. However, only a maximum of a 2-fold difference was noted (figure 3.20.1.1). The effect of stearic acid and cholesteryl hemisuccinate on adriamycin toxicity in the cells was also investigated, to determine if alterations in membrane properties could increase in the toxicity profile of adriamycin. When the toxicity of adriamycin was studied, in the presence of a non-toxic concentration of cholestryl hemisuccinate ($IC_{95}$), no significant enhancement of toxicity was noted in the DLKP and DLKPA10 cell lines (Figure 3.20.1.2). The addition of stearic acid did not significantly alter the toxicity of adriamycin in the parental DLKP cells, although a slight increase was observed in the DLKPA10 cells (Figure 3.20.1.2).

### 3.20.2 Effect of stearic acid and cholesteryl hemisuccinate on adriamycin accumulation

The cellular concentration of adriamycin in the DLKP and DLKPA10 cells was determined in the presence of stearic acid and cholestryl hemisuccinate over a time course of four hours. Following coincubation with stearic acid (100$\mu$M), no significant increase in adriamycin accumulation was observed in either the parental DLKP cells or the DLKPA10 cells over the time period studied. The addition of cholestryl hemisuccinate (100$\mu$M) resulted in a slight increase in adriamycin accumulation in the DLKPA10 cells while having no observable effect on adriamycin accumulation in the DLKP cells (Figure 3.20.2). When the concentration of stearic acid and cholestryl hemisuccinate were increased (concentrations up to 500$\mu$M), no further increase in adriamycin accumulation was observed in either the parental DLKP or the DLKPA10 cells.

a.

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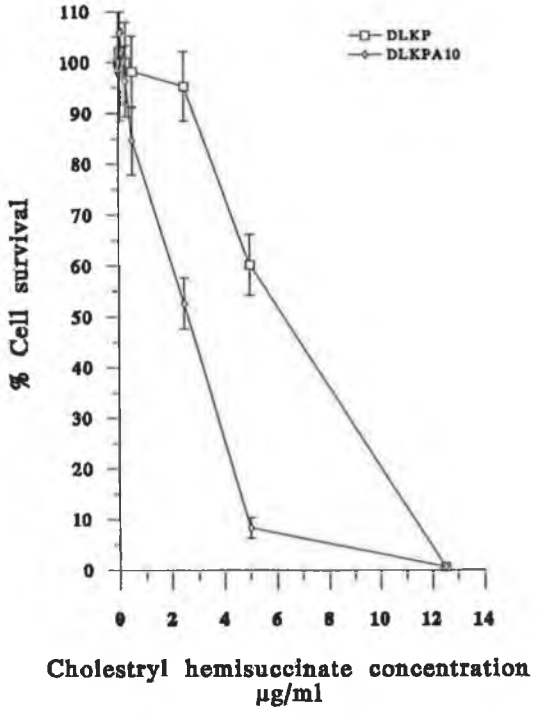
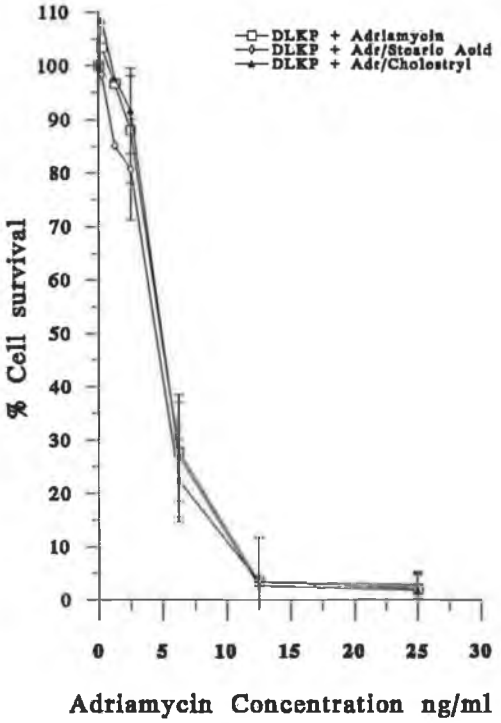



**Figure 3.20.1.1** The toxicity profiles of stearic acid (a) and cholestryl hemisuccinate (b) in the DLKP and DLKPA10 cell lines.

a.




b.

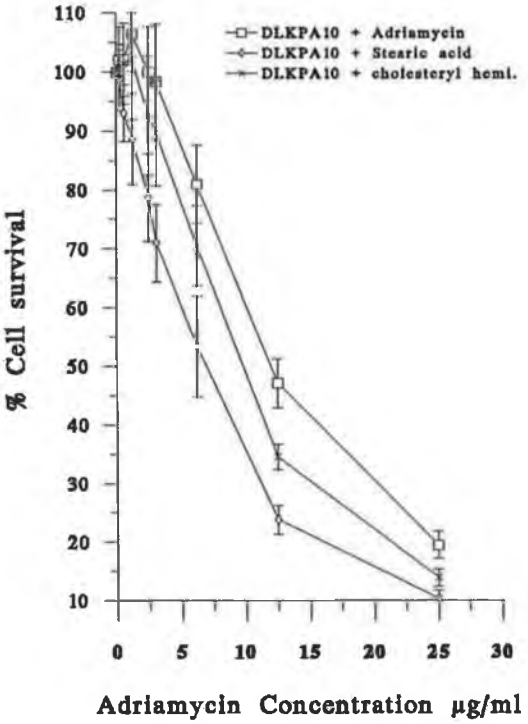



**Figure 3.20.1.2** The effect of stearic acid and cholestryl hemisuccinate on the toxicity profile of adriamycin in the DLKP (a) and DLKPA10 (b) cell lines.

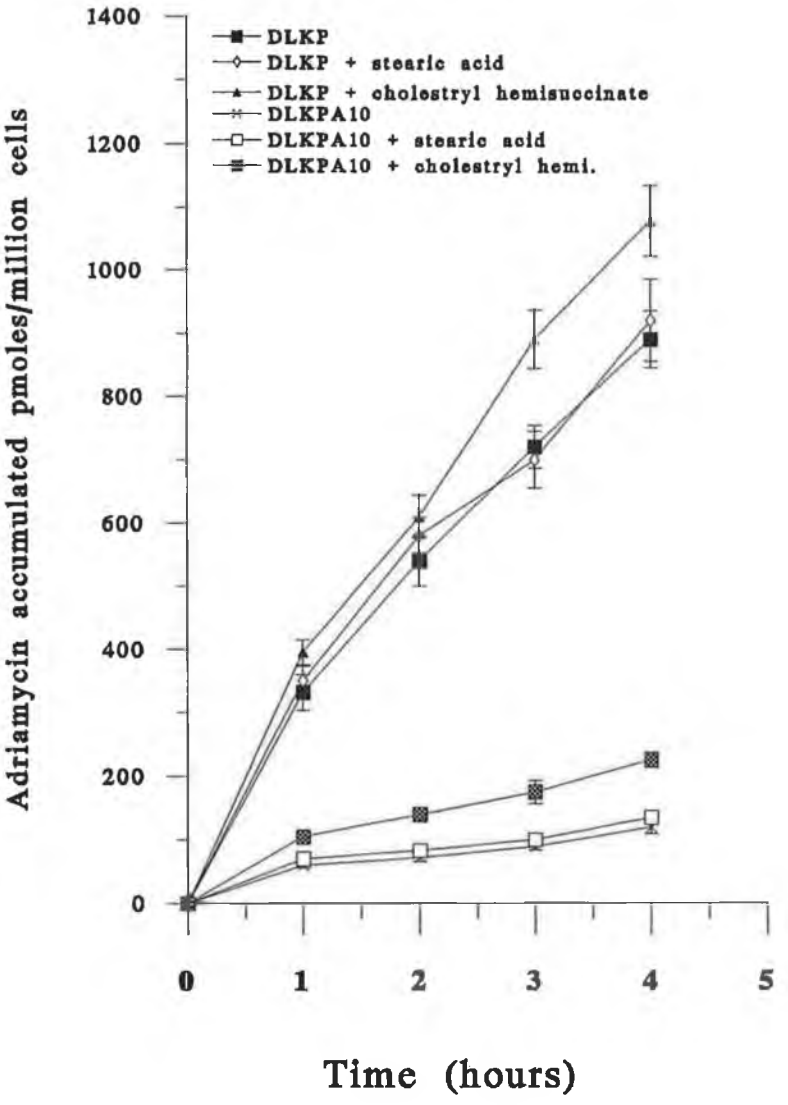



**Figure 3.20.2** The effect of stearic acid (100$\mu$M) and cholestryl hemisuccinate (100$\mu$M) on the time course of adriamycin accumulation in the DLKP and DLKPA10 cell lines.

### 3.21 Immunization and fusions

Since the adriamycin transport and circumvention work described earlier indicated that mechanisms other than P-glycoprotein overexpression may be involved in adriamycin exclusion in the DLKPA10 cell line, an attempt was made to identify antigens overexpressed in the DLKPA10 cells relative to the parental cell line. Monoclonal antibodies were raised against the DLKPA10 cell line to establish if overexpression of a novel antigen was involved in the accumulation defect observed in this cell line. An immunization programme was carried out using whole cell extracts of the resistant cell line DLKPA10, as described in section 2.13.1. Following the immunizations, three separate fusion were carried out using the murine myeloma cell line, SP2 (section 2.13.2). Of the three fusions carried out, the first displayed a low fusion rate of 25%-30% and while positive clones were obtained, no significant difference in the immunoreactivity was observed when the clones were screened against the immunogen (DLKPA10) and the DLKP parental cells. The second fusion proved unsuccessful due to the condition of the SP2 cells, while the third resulted in a fusion rate of approximately 65%. When the clones produced from the third fusion were screened using the ELISA method, against the parental DLKP and the resistant DLKPA10 cells, only a small number of the clones tested positive. A number of these hybridomas differentiated before the screening process was carried out, while other clones stopped producing antibodies during the screening process. One hybridoma was selected for further characterisation and was designated D8-8.

#### 3.21.1 Screening of the selected hybridoma by ELISA

The supernatent from the D8-8 hybridoma was screened against the parental DLKP and DLKPA10 cell lines, using the ELISA method, as described in section 2.13.3. The D8-8 hybridoma was found to be more positive against the parental cell line than the immunogen, DLKPA10. Strong immunoreactivity was observed with the DLKP cells. The selected hybridoma was then grown up as ascitic fluid in Balb/c mice and the antibody titre determined through serial dilution (1:10 - 1:10000 dilutions) of the ascitic fluid, again using the ELISA method. A suitable working dilution for the D8-8 hybridoma was found to be a 1:100 dilution of the ascitic fluid. The ascitic fluid was aliquoted and stored at -20$^0$C, until required.

### 3.21.2 Determination of antibody class

The supernatent from the selected hybridoma was used to determine the class and sub-class of the antibodies produced. A monoclonal antibody isotyping kit (Serotec) was employed to determine the class of antibody, as described in section 2.14. Table 3.21.2 represents the profile of the antibody class and subclass for the D8-8 hybridoma. The results show that the D8-1 hybridoma produces antibodies of the IgG class, with subclass 1.

| Class subclass | D8-8 |
|---|---|
| IgG1 | + |
| IgG2a | - |
| IgG2b | - |
| IgG3 | - |
| IgA | - |
| IgM | - |
| positive | + |
| negative | - |

**Table 3.21.2** Antibody class, subclass determination of the D8-8 antibody

### 3.21.3 Protein Analysis of cell membrane preparations

Purified membrane fractions of the immunogen, DLKPA10 and the parental DLKP cells were prepared and freeze dried, as described in section 2.7.2. The protein concentrations of the preparations were determined from standard curves obtained from the BCA protein assay (section 2.7.4).

### 3.21.3.1 SDS PAGE electrophoresis and western blotting

The DLKP and DLKPA10 membrane proteins were separated on 7.5% polyacrylamide denaturing gels and transferred to nitrocellulose paper, as described in section 2.7.6. Prestained molecular weight markers were run simultaneously with the membrane proteins. Following the transfer of the membrane protein to nitrocellulose, the blotted proteins were probed with the D8-8 antibody. Figure 3.21.3.1 represents the results obtained for the D8-8 antibody (1:100 dilution of ascitic fluid) with a 7.5% polyacrylamide gel. The results obtained illustrate that the D8-8 antibody preferentially binds to an antigen of approximately 180 kilodaltons, although it exhibits binding only in the DLKP membrane protein, under the experimental conditions employed.

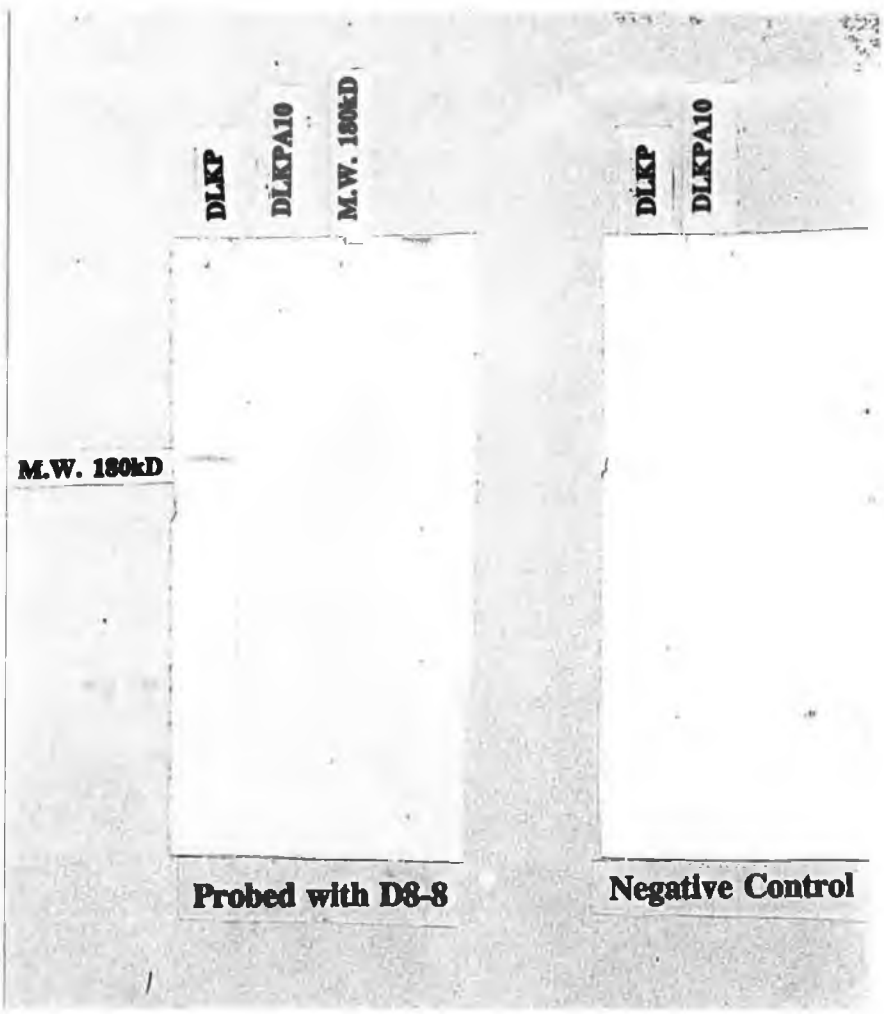



**Figure 3.21.3.1** Western blot analysis of the D8-8 antibody in cell membrane preparations of the DLKP and DLKPA10 cell variants.

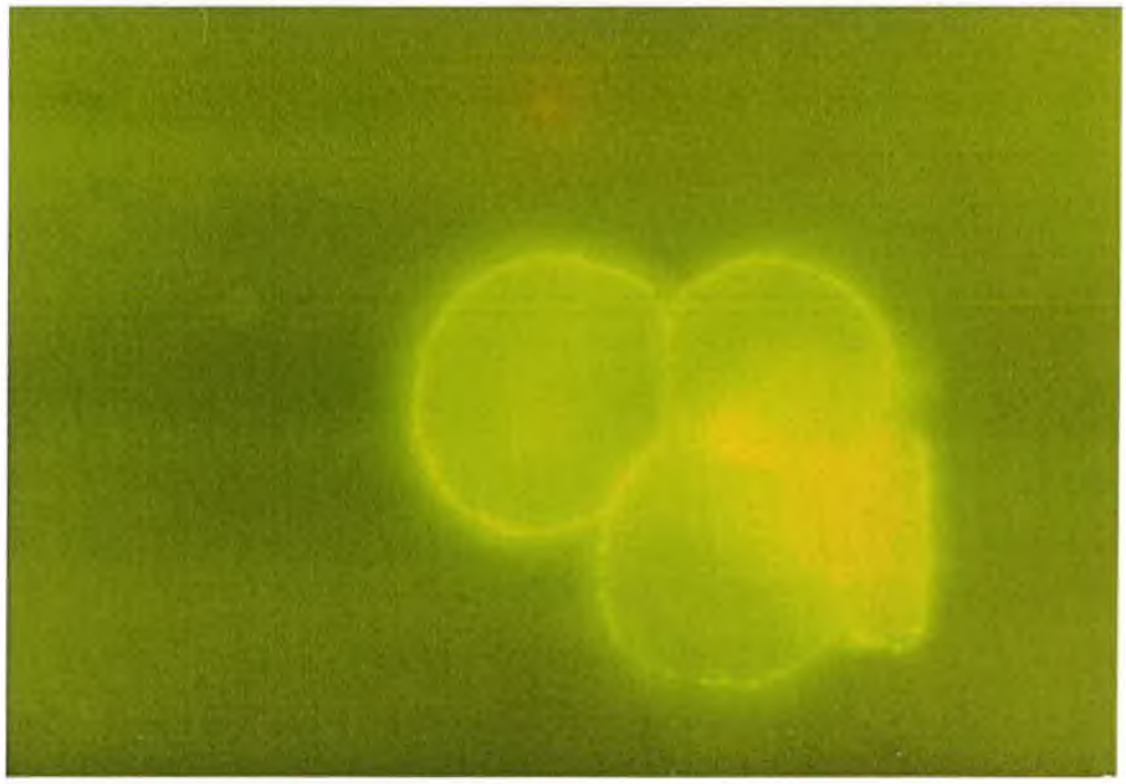
### 3.21.4 Immunological Studies

#### 3.21.4.1 Indirect immunofluorescence studies

Immunofluorescence was carried out on live cells to detect the presence of cell surface antigens on the DLKP and DLKPA10 cells, as described in section 2.9.1. The D8-8 ascitic fluid (1:100 dilution) was used as the primary antibody in the study. The EP16 monoclonal antibody, which is specific for a cell surface antigen on normal human epithelial cells, was included in the study as a positive control, with the SCC-9 cell line (a cell line derived from human squamous carcinoma of the tongue). When the EP16 antibody was used as the primary antibody, cell surface fluorescence was clearly visible in the SCC-9 cells (Figure 3.21.4.1). Following exposure to the D8-8 antibody, the DLKP cells showed only faint surface fluorescence. However, cell surface fluorescence was clearly visible in the DLKPA10 cells. Figure 3.21.4.2 illustrates the results obtained with the D8-8 antibody when exposed to the DLKP and DLKPA10 cells. The results show that the D8-8 antibody recognised a surface antigen on the DLKPA10 cells, producing a ring of fluorescence around the cell surface. The faint fluorescence observed on the surface of the DLKP cells was comparable to that seen on the negative control cells, in which PBS was used as the primary antibody.

Live cell immunofluorescence was also performed on the SKMES-1, SKMES-1/ADR, OAW42-S and OAW42-A cell lines, with the D8-8 antibody. Intense fluorescence was clearly visible on the surface of both the parental SKMES-1 and the resistant SKMES-1/ADR cell lines following exposure to the antibody. Differences in the intensity of fluorescence in the two cell lines, however, were not distinguishable (figure 3.21.4.3). Figure 3.21.4.4 illustrates the surface staining of the D8-8 antibody on the human ovarian cancer cell lines, OAW42-S and OAW42-A. The results show that the antibody binds to the surface of both cell lines thus producing intense fluorescence. Although as with the results obtained for the SKMES-1 cell lines, differences in the intensity of fluorescence were not distinguishable. Slight autofluorescence was observed for the negative controls (PBS) for each of the cell lines, although relative to the test cell lines, the intensity of the fluorescence observed was low.

a.



b.

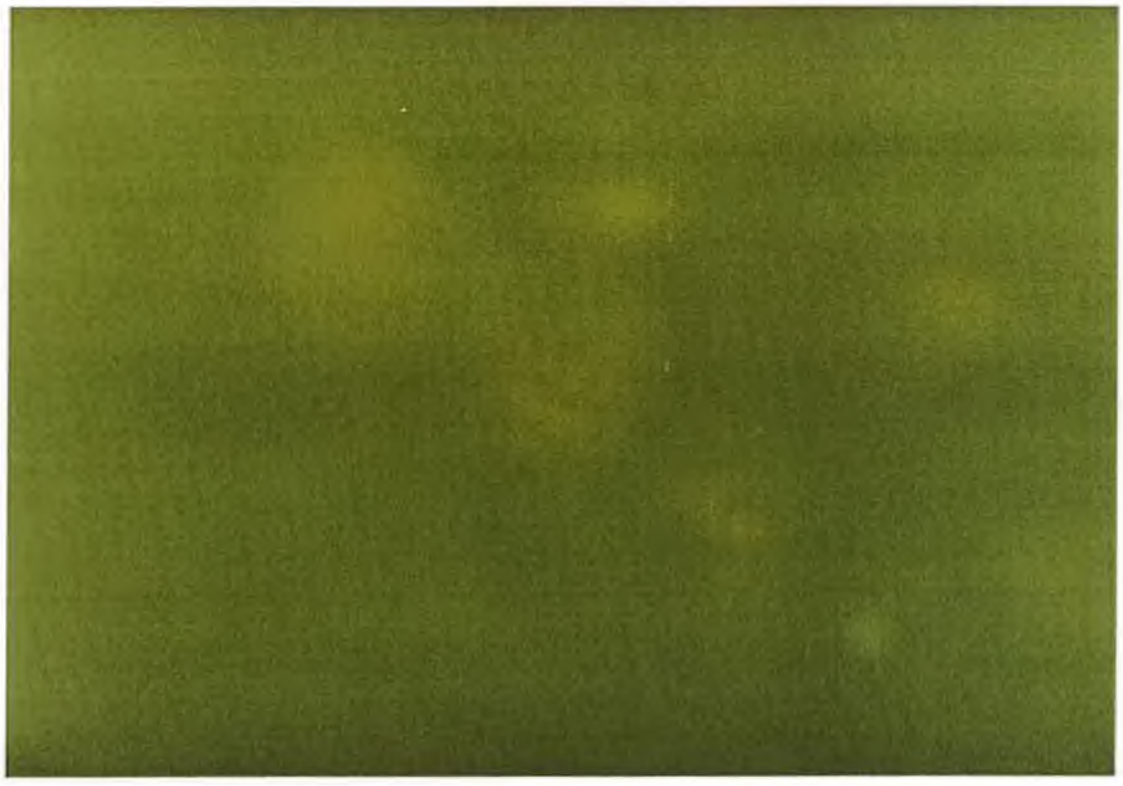
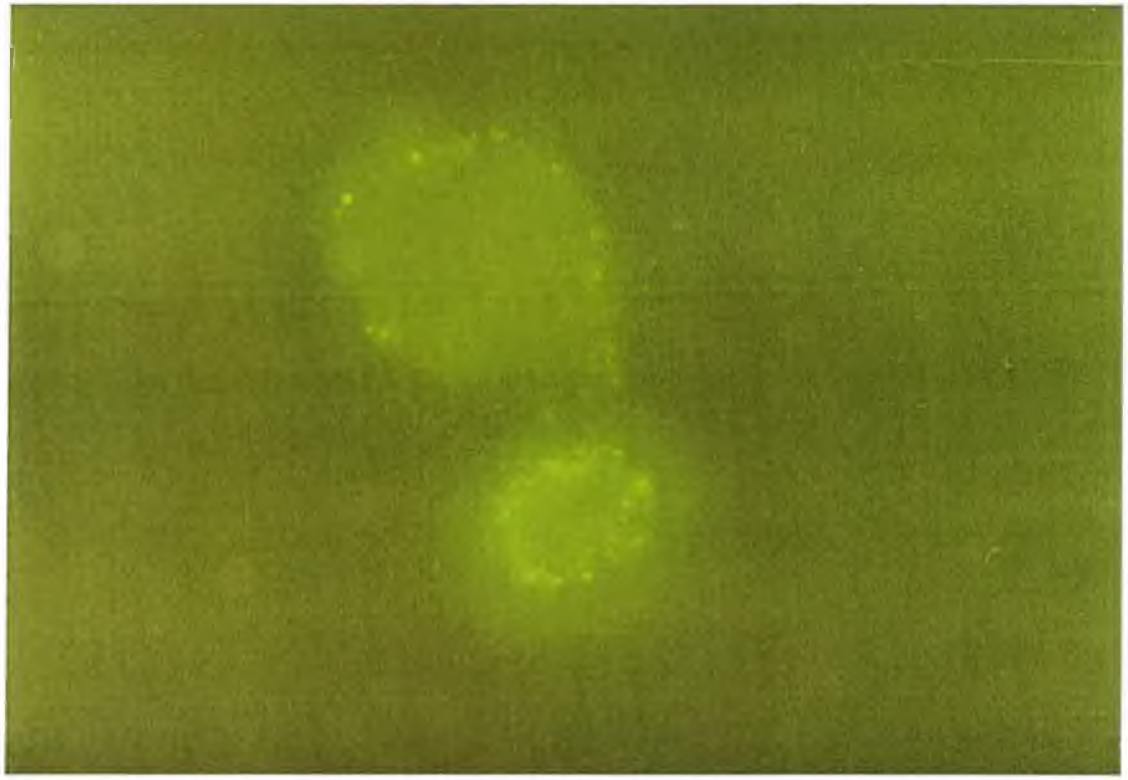


**Figure 3.21.4.1** Live cell immunofluorescence of the human squamous carcinoma tongue cell line, SCC-9, with the EP16 monoclonal antibody (a); negative control (b).

a.



b.

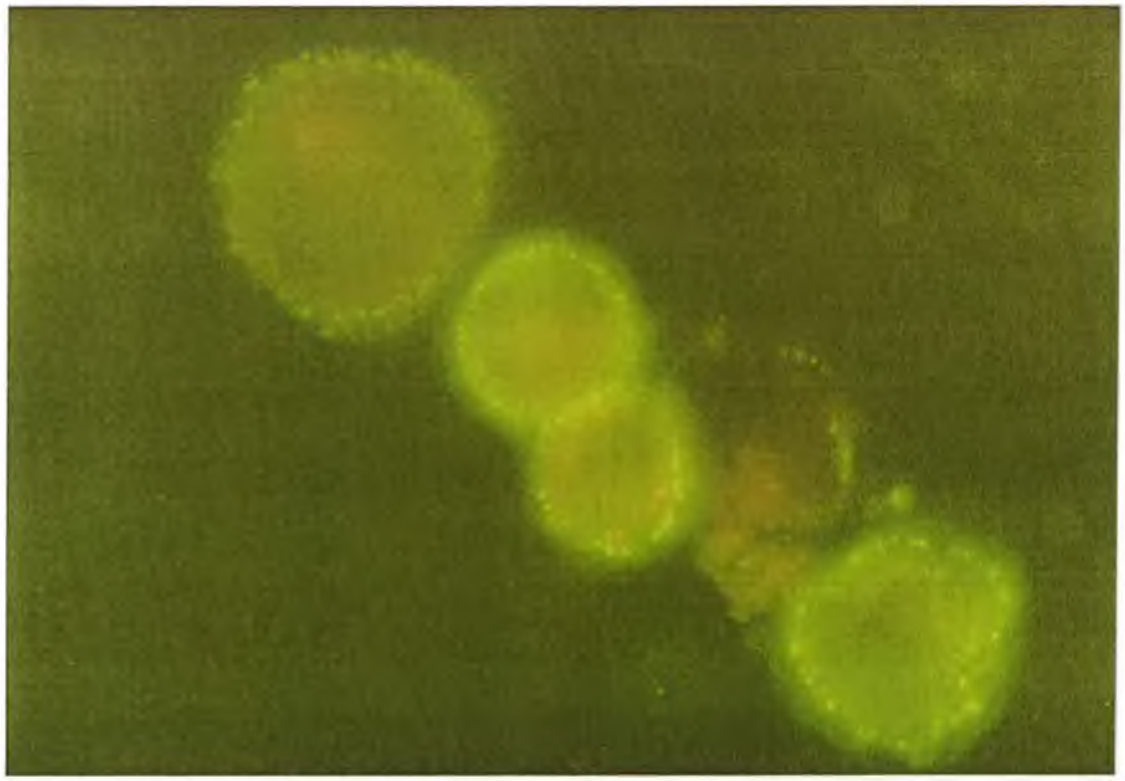
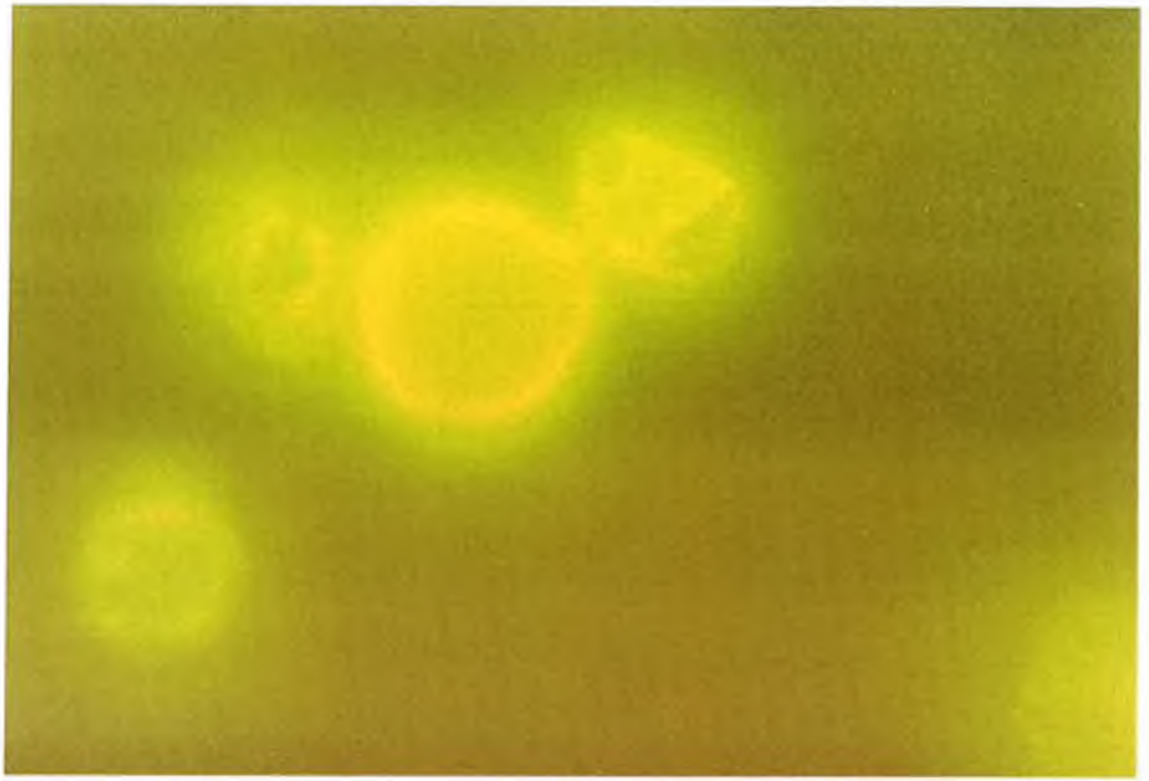


**Figure 3.21.4.2** Live cell immunofluorescence of the DLKP (a) and DLKPA10 (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

a.



b.

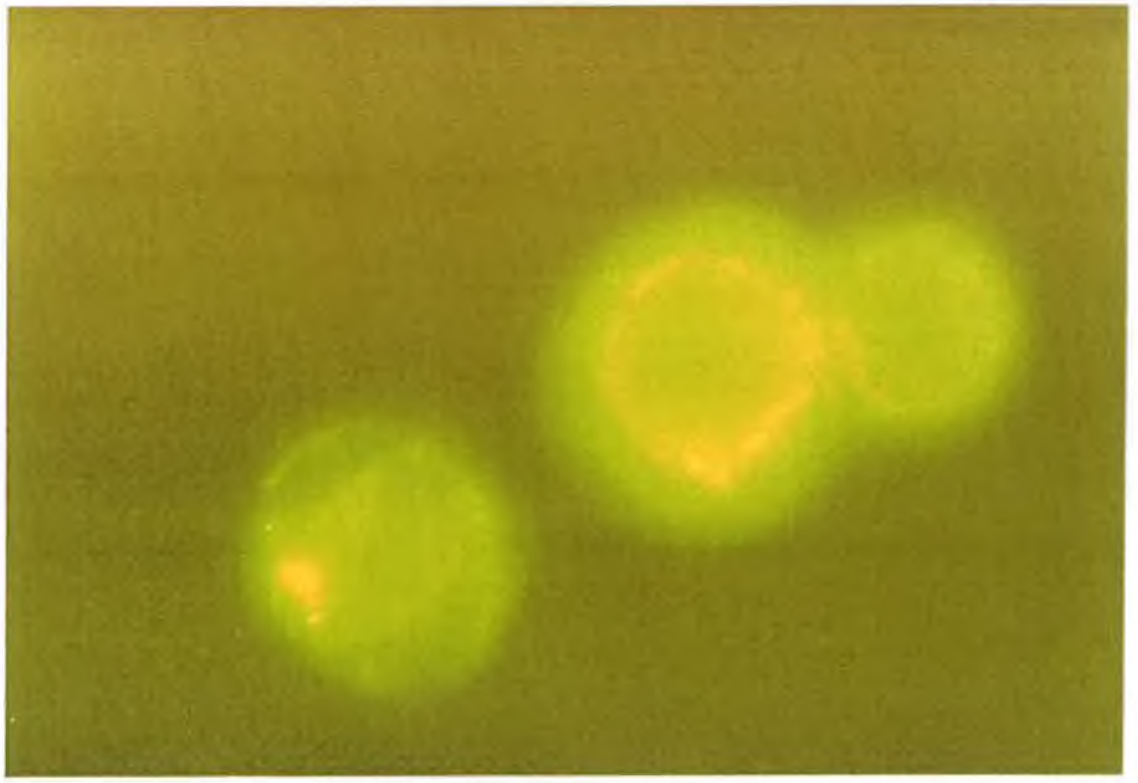
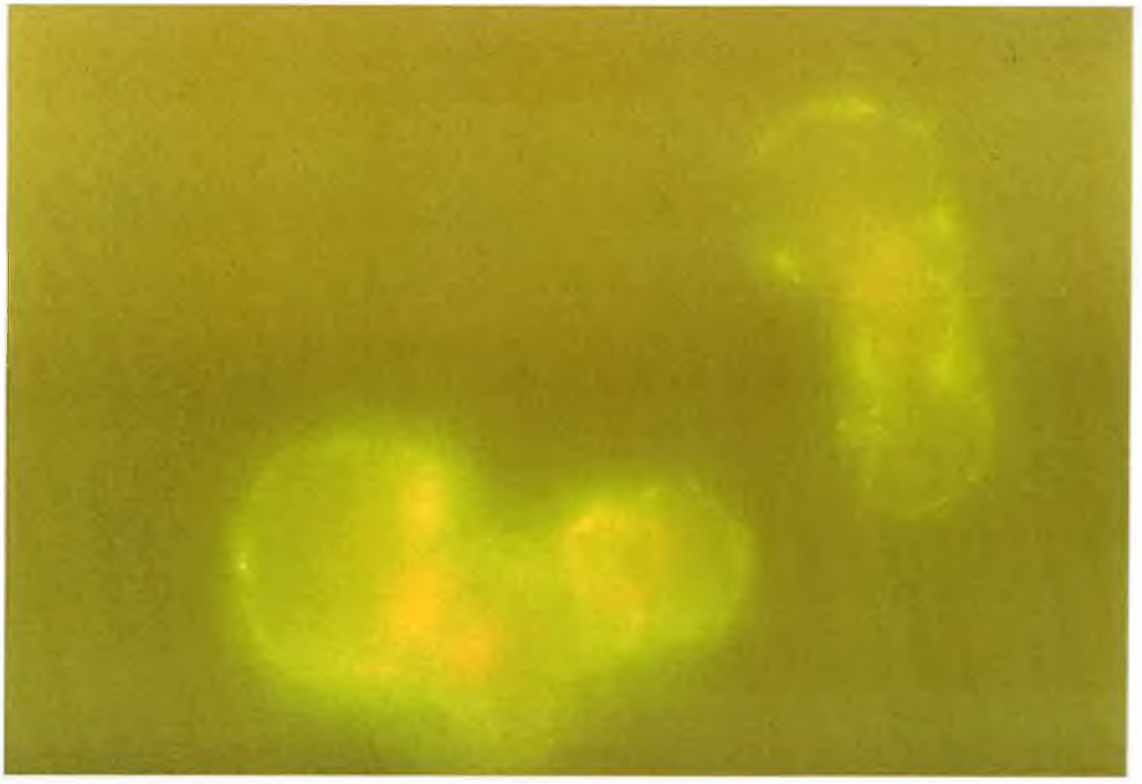


**Figure 3.21.4.3** Live cell immunofluorescence of the SKMES-1 (a) and SKMES-1/ADR (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

a.



b.

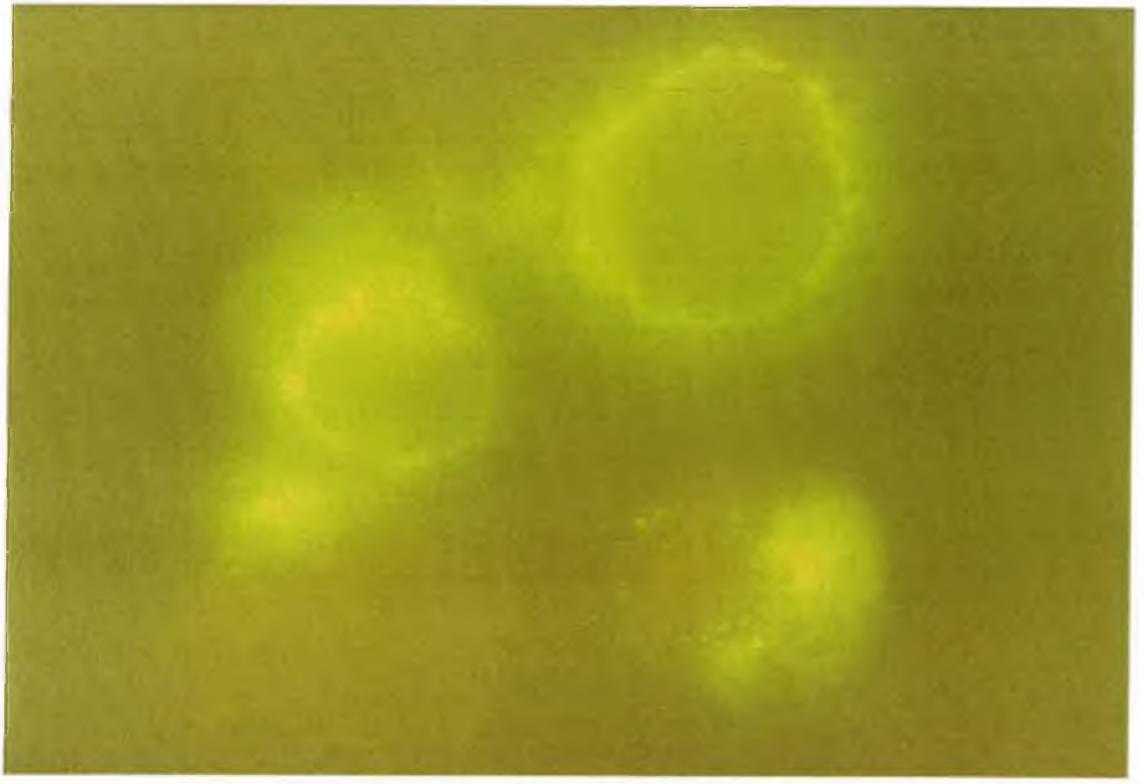


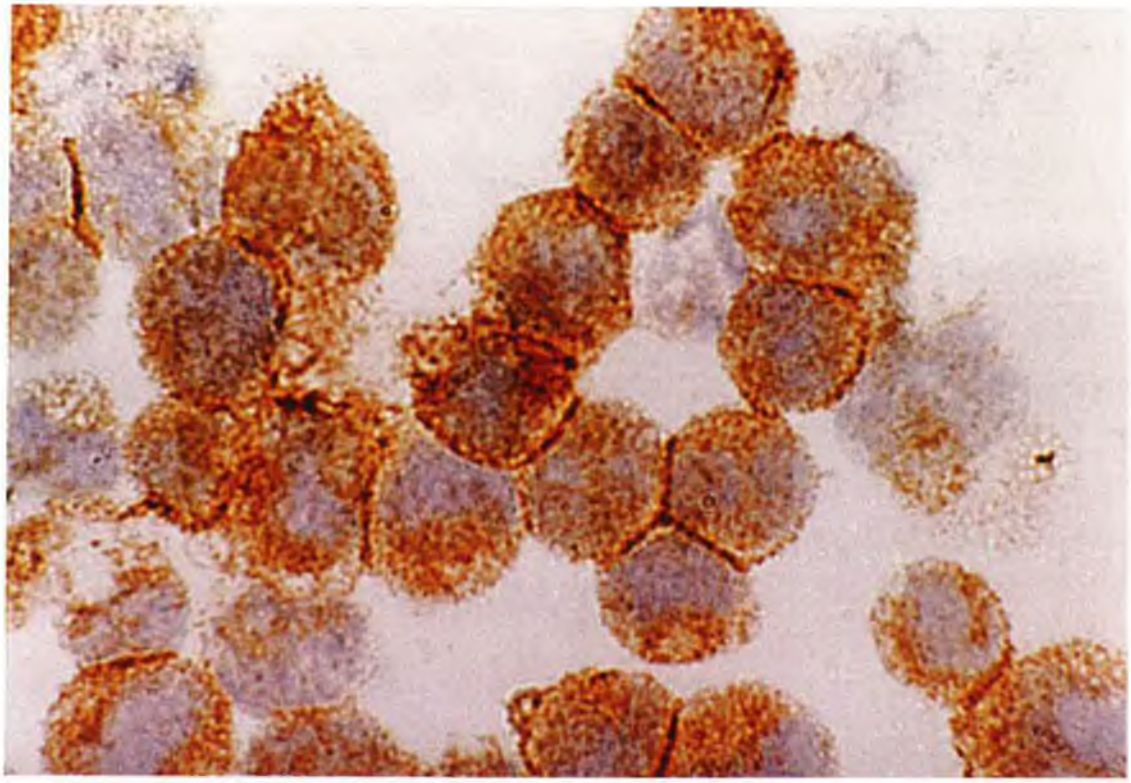
**Figure 3.21.4.4** Live cell immunofluorescence of the OAW42-S (a) and OAW42-A (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

### 3.21.4.2 Immunohistochemical studies

The immunoreactivity of the D8-8 antibody was also studied against the DLKP / DLKPA10, the SKMES-1 / SKMES-1/ADR and the OAW42-S / OAW42-A cell lines. Cytospin preparations of each of the cell lines were prepared and the cells fixed in acetone, as described in section 2.9. Immunohistochemal analysis was carried out on the cytospin preparations to detect the presence of internal and membrane associated antigens. When the DLKP and DLKPA10 cells were incubated with the D8-8 (1:100 dilution of ascitic fluid), the antibody displayed the strongest immunoreactivity against the DLKP cells. Positive cytoplasmic and cell surface staining was clearly visible in the majority of the cells viewed. The DLKPA10 cells did show some faint staining with the D8-8 antibody, although relative to the DLKP cells the proportion of cells stained and the intensity of staining was low (Figure 3.21.4.5). Similar staining patterns to the DLKP and DLKPA10 cell lines were observed with the OAW42-S and OAW42-A cell lines. Cytoplasmic staining was observed in approximately 75% of the OAW42-S sensitive cells. However, the intensity of staining varied within the cell population. Surface staining with the D8-8 antibody was also noted in the OAW42-S cells. A large percentage of the OAW42-A resistant cells also demonstrated positive immunoreactivity with the D8-8 antibody, however, the overall intensity staining was substantially less than that observed in the sensitive cells (figure 3.21.4.6). When the reactivity of the D8-8 antibody with the SKMES-1 and SKMES-1/ADR cell lines was investigated, cytoplasmic staining was observed in both cell lines. A typical staining pattern for the D8-8 antibody in SKMES-1 and SKMES-1/ADR cells is presented in figure 3.21.4.7. The staining intensity appears slightly greater in the parental SKMES-1 cells.

The immunoreactivity of the D8-8 antibody was also studied in 3 clones, isolated from the parental DLKP cell line. The results obtained were compared with those obtained from the parental cells. The three clones showed positive staining with the D8-8 antibody. However, variations in the staining intensity were observed. The DLKP-I and the DLKP-SQ clones showed very similar staining patterns, with positive staining observed in the majority of cells. The D8-8 appear to react with a larger percentage of cells in these two clones than in the parental DLKP cells. The intensity of the staining was also slightly greater in these cells. The DLKP-M clone was found to have a lot less staining than the parental cells and the other two clones. The staining observed was also less intense than in the DLKP cells.

a.



b.

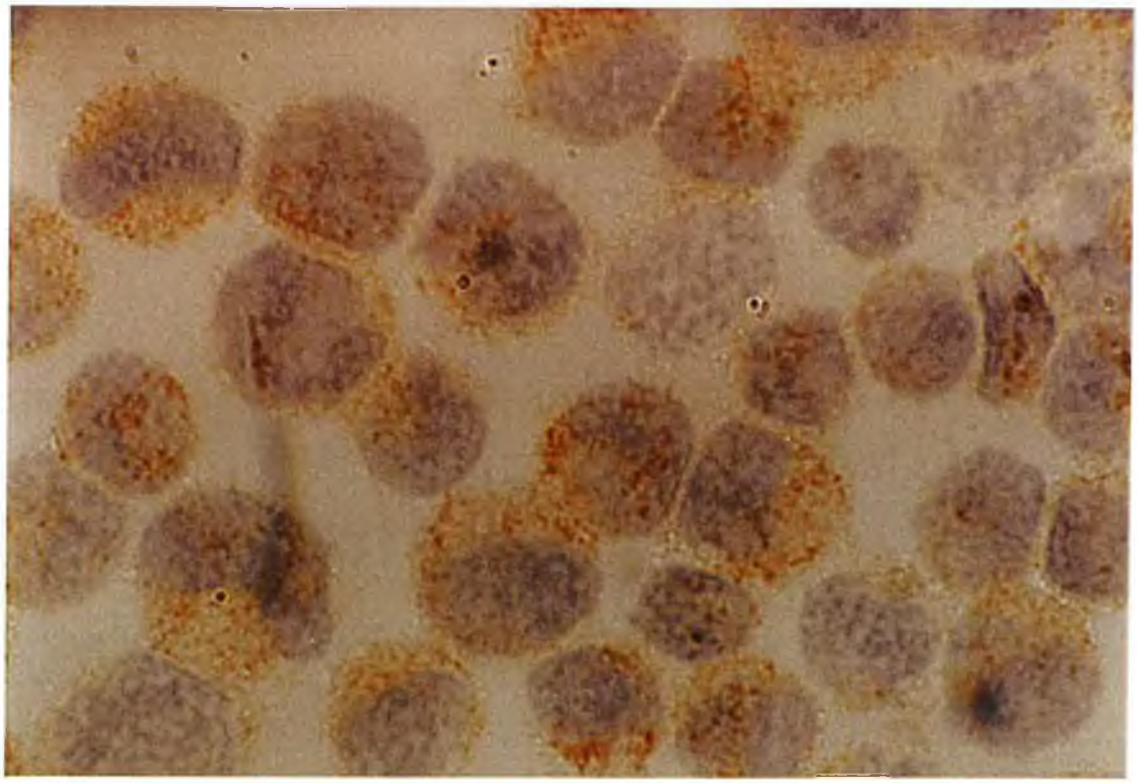
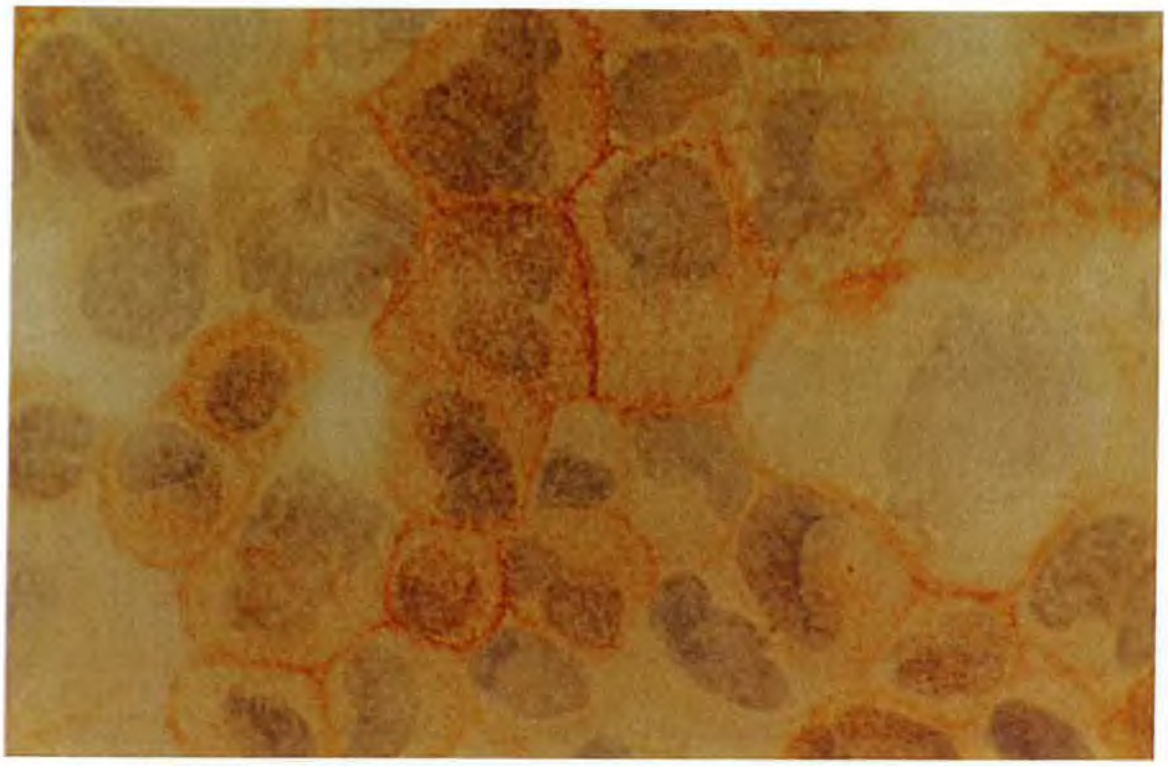


**Figure 3.21.4.5** Immunocytochemical staining of the DLKP (a) and DLKPA10 (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

a.



b.

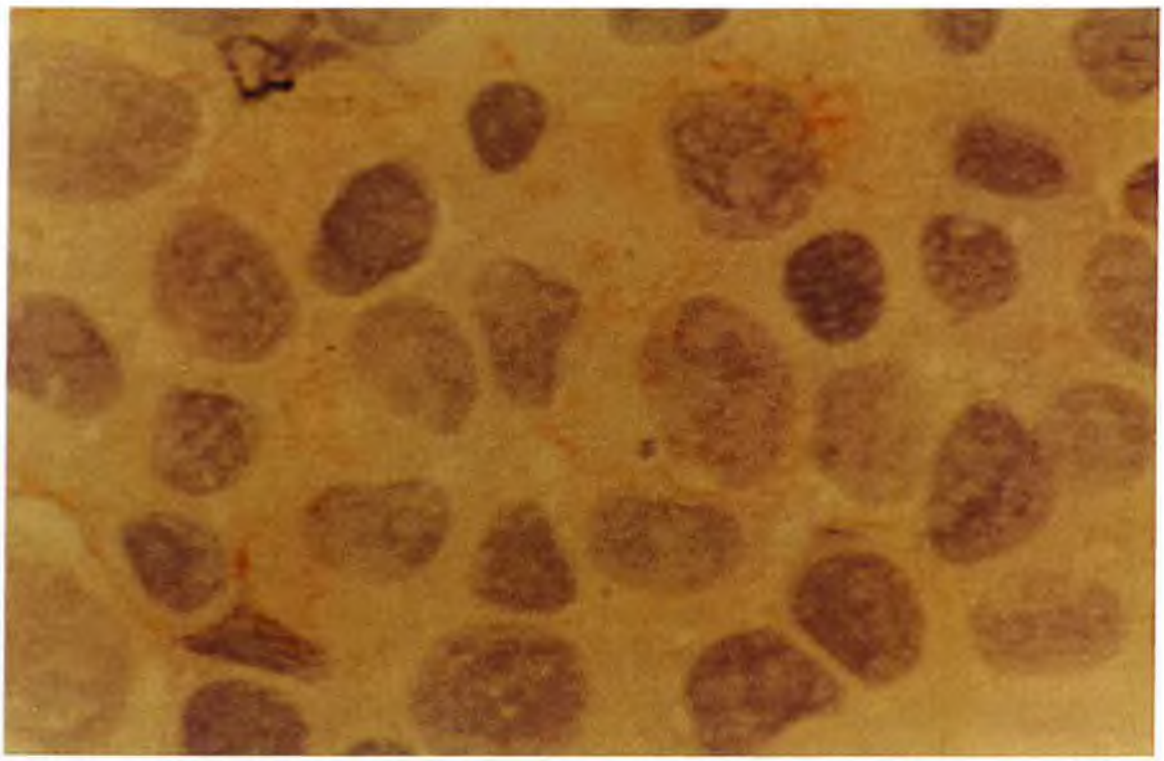
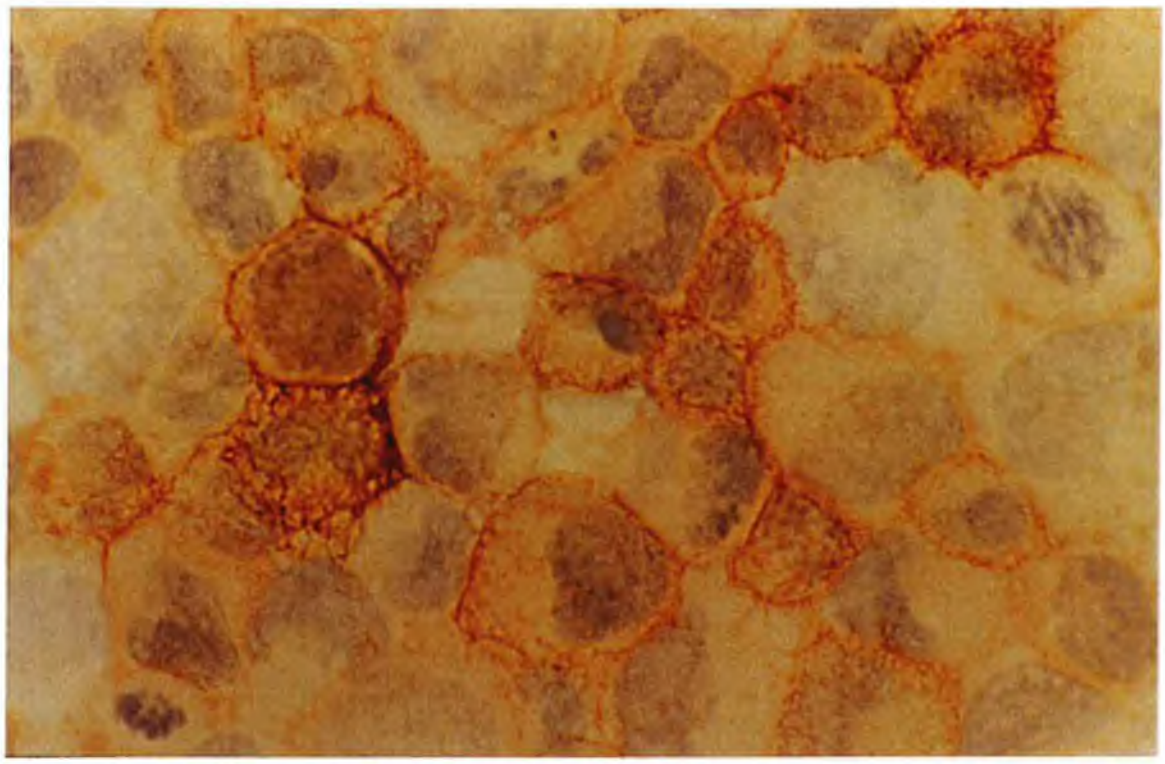


**Figure 3.21.4.6** Immunocytochemical staining of the OAW42-S (a) and OAW42-A (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

a.



b.

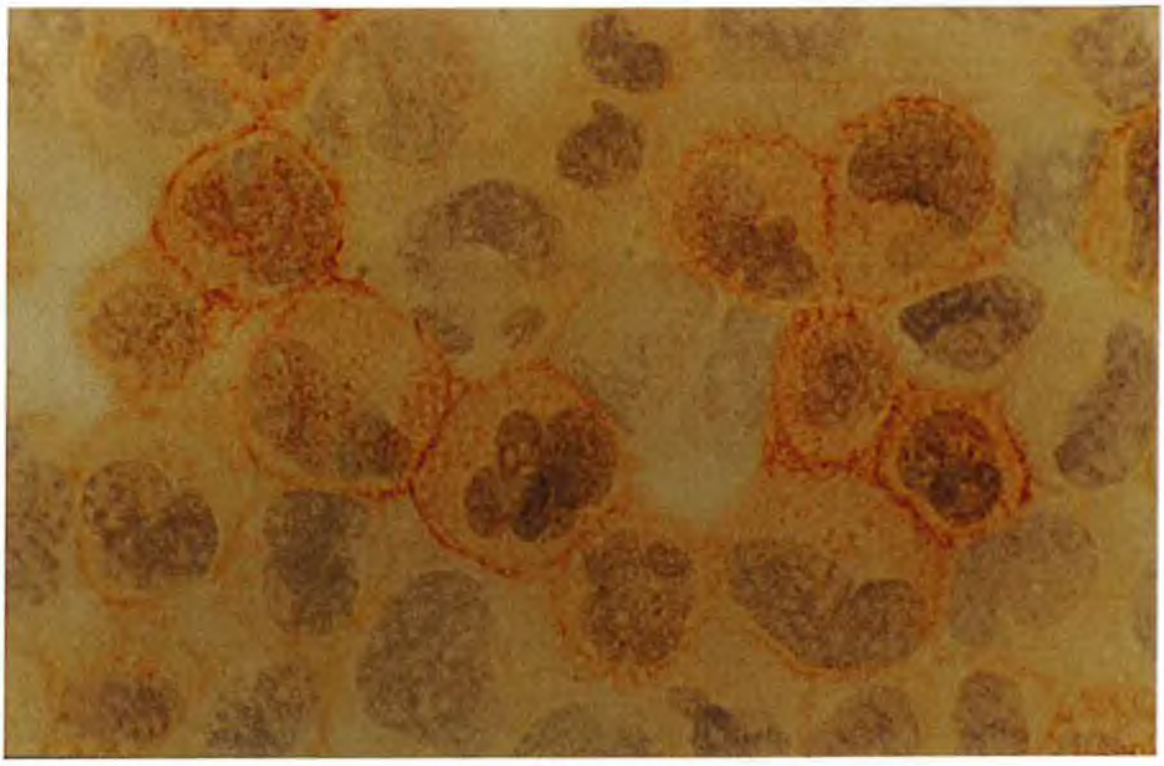


**Figure 3.21.4.7** Immunocytochemical staining of the SKMES-1 (a) and SKMES-1/ADR (b) cell lines with 1:100 dilution of the D8-8 ascitic fluid.

# *4. Discussion*

## 4.1 Platinum resistance

Cisplatin and its analogues are among the most widely used chemotherapeutic agents, either alone or in combination with other agents. These agents have proved to be effective in the treatment of ovarian, testicular, head-and-neck, non-small cell lung and brain tumours (Rosenberg, 1985). However, many of these tumours which are initially responsive to chemotherapy develop resistance to cisplatin during therapy leading to treatment failure. Cancer patients treated with cisplatin have approximately a 50% response rate. Within 12 to 24 months, however, the majority of these patients relapse. At present, the dominant mechanism involved in clinically acquired cisplatin resistance is unknown. However, a significant number of platinum resistant cell lines have now been developed which have proved useful tools in the study of acquired resistance in cancer therapy. Table 4.1 presents a number of cisplatin resistant human cell lines that have been established and used in the study of cisplatin resistance. These cell lines were developed *in vitro* from a number of tumour types, including ovarian carcinomas, lung carcinomas, colon carcinomas, teratocarcinomas, breast carcinomas, bladder carcinomas and head-and-neck carcinomas. Cisplatin resistant cells display a unique cross resistance profile to multiple agents including antimetabolites, such as 5-fluorouracil and methotrexate, topoisomerase inhibitors such as camptothecin and etoposide and DNA polymerase inhibitors such as azidothymidine. This "atypical" multidrug resistance is distinct from the classical multidrug resistance which usually involves resistance to the anthracyclines and vinca alkaloids and is frequently due to the overexpression of the P-glycoprotein membrane associated efflux pump.

The cell lines resistant to cisplatin have been shown to have multifactorial mechanisms of resistance. Four biological alterations capable of producing cisplatin resistance have been reported and include decreased cellular accumulation of cisplatin (Gately and Howell, 1993), increased levels of glutathione or increased glutathione-S-transferase activity (Waxman, 1990), increased levels of intracellular metallothionein (Saijo and Lazo, 1993) and enhanced DNA repair (Johnson *et al.*, 1994a). Mechanisms that have been implicated in cisplatin resistance include alterations in mitochondrial membrane potential and signal transduction pathways, oncogene expression (in particular c-*fos* and c-*jun* gene expression) and alterations in the expression of the nuclear enzymes, topoisomerase I and II, DNA polymerases and thymidylate synthase, all of which are involved in DNA repair.

| Cell Line | Description | Reference |
|---|---|---|
| PC10-B3<br>PC10-E5 | Human lung squamous carcinoma | Katabami *et al.*, 1993 |
| PC14/CDDP | Human non-small-cell lung carcinoma | Ohmori *et al.*, 1993 |
| GLC4-CDDP | Human small-cell lung carcinoma | Hosper *et al.*, 1988 |
| SW2/CP | Human small-cell lung carcinoma | Kelley *et al.*, 1988 |
| H69/$CDDP_{0.2}$<br>H69/CDDP | Human small-cell lung carcinoma | Hong *et al.*, 1988<br>Kasahara *et al.*, 1991 |
| SL6/CP | Human large cell lung carcinoma | Kelley *et al.*, 1988 |
| Tera-CP | Human teratocarcinoma | Timmer-Bosscha *et al.*, 1993 |
| HeLa CA<br>HeLa CK | Human cervical carcinoma | Osmak and Eljuga, 1993 |
| 2008 C13* | Human ovarian carcinoma | Andrews *et al.*, 1985 |
| $A2780^{cp}$<br>$A2780^{cp8}$<br>A2780/CP20<br>A2780/CP70<br>A2780/C30<br>A2780/C50<br>A2780/C80<br>A2780/C100<br>A2780/C200 | Human ovarian carcinoma | Masuda *et al.*, 1988<br>Behrens *et al.*, 1987<br>Godwin *et al.*, 1992 |
| COV413B-PtR | Human ovarian carcinoma | Kuppen *et al.*, 1988 |
| $41M_{cis}R_2$<br>$41M_{cis}R_4$<br>$41M_{cis}R_6$ | Human ovarian carcinoma | Loh *et al.*, 1992 |
| LoVo CP2.0<br>LoVo RT | Human colon carcinoma | Yang *et al.*, 1993 |
| SCC-25/CP | Human carcinoma of the tongue | Teicher *et al.*, 1986 |
| KB-rc | Human oral epidermoid carcinoma | Hori *et al.*, 1993 |
| Hep2 CA3<br>Hep2 CK2 | Human larynx carcinoma | Osmak, 1992 |
| K562 DDP | Human erythroleukaemia | Shionoya *et al.*, 1986 |
| MCF-7/CP | Human breast carcinoma | Kelley *et al.*, 1988 |
| G3361/CP | Human melanoma | Kelley *et al.*, 1988 |

**Table 4.1** Human cisplatin resistant cell lines

One of the aims of this thesis was to establish novel platinum resistant cell variants and to investigate their underlying mechanisms of resistance. The platinum analogue, carboplatin, was chosen as the selecting agent for the procedure. Carboplatin has been shown to have a similar spectrum of antitumour activity to cisplatin. However, it causes considerably less nephrotoxicity and neurotoxicity compared to cisplatin. Consequently, it is now frequently used in cancer therapy, in particular for the treatment of ovarian and lung cancers (Gore *et al.*, 1989; Raghaven *et al.*, 1994). As carboplatin is not in fact a more active anticancer agent than cisplatin, its role in cancer therapy is primarily as an alternative to cisplatin in patients with pre-existing renal dysfunction and also in patients with a clear predisposition to neurotoxicity or ototoxicity. A selection process was initiated to establish carboplatin resistant variants of the human cell lines, DLKP and OAW42. The DLKP cell line was derived from a poorly differentiated lymph node metastases of a squamous cell carcinoma of the lung (Law *et al.*, 1992) and the OAW42 cell line was established from the ascites of a patient with serous cystadenocarcinoma of the ovary (Wilson, 1984). While platinum resistant variants were successfully isolated from the DLKP cells, the OAW42 cells did not adapt to growth in the drug and therefore the selection process was discontinued.

Three carboplatin resistant variants were isolated by continuous exposure of the DLKP parental cells to increasing concentrations of the selecting agent. The three variants, DLKPC 6.2, DLKPC 14 and DLKPC 25 were selected to 6.2$\mu$g/ml, 14$\mu$g/ml and 25$\mu$g/ml of carboplatin respectively. The cells readily adapted to growth in the drug and the selection procedure was carried out over a period of approximately one year. To verify that the variants were derived from the DLKP cells, DNA fingerprint analysis was carried out on DNA extracted from both the parental DLKP cells and also the highest resistant variant, DLKPC 25. The results obtained showed that the DLKPC 25 cells shared almost identical bands with the parental cells implying that the DLKPC 25 cells must indeed have originated from the parental DLKP cell line. Only one extra band was visible in the DLKP cells with the 33.15 locus probe. Since the DLKPC 25 variant was derived from the DLKPC 6.2 and DLKPC 14 variants it was concluded that these variants also originated from the DLKP cells. The morphology of each of the carboplatin resistant variants was also investigated to determine if exposure to the drug resulted in any significant alteration. The variants were found to be similar to the parental cells with all three variants displaying the squamous type cell population evident in the DLKP parental cells.

## 4.2 Cross resistance profile in the DLKPC variants

Cross resistance studies of the variants revealed that all three DLKPC variants had similar resistance profiles. They were found to be resistant to the selecting agent, carboplatin (4-, 8.3- and 15.8-fold) and also cross resistant to cisplatin (5.1-, 12.2- and 25.1-fold). A low level of cross resistance was also observed to the topoisomerase II inhibitor, VP16, in particular with the DLKPC 25 variant (2-fold). However, there was no significant resistance or sensitivity to the classical MDR anticancer agents, adriamycin and vincristine or to the antimetabolite 5-fluorouracil. The results obtained were similar to results previously reported for cisplatin resistant cell lines. Generally cisplatin resistant cell lines exhibit cross resistance to platinum agents, including cisplatin, carboplatin, transplatin and ormaplatin while showing little cross resistance to the classical MDR drugs (table 4.2). In a study on the human small cell lung cancer cell line, H69/CDDP, that was selected for resistance with cisplatin, the cells were found to be cross resistant to the cisplatin analogue, cis-diammine(glycolato)platinum while showing no resistance to adriamycin, vincristine or VP16 (Kasahara *et al.*, 1991). The cisplatin resistant ovarian cell lines, A2780/CP20 and A2780/CP70 were also shown to be resistant to both cisplatin and carboplatin although these cell lines also exhibited resistance to VP16. Only a low level of resistance was observed with adriamycin (Hamaguchi *et al.*, 1993). In a study on the cisplatin resistant teratocarcinoma cell line, Tera-CP, the cells were found to be cross resistant to the platinum analogues and also to 5-fluorouracil but showed only a low level of resistance to adriamycin and the topoisomerase II inhibitor, VM-26 (Timmer-Bossacha *et al.*, 1993). The cross resistance profile of the platinum resistant human Burkitt lymphoma cell line, Raji/CP and the human head-and-neck carcinoma cell line SCC-25/CP revealed that both cell lines were cross resistant to the platinum agents. The SCC-25/CP cells also exhibited cross resistance to methotrexate, while the Raji cell exhibited approximately a 2-fold resistance to 5-fluorouracil. The two cell lines were found to be hypersensitive to adriamycin and vincristine toxicity (Teicher *et al.*, 1988). Similar toxicity profiles were reported for platinum resistant rodent cell lines. In a study on chinese hamster ovary cells, CHO/CDP-2, Saburi *et al.* (1989) reported that the cells exhibited cross resistance to cisplatin, carboplatin and melphalan, but not to adriamycin, VP16 or 5-fluorouracil. Cross resistance to cisplatin and carboplatin was also observed in the platinum resistant murine cells, L1210PtR4 and L1210DDP5, but again no cross resistance to adriamycin was observed (Kraker and Moore, 1988).

| Cell line | Cisplatin | Carboplatin | Methotexrate | Adriamycin | Vincristine | VP16 | VM-26 | 5-fluorouracil | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Raji/CP** | 7.3 | ND | 1.6 | 0.2 | 0.2 | ND | ND | 1.9 | Teicher *et al.*, 1986 |
| **SCC-25/CP** | 12.0 | ND | 23.8 | 0.6 | 0.9 | ND | ND | 0.6 | Teicher *et al.*, 1986 |
| **GLC4-CDDP** | 6.4 | 1.5 | ND | 4.0 | 0.48 | ND | ND | ND | Hosper *et al.*, 1988 |
| **L1210DDP5** | 25.8 | 9.0 | ND | 0.6 | ND | ND | ND | ND | Kraker and Moore, 1988 |
| **P388PtR4** | 24.1 | 5.4 | ND | 0.5 | ND | ND | ND | ND | Kraker and Moore, 1988 |
| **CHO/CDP-2** | 10.0 | 5.0 | ND | 1.0 | ND | 1.0 | ND | 0.9 | Saburi *et al.*, 1989 |
| **LoVo/CP2.0** | 6.4 | ND | ND | 2.6 | 7.8 | 1.1 | ND | 2.6 | Yang *et al.*, 1993 |
| **Tera-CP** | 3.7 | ND | ND | 1.3 | 1.3 | ND | 1.3 | 2.3 | Timmer-Bossacha *et al.*, 1993 |
| **A2780/CP20** | 5 | 21 | ND | 3.0 | ND | 8.0 | ND | ND | Hamaguchi *et al.*, 1993 |
| **A2780/CP70** | 17 | 24 | ND | 6 | ND | 22.2 | ND | ND | Hamaguchi *et al.*, 1993 |
| **T24DDP10** | 8.4 | ND | ND | 0.8 | 8.1 | 1.1 | ND | ND | Kotoh *et al.*, 1994 |

ND - not determined

**Table 4.2** Fold-resistance profile of a number of human and rodent platinum resistant cell lines with respect to the parental cell line

## 4.3 Mechanisms of resistance in DLKPC variants

### 4.3.1 Glutathione and glutathione-S-transferases

Glutathione is one of the most prevalent cellular sulfhydryl peptides and has been shown to be involved in many cellular functions, including drug metabolism, intracellular detoxification and protection from oxidative stress (Arrick and Nathan, 1984). Numerous studies have reported increased glutathione levels in cisplatin resistant cell lines, although its role in contributing to drug resistance remains unclear. The anticancer agent, cisplatin, is sufficiently electrophilic to react with sulfhydryl containing nucleotides, consequently glutathione may play a role in modulating cisplatin cytotoxicity. Several investigators have reported a significant correlation between cellular glutathione levels and cisplatin resistance, although in the majority of studies, increases in glutathione levels were less than the corresponding increase in cisplatin resistance. The GLC4 human small cell lung carcinoma cell line, resistant to cisplatin, was found to exhibit a 3.4-fold increase in glutathione levels which was associated with a 6.4-fold increase in cisplatin resistance (Hosper *et al.*, 1988). Batist *et al.* (1986) reported a 3.2-fold increase in glutathione levels in the cisplatin resistant human carcinoma cell line, A2780/CP, which was 14 fold resistant to the selecting agent. The BE human colon carcinoma cell line, resistant to cisplatin, showed a 3-fold increase in glutathione levels which was associated with a 5-fold increase in cisplatin resistance (Fram *et al.*, 1990). These results would suggest that, although elevated levels of glutathione may play a role in mediating platinum resistance, other mechanisms of resistance must also be involved in order to account for the higher levels of cisplatin resistance observed in the cell lines.

To investigate the possible role of glutathione in the resistance phenotype observed in the carboplatin resistant DLKPC variants, the effect of the glutathione biosynthesis inhibitor, BSO, on the cytotoxicity of carboplatin was studied. A number of studies have reported a substantial reversal of resistance in cisplatin resistant tumour cell lines following treatment with non-toxic concentrations of BSO. The cell lines involved all contained elevated levels of glutathione. Increased glutathione levels were reported from a study on the cisplatin resistant cell line, GLC4-CDDP. When the resistant cells were treated with BSO, a decrease in the level of glutathione and an increase in the cytotoxicity of cisplatin was observed (Meijer *et al.*, 1990). Oldenburg *et al.* (1994) also found an increase in the

2,4-dinitrobenzene as the substrate for the GST enzyme. The results obtained showed a slight decrease in activity in the DLKPC 6.2 and DLKPC 14 variants relative to the parental DLKP cells. However, the level of activity in the DLKPC 25 variant was comparable to the parental cells. A maximum of a 1.5 fold decrease was observed in the lower resistant cell line, thus suggesting that alterations in GST activity does not play a major role in drug resistance in the DLKPC resistant sublines. These results were also confirmed from studies with the GST inhibitor, ethacrynic acid. Ethacrynic acid has been reported to cause a reduction in the levels of GST activity and an increase in the cytotoxicity of a number of alkylating agents in resistant cells lines (Tew *et al.*, 1988; Hoffman *et al.*,1995). However, no significant alteration in the cytotoxicity of carboplatin was observed in the DLKPC variants following treatment with ethacrynic acid.

### 4.3.2 Metallothionein

Metallothioneins (MTs) comprise a family of small, thiol-rich metalloproteins having molecular weights of 6000-7000 daltons. They are involved in $Zn^{2+}$ and $Cu^{2+}$ homeostasis and in the binding and detoxification of heavy metals (Hamer, 1986). Metallothioneins are composed of approximately 30% cysteine and can account for a large percentage of the intracellular thiol content. The observation that metallothioneins play a role in heavy metal detoxification led to investigations exploring the relationship between cellular metallothionein levels and sensitivity to cisplatin. Since metallothioneins are rich in thiol groups they are likely targets for electrophilic agents such as cisplatin and other heavy metals. Although the majority of these intracellular thiols are bound to $Zn^{2+}$, it appears that they are still capable of reacting with platinum compounds and thus may serve as intracellular reservoirs, inactivating incoming cisplatin. Several reports have implicated metallothionein as a major cause of cellular resistance to cisplatin and other alkylating agents. Generally, cells resistant to cisplatin also exhibited cross resistance to the heavy metal, cadmium chloride. Consequently, resistance to the cytotoxic effects of cadmium chloride has been used as an indicator of increased intracellular metallothionein content. Overexpression of metallothionein has also been studied by Northern blotting, Western blotting, immunocytochemistry and radioactivity studies. Early studies by Bakka *et al.* (1981) reported that human epithelial cells and mouse fibroblasts, selected for resistance to cadmium chloride and containing large amounts of metallothioneins, were also cross

resistant to cisplatin. Numerous studies have since reported overexpression of metallothionein in cell lines selected directly for cisplatin resistance. Table 4.3 presents the metallothionein content in a number of cell lines resistant to cisplatin. Teicher *et al.* (1987) demonstrated a 2-fold increase in cadmium chloride resistance in the human head-and-neck carcinoma cell line, SCC-25/CP. The increase in the level of resistance correlated well with the increase observed in the total protein sulfhydryl content in the cells. Later studies by Kelley *et al.* (1988) reported approximately a 4.4-fold increase in metallothionein content in the SCC-25/CP cell line as determined by an indirect competitive ELISA technique. Metallothionein levels have also been studied in two human ovarian cell lines, 2008 and COLO 316, that were resistant to cadmium chloride. The 2008 and COLO 316 cells exhibited a 3.2-fold and 1.2-fold increase in cadmium chloride resistance and were shown to be 4.1-fold and 3.3-fold cross resistant to cisplatin respectively. Metallothionein levels were determined by analysis of radiolabelled $^{203}HgCl_2$ binding assays and an increase was reported in both cell lines. The 2008 cells exhibited a 23-fold increase in metallothionein levels, while the COLO 316 cells exhibited a 9-fold increase (Andrews *et al.*, 1987).

In a comprehensive study by Kelley and coworkers (1988) metallothionein levels and expression of metallothionein mRNA levels were investigated in a number of platinum resistant human and murine cell lines. Increased metallothionein levels were detected in the human melanoma cell line, G3361/CP, the human small cell lung carcinoma cell line, SW2/CP, the human large cell lung carcinoma SL6/CP cell line and the murine leukaemia cell line, L1210/CP, compared to levels observed in the corresponding parental cell lines. An increase in metallothionein levels was also reported in the carboplatin resistant L1210 cell line and the cadmium chloride resistant head-and-neck carcinoma cell line, A-253/CD. The elevated levels of metallothioneins correlated well with the level of cisplatin resistance in some of the cell lines studied, including the SW2/CP and the L1210/DACH cell lines. The cisplatin resistant ratio for the SW2/CP cell line was found to be 4.5, while the increase in metallothionein content was found to be 5.1-fold. The L1210/DACH cell line exhibited a 2.74-fold increase in metallothionein levels, which was associated with a 2.7-fold increase in cisplatin resistance. The results obtained for both cell lines would indicate that metallothioneins play a major role in mediating the resistant phenotype observed in the cells. However, results obtained from other cell lines in this study suggests that although metallothioneins may be involved in mediating resistance, other mechanisms must also play a role in order to account for the high level of cisplatin resistance observed. One such

example, where alternative mechanism may be involved is the cisplatin resistant murine leukaemia cell line, L1210/CP. This particular cell line, while shown to have a 44-fold increase in cisplatin resistance only displayed a 13.3-fold increase in metallothionein levels. The cisplatin resistant human small cell lung carcinoma cell line, H69/CDDP has also been shown to have elevated levels of metallothionein relative to the parental H69 cells. Results obtained from radiolabelled $^{203}Hg$ binding assays showed approximately a 2.3-fold increase in Hg binding to metallothionein, thus representing a 2.3-fold increase in metallothionein levels, while Northern blot analysis revealed a 4.3-fold increase in metallothionein mRNA expression (Kasahara *et al.*, 1991). Overexpression of metallothionein levels in tumour samples and established cell lines has also been detected by immunohistochemical and immunocytochemical techniques. In a study by Kondo *et al.* (1995) metallothionein overexpression was detected in a number of prostatic tumour cell line by antibodies which recognized all isoforms of human metallothionein. Overexpression of metallothionein has also been demonstrated immunohistochemically, using a number of monoclonal anti-metallothionein antibodies, in colerectral adenocarcinoma cells (Ofner *et al.*, 1994), in human thyroid tumour cells (Nartey *et al.*, 1987) and in childhood acute lymphoblastic leukaemia cells (Sauerberg *et al.*, 1994).

The role of metallothionein in drug resistance in the DLKPC variants was investigated by two methods, cadmium chloride toxicity and immunocytochemical analysis. Cadmium chloride toxicity was studied in the parental DLKP cells and in the three carboplatin resistant variants. All three carboplatin resistant variants were shown to be cross resistant to cadmium chloride, although the results obtained demonstrated a different toxicity profile in each variant. The DLKPC 6.2 variant exhibited a 2.7-fold increase in cadmium chloride toxicity which was associated with a 5.1-fold increase in cisplatin resistance. The DLKPC 14 variants, which was 12.2-fold resistant to cisplatin, was shown to have a 9-fold increase in cadmium toxicity. A 10-fold increase in cadmium chloride toxicity was observed in the highest cisplatin resistant variant, DLKPC 25 (25.1-fold resistant to cisplatin). When immunocytochemical analysis was performed on the DLKPC variants immunoreactivity was clearly shown to be related to the level of resistance in the variants. The three sublines displayed positive immunoreactivity for metallothionein expression, although the intensity of cellular staining differed in each variant. Metallothionein expression was predominantly observed in the cytoplasm of the cells, as found in other reports published. A low level of staining was observed in approximately 30% of the DLKPC 6.2 cells, while approximately

80% of the DLKPC 14 cells reacted postively with the anti-metallothionein antibody. The intensity of staining was also found to be greater in the DLKPC 14 cells. Intense cytoplasmic staining was observed in the majority of the DLKPC 25 cells, thus indicating a high level of metallothionein expression. Overall the results, indicated an overexpression of metallothionein which may be involved in mediating drug resistance in the DLKPC variants. The involvement of alternative resistance mechanisms may also explain the results obtained with the DLKPC 25 variant. Although the DLKPC 25 variant displayed higher resistance to carboplatin and cisplatin than the DLKPC 14 variant, only a slight difference (1.2-fold increase) was observed in cadmium chloride toxicity.

| Cell line | Tumour type | Fold Resistance | MT content fold increase | Reference |
|---|---|---|---|---|
| **2008** | Human ovarian carcinoma | 4.1 | 23 | Andrews *et al.*, 1987 |
| **COLO 316** | Human ovarian carcinoma | 3.3 | 9 | Andrews *et al.*, 1987 |
| **SCC-25/CP** | Human head and neck carcinoma | 7.1 | 4.4 | Kelley *et al.*, 1988 |
| **G3361/CP** | Human melanoma | 6.7 | 2.0 | Kelley *et al.*, 1988 |
| **SW2/CP** | Human small-cell lung carcinoma | 4.5 | 5.1 | Kelley *et al.*, 1988 |
| **SL6/CP** | Human large cell lung carcinoma | 2.5 | 3.4 | Kelley *et al.*, 1988 |
| **MCF-7/CP** | Human breast carcinoma | 2.5 | 0.95 | Kelley *et al.*, 1988 |
| **L1210/CP** | Murine leukaemia | 44 | 13.3 | Kelley *et al.*, 1988 |
| **H69/CDDP** | Human small-cell lung carcinoma | 11 | 2.3 | Kasahara *et al.*, 1991 |

**Table 4.3** Role of metallothionein in cisplatin resistance

### 4.3.3 P-glycoprotein, topoisomerase, LRP and MRP expression

In an attempt to further identify the mechanism of resistance in the DLKPC resistant variants, alterations in the levels of a number of cellular proteins were investigated. The level of P-glycoprotein, topoisomerase I, topoisomerase II, lung resistance protein (LRP) and multidrug resistant associated protein (MRP) was studied. P-glycoprotein is a plasma membrane associated efflux pump that actively extrudes drug from the cells. Although overexpression of P-glycoprotein is usually associated with resistance to the classical MDR drugs (anthracyclines, vinca alkaloids and epipodophyllotoxins) studies by Yang and coworkers (1993) have reported alterations in P-glycoprotein levels in the cisplatin resistant cell line, LoVo CP2.0. This cell line was selected by continuous exposure to cisplatin and was found to be 6.4-fold resistant to the drug. Immunocytochemical studies and Western blot analysis, using the C219 monoclonal antibody, revealed an increased level of P-glycoprotein in the LoVo CP2.0 cells relative to the parental cells, although Northern blot analysis demonstrated no increase in mRNA levels. Since the LoVo CP2.0 cells were also shown to have elevated levels of metallothionein, glutathione and glutathione S-transferase mRNA, the authors suggested that P-glycoprotein mediated MDR and cisplatin resistant phenotype could coexist in cells with primary resistance to cisplatin. To determine if P-glycoprotein mediated MDR and a cisplatin resistant phenotype coexisted in the DLKPC resistant variants, Western blot analysis was performed on purified membrane preparations of the DLKPC cells, using the C219 monoclonal antibody. Analysis was also carried out on preparations from the parental DLKP cell line and the adriamycin resistant variant, DLKPA10. Overexpression of P-glycoprotein was detected in the DLKPA10 cell line (positive control), however no detectable levels of P-glycoprotein was observed in the parental cells or in any of the DLKPC variants. These results therefore suggested that overexpression of P-glycoprotein was not involved in cisplatin resistance in the DLKPC variants.

Topoisomerases are nuclear enzymes that catalyze the interconversion of topological forms of single and double strand DNA. The enzymes are involved in processes related to cell growth and division and appear to be structural and functional components of the cell nucleus (Liu *et al.*, 1983). Eukaryotic topoisomerases have been categorized into two types. The type I enzymes make transient single strand breaks in DNA, while the type II enzymes make double strand breaks (Wang, 1985). Topoisomerase enzymes have been

implicated as the target for a number of antitumour agents, including adriamycin, daunorubicin, VP16, VM-26 and camptothecin. Camptothecin appears to be a specific inhibitor for the DNA topoisomerase I enzyme (Hsiang and Liu, 1988), while the other drugs inhibit DNA topoisomerase II enzymes. Decreased activity of the topoisomerases has been associated with drug resistance in a number of cell lines. Although resistance, due to alterations in the topoisomerase enzymes is usually associated with drugs such as VP16, VM-26 and camptothecin, recent reports have suggested a role for topoisomerases in mediating cisplatin resistance in a number of platinum resistant cell lines. Barret *et al.* (1994) demonstrated a 3-fold increase in DNA topoisomerase II activity in nuclear extracts of the cisplatin resistant murine leukaemia cell line, L1210/10, compared to the parental L1210 cells. However, no alteration in the activity of the topoisomerase I enzymes was observed. Topoisomerase II activity was also studied in two other cisplatin resistant sublines of the L1210 cells. The L1210/DDP5 and L1210/DDP8 cells were established *in vivo* (5mg/kg and 8mg/kg) and grown for several weeks in culture. Topoisomerase II activity was shown to be higher in the cisplatin resistant lines than in the parental cells. The L1210/DDP8 resistant line, exhibited the highest level of topoisomerase II activity. Elevated levels of glutathione-S-transferase was also reported in the L1210/DDP5 and L1210/DDP8 cell lines suggesting that glutathione metabolism as well as increased levels of topoisomerase II activity were involved in mediating resistance in both cell lines (Mestagh *et al.*, 1994). Elevated levels of DNA topoisomerase I enzyme has also been demonstrated in cisplatin resistant cell lines. Studies by Kotoh *et al.* (1994) on three cisplatin resistant variants of the human bladder cell line, T24, showed an increase in topoisomerase I cellular levels. Increased levels of mRNA and protein was detected by immunoblot and Northern blot analysis respectively. The cisplatin variants were also demonstrated to exhibit collateral sensitivity to camptothecin derivatives that target DNA topoisomerase I.

The possible involvement of topoisomerase enzymes in the resistant phenotype of the DLKPC variants was investigated. Toxicity studies showed that the variants exhibited slight cross resistance to the topoisomerase inhibitor, VP16. Consequently, the level of topoisomerase II in the highest resistant variant, DLKPC 25, was investigated by Western blot analysis. The results showed that although topoisomerase II was detected in the resistant cell line, there was no significant difference observed relative to the DLKP parental cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis also demonstrated

no detectable alteration in the level of topoisomerase II mRNA in any of the DLKPC variants (Lorraine O'Driscoll, personal communication). To investigate the role of topoisomerase I in mediating resistance in the DLKPC variants, the toxicity of the topoisomerase I inhibitor, camptothecin was studied. The results showed that camptothecin exhibited slightly lower toxicity in all three variants relative to the parental cells. However, only a maximum of a 2-fold decrease was observed in the DLKPC 25 variant. RT-PCR analysis again revealed no significant alteration in topoisomerase I mRNA levels in the platinum resistant variants (Lorraine O'Driscoll, personal communication). Results therefore from studies on topoisomerase I and II levels would suggest that topoisomerases do not play a major role in the resistant phenotype in the DLKPC variants. However, further studies are required to determine the level of enzyme activity before the involvement of topoisomerases can be completely ruled out, since Barret and coworkers (1994) reported an increase in topoisomerase II activity in the cisplatin resistant variant, L1210/10, without a corresponding increase in protein or mRNA levels, as determined by immunoblotting and Northern blot analysis respectively.

The expression of the 110 kDa protein, LRP (lung resistance protein) was studied in the DLKPC variants to determine if LRP was involved in the resistance phenotype of the cells. LRP is associated with low level resistance and has been shown to be overexpressed in a number of multidrug resistant cell lines (Scheper *et al.*, 1993). However, to date no studies suggesting a possible role for LRP in cisplatin resistance have been reported. LRP expression was detected by immunocytochemical analysis in all three DLKPC resistant variants, although no significant difference was observed in the level of immunoreactivity, relative to the parental cell line. Immunocytochemical studies on the expression of the 190 kDa protein, MRP (multidrug resistant related protein) revealed no alteration in the level of MRP in any of the DLKPC resistant variants, thus suggesting that MRP was not involved in cisplatin resistance in these cell lines. Further evidence to support this theory was obtained from RT-PCR studies, where no significant difference was observed in the MRP mRNA levels (Lorraine O'Driscoll, personal communication). MRP has been shown to be associated with resistance in some non-P-glycoprotein MDR cell lines (Cole *et al.*, 1992b), however, to date no studies implicating the involvement of MRP in cisplatin resistance has been reported. In a study by Hamaguchi *et al.* (1993) on the expression of MRP in a series of cisplatin resistant sublines of the ovarian cell line, A2780, no detectable levels of MRP was observed by either Northern blot or Southern blot analysis.

### 4.3.4 Cisplatin accumulation studies

Decreased intracellular cisplatin accumulation has been reported in many, but not all, cisplatin resistant cell lines (Andrews and Howell, 1990; Andrews *et al.*, 1991; Loh *et al.*, 1992 and Ohmori *et al.*, 1993). Generally, the decreased level of cisplatin accumulation did not correlate with the level of cisplatin resistance, indicating that other mechanisms of resistance were also involved. Several reports have shown that cisplatin enters the cells through passive diffusion, since saturation level was not reached, even at high platinum concentrations (Gale *et al.*, 1973; Ogawa *et al.*, 1975). However, reports also suggest that cisplatin transport may be mediated by an active carrier mechanism. Energy and sodium dependence along with inhibition of cisplatin transport by the $Na^+K^+$ATPase inhibitor, ouabain points to the presence of an active uptake mechanism. However, changes in membrane phospholipid structure might also contribute to decreased passive diffusion.

$Na^+K^+$-ATPases have been postulated to play a central role in cisplatin accumulation. The enzyme regulates the transmembrane $Na^+$ gradient, which in turn has been found to regulate cisplatin accumulation (Kawai *et al.*, 1987; Andrews *et al.*, 1991). In a study by Andrews and coworkers (1991) ouabain, was shown to decrease cisplatin accumulation by approximately 50% in the human ovarian cell line, 2008. Similar inhibition of cisplatin accumulation was observed when the $Na^+K^+$-ATPase was blocked by ATP depletion or by incubating the cells in $K^+$ medium. Further studies demonstrated that the cisplatin resistant subline, 2008/CDDP, was cross resistant to ouabain, as indicated by continuous exposure clonogenic assays. The 2008/CDDP cells that were approximately 3-fold resistant to cisplatin were found to be 2.3-fold resistant to ouabain. The authors suggested that the decrease in cisplatin accumulation in the 2008/CDDP cells was due to direct inhibition of $Na^+K^+$-ATPase and dissipation of the $Na^+$ gradient, which then altered cisplatin influx. In contrast, several studies have also shown decreased cisplatin accumulation in resistant cell lines that was not mediated by $Na^+K^+$-ATPase inhibition. Loh *et al.* (1992) demonstrated reduced drug accumulation in the cisplatin resistant cell line, $41M_{cis}R6$, relative to the parental cell line. However, the $41M_{cis}R6$ cells did not exhibit cross resistance to ouabain, suggesting that the decreased accumulation in the resistant cells was probably not regulated by alterations in $Na^+K^+$-ATPase. Studies on the non-small cell lung carcinoma cell line, PC-14, also showed reduced drug accumulation in the cisplatin resistant subline, PC-14/CDDP. Although ouabain was demonstrated to decrease accumulation in the parental

cells it had no effect on cisplatin accumulation in the resistant cell line. Lack of cross resistance to ouabain was observed in the PC-14/CDDP cells suggesting that $Na^+K^+$-ATPases were not involved in the decreased cisplatin accumulation in the resistant cells.

Ouabain toxicity studies in the DLKP parental cells and DLKPC resistant variants revealed that the DLKPC cells exhibited slight cross resistance to ouabain relative to the parental cells. The DLKPC 25 variant was found to exhibit the highest cross resistance exhibiting approximately a 2-fold resistant to the $Na^+K^+$-ATPase inhibitor. Since neither cisplatin nor carboplatin accumulation was directly determined in the DLKPC variants, the involvement of $Na^+K^+$-ATPase in the resistant phenotype in the DLKPC cells remains to be clarified. However, results obtained from metallothionein studies showed that although metallothioneins appeared to play a role in platinum resistance, other mechanisms must also have been involved. This could sugest that reduced cisplatin accumulation may account for the non-metallothionein mediated resistance observed in the resistant cells. Since the DLKPC cells exhibited slight cross resistance to ouabain, $Na^+K^+$-ATPases inhibition may be involved.

Reduced platinum accumulation could, alternatively, be due to decreased membrane permeability of the resistant cells. A number of studies have shown that agents capable of altering membrane permeability could increase cisplatin toxicity in resistant cell lines. The antifungal agent, amphotericin B, has been demonstrated to increase cisplatin toxicity by increasing membrane permeability and thus the intracellular concentration of the drug. Amphotericin B has a unique mechanism of action, related to its affinity for sterols in the cell membrane. Studies have shown that amphotericin B binds to cholesterol and forms pores in the cell membrane, increasing permeability and facilitating uptake of various molecules (Presant *et al.*, 1981). Morikage and coworkers (1993) reported an increase in cisplatin cytotoxicity in the human lung cancer cell line, PC-14/CDDP following treatment with amphotericin B. An increase in cisplatin accumulation was also observed in the resistant cell line which led to the suggestion that the increase in platinum accumulation was at least in part responsible for the mechanism of the sensitizing effect. Amphotericin B has also been shown to increase cisplatin accumulation in the human ovarian cell line, HRA/CDDP. The increase in accumulation was associated with a 12.1-fold increase in cisplatin toxicity when treated with amphotericin B (Kojima *et al.*, 1994). Studies by Sharp *et al.* (1994) reported potentiation of cisplatin and carboplatin cytotoxicity by amphotericin

B in two human platinum resistant ovarian cell lines, $41M_{cis}R6$ and HX/62. Previous studies had shown that resistance in both cell lines was due primarily to reduced platinum uptake. No significant potentiation was observed in the parental cell lines or in the resistant cell line, $CH1_{cis}R6$, where reduced drug accumulation did not play a role in mediating cisplatin resistance. The potentiation effect of amphotericin B was shown to be lower with carboplatin than cisplatin, although, as in the case of cisplatin, the $41M_{cis}R6$ and MX/62 cell lines were the only cell lines where potentiation was observed. The effect of amphotericin B on carboplatin toxicity were not found to be statistically significant.

Treatment of the DLKPC variants with non-toxic concentrations of amphotericin B did not significantly alter the toxicity of carboplatin in any of the variants. The results could indicate that decreased membrane permeability was not involved in platinum resistance in the DLKPC cells or alternatively that amphotericin B selectively potentiates platinum drug sensitivity in resistant cells exhibiting a reduction in drug accumulation. Morikage and coworkers (1993) also showed a similar lack of sensitization by amphotericin B with the lipophilic platinum analogue, ormaplatin.

## 4.4 Drug accumulation studies in P-glycoprotein MDR cell lines

Decreased drug accumulation has been implicated as the mechanism of resistance in many MDR cell lines, although for several years it was not clear whether decreased accumulation reflected decreased drug uptake or increased drug efflux. Observations however, that exposure of MDR cells to metabolic inhibitors, including sodium azide and 2-deoxy-D-glucose, led to increased intracellular drug levels, strongly suggested that decreased accumulation was in fact due to accelerated drug efflux (Dano, 1973). It has since been demonstrated that reduction in the cellular accumulation of antitumour agents usually occurs as a result of overexpression of the *mdr1* gene, which encodes the transmembrane protein pump, P-glycoprotein. Studies have shown that P-glycoprotein can bind diverse cytotoxic agents and extrude them from the cells in an energy requiring reaction, thus reducing the cellular concentration of the drug (Endicott and Ling, 1989). Several observations have supported the proposal that P-glycoprotein is the transporter responsible for the multidrug resistant phenotype in many MDR cell lines. Photoaffinity labelling studies, using photoactive analogues of vincristine, have shown that P-glycoprotein can bind drugs that are transported out of MDR cells (Cornwell *et al.*, 1986). Analysis of the structure of P-glycoprotein also revealed that the peptide has conserved structural features which were consistent with it being an energy dependent transporter. In addition, numerous reports have also shown that development of MDR is accompanied by amplification of the *mdr1* gene while transfection of the cDNAs for human and mouse *mdr1* into drug sensitive cells resulted in the expression of the multidrug resistant phenotype (Gros *et al.*, 1986b; Ueda *et al.*, 1987).

Early studies on drug accumulation in multidrug resistant cell lines were predominantly carried out using rodent cell lines. A number of techniques were employed in these studies to determine drug accumulation, including autoradiography, spectrofluorometric techniques and radioactivity studies. Since several of the anthracyclines were found to be naturally fluorescent under ultraviolet illumination (Egorin *et al.*, 1974), the accumulation of these agents could easily be determined by spectrofluorometric analysis. The drugs could also be radiolabeled and thus accumulation could be determined by measuring the cellular concentration of radiolabelled drug by scintillation counting. Initial drug accumulation studies were carried out on chinese hamster ovary cell lines. Biedler and Riehm (1970) demonstrated by autoradiography the uptake of actinomycin D in sensitive wild-type and

a number of resistant variants of the chinese hamster ovary CLM/AD cell line. The procedure involved labelling the cells with tritiated actinomycin D and counting the number of grains per nucleus in each of the cell lines studied. The results obtained demonstrated that uptake of drug, in terms of mean number of grains per nucleus, was diminished in the resistant cells and also that the degree of resistance was inversely related to the degree of nuclear labelling by actinomycin D. A reduction in the cellular accumulation of drug was also evident in a number of resistant variants of the Ehrlich ascites tumour cell line (Dano, 1973). The accumulation of the anthracycline, daunomycin, was studied in a number of resistant variants of the EHR 2 cell line. These variants were selected for resistance to daunomycin, adriamycin, vincristine and vinblastine. Daunomycin accumulation was analysed spectrofluorometrically by determining the daunomycin content in the medium and subtracting it from the total amount added to the cells. Daunomycin was found to be highly concentrated in the cells, although lower levels were detected in the resistant variants than in the parental cells. Ehrlich ascites tumour cells selected for resistance to adriamycin, vincristine and vinblastine, all of which exhibited cross resistance to daunomycin, showed the same low level of daunomycin accumulation as the cells selected by treatment with the drug itself. Accumulation of daunomycin was greatly enhanced in the resistant variants by the metabolic inhibitors, 2-deoxyglucose and iodoacetate, suggesting an active extrusion of the drug from the resistant cells.

Daunomycin uptake in Ehrlich ascites tumour cells was also investigated by Skovsgaard and coworkers (1978b). These studies involved spectrofluorometric analysis, although, the cellular uptake of daunomycin was determined by measuring the total drug fluorescence extracted from the cells as opposed to measuring the drug content in the medium. The results obtained were consistent with those previously reported by Dano (1973), in which the resistant variants accumulated less drug than the parental cells. These studies also demonstrated increased drug uptake in the cells following treatment with the metabolic inhibitor, sodium azide, which again suggested an active efflux mechanism. A number of anthracycline accumulation studies have also been carried out on sensitive and resistant sublines of the murine leukaemia P388 cell line. Initial studies by Inaba and Johnson (1978) reported a decrease in drug accumulation in the adriamycin resistant and the daunorubicin resistant cell lines, P388/ADR and P388/DAU, when compared with the sensitive parental cells. The uptake was determined by measuring the cellular levels of radiolabelled drug ($^{3}$H-daunorubicin and $^{14}$C-adriamycin) in each of the cell lines studied. Further studies revealed that drug uptake was accelerated by metabolic inhibitors (sodium azide, 2,4-

dinitrophenol, oligomycin and vainomycin) in the resistant variants to a level comparable to the parental cells. Furthermore, in sensitive and resistant cells preloaded with adriamycin or daunorubicin, drug efflux was inhibited by the addition of 2,4-dinitrophenol. These results suggested that an active outward transport mechanism for anthracyclines was present in the P388 leukaemia cells and that enhanced activity of this efflux process rendered the cells highly resistant to the cytotoxic effects of adriamycin and daunomycin (Inaba *et al.*, 1979). Studies on the accumulation of the vinca alkaloids have also demonstrated a reduction in the cellular content of the drug in resistant cell lines. The majority of studies to date have used radioactivity to determine the cellular accumulation of the vinca alkaloids. Skovsgaard (1978a) reported a decrease in the cellular accumulation of radiolabeled vincristine ([$^3$H]VCR) in the vincristine resistant Ehrlich cell line, EHR2/VCR+ relative to the parental cell line. Breier *et al.* (1994) reported a decrease in vincristine accumulation in two resistant variants of the murine leukaemia cell line, L1210, when compared with the sensitive cell line. A decrease in the accumulation of labelled vincristine was also observed in the vincristine resistant murine leukaemia P388/VCR cell line (Tsuruo *et al.*, 1981).

Drug accumulation studies on human cell lines were initially carried out in the mid 1980s by Fojo and coworkers (1985) on a number of resistant variants of the human colon carcinoma cell line, KB. Four resistant variants were selected, with increasing concentrations of colchicine, and were shown to be cross resistant to the classical MDR drugs (adriamycin, vincristine, vinblastine and actinomycin D). Drug uptake was determined in each of the four resistant variants by radiolabeled studies and the results obtained indicated that drug accumulation decreased with increasing level of resistance. This relationship was seen most clearly with colchicine, vincristine, vinblastine and daunorubicin and to a lesser extent with actinomycin D. Drug efflux studies revealed that efflux occured rapidly in all the variants, although in the more resistant variants a greater percentage of the drug was released more rapidly. These findings again suggested that decreased accumulation was due to active efflux of the drug rather than decreased uptake. Several studies have since reported a reduction in the cellular drug accumulation in human resistant cell lines. In the majority of studies, drug uptake was determined either spectrofluorometrically or by radioactivity analysis of labelled compounds. However, more recent reports have demonstrated drug accumulation defects in resistant cell lines by flow cytometry and high-preformance liquid chromatography (HPLC). In one such study, Wiebe *et al.* (1992) reported a reduction in adriamycin accumulation in the resistant human breast

cancer cell line, MDA A-1. Cellular levels of the drug were quantified by HPLC following extraction of the drug from the cells. Total adriamycin accumulation, as measured by the area under the concentration curve, was shown to be reduced in the resistant MDA A-1 cells, relative to the parental cells. Flow cytometry was also used in this study to determine adriamycin retention in the sensitive MDA 231 and resistant MDA A-1 cells. Flow cytometry has also been utilized to study drug retention in chinese hamster ovary CHrC5 cells (Bruno and Slate, 1990), in the murine P388/Adr leukaemia cell line (Gupta *et al.*, 1994), in human leukaemia HL60/Adr cells (Gupta *et al.*, 1994) and in the human K562 leukaemia cell line (Mechetner and Roninson, 1992).

### 4.4.1 Drug accumulation studies in SKMES-1 and SKMES-1/ADR cells

A major focus of this thesis was to investigate the transport of cytotoxic agents in a number of human MDR cell lines. Studies were carried out using the anthracycline adriamycin and the vinca alkaloid vincristine, both of which are classified as classical MDR drugs. Adriamycin uptake was determined spectrofluorimetrically following direct extraction of the drug from the cells, as detailed in section 2.10. Vincristine transport was determined by measurement of the cellular level of radiolabeled vincristine ($^{3}$H-Vincristine). Initial studies were carried out on the human lung squamous carcinoma cells line, SKMES-1 and its resistant variant, SKMES-1/ADR. The resistant cell line was established by continuous exposure of the cells to increasing concentration of adriamycin. It was found to exhibit approximately a 45-fold increase in resistance to the selecting agent, relative to the parental SKMES-1 cells. Toxicity studies, using a number of anticancer agents, revealed that the SKMES-1/ADR cells line was cross resistant to the vinca alkaloid, vincristine (50-fold) and the semi-synthetic derivative of podophyllotoxin, VP16 (3-fold). A low level of cross resistance to carboplatin (2-fold) was also noted. In order to determine if the SKMES-1/ADR cells exhibited the classical MDR phenotype, Western blot analysis of P-glycoprotein expression was performed. The results obtained demonstrated an overexpression of P-glycoprotein in the SKMES-1/ADR, while only a very low level of P-glycoprotein was detected in the SKMES-1 parental cells.

To further investigate the role of P-glycoprotein overexpression on the SKMES-1/ADR cells, adriamycin accumulation studies were carried out on the parental and resistant cell lines. The aim of these studies was to determine if P-glycoprotein expression was associated with a reduction in drug accumulation in the SKMES-1/ADR cells. Results obtained from basic uptake assays indicated decreased adriamycin accumulation in the SKMES-1/ADR cells, relative to the parental cells. An approximate 4-fold decrease in the cellular accumulation of the drug was observed in the SKMES-1/ADR cells over a period of four hours. To determine whether reduced accumulation resulted from decreased uptake or enhanced efflux, the effect of energy inhibition on accumulation was studied. When the cells were treated with the metabolic inhibitors, sodium azide and 2-deoxyglucose, in glucose free medium a marked increase in cellular accumulation was observed. The level of drug accumulation in the SKMES-1/ADR cells after three hours under these conditions was comparable to the level observed in the parental cells, thus suggesting inhibition of the active efflux mechanism since depletion of ATP levels would ultimately result in inhibition of the P-glycoprotein efflux pump. When glycolysis and oxidative phosphorylation was restored by addition of glucose a pronounced extrusion of adriamycin was observed in the SKMES-1/ADR cells again implicating the involvement of the P-glycoprotein efflux pump.

Adriamycin accumulation was also investigated in the SKMES-1 variants, when the cells were at different confluency levels. The purpose of this study was to determine if drug accumulation was cell density dependent. Accumulation studies revealed a significant decrease in the cellular concentration of drug in confluent SKMES-1 and SKMES-1/ADR cells. Adriamycin accumulation was shown to be greater in cells that were plated at a low density. The results correlated with several studies which reported that confluent cancer cells in culture were more resistant to anticancer drugs that non confluent cells, even where the incubation medium was regularly renewed and if the cells were incubated with drug as monolayers, in the presence of controlled pH and atmosphere. Confluency dependent resistance has been attributed to either a decrease in drug accumulation or to a reduction of cell proliferation (Valeriote *et al.*, 1975; Drewinko *et al.*, 1981; Chambers *et al.*, 1981). Confluent cells, grown in monolayers, are tightly attached and are therefore exposed to the incubation medium by a smaller surface of plasma membrane than nonconfluent cells. It could be suggested that reduced drug accumulation was simply due to geometric parameters, however a number of alternative mechanisms have been attributed to reduced drug accumulation in confluent cells. Studies have shown that plasma membranes of confluent

cells appear less permeable to passively diffusing molecules than those of nonconfluent cells. This could account for decreased drug uptake in the cells and consequently a reduction in the cellular levels of the drug (Pelletier *et al.*, 1990). A significant decrease in membrane fluidity has also been reported in confluent cells (Dimanche-Boitrel *et al.*, 1992). Since membrane fluidity can influence the permeability of the membrane to molecules, alterations in fluidity could also result in decreased drug accumulation.

The effect of the calcium channel antagonist, verapamil, on adriamycin accumulation was investigated in the parental SKMES-1 and resistant SKMES-1/ADR cells, to determine if it could restore drug accumulation in the resistant cells. Early studies by Friche *et al.* (1987) demonstrated an enhancement of daunorubicin accumulation by verapamil in resistant cells, to the same level as in the wild-type cells. Tsuruo *et al.* (1981) also reported an enhancement of drug accumulation in P388 resistant cells, to a level comparable to the parental cells, following treatment with verapamil. Numerous reports have since described the effectiveness of verapamil in restoring cellular levels of anticancer drugs in MDR cell lines and thus reversing P-glycoprotein resistance. The exact mechanism by which verapamil restores drug accumulation in MDR cells is unknown, although there is substantial evidence to suggest that the effect is not directly related to alterations in calcium flux or to an effect on calcium channels. Studies by Roberts *et al.* (1987) demonstrated increased drug accumulation in rat glioblastoma cells following treatment with verapamil. They reported that verapamil was still active in the absence of calcium and in the presence of the calcium channel blockers, EGTA and manganese ions, in the culture medium. The results suggested that the action of verapamil on drug resistance was independent of the calcium fluxes of the cells and was not due to effects on calcium channels. Studies by Naito and Tsuruo (1989) demonstrated that verapamil inhibited vincristine binding to plasma membrane preparations from the adriamycin resistant K562/ADM cell line. A substantial amount of vincristine binding was still observed following the addition of high concentration of calcium or the chelating agent [ethylenebis(oxyethenenitrilo)]tetraacetic acid. These findings indicated that calcium ions were not required for vincristine binding and that verapamil competitively inhibited the vincristine binding without the involvement of calcium ions. In photoaffinity labelling studies, competition of verapamil with a vinblastine photoactive analogue for binding to P-glycoprotein has been demonstrated (Safa *et al.*,1986). Further studies using photoactive analogues of verapamil demonstrated that verapamil binds directly to the P-glycoprotein molecule, implying that verapamil may

directly interact with P-glycoprotein and inhibit drug efflux from the cells (Safa, 1988). The mode of inhibition and possible involvement of calcium in the process, however, has not yet been established. When the SKMES-1 and SKMES-1/ADR cell lines were treated with verapamil, a marked increase in adriamycin accumulation was observed in the resistant SKMES-1/ADR cells. The level of cellular accumulation of the drug was comparable to the parental cell line, indicating that verapamil was a very effective agent at restoring drug accumulation levels in these cells. Since the cells were shown to overexpress P-glycoprotein, verapamil may therefore act by competing with the drug for P-glycoprotein binding sites and thus inhibit adriamycin efflux. A slight increase in adriamycin accumulation was also observed in the SKMES-1 parental cells following treatment with verapamil. This result can be explained by the low level of P-glycoprotein expression also detected in the parental cells.

The effect of the immunosuppressive agent, cyclosporin A, on adriamycin accumulation was also investigated in the SKMES-1 variants. Cyclosporin A has been reported to act as a chemosensitizer in many MDR cell lines, by restoration of cellular levels of drug. The mechanism of action of cyclosporin is unclear, although as is the case with verapamil it is thought to modulate MDR by interacting with drug binding sites of P-glycoprotein. Reports have shown that cyclosporin effectively inhibits binding of anticancer drugs to P-glycoprotein (Slatter *et al.*, 1986; Twentyman *et al.*, 1987). Photoaffinity labelling studies have also demonstrated the binding of a photoactive analogue of cyclosporin to P-glycoprotein and shown inhibition of binding by cyclosporin A, cyclosporin H and verapamil (Foxwell *et al.*, 1989). These result suggest that the mechanism of action of cyclosporin is related to competitive inhibition of P-glycoprotein, although several reports have also implicated a role for cyclophilin in the mechanism of action of cyclosporin A. Cyclophilins are cytoplasmic proteins of approximately 18 kDa. Amino acid sequence analysis has revealed two regions containing consensus sequences for ATP binding (Harding *et al.*, 1986). Handschumacher and coworkers (1984) reported that 70-80% of cyclosporin A was located in the cytosol of cells as a result of binding to the cyclophilins. Several laboratories subsequently demonstrated that the degree of binding of a number of cyclosporin analogues to cyclophilin closely paralleled their immunosuppressive activities, thus suggesting a central role for cyclophilin in the action of cyclosporins (Quesniaux *et al.*, 1987). The role of cyclophilin in the chemosenitizing effect of cyclosporin however, remains to be clarified. Cyclosporin A proved to be very effective at restoring adriamycin

accumulation in the SKMES-1/ADR cell line. The cellular level of drug after four hours was greater than the level observed in the parental cells. The addition of cyclosporin A also caused a slight increase in adriamycin accumulation in the parental cells which was consistent with the results obtained with verapamil. The results indicate that cyclosporin A was more effective than verapamil at enhancing accumulation in the SKMES-1/ADR cells, which could suggest that cyclosporin A caused greater inhibition of the P-glycoprotein efflux pump. Alternatively, since cyclosporin A has been shown to affect the physico-chemical properties of the plasma membrane by binding to phospholipid vesicles (Hayes *et al.*, 1985), the additional increase in drug accumulation could be due the direct effect of cyclosporin A on the cell membrane. Cyclosporin A has also been reported to depolarize cytoplasmic membrane potential. Matyus *et al.* (1986) demonstrated a decrease in membrane potential of murine lymphocytes in the presence of cyclosporin A, as determined by flow cytometry and fluorescence spectroscopy. The results indicated that cyclosporin A decreased the apparent freedom of motion of membrane lipids, which may have been responsible for changes in ion flux. Alterations in ion fluxes may alter membrane permeability and thus influence drug uptake, resulting in increase cellular drug accumulation.

Vincristine accumulation studies also revealed a reduction in the accumulation of vincristine in the SKMES-1/ADR resistant cells, relative to the parental SKMES-1 cell line. Approximately a 4-fold decrease in accumulation was observed in the resistant cells over a three hour period which was consistent with the results obtained from adriamycin accumulation studies. The effect of cyclosporin A on vincristine accumulation was also investigated and the results demonstrated that cyclosporin A completely restored accumulation in the resistant cells. Cyclosporin A was also shown to be more effective at restoring vincristine accumulation than adriamycin accumulation in the SKMES-1 variants. The maximum cellular drug level was increased by approximately 11-fold in the SKMES-1/ADR cells and approximately 2.6-fold in the parental cells, following treatment with cyclosporin A. Since photoaffinity labelling studies have demonstrated vincristine binding to P-glycoprotein, the reduction in vincristine accumulation in the SKMES-1/ADR also appears to be associated with overexpression of the P-glycoprotein efflux pump. Overall the results obtained from studies on the SKMES-1/ADR cells would indicate that the cell line is an example of a classical MDR cell line, displaying resistance to a number of the MDR associated drugs including adriamycin, vincristine and VP16. P-glycoprotein

overexpression was associated with a reduction in the cellular accumulation of both adriamycin and vincristine in the resistant cells. Restoration of cellular drug levels was observed when the cells were treated with metabolic inhibitors, verapamil and cyclosporin A which is consistent with classical MDR cell lines.

### 4.4.2 Adriamycin accumulation studies in the T24 variants

Adriamycin accumulation was also studied in variants of the human bladder carcinoma cell line, T24. The parental T24 cells were obtained from the American Type Culture Collection and the MDR resistant sublines, T24A and T24V were established by continuous exposure to increasing concentrations of adriamycin and VP16 respectively. Toxicity studies on the resistant variants revealed that while both variants exhibited resistance to adriamycin and VP16 (the T24V cells were found to be more resistant to adriamycin and VP16 than the T24A cells) the cells showed no significant cross resistance or sensitivity to cisplatin or 5-fluorouracil. The variants therefore appeared to be cross resistant to the classical MDR drugs. To determine if P-glycoprotein was overexpressed in the resistant variants, Western blot analysis was carried out on purified membrane fractions of the parental and resistant cells. Immunoreactive P-glycoprotein was detected in the T24A and T24V cell lines, however only a very low level was detected in the sensitive T24 cells (Cleary *et al.*, 1995).

In order to determine if P-glycoprotein overexpression was associated with a reduction in the cellular levels of drug, adriamycin accumulation was studied in the three T24 variants. The results obtained demonstrated that adriamycin accumulation was significantly reduced in both the T24A and T24V cells, when compared with the parental cells. Although the T24V cell line exhibited greater resistance to the MDR drugs, the T24A cells were found to accumulate the lowest level of drug within the four hour period studied. The effect of verapamil and cyclosporin A on the intracellular accumulation of adriamycin was also investigated in the T24A and T24V cell lines. A significant increase in drug accumulation was observed in the variants following treatment with verapamil and cyclosporin A, although both appeared to be more effective at restoring accumulation in the T24A variant. The maximum level of accumulation in the resistant cells was greater than the level observed in the parental cells, implying that the accumulation deficit in the T24A and T24V

cells was due to mechanism inhibited by verapamil and cyclosporin A, probably overexpression of the P-glycoprotein efflux pump. The results would indicate that, as with the SKMES-1/ADR cell, the T24A cell line is an example of a classical MDR cell line, although further studies are required to fully characterise the mechanisms of resistance in the T24V cell line. P-glycoprotein overexpression appears to play a major role in the resistant phenotype, although other mechanisms of resistance may also be involved in the T24V cell line to account for the higher level of drug resistance observed in this variant.

### 4.4.3 Adriamycin accumulation studies in the OAW42 variants

The parental OAW42 cells were obtained from the ECACC (European collection of animal cell cultures) and a number of variants were established from a spontaneous MDR resistant strain (OAW42-SR) of the parental cell line. Two adriamycin resistant variants, OAW42-A1 and OAW4-A, were established by continuous exposure of the OAW42-SR cells to drug. An adriamycin sensitive variant, OAW42-S, a clonal subpopulation of the OAW42-SR was also included in the study. Toxicity studies, using a number of anticancer agents, revealed that the three adriamycin resistant variants also displayed cross resistance to vincristine and VP16 but exhibited no significant resistance or sensitivity to cisplatin and 5-fluorouracil. Increases of approximately 8.3-fold, 14-fold and 29-fold, relative to the sensitive OAW42-S cells, were observed in adriamycin resistance in the OAW42-SR, OAW42-A1 and OAW42-A cell lines respectively. Western blot analysis was also carried out to investigate the role of P-glycoprotein in the resistant phenotype in each of the four OAW42 variants. While P-glycoprotein expression was not detected in the OAW42-S cells, immunoreactive P-glycoprotein was evident in the three resistant variants. P-glycoprotein expression was shown to be related to the level of resistance in the cells. The OAW42-SR cells were found to express the lowest level of P-glycoprotein, while the highest level was detected in the OAW42-A cells (Moran *et al.*, 1995).

Adriamycin accumulation was also found to be related to the level of resistance in the OAW42 variants. A significant reduction in the cellular accumulation of drug was observed in the resistant variants, when compared with the sensitive OAW42-S cells. The maximum level of adriamycin accumulation was shown to be similar in the OAW42-SR and OAW42-A1, while the OAW42-A cells accumulated a lower level of drug. Treatment with

verapamil and cyclosporin A resulted in a marked increase in adriamycin accumulation in the three resistant variants. The cellular accumulation of the drug was enhanced to a level greater than that observed in the sensitive OAW42-S cells. These results would indicate that the reduction in drug accumulation was predominately P-glycoprotein mediated in the OAW42-A1 and OAW42-A variants and that the resistant phenotype of the cells can be attributed to P-glycoprotein overexpression. However, other mechanisms must also be involved in mediating resistance in the OAW42-SR variant, since the reduction in adriamycin accumulation does not appear to be consistent with the level of P-glycoprotein detected in the cells. Although drug accumulation was similar for the OAW42-SR and OAW42-A1 variants, a lower level of P-glycoprotein was detected in the OAW42-SR cells. Immunocytochemical studies have shown that the OAW42-SR cells overexpress the 110 kDa, LRP protein (Moran *et al.*, 1995). LRP expression has been demonstrated in a number of non-P-glycoprotein cell line, all of which were found to be defective in the cellular accumulation of drug (Scheper *et al.*, 1993). The authors suggested that the LRP protein may be involved in mediating resistance, since reversal of drug resistance in the SW1573/2R120 cell line was associated with a decrease in expression of the protein. Overexpression of LRP may therefore be associated with the non-P-glycoprotein reduction in adriamycin accumulation in the OAW42-SR cell line.

## 4.5 Adriamycin localisation studies in P-glycoprotein MDR cell lines

In addition to a reduction in drug accumulation, many MDR cell lines also exhibit altered distribution and compartmentalisation of drug in vesicles, away from the target site. This had led to numerous studies investigating the distribution of adriamycin in wild type and multidrug resistant cell lines. The majority of studies have involved fluorescent microscopy to detect adriamycin localisation since a number of the anthracyclines were found to be inherently fluorescent (Egorin *et al.*, 1974) and therefore could be easily visualized under UV illumination. Cytofluorescence studies on the cellular localisation of adriamycin in MDR cell lines were first described by Chauffert *et al.* (1984) on adriamycin resistant rat colon cancer cells. The studies revealed that initially adriamycin accumulated rapidly in the nucleus, however, when the cells were exposed to the drug for longer incubation periods adriamycin was localised primarily within the cytoplasmic region. These findings indicated that the inherently resistant cells were capable of removing adriamycin from the nucleus and distributing it in the cytoplasm, away from its main site of action. Consequently, the cells were able to survive and grow in the presence of the drug. Numerous reports have since demonstrated altered drug distribution in MDR cell lines and have associated the alteration with acquired resistance in the cells. In general, while adriamycin has been shown to be primarily associated with the nucleus of sensitive wild type cells, the drug is localised primarily within the cytoplasmic region in resistant cell lines (Willingham *et al.*, 1986; Hindenburg *et al.*, 1987; Versantvoort *et al.*, 1993; Barrand *et al.*, 1993).

### 4.5.1 Adriamycin localisation studies in SKMES-1 and SKMES-1/ADR cells

One of the aims of this thesis was to investigate the subcellular distribution of adriamycin in a number of MDR cell lines, including the SKMES-1/ADR cell line and the OAW42 resistant variants, to determine if the acquired resistance observed in the cells was associated with altered drug distribution. Fluorescence microscopy studies were initially carried out on the sensitive SKMES-1 and its MDR variant, SKMES-1/ADR and the results obtained were found to be consistent with results previously reported on adriamycin distribution in MDR cells. When the parental cells were viewed following exposure to adriamycin, intense fluorescence was clearly visible in the nucleus of the cells indicating the localisation of the drug within the nucleus. In contrast, while faint nuclear fluorescence was observed in the

SKMES-1/ADR cells, the majority of adriamycin fluorescence appeared to be localised within the cytoplasmic region.

While the exact mechanism by which drug distribution is altered in MDR cell lines remains to be clarified, the most likely explanation, however, to account for the altered distribution centers on the overexpression of P-glycoprotein. Anthracyclines appears to enter both the wild type sensitive and resistant cells passively, in an energy independent process. Studies have shown that entry of the anthracycline, daunorubicin into both sensitive and resistant cells was unaffected by the metabolic inhibitor, sodium azide (Gervasoni *et al.*, 1991). Once the drug has entered the cells, it appears that the sensitive cells continue to accumulate drug in their nuclei, while the P-glycoprotein present in the resistant cells actively extrudes the drug from the cells. Consequently, a high cytoplasmic concentration of the drug is never achieved and thus nuclear accumulation of adriamycin does not occur. Since P-glycoprotein has been shown to be overexpressed in the resistant SKMES-1/ADR cells, the results suggest that P-glycoprotein was involved in the altered drug distribution pattern in the resistant cells.

To further investigate the role of P-glycoprotein in the SKMES-1/ADR cells, the effect of a number of known P-glycoprotein inhibitors on adrimaycin distribution was studied, to determine if nuclear fluorescence could be restored in the resistant cells. The metabolic inhibitors, sodium azide and 2-deoxyglucose, were both found to restore nuclear fluorescence in the resistant cells, to a level comparable with the parental SKMES-1 cells. The results confirm the involvement of an energy dependent efflux mechanism, probably P-glycoprotein, since depletion of ATP levels would result in inhibition of the P-glycoprotein efflux pump. These findings are in agreement with a number of reports that have demonstrated increased nuclear fluorescence in MDR cell lines, following treatment with metabolic inhibitors. In one such study, Chauffert *et al.* (1984) reported an increase in nuclear fluorescence in the inherent adriamycin resistant colon carcinoma cells that were exposed to daunorubicin, in the presence of sodium azide. Studies by Gervasoni *et al.* (1991) have also demonstrated complete restoration of nuclear fluorescence following treatment with sodium azide in a number of MDR resistant cell lines, including the human breast cell line, MCF-7/ADR, the human leukaemia cell line, P388/ADR and the human colon cell line, KBV-1. Since previous studies had shown that the resistant cells all expressed P-glycoprotein, it was suggested that the increase in nuclear fluorescence in each

of the cell lines was associated with inhibition of the P-glycoprotein efflux pump. 2-deoxyglucose has also been shown to restore nuclear fluorescence in MDR cell lines, indicating that this agent is also an effective inhibitor of P-glycoprotein, by depletion of cellular ATP levels (Hindenburg *et al.*, 1989).

Reports have suggested that verapamil and cyclosporin A can also restore nuclear fluorescence in P-glycoprotein MDR cell lines most likely by binding to P-glycoprotein and inhibiting drug efflux. Early studies by Chauffert *et al.* (1984) demonstrated an increase in nuclear fluorescence in resistant rat colon cells, when treated with verapamil. Verapamil was also shown to increase nuclear fluorescence in the resistant human colon cell line, KB-C4 (Willingham *et al.*, 1986). The authors suggested that verapamil restored nuclear fluorescence by inhibiting the binding of daunorubicin to an efflux channel or a binding site on the inside of the plasma membrane. This binding site has subsequently been shown to be P-glycoprotein thus implicating the role of P-glycoprotein in drug distribution in MDR cells. Numerous studies have since demonstrated increased nuclear fluorescence in MDR cell lines treated with verapamil (Hindenburg *et al.*, 1987; Schuurhuis *et al.*, 1993; Versantvoort *et al.*, 1993; Sebille *et al.*, 1994; Takeda *et al.*, 1994). Verapamil also proved to be a very effective agent at restoring nuclear fluorescence in the SKMES-1/ADR cells. Treatment with verapamil resulted in an increase in fluorescence to a level comparable to the parental SKMES-1 cells. Since studies have shown that verapamil acts by binding to P-glycoprotein, these results would suggest that altered drug distribution in the SKMES-1/ADR was associated with enhanced efflux of the drug by P-glycoprotein. Cyclosporin A was found to be even more effective than verapamil at restoring nuclear fluorescence in the SKMES-1/ADR. Following treatment with cyclosporin, the intensity of nuclear fluorescence in the resistant cells was greater than the intensity observed in the parental cells. Although fewer studies have been carried out using cyclosporin A, these results are consistent with a number of reports that have demonstrated an increase in nuclear fluorescence in MDR cells when exposed to cyclosporin A (Hindenburg *et al.*, 1987; Sebille *et al.*, 1994). These findings suggests that, as is the case for verapamil, restoration of nuclear fluorescence is due to inhibition of the P-glycoprotein efflux pump.

### 4.5.2 Adriamycin localisation studies in the OAW42 variants

Drug distribution in the OAW42 variants was shown to be inversely related to the level of adriamycin resistance in each variant (Moran *et al.*, 1995). Intense nuclear fluorescence was observed in the OAW42-S cells, following exposure to the drug. This corresponded to the localisation of drug in the nucleus of sensitive cells. Nuclear fluorescence was observed in the OAW42-SR and OAW42-A1 cells, although the intensity of fluorescence was less than that noted in the sensitive cells. The fluorescence intensity appeared to be slightly less in the OAW42-A1 cells, although due to the inherent limitations of the fluorescence microscopy technique, differences in nuclear fluorescence were difficult to determine. The OAW42-A1 cells have been shown to be slightly more resistant to adriamycin than the OAW42-SR cells. It is therefore possible that the differences in resistance levels was reflected in the subcellular distribution of the drug. Only faint nuclear fluorescence was observed in the OAW42-A cells, which exhibit the highest resistance to MDR drugs. Distinct regions of cytoplasmic fluorescence were also visible in the OAW42-A1 and OAW42-A cell lines, consistent with the localisation of drug within cytoplasmic vesicles in resistant cells (Chauffert *et al.*, 1984; Willingham *et al.*, 1986; Hindenberg *et al.*, 1987; Schuurhuis *et al.*, 1991; Coley *et al.*, 1993; Meschini *et al.*, 1994).

The level of nuclear fluorescence was also found to correspond to the level of P-glycoprotein expression in each of the variants, suggesting a role for P-glycoprotein in altering drug distribution in these cell lines. A low level of P-glycoprotein was detected in the spontaneous resistant OAW42-SR cells and also in the low level, adriamycin resistant, OAW42-A1 cells. The decrease in nuclear fluorescence could, therefore, be associated with overexpression of the P-glycoprotein efflux pump which would result in a decrease in the cellular concentration of drug. The OAW42-A cells were shown to express a high level of P-glycoprotein which could be responsible for the marked decrease in nuclear fluorescence observed in the cells. Treatment with both verapamil and cyclosporin A resulted in an increase in nuclear fluorescence in the resistant cell lines. The fluorescence intensity in the OAW42-SR, OAW42-A1 and OAW42-A cells was comparable to the level observed in the OAW42-S sensitive cells, although again it was difficult to determine which variant exhibited the highest level of nuclear fluorescence. Overall, the findings would suggest that as in the case of the SKMES-1/ADR cell line altered drug distribution in the OAW42 variants can be attributed to overexpression of the P-glycoprotein efflux pump.

### 4.5.3 Adriamycin localisation studies in the T24 variants

Studies on the subcellular distribution of adriamycin in the human bladder carcinoma T24 cell line revealed that the drug was localised, predominately in the nucleus of the cells. Distinct regions of fluorescence were discernable within the nuclei corresponding to adriamycin binding to chromosomal DNA. In contrast, when the adriamycin selected variant, T24A, was exposed to the drug for a short time period, the cells showed a mixture of faint nuclear and faint cytoplasmic fluorescence, while longer exposure to the drug resulted in a significant increase in the proportion of cells where only cytoplasmic fluorescence was observed. Similar observations were noted in the VP16 selected, T24V cells when exposed to the drug. Since P-glycoprotein has been shown to be overexpressed in both the T24A and T24V cells, these results would suggest that adriamycin was extruded from the cells by the P-glycoprotein efflux pump, thus inhibiting the binding of the drug to nuclear DNA. Further evidence supporting the involvement of P-glycoprotein was obtained from studies with verapamil and cyclosporin A, since both agents were shown to completely restore adriamycin nuclear fluorescence in the T24A and T24V cells (Cleary *et al.*, 1995).

### 4.5.4 Confocal laser microscopy

Preliminary studies were also carried out using confocal laser scanning microscopy to investigate the subcellular distribution of adriamycin in the SKMES-1 and SKMES-1/ADR cell lines. Confocal scanning microscopy is an optical microscopic technique offering significant advantages over conventional fluorescence microscopy. In laser scanning microscopy the sample is scanned by a diffraction beam of laser light. The light is transmitted or reflected, by the in-focus illuminated volume element. Alternatively, the fluorescence emission is excited within by the incident light and is focused as in the first case onto a photodetector. As the illuminating spot is scanned over the sample, the electrical output from the detector is displayed at the appropriate spatial position on a monitor, thus building up a two-dimensional image (Shotton, 1989). Several studies have recently been published demonstrating the use of this technique in the study of the subcellular localisation of anthracyclines in MDR cell lines. Confocal laser microscopy has a number of advantages over conventional fluorescence microscopy, principally in terms of

its greater resolution and elimination of autofluorescence (White *et al.*, 1987). Confocal laser microscopy markedly reduces observed fluorescence overlap and scatter, thus enabling the precise location of the drug to be determined. This technique also allows the quantification of ratios of nuclear fluorescence to cytoplasmic fluorescence (N/C ratio) which have been extensively used to evaluate the effect of various agents on drug distribution. The N/C ratios cannot be accurately determined by conventional microscopy. Confocal scanning laser microscopy, allowing the optical sectioning of the cells, represents a suitable tool for the fine intracellular localisation of fluorescent substances and consequently the technique has been used to study subcellular anthracycline localisation in a number of P-glycoprotein MDR cell lines. These include the chinese hamster ovary $CH^RC5$ cell line (Schuurhuis *et al.*, 1989), the human breast MCF-7 resistant cell lines (Schuurhuis *et al.*, 1989; Gervasoni *et al.*, 1991; Meschini *et al.*, 1994), the human leukaemia P388/ADR cell line (Gervasoni *et al.*, 1991) and the murine mammary tumour cell line, EMT6/AR1.0 (Coley *et al.*, 1993).

Adriamycin distribution in the SKMES-1 and SKMES-1/ADR cells was studied by confocal laser microscopy and, although similar results to the fluorescence microscopy studies were obtained, the resolution was found to be greatly improved. Nuclear fluorescence was observed in the parental SKMES-1 cells following exposure to adriamycin. The drug appeared to be located in distinct regions within the nuclei, most likely involving chromosomal DNA. Areas within the nucleus were also found to display no fluorescence, which was not evident with fluorescence microscopy where significant fluorescence overlap was observed in the nuclei of the SKMES-1 cells. No cytoplasmic fluorescence was visible in the majority of the SKMES-1 cells viewed, although again this was not evident from previous fluorescence microscopy studies due to the fluorescence overlap and out-of-focus blur. The resolution of the images obtained for adriamycin distribution was also much improved in the SKMES-1/ADR cells. Regions of faint nuclear fluorescence were clearly visible when the cells were exposed to adriamycin and viewed by confocal laser microscopy. In contrast, these distinct areas of fluorescence were not discernable by conventional fluorescence microscopy. The results also demonstrated regions of intense cytoplasmic fluorescence in the SKMES-1/ADR cells, which was consistent with the localisation of drug in cytoplasmic vesicles. However, observations from the fluorescence microscopy studies suggested an overall distribution of the drug within the cytoplasm.

The addition of verapamil and cyclosporin A was shown to cause a marked increase in adriamycin nuclear fluorescence in the SKMES-1/ADR cells. Following treatment with the circumvention agents, the intensity of nuclear fluorescence was greater in the SKMES-1/ADR cells than in the untreated parental cells. The results clearly demonstrated that the fluorescence was greater in the resistant cells, thus indicating a complete restoration of nuclear levels of the drug. Although the fluorescence microscopy studies also showed an increase in nuclear fluorescence in the SKMES-1/ADR cells, the actual intensity of the fluorescence could not be determined. An additional observation from the confocal laser microscopy work that was not evident from the conventional fluorescence microscopy studies was that no cytoplasmic fluorescence was visible in the majority of the SKMES-1/ADR cells following treatment with the circumvention agents. This finding is consistent with the redistribution of drug from the cytoplasmic regions to the nucleus. Overall, the results clearly show that confocal laser scanning microscopy is a powerful tool for studying the subcellular localisation of anticancer agents in MDR cell lines. Although similar information can be obtained from conventional fluorescence microscopy, its use in determining the precise intracellular location of anticancer drugs in MDR cells is limited.

## 4.6 Drug accumulation studies in DLKP and DLKPA10 cells

The accumulation of anticancer drugs was investigated in the human lung squamous carcinoma cell line, DLKPA10. This cell line was derived from the adriamycin resistant DLKP-A cell line, established by exposure of the parental DLKP cells to increasing concentrations of adriamycin (Clynes *et al.*, 1992). The DLKPA10 cell line was established by further exposing the DLKP-A cells to increasing concentrations of the drug, to a final concentration of 10$\mu$g/ml. The cells readily adapted to growth in the higher drug concentrations, which was probably attributable to a more resistant population present in the DLKP-A cells, that was subsequently selected out. Toxicity studies revealed that, in addition to exhibiting resistance to the selecting agent, the DLKPA10 cells were also cross resistant to the MDR drugs, vincristine and VP16 (approximately 3000-fold and 63.3-fold respectively) but showed no significant resistance or sensitivity to the anticancer agents, cisplatin or 5-fluorouracil. The DLKP-A cells were also shown to exhibit a similar cross resistance profile although the level of resistance observed for vincristine and VP16 was substantially lower (approximately 36-fold and 13-fold respectively) than in the DLKPA10 cell line (Clynes *et al.*, 1992). Western blot analysis of purified membrane preparations illustrated that the DLKPA10 cell line overexpressed the 170 kDa membrane protein, P-glycoprotein, usually associated with mediating resistance in classical MDR cell lines (Endicott and Ling, 1989; Clynes *et al.*, 1992; Nielsen and Skovsgaard, 1992). A low level of P-glycoprotein was also detected in the parental DLKP cells.

To further investigate the role of P-glycoprotein in mediating resistance in the DLKPA10 cell line, the accumulation of adriamycin and vincristine was studied in the parental and resistant cells. Drug uptake studies illustrated a marked reduction in the cellular concentration of both adriamycin and vincristine in the DLKPA10 resistant cells relative to the parental DLKP cell line. A decrease in accumulation of approximately 16-fold was observed for both drugs in the DLKPA10 cells. Similar to the results obtained for the SKMES-1 variants, drug accumulation in both the parental and resistant cells was found to be cell density dependent, with confluent cells accumulating a lower level of the drug than non-confluent cells. This confluence dependent resistance was most evident in the DLKPA10 cells. The initial results obtained suggested that, similar to the classical MDR cell lines, overexpression of the P-glycoprotein pump was associated with the observed reduction in drug accumulation, by extruding the drug from the cells thereby reducing the

cellular concentration of the drug.

The effect of the circumvention agents, verapamil and cyclosporin A, on drug accumulation was also investigated. The purpose of this work was to establish whether these agents could reverse the accumulation defect that was observed in the DLKPA10 resistant cells and thus verify the role of P-glycoprotein in mediating drug resistance. Verapamil was shown to enhance the cellular accumulation of both adriamycin and vincristine in the DLKPA10 cells, although the maximum level of drug accumulated was only approximately 45% and 28% of the level observed in the parental DLKP cells for adriamycin and vincristine respectively. Cyclosporin A has consistently been shown to be more effective than verapamil at altering drug accumulation (Boesch *et al.*, 1991; Sebille *et al.*, 1994). Consequently, drug accumulation, following treatment with cyclosporin A, was determined. Although cyclosporin A proved to be more effective than verapamil, the maximum level of drug accumulation for adriamycin and vincristine was still only approximately 50% and 31% of the level observed in the DLKP cells. Treatment with higher concentrations of the circumvention agents or exposure for longer time periods, did not significantly alter drug accumulation, although an increase was observed following pretreatment with cyclosporin A. Adriamycin accumulation was restored to approximately 60% of the level observed in the parental cells, indicating that cyclosporin A pretreatment was more effective than cotreatment at restoring the cellular levels of drug. These results are consistent with other reports published demonstrating higher drug retention levels in pretreated cells. Boesch and Loor (1994) reported that the cellular concentration of daunomycin was higher in the murine resistant MDR, P388 cells, following pretreatment with cyclosporin A than in cells cotreated with the circumvention agent. Similar accumulation patterns were also demonstrated with verapamil. Whilst the initial results suggested the involvement of P-glycoprotein in mediating resistance in the DLKPA10 cells, the results obtained from the verapamil and cyclosporin A studies indicate that overexpression of P-glycoprotein can not fully explain the accumulation defect in the DLKPA10 cells. Other mechanisms must therefore, play a role in mediating resistance to account for the apparent non-P-glycoprotein accumulation defect observed in the cell line.

The effect of a number of metabolic inhibitors on drug accumulation was investigated to verify that the reduction in drug accumulation was associated with enhanced active drug efflux and to determine if complete restoration of drug accumulation could be achieved in

the DLKPA10 cell line. Numerous studies have shown that metabolic inhibitors, including 2-deoxyglucose, sodium azide, dinitrophenol and valinomycin, are effective at reversing the drug accumulation defect in classical MDR cells, most likely through inhibition of the P-glycoprotein efflux pump (Dano, 1973; Skovsgaard, 1978b; Inaba *et al.*, 1979). Since P-glycoprotein is energy dependent, depletion of ATP levels would inhibit the activity of the pump and thereby increase the cellular drug concentration. The effect of the inhibitors, sodium azide, 2-deoxyglucose and antimycin A on drug accumulation was investigated in the DLKP and resistant DLKPA10 cell lines. Initial studies with sodium azide and 2-deoxyglucose revealed that, while both agents increased drug accumulation in the resistant cells, the accumulation could not be restored to a level comparable to the parental DLKP cells. These finding suggested that a non-ATP-dependent mechanism of drug efflux was involved in mediating resistance in the DLKPA10 cell line. However, studies with antimycin A disproved this hypothesis, since treatment with antimycin A proved to be very effective at restoring the cellular concentration of drug. The maximum level of drug accumulated in the DLKPA10 cells was greater than the level observed in the parental cell line, thus showing a complete reversal of the accumulation defect in the resistant cells. A significant increase was also observed in adriamycin and vincristine accumulation in the parental DLKP cells following treatment with antimycin A. Since only a low level of P-glycoprotein was detected in the DLKP cells, the results could indicate that depletion of ATP resulted in increased accumulation by mechanisms other than P-glycoprotein. To verify that the enhanced drug accumulation in both the sensitive and resistant cells following treatment with antimycin A was not a consequence of disruption of membrane permeability which would subsequently result in increased drug uptake, cell viability was determined using trypan blue. The results obtained showed that the trypan blue was excluded from the cells that were treated with antimycin A indicating that the membrane was impermeable to the dye. These findings therefore imply that increased drug accumulation in the DLKPA10 cells was not due to an alteration in membrane permeability.

The results obtained from these studies indicate that, of the three compounds tested, antimycin A was the most effective inhibitor of ATP production and thus the most effective at inhibiting active drug efflux. These findings could be attributable to the different sites of action, since each compound has been shown to act at different stages of ATP production. 2-deoxyglucose acts by competing with glucose and thereby inhibiting the glycolytic pathway. Consequently, there is a reduction in the amount of pyruvate available

to enter the citric acid cycle, which leads to a reduction in NADH production (figure 4.6.1). This ultimately leads to a reduction in ATP production. Sodium azide and antimycin A both act by inhibiting the electron transport chain. The free energy necessary to generate ATP is extracted from the oxidation of NADH and $FADH_2$ by the electron transport chain, with consequent inhibition of electron transport resulting in inhibition of ATP production. The electron transport chain is comprised of a series of four protein complexes through which electrons pass from lower to higher standard reduction potential. Electrons are carried from complex I and II to complex III by coenzyme Q (CoQ or ubiquinone) and from complex III to complex IV by the membrane protein, cytochrome C (figure 4.6.2). The change in standard reduction of an electron pair as it successively traverses complexes I, III and IV generates sufficient free energy to power the synthesis of an ATP molecule. Antimycin A has been shown to block the transfer of electrons from ubiquinone to cytochrome C, while sodium azide blocks the reduction of oxygen catalysed by cytochrome oxidase (cytochrome $aa_3$). If antimycin A blocks the action of ubiquinone the transfer of electrons from carriers I and II to carrier III would be inhibited. The carriers before ubiquinone, would become reduced, while all the subsequent carriers would become fully oxidized. As a result, ATP production would be inhibited from the ubiquinone stage of the electron transport chain. Since sodium azide blocks the transport chain at a later stage and only the oxidation of cytochrome C by oxygen is affected, therefore only one stage of ATP production is inhibited. This would therefore imply that whilst sodium azide does block ATP production, it is not as effective as antimycin A. Consequently, this study has found that sodium azide may not deplete cellular ATP levels within the limited time period studied and thus the P-glycoprotein may have sufficient energy supply to actively pump the drug from the cells. Inhibition of glycolysis may also be ineffective at depleting the cellular levels of ATP and may therefore be ineffective at inhibiting P-glycoprotein. Although glycolysis may be inhibited, other metabolic pathways including amino acid breakdown, lipid metabolism and protein metabolism, would still be functional and could ultimately feed into the electron transport chain and lead to ATP production (figure 4.6.2).

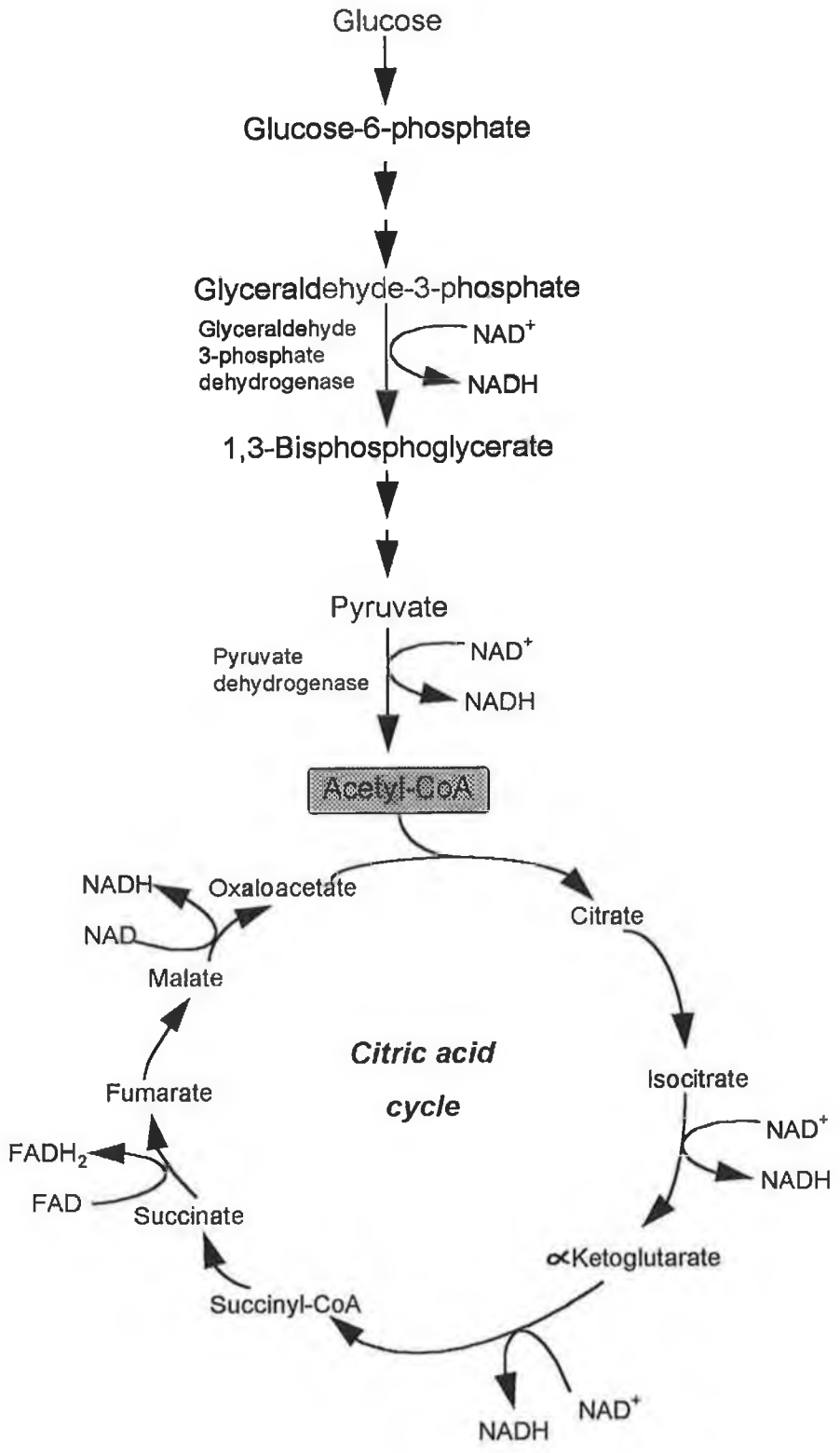



**Figure 4.6.1** The sites of electron transfer that form NADH and $FADH_2$ in glycolysis and the citric acid cycles

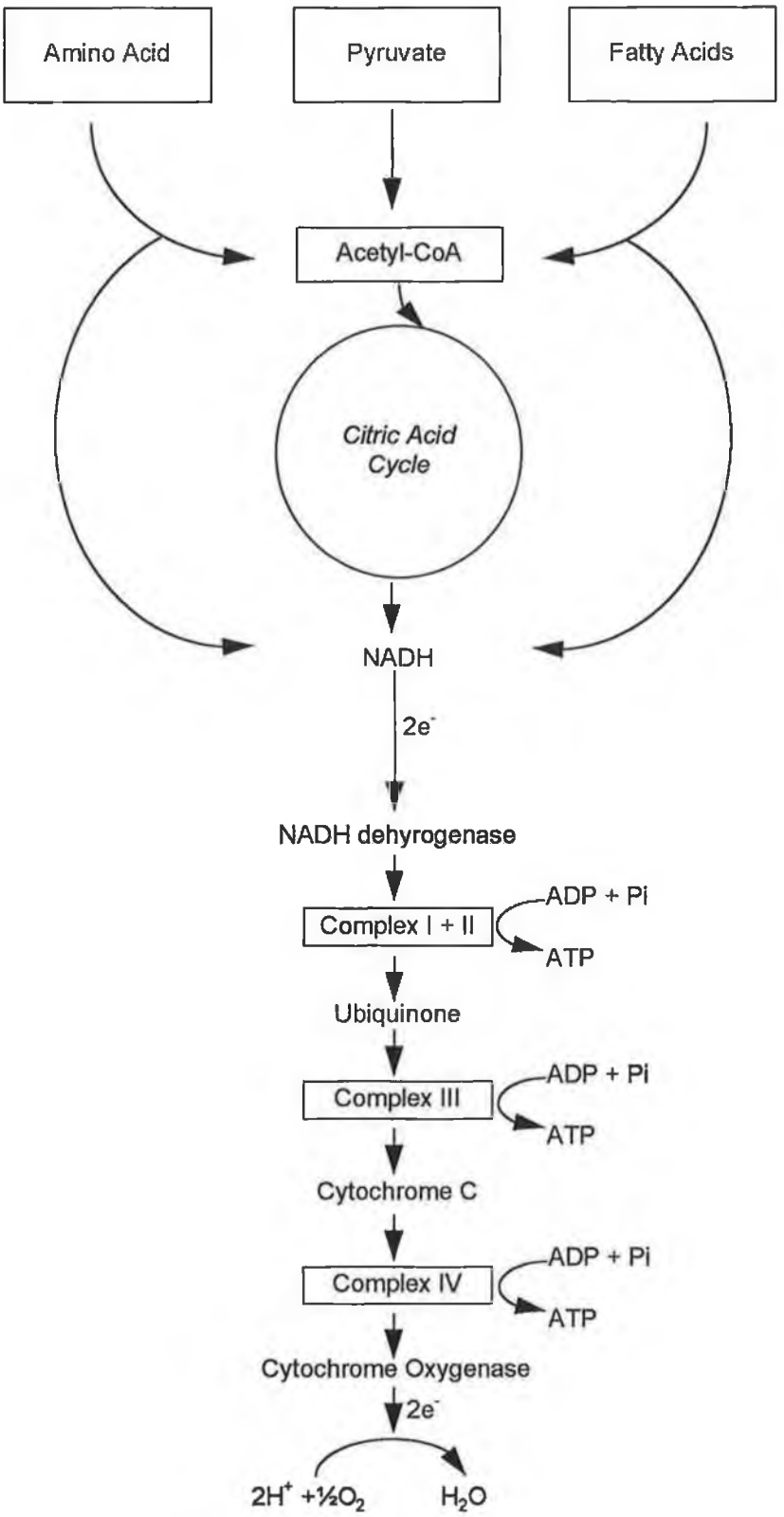



**Figure 4.6.2** Stages in cell respiration. Stage 1: mobilisation of acetyl-CoA from glucose, fatty acids and some amino acids, Stage 2: the citric acid cycle and Stage 3: electron transport and oxidative phosphorylation

The efflux of adriamycin and vincristine from the parental DLKP and the resistant DLKPA10 cells was studied, following preloading of the cells in the presence of antimycin A, since this agent was found to fully restore drug accumulation in the DLKPA10 cells. When the cells were reintroduced into glucose containing medium, rapid drug efflux was observed in both cell lines, which again suggested the presence of an active efflux mechanism. Cyclosporin A was demonstrated to significantly decrease adriamycin and vincristine efflux in the DLKPA10 cell line, which was consistent with other published reports which suggested that cyclosporin A inhibits drug efflux by binding to the P-glycoprotein molecule. Meador *et al.* (1987) reported a decrease in daunorubicin efflux in Ehrlich carcinoma cells and also in hepatoma 129 cells, following treatment with cyclosporin A. Studies by Sebille and coworkers (1994) also demonstrated a decrease in THP-adriamycin efflux in MDR K562 cells in the presence of cyclosporin A. Verapamil has also been reported to inhibit drug efflux in MDR cell lines, although generally it has been shown to be less effective than cyclosporin A (Tsuruo *et al.*, 1981; Klohs *et al.*, 1988; Boesch *et al.*, 1994; Gruol *et al.*, 1994; Julia *et al.*, 1994). Cyclosporin A did not significantly alter adriamycin efflux in the DLKP parental cells indicating a cyclosporin A independent efflux mechanism. There was also no significant alteration in vincristine efflux in the parental cells following treatment with cyclosporin within the first 60 minutes, however a slight increase in efflux was observed after this time.

Another agent which has been shown to increase cellular levels of drug in MDR cell line is the carboxylic ionophore, monensin. Studies were carried out to investigate the effect of monensin on drug accumulation and efflux in the DLKP and DLKPA10 cell lines. Several reports have shown that ionophores increase the cytotoxicity of anticancer agents in MDR cells by selectively increasing the cellular accumulation of the drug. The ionophores, monensin and nigericin, have been demonstrated to increase daunorubicin accumulation in daunorubicin resistant Ehrlich ascites tumour cells (EHR2/DNR+) in a dose dependent manner, while having only a negligible effect on daunorubicin accumulation in the wild type cells (Sehested *et al.*, 1988). Studies by Klohs and Steinkampf (1988) revealed that increased adriamycin accumulation was observed in the adriamycin resistant murine leukaemia cell line, P388, following treatment with both monensin and nigericin. The increase was shown to be associated with inhibition of drug efflux which resulted in increased cellular retention of the drug. Increased adriamycin accumulation was also observed in the human KB/MDR cells when exposed to monensin. However, monensin did

not significantly alter cellular levels of the drug in the parental KB cells (Ling *et al.*, 1993). The exact mechanism by which monensin and other ionophores increase the cellular accumulation of anticancer agents is as yet unknown. However, studies have suggested that these agents act by inhibiting the P-glycoprotein mediated efflux of the drug, thus increasing the cellular drug levels. A number of laboratories have examined the possible involvement of P-glycoprotein in ionophore induced drug accumulation in MDR cell lines. The interaction of various ionophore antibiotics with P-glycoprotein has been investigated by Naito *et al.* (1991). The results obtained indicated that a number of ionophores, including monensin, nigericin and valinomycin inhibited the binding of radiolabelled vincristine to the plasma membrane isolated from resistant K562 cells. The studies also showed that these agents reduced the photoaffinity labelling of P-glycoprotein by azidopine, thus suggesting competitive binding of the ionophores to the P-glycoprotein binding sites. Further evidence supporting the involvement of P-glycoprotein was obtained from studies by Borrel *et al.* (1994), which demonstrated that monensin and nigericin inhibited the P-glycoprotein mediated efflux of tetrahydrophranyl-adriamycin (THP-adriamycin) in resistant K562 cells.

In our studies, monensin also proved to be an effective agent at enhancing adriamycin accumulation in the resistant DLKPA10 cells, while only having a negligible effect on drug accumulation in the parental DLKP cells. A marked increase in the cellular concentration of adriamycin was observed in the DLKPA10 cells, although the maximum level was significantly less than that observed in the parental cells. Pretreatment with monensin was found to result in a greater enhancement of drug accumulation in the resistant cells, although complete restoration of drug levels was not observed. An increase in vincristine accumulation was also noted in the DLKPA10 cells, following treatment with monensin although the results obtained would suggest that it was much less effective at enhancing vincristine accumulation than adriamycin accumulation. These findings tend to confirm results obtained from monensin toxicity studies. The effect of monensin on the cytotoxicity of a number of anticancer agents was investigated in the DLKPA10 cell line, the results showing that, although monensin increased the cytotoxicity of adriamycin and VP16 it did not significantly alter the toxicity profile of vincristine in the cells. The cause of this apparent drug specific effect is unknown, although, since both adriamycin and VP16 are topoisomerase II inhibitors, the specific effect of monensin may be related to topoisomerase inhibition. Alternatively, since vincristine has a higher molecular weight and a more complex structure than either adriamycin or VP16, it may be possible that monensin does

not interact with vincristine. Monensin was also found to decrease drug efflux in the DLKPA10 cells, following preloading of the cells. A marked inhibition of adriamycin efflux was observed when the cells were exposed to monensin, which was consistent with reports published previously. Although treatment with monensin also resulted in a decrease in vincristine efflux, its effect was not as great as that observed with adriamycin efflux. The results obtained from the monensin studies could suggest that the increase in the cellular accumulation of the drug was attributable to the P-glycoprotein efflux pump, since inhibition of the pump would inhibit drug efflux and thus increase cellular levels of the drug. However, monensin may also increase drug accumulation by an alternative mechanism since studies have shown that monensin is also effective at altering cellular drug accumulation in non-P-glycoprotein MDR cell lines (Marquardt and Center, 1992).

Overall, the results obtained indicated that monensin was more effective than cyclosporin A at enhancing drug accumulation and decreasing drug efflux, which suggests that in addition to interacting with P-glycoprotein present in the DLKPA10 cells, monensin may also act through another mechanism, unaffected by cyclosporin A. The observation that monensin had only negligible effect on the parental cells, while cyclosporin A increased drug accumulation in these cells, further supports the involvement of a non-P-glycoprotein mechanism. Combination treatment with monensin and cyclosporin A proved to be more effective at increasing adriamycin accumulation in the DLKPA10 cells than treatment with either monensin or cyclosporin A alone. This observation could suggest that these agents have different mechanisms of action. It has been proposed that, in addition to the P-glycoprotein mediated drug efflux mechanism, an alternative transport system, referred to as acidic vesicular trafficking system, may be involved in mediating resistance in MDR cell lines. Reports have indicated that the MDR phenotype may in some way be related to an increase in plasma membrane traffic which would result in increased drug efflux in resistant cells (White and Hines, 1984; Sehested *et al.*, 1988; Ling *et al.*, 1993). Endosomal drug trapping, followed by vesicular extrusion to the extracellular medium, has been suggested as a possible mechanism for such cellular drug resistance. Studies have shown that monensin and other ionophores can disrupt acidic vesicular traffic by increasing the pH environment of the membrane lumen (Tarkakoff, 1987; Molleuhauer *et al.*, 1990). It is therefore possible that monensin increases drug accumulation in the DLKPA10 cells by a combination of P-glycoprotein inhibition and disruption of acidic vesicular traffic.

The results obtained, thus far, from studies on the DLKPA10 cell line would suggest that, although the cells are cross resistant to the classical MDR drugs and overexpress P-glycoprotein, other resistance mediating mechanisms must also be involved to account for the cyclosporin A/verapamil independent accumulation defect in the cells. Further studies were carried out in an attempt to determine these additional mechanism(s). A number of drug selected cell lines have been reported to exhibit the MDR phenotype without the overexpression of P-glycoprotein and are termed non-P-glycoprotein MDR (McGrath *et al.*, 1989; Kuiper *et al.*, 1990; Coley *et al.*, 1991). To date, at least two drug resistance mechanisms have been detected in non-P-glycoprotein MDR. The first mechanism involves an alteration in topoisomerase II activity, while the second mechanism relates to a decreased cellular drug concentration at the target due to a decreased accumulation of the drug and/or altered distribution of the drug (Center, 1993). In several of the non-P-glycoprotein MDR cell lines, drug accumulation was shown to be decreased due to an enhanced drug efflux (Slovak *et al.*, 1988; Marquardt *et al.*, 1990; Coley *et al.*, 1991; Versantvoort *et al.*, 1992). This enhanced efflux was blocked when cellular ATP synthesis was completely inhibited by metabolic inhibitors, indicating the presence of active drug transport in non-P-glycoprotein MDR cell lines. Since the DLKPA10 was found to possess an active non-P-glycoprotein drug efflux mechanism the possible involvement of an alternative transporter protein in drug efflux was investigated in the cells.

Studies were carried out to investigate the role of MRP in mediating drug resistance in the DLKPA10 cells. The MDR-associated protein (MRP) gene has recently been cloned and reports have suggested that the gene is likely to encode a drug transport protein in many non-P-glycoprotein cell lines (Cole *et al.*, 1992). Transfection of the MRP gene has been shown to confer the MDR phenotype in drug sensitive HeLa cells, without P-glycoprotein overexpression (Grant *et al.*, 1994). These MRP transfections were also shown to overexpress a 190 kDa membrane protein, which is known to be overexpressed in a number of other non-P-glycoprotein MDR cell lines, including the HL60/ADR, GLC4/ADR and COR-L23/R cell lines (Barrand *et al.*, 1993; Zaman *et al.*, 1933; Krishnamachary and Center, 1994). These cell lines have the common characteristic of decreased drug accumulation. Studies have demonstrated that decreased daunorubicin accumulation in cell lines overexpressing the 190 kDa protein could be reversed by the isoflavonoid genistein, a tyrosine kinase inhibitor (Akiyama *et al.*, 1987). It was also illustrated that genistein did not significantly alter drug accumulation in P-glycoprotein MDR cell lines (Versantvoort

*et al.*, 1993). Since genistein appears to have a differential effect in non-P-glycoprotein cell lines, it has been selected as a probe for non-P-glycoprotein mediated MDR cell lines. Versantvoort *et al.* (1994) studied the effect of genistein on daunorubicin accumulation in the GLC4/ADR MDR cell line. This cell line had been shown to overexpress the MRP gene, which was likely to be the gene encoding the drug transporter present in the cells. Genistein was found to inhibit the enhanced efflux of daunorubicin in the GLC4/ADR cells, suggesting that the agent was a competitive inhibitor of daunorubicin transport possibly by interacting with the drug transport protein. Although the exact mechanism of action of genistein is unknown, it is unlikely that a tyrosine kinase step is involved (Versantvoort *et al.*, 1993). Studies were carried out to investigate the effect of genistein on adriamycin accumulation in the DLKP and DLKPA10 cell lines. However, the results did not illustrate a significant alteration in drug accumulation in either the parental or resistant cells following treatment with genistein. This would suggest that the DLKPA10 cells do not overexpress the transporter protein that is present in a number of MRP positive cell lines. Since it is generally accepted that the MRP gene encodes the transport protein in these cell lines, the results would seem to imply that MRP is not involved in mediating the non-P-glycoprotein resistance in the DLKPA10 cells. Further evidence to support the non-involvement of MRP was obtained from immunocytochemical analysis and drug distribution studies, which showed no overexpression of the protein in the DLKPA10 cell line and no alteration in the cellular localisation of adriamycin respectively.

### 4.6.1 Drug accumulation studies in the DLKPA clones

A number of clones were isolated from the adriamycin resistant cell line, DLKP-A, and were shown to display varying resistance to the drug (M Heenan, PhD thesis). A total of nine clones were isolated, four of which were selected for drug accumulation studies to establish if any of the clones showed similar accumulation patterns to the DLKPA10 cell line. Adriamycin accumulation studies revealed a significant difference in the cellular concentration of the drug in each of the clones. The DLKPA 2B cells accumulated the highest level of adriamycin, while the lowest level of adriamycin accumulation was noted in the DLKPA 5F cells. An intermediate level of accumulation was observed in the DLKPA 6B and DLKPA 11B cells. The level of drug accumulation was shown to be inversely related to the level of resistance in each clone. The DLKPA 5F clone exhibited the greatest resistance to adriamycin (approximately 330-fold), followed by the DLKPA 6B (approximately 95-fold) and DLKPA 11B cells (approximately 86-fold) and finally the DLKPA 2B cells which displayed approximately 37-fold resistance to adriamycin. Since the clones were also found to exhibit cross resistance to the MDR drugs, vincristine and VP16 (M Heenan, PhD thesis), the results would therefore suggest that the accumulation defect in the clones was associated with overexpression of the P-glycoprotein efflux pump.

In contrast, further studies revealed that the circumvention agents, verapamil and cyclosporin A were ineffective at reversing the accumulation defect in two of the clones, indicating that the resistance can not be fully explained by P-glycoprotein overexpression. Treatment with verapamil resulted in restoration of adriamycin accumulation in the DLKPA 2B and DLKPA 5F clones to levels comparable to the parental DLKP cells. Similar results were obtained following treatment with cyclosporin A, suggesting that the reduction in drug accumulation in these clones was associated with P-glycoprotein overexpression. Both agents also enhanced adriamycin accumulation in the DLKPA 6B and DLKPA 11B cells, although only to a maximum of approximately 70% of the level observed in the parental cells. These findings suggested that, while P-glycoprotein appears to play a major role in mediating resistance in the DLKPA 6B and DLKPA 11B cells, other mechanisms must also be involved to account for the cyclosporin A/verapamil independent accumulation defect observed in the cells. The accumulation studies in the DLKPA 6B and DLKPA 11B cells revealed similar patterns to that observed in the DLKPA10 cells which could suggest that the DLKPA10 cells were selected out from the DLKPA 6B/DLKPA 11B population. However, further studies need to be carried out to confirm this.

## 4.7 Adriamycin localisation studies in DLKP and DLKPA10 cells

Detailed studies were carried out to investigate the distribution of adriamycin in the resistant DLKPA10 cells to determine if, in addition to a reduction in drug accumulation the cells also exhibited altered distribution of the drug. Initial fluorescence microscopy studies revealed that adriamycin was localised predominantly in the nucleus of the parental DLKP cells, following a two hour exposure to the drug. However, the DLKPA10 resistant cells displayed an intracellular localisation of the anticancer agent which was notably different from the parental cells. When the DLKPA10 cells were exposed to the drug for the same time period, faint nuclear fluorescence was observed in a small proportion of the cells, with the majority of the cells showing only cytoplasmic fluorescence. These results were therefore consistent with several reports that had demonstrated altered drug distribution patterns in MDR cell lines (Chauffert *et al.*, 1984; Willingham *et al.*, 1986; Hindenburg *et al.*, 1987; Gervasoni *et al.*, 1991; Schuurhuis *et al.*, 1991; Coley *et al.*, 1993). Drug distribution was also studied in the DLKPA10 cells, following exposure of the cells to a higher concentration of the drug. Although a marked increase in cytoplasmic fluorescence was observed in the cells, no corresponding increase in nuclear fluorescence was noted. Similar results were obtained when the cells were exposed to the drug for longer incubation periods, thus eliminating the possibility that nuclear drug exclusion was due to either time restraints or concentration effects. Adriamycin fluorescence appeared to be localised in distinct regions within the cytoplasm, which were more evident when the cells were exposed to high concentrations of the drug. Regions of very intense fluorescence were also visible in the perinuclear region following longer drug exposure times.

Confocal laser scanning microscopy studies were also performed to verify the results obtained through fluorescence microscopy. Similar to the observations in the SKMES-1 and SKMES-1/ADR cell lines, the resolution of the fluorescence images was greatly improved. Subsequently the exact localisation of adriamycin could be determined. As expected, adriamycin was shown to be localised within distinct regions of the nucleus in the parental DLKP cells, confirming the interaction of adriamycin with chromosomal DNA. Adriamycin appeared to be exclusively located within the cytoplasm of the DLKPA10 resistant cells. Distinct regions of adriamycin fluorescence were clearly visible within the cytoplasm, particularly in regions close to the nucleus. In addition, intense adriamycin fluorescence was visible at the nuclear envelope.

Studies were also carried out to determine if nuclear drug exclusion observed in the DLKPA10 cells, was a consequence of reduced cellular drug concentration in these cells. Quantitative drug accumulation studies revealed that the cellular concentration of adriamycin in the DLKPA10 cells, after four hour exposure to the drug was equivalent to the concentration of drug in the parental DLKP cells after exposure for just five minutes. Subsequently, adriamycin distribution was studied in the DLKP and DLKPA10 cells, following incubation periods of five minutes and four hours respectively. The results however, illustrated that despite equivalent intracellular concentrations, different distribution patterns were observed in the cell lines. Faint nuclear fluorescence was detected in the parental DLKP cells, while intense cytoplasmic fluorescence was clearly visible in the resistant cells. These findings suggest that the reduced intracellular levels of the drug were not principally responsible for the decrease in nuclear fluorescence and that alternative mechanisms are involved in nuclear drug exclusion in the DLKPA10 cells.

In order to determine if the altered drug distribution pattern in the DLKPA10 cells was associated with P-glycoprotein overexpression, the effect of a number of P-glycoprotein inhibitors on the subcellular localisation of adriamycin was investigated. Verapamil and cyclosporin A were both shown to enhance nuclear fluorescence in the resistant cells, although the intensity of nuclear fluorescence was less than that observed in the parental DLKP cells. Both agents were also found to increase nuclear fluorescence in the parental cells. Results obtained from confocal laser microscopy studies demonstrated that, although cyclosporin A increased nuclear fluorescence in the DLKPA10 cells, distinct regions of fluorescence were still clearly visible in the cytoplasm, indicating partial restoration of nuclear fluorescence in the resistant cells. These findings suggested that, while P-glycoprotein was probably associated with altered drug distribution in the DLKPA10 cells, other non-P-glycoprotein mechanisms must also play a role, to fully explain the decrease in adriamycin nuclear fluorescence. A number of studies have shown that verapamil and cyclosporin A do not significantly alter drug distribution in non-P-glycoprotein MDR cell lines. In a study by Barrand *et al.* (1993) daunorubicin was shown to be confined to cytoplasmic perinuclear regions in the non-P-glycoprotein resistant human lung cell line, COR-L23/R. The addition of cyclosporin A resulted in only a slight increase in cytoplasmic fluorescence but had no effect on nuclear fluorescence while treatment with verapamil resulted in a slight increase in both cytoplasmic and nuclear fluorescence. Verapamil was also shown to more effectively modulate adriamycin nuclear fluorescence

in P-glycoprotein positive resistant variants of the human non-small cell lung cancer cell line, SW1573, than in non-P-glycoprotein resistant variants (Schuurhuis *et al.*, 1991).

The effect of the calmodulin inhibitor, trifluoroperazine, on the subcellular distribution of adriamycin was also investigated in the DLKPA10 cells, since studies have shown that calmodulin inhibitors can increase anthracycline retention in MDR cells. Calmodulin is a $Ca^{2+}$ binding protein that participates in numerous $Ca^{2+}$ regulatory processes. It has four high affinity $Ca^{2+}$ binding sites, two on each of its globular domains. The binding of $Ca^{2+}$ to any of these four binding sites triggers a large conformational change in that domain, which is thought to expose a nonpolar surface. The hydrophobic interactions of calmodulin's target proteins with one or both of these nonpolar surfaces is believed to mediate calmodulin's $Ca^{2+}$dependent regulatory processes. Several reports have demonstrated modulation of the cytotoxicity of anthracyclines and vinca alkaloids by calmodulin inhibitors. Reduction in cytotoxicity was shown to be associated with enhanced drug accumulation and cellular retention levels (Tsuruo *et al.*, 1982; Ganapathi and Grabowski, 1983; Ganapathi and Grabowski, 1988). The exact mechanism by which calmodulin inhibitors act is unknown, although it has been suggested that enhanced drug accumulation may be due to the membrane perturbing effect of these calcium modifiers. $Ca^{2+}$-calmodulin activates the $Ca^{2+}$-ATPase of the plasma membrane, which regulates the cellular concentration of $Ca^{2+}$. Inhibition of the $Ca^{2+}$-ATPase would subsequently lead to alterations in the cellular levels of calcium, which may also play a role in modulating drug resistance. Our studies, however, showed no significant alteration in the subcellular distribution or retention of adriamycin in the DLKPA10 cells, thus implying that $Ca^{2+}$ calmodulin complexes do not play a major role in drug distribution in this resistant cell line.

Adriamycin distribution was also investigated in the DLKP and DLKPA10 cells, following treatment with the metabolic inhibitors, sodium azide, 2-deoxyglucose and antimycin A, to determine if inhibition of ATP production could restore nuclear adriamycin levels in the DLKPA10 cells. Initial findings revealed that both sodium azide and 2-deoxyglucose had a toxic effect on the parental cells causing cell lysis in a large percentage of the cells. However, neither compound appeared to be toxic to the resistant cells within the time period studied. These results suggested that the DLKP cells were more susceptible than the DLKPA10 cells to metabolic inhibition. It could be possible that greater levels of ATP are

produced in the resistant cells, associated with the presence of the energy dependent P-glycoprotein pump. Since cellular ATP levels would therefore be lower in the sensitive cells, depletion of ATP levels would occur more rapidly in these cells, resulting in cell lysis. It has been reported that human breast cancer cells (MCF-7) with acquired resistance to adriamycin exhibit an enhanced glycolysis rate (approximately 3-fold) when compared to their parental cells, confirming the occurrence of some modification in their energy metabolism, following the onset of the resistant phenotype (Cohen and Lyon, 1987). More recent studies have also demonstrated alterations in glucose metabolism in drug resistant LoVo cells. A significant increase in the oxidative pathway of glucose metabolism as well as in acetyl-CoA production was observed in adriamycin resistant LoVo cells. The number of glucose carbon atoms metabolised in the resistant cells, through the pentose phosphate pathway, was also significantly higher than in adriamycin sensitive cells (Fanciulli *et al.*, 1993). These findings could therefore suggest a modified glucose metabolism in the DLKPA10 cells, however each resistant cell line might develop its own metabolic alterations so further studies need to be carried out to determine if increased metabolism is a characteristic of the DLKPA10 cell line.

Fluorescence microscopy studies demonstrated intense adriamycin nuclear fluorescence in viable DLKP cells, following exposure to each of the three inhibitors. While the fluorescence intensity appeared similar in sodium azide and 2-deoxyglucose treated cells and was comparable to levels observed in cells exposed only to the drug, nuclear fluorescence in antimycin A treated cells was greater than in cells treated with the drug alone. These findings were consistent with the results obtained from the quantitative adriamycin accumulation studies, where antimycin A was shown to significantly increase adriamycin accumulation in the DLKP parental cells. When the DLKPA10 cells were exposed to adriamycin in the presence of sodium azide and 2-deoxy-glucose, an increase in cytoplasmic fluorescence was observed, although no corresponding increase in nuclear fluorescence was noted. Adriamycin appeared to be localised in distinct areas within the cytoplasm, in particular in the perinuclear region of the cells. These results were unexpected, since several studies had reported that treatment with these agents resulted in redistribution of drug, from the cytoplasmic regions to the nucleus, in a number of MDR cell lines (Chauffert *et al.*, 1984; Willingham *et al.*, 1986; Maymon *et al.*, 1994). Pretreatment of the DLKPA10 cells with the inhibitors did, however, result in an enhancement of adriamycin nuclear fluorescence. Although a marked increase in nuclear fluorescence was

observed, the intensity was less than that noted in the parental cells. A decrease in cytoplasmic fluorescence was also observed, probably due to the redistribution of the drug to the nucleus. While it would appear from these observations that pretreatment with the inhibitor is necessary to alter the subcellular distribution of adriamycin in the resistant cells, further work showed that cotreatment with antimycin A effectively restored nuclear levels of adriamycin in the DLKPA10 cells. Intense nuclear fluorescence was clearly visible in all cells viewed and the fluorescence intensity appeared to be greater than that observed in the parental DLKP cells. These findings confirm results obtained from quantitative studies, where it was found that antimycin A was more effective than either sodium azide of 2-deoxyglucose at increasing adriamycin concentrations in the DLKPA10 cells. Antimycin A completely reversed the accumulation defect, while sodium azide and 2-deoxyglucose only partially restored drug accumulation in the resistant cells. These results therefore reiterate that treatment with sodium azide and 2-deoxyglucose does not completely deplete ATP stores in the DLKPA10 cells and that consequently there may be sufficient ATP available to drive the P-glycoprotein efflux pump. In contrast, antimycin A appears to effectively deplete ATP levels thus inhibiting P-glycoprotein mediated drug efflux.

Additional studies were carried out to investigate the effect of antimycin A on adriamycin pretreated DLKPA10 cells. Following preloading, distinct regions of intense cytoplasmic fluorescence were clearly visible in the cells, particularly in areas close to the nucleus. When the cells were treated with antimycin A, a marked increase in nuclear fluorescence was observed in the majority of cells viewed. A significant decrease in cytoplasmic fluorescence was also noted following exposure to antimycin A, demonstrating the redistribution of drug from the cytoplasmic vesicles to the nucleus. These findings suggest that antimycin A can in some way disrupt the cytoplasmic vesicles and thus prevent the localisation of drug within the cytoplasm. Since depletion of ATP levels would also inhibit P-glycoprotein mediated drug efflux, adriamycin would be redistributed to the nucleus. The mechanism by which antimycin A can apparently disrupt cytoplasmic vesicles is unclear, although it is possible that it could be due to pH alterations within the cells, since it was noted that the addition of antimycin A caused an increase in the pH of the culture medium. Studies have shown that shifts in intracellular pH leads to alterations in drug accumulation and distribution in MDR cells (Simon *et al.*, 1994), although further work needs to be carried out to determine if antimycin A induced drug redistribution is due to pH alterations in the DLKPA10 cells.

A number of reports have also demonstrated that ionophores, including monensin and nigericin can reduce the cytotoxicity of anthracyclines in MDR cell lines by increasing the cellular accumulation and altering the subcellular distribution of the drug. Ionophores are a heterogenous group of antibiotics capable of altering the membrane permeability of small inorganic ions. There are two basic types of ionophores; channel forming and carrier ionophores (Figure 4.7.1). Channel forming ionophores (*e.g.* gramicidin) form trans membrane channels or pores through which their selected ions can diffuse. Carrier ionophores can in turn be divided into two groups: those such as valinomycin and nonactin which transport cations as lipid-soluble charged complexes and those such as nigericin and monensin (figure 4.7.2), which contain a charged carboxyl group and transport cations as lipid-soluble, electrically neutral complexes. In our studies both monensin and nigericin were shown to enhance nuclear fluorescence in the DLKPA10 cells. Similar to observations with cyclosporin A and verapamil treatment, the intensity was not as great as that observed in the parental DLKP cells, although monensin did appear to be more effective at increasing nuclear accumulation of the drug. Regions of cytoplasmic fluorescence were also visible, although the intensity and quantity of the vesicles detected appeared less than that noted when the cells were exposed only to adrimaycin.

Considerable confusion exists regarding the exact mechanism(s) by which ionophores bring about their effect in resistant cells. A number of studies have shown that ionophores act by inhibiting the P-glycoprotein mediated efflux of anthracyclines and thereby increasing cellular levels of the drug. Naito *et al.* (1991) reported that a number of ionophores, including monensin and nigericin, inhibited the binding of vincristine to the plasma membrane of resistant K562 cells. They also showed that photoaffinity labelling of P-glycoprotein by azidopine was markedly reduced in the presence of the ionophores. In studies by Borrel *et al.* (1994) THP-adriamycin accumulation was found to be enhanced in resistant cells, following treatment with monensin. This effect was caused by inhibition of the P-glycoprotein mediated efflux of THP-adriamycin. Inhibition of P-glycoprotein by monensin could also contribute to the increase in nuclear fluorescence in the resistant DLKPA10 cells. However, since monensin proved to be more effective than cyclosporin A at enhancing nuclear adriamycin accumulation, it would appear that monensin also exerts its effect in the DLKPA10 cells by a non-P-glycoprotein mechanism. It has also been proposed that ionophores can alter drug distribution in MDR cells by disrupting intracellular vesicular traffic. The involvement of an acidic vesicular traffic transport system in MDR

cell lines was described independently by Sehested *et al.* (1987a) and Beck *et al.* (1987), who suggested that the lysosomal system in MDR cells was involved in drug efflux and that drugs such as adriamycin and vinblastine, which are weak bases, could become trapped in these acidic compartments by protonation. According to this proposal, these lysosomal vesicles then migrate to the plasma membrane where they fuse and extrude their contents to the outside. Evidence supporting the involvement of acidic vesicular traffic has been obtained from a number of studies, which have shown that agents which are known to interact with acidic compartments could potentiate the cytotoxicity of anticancer agents and/or inhibit anthracyclines efflux (Klohs and Steinkampt, 1988; Sehested *et al.*, 1988; Marquardt and Center, 1992). Reports have also demonstrated an increase in plasma membrane traffic (endo/exocytosis) in MDR cell lines which could be attributed to the acidic vesicular traffic transport system in the cells. Sehested *et al.* (1987a) reported a significant increase in plasma membrane traffic in four Ehrlich ascites tumour cell lines resistant to daunorubicin, adriamycin, vincristine and vinblastine respectively. Further studies also demonstrated an increase in plasma membrane traffic in daunorubicin resistant P388 leukaemia cells (Sehested *et al.*, 1987b).

The mechanism by which ionophores disrupt intracellular vesicular traffic is unclear, although it is generally believed that these agents act by abolishing the $Na^+K^+$ gradient, which would lead to an increase in the pH environment in the membrane lumen and subsequent disruption of the acidic vesicles. The majority of studies to date have used either monensin or nigericin to investigate the effect of ionophores on acidic vesicles. It has been proposed that these agents insert into membranes and facilitate the exchange of $Na^+$ or $K^+$ ions for protons. (Monensin has been shown to have a greater affinity for $Na^+$ while nigericin has a greater affinity for $K^+$). The loss of protons would subsequently result in an increase in the intracellular pH environment, disruption of the acidic compartments and a decrease in drug efflux. The subcellular adriamycin distribution pattern observed in the DLKPA10 cells could also implicate the involvement of an acidic vesicular traffic transport system in the resistant cells. Confocal laser microscopy studies clearly demonstrated the presence of fluorescent vesicles within the cytoplasm which is consistent with the localisation of drug within cytoplasmic acidic vesicles. Since both monensin and nigericin enhanced adriamycin nuclear fluorescence and decreased the quantity of cytoplasmic vesicles in the DLKPA10 cells, it is possible that these agents disrupted acidic vesicular traffic and thus inhibited drug efflux.

It has been suggested that the cytotoxic actions of drugs, such as monensin and nigericin may be due to a decrease in cellular ATP levels (Rotin *et al.*, 1987; Mariani *et al.*, 1989). Reduced cellular ATP levels could ultimately affect the subcellular localisation of anthracyclines in a number of ways. Depletion of ATP levels would result in inhibition of the P-glycoprotein efflux pump, thus increasing the cellular concentration of the drug. Alternatively, studies have shown that vacuolar ATPases are involved in driving the uptake of drug into constituted organelles. Moriyama *et al.* (1993) demonstrated the uptake of radiolabelled daunorubicin in acidic vesicles, that were reconstituted with purified F-type $H^+$-ATPase, upon addition of ATP. Further studies showed that the accumulation of both adriamycin and daunorubicin in acidic organelles of cultured BNL CL.2 cells was decreased by inhibiting vacuolar ATPase, thus indicating energy dependent uptake. If this system was representative of the uptake of anticancer agents in MDR cells, depletion of ATP levels would subsequently inhibit the uptake of drug into cytoplasmic vesicles. These findings suggest that depletion of ATP levels by monensin may increase nuclear fluorescence in a number of ways. However, it is unlikely that monensin alters the subcellular localisation of adriamycin in the DLKPA10 by inhibition of vacuolar ATPases. Treatment of these cells with the vacuolar ATPase inhibitor, bafilomycin A1, did not significantly alter adriamycin distribution in these cells. Although a number of studies have demonstrated altered intracellular distribution of anthracyclines following treatment with bafilomycin A1 (Marquardt and Center, 1991; Rhodes *et al.*, 1994), it did not appear to significantly affect the localisation of adriamycin within cytoplasmic vesicles in the DLKPA10 cells.

It seems clear from these findings that monensin may alter the subcellular distribution of adriamycin in the DLKPA10 cells by a number of mechanisms. It is possible that it acts by P-glycoprotein inhibition, although other non-P-glycoprotein mechanisms must also be involved to account for the observation that monensin was more effective than the P-glycoprotein inhibitor, cyclosporin A, at enhancing nuclear fluorescence. The results obtained from the microscopy studies confirm the findings from the quantitative accumulation studies, where it was found that pretreatment with monensin almost restored adriamycin accumulation in the DLKPA10 cells to the level observed in the parental cells. These results would therefore suggest that, in addition to P-glycoprotein inhibition, monensin acts by disrupting acidic vesicular traffic within the cells. However, further studies need to be carried out to identify the mechanisms by which monensin and nigericin can increase drug accumulation in the DLKPA10 cell line.

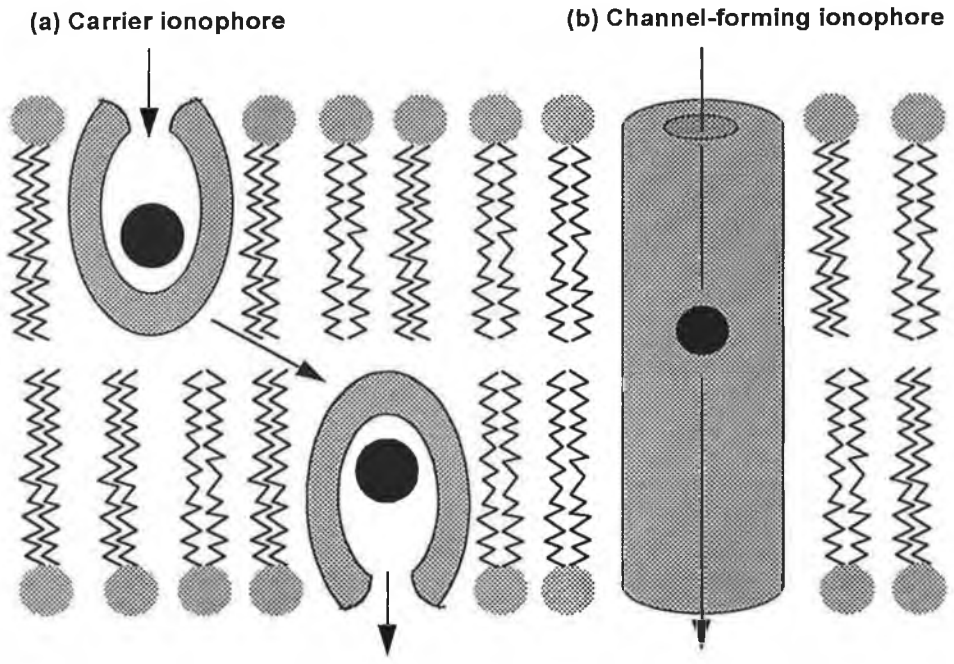



**Figure 4.7.1** The ion transport modes of ionophores: (a) carrier ionophores transport ions by diffusing through the lipid bilayer. (b) Channel-forming ionophores span the membrane with a channel through which ions can diffuse

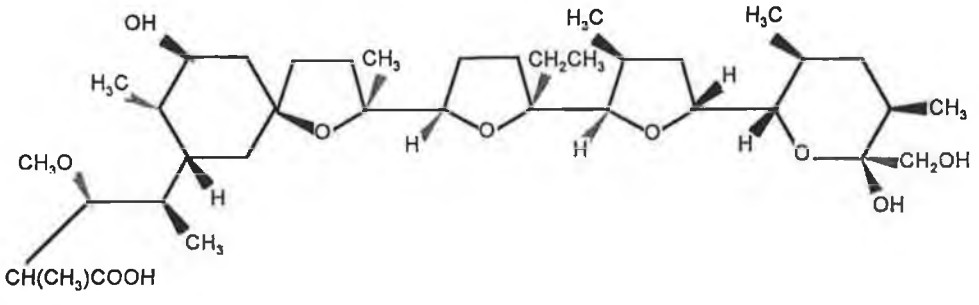



**Figure 4.7.2** Structure of the ionophore monensin

The detection of the cytoplasmic vesicles in the DLKPA10 cells prompted a number of studies to determine if adriamycin was actually localised within cellular organelles (*e.g.* lysosomes or Golgi apparatus). A number of intracellular sites have been postulated for adriamycin localisation in MDR cell lines. Since reports had shown that lysosomal inhibitors could cause redistribution of anthracyclines in MDR cells, the role of lysosomes in mediating resistance in the DLKPA10 cells was investigated. Lysosomes are membrane-encapsulated organelles that contain approximately fifty hydrolytic enzymes, including a variety of proteases known as cathepsins. The function of lysosomes is to digest materials ingested by endocytosis and to recycle cellular components. The lysosome maintains an internal pH of approximately 5 and the enzymes have acidic pH optima. Lysosomes recycle intracellular constituents by fusing with membrane enclosed regions of cytoplasm, known as autophagic vacuoles, subsequently breaking down their content. They similarly degrade substances taken up by the cells through endocytosis. Since the lysosomes are acidic organelles, it is possible that weak bases such as adriamycin and the vinca alkaloids could become localised within these compartments.

To determine if adriamycin was localised within lysosomes in the DLKPA10 cells, the effect of lysosotropic inhibitors, chloroquine and methylamine, on the subcellular distribution of the drug was investigated. These agents, both of which are weak bases, freely penetrate the lysosomes in uncharged form. However, they accumulate in a charged form, thereby increasing the intralysosomal pH and inhibiting lysosomal function. Lysosotropic agents strongly prefer to stay in aqueous phases, thus phospholipid membranes become a barrier to any incoming substances (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981). Early studies showed that lysosotropic agents, including chloroquine and methylamine, along with other agents known to interact with lysosomes, could reverse drug resistance to the anthracyclines and vinca alkaloids in MDR cells (Shiraishi *et al.*, 1986a; Zamora and Beck, 1986). These studies also demonstrated an increase in drug accumulation together with a reduction in drug efflux in the resistant cells following treatment with these agents. Klohs and Steinkampf (1988) also reported increased drug accumulation in P388R cells, following treatment with a number of agents known to interact with lysosomes. Recent studies by Dubowchik *et al.* (1994) showed that a number of lipophilic nitrogenous bases exhibited potent reversal of adriamycin resistance in the HCT116-VM46 cell line. These agents were also shown to neutralise the activity of lysosomes. In our studies, chloroquine and methylamine did not appear to significantly

the subcellular localisation of adriamycin in the DLKPA10 cells. If these vesicles were being actively transported to the extracellular environment in order to facilitate decreased intracellular drug levels, then inhibition of the vesicular traffic by brefeldin A could lead to increased intracellular drug levels and subsequent enhancement of cytotoxicity. Brefeldin A is a fungal metabolite which has been shown to inhibit the transport of membrane and secretory proteins from the endoplasmic reticulum to the Golgi apparatus in various cell types. In addition, brefeldin A has also been found to disrupt the Golgi apparatus by disassembly of the cisternal stacks (Fujiwara *et al.*, 1988; Ulmer and Palade, 1989; Marquardt and Center, 1992). The mode of action of brefeldin A is unknown, although it is thought to affect Golgi specific coat proteins that may be involved in maintaining the structural integrity of the organelle and in regulating membrane transport in the secretory pathway (Serafini *et al.*, 1991). Treatment with brefeldin A has been demonstrated to alter the subcellular distribution of anthracyclines in the large cell lung carcinoma COR-L23/R cell line. Rhodes *et al.* (1993) reported a redistribution of the drug from the cytoplasm to the nucleus following exposure to brefeldin A. The redistribution was accompanied by a decrease in perinuclear fluorescence, which suggested that the drug was localised within the Golgi apparatus. Quantitative studies showed an increase in the cellular concentration of daunorubicin in COR-L23/R cells treated with brefeldin A, indicating a decrease in drug efflux. It has been proposed that brefeldin A inhibited the Golgi apparatus membrane trafficking system and subsequent drug efflux, resulting in increased accumulation of the drug.

Brefeldin A did not appear to significantly alter the subcellular distribution of adriamycin in the DLKPA10 cells within the time period studied. Adriamycin fluorescence was still clearly visible within the perinuclear region, although, due to the inherent limitation of the microscope any alterations in fluorescence intensity was difficult to determine. Quantitative studies revealed that brefeldin A was also ineffective at altering drug accumulation in the resistant cells. No significant increase in the cellular concentration of drug was observed in the cells when exposed to brefeldin A. Adriamycin accumulation and distribution was also studied in the DLKPA10 cells, following pretreatment with brefeldin A. However, the results showed that pretreatment did not appear to be any more effective than cotreatment at altering drug accumulation and localisation in the resistant cells. The observation that brefeldin A does not appear to effect drug distribution in the DLKPA10 cells suggests that the Golgi apparatus membrane trafficking system does not play a major role in drug efflux

in these cells. It may, however, be possible that the concentration of brefeldin A was not sufficient to actually disrupt the Golgi apparatus. Due to the toxicity of brefeldin A on the DLKPA10 cells, the concentration used in these studies was less than that used in previous reports, although studies have shown that brefeldin A was also ineffective at altering daunorubicin distribution in resistant HL60/ADR cells (Marquardt and Center, 1992).

### 4.7.1 Adriamycin localisation studies in DLKPA clones

Preliminary studies were also carried out to investigate the subcellular distribution of adriamycin in the DLKPA clones. The results obtained clearly indicated different adriamycin fluorescence patterns in each of the four clones studied. The level of nuclear fluorescence was found to be inversely related to the resistance levels in the cells. One unexpected observation from these studies, however, was that all the clones displayed heterogenous fluorescence staining. Nuclear fluorescence was clearly visible in the DLKPA 2B cells, although the intensity of staining varied within the cell population. While approximately 65% of the cell population displayed intense fluorescence, the remaining cells had only faint nuclear fluorescence. A mixed fluorescence pattern was also observed in both the DLKPA 6B and DLKPA 11B clones, with approximately 50% and 35% of the cells respectively displaying quite intense nuclear fluorescence. Cytoplasmic fluorescence was observed in the remaining cells and appeared to be localised in distinct regions. The majority of the DLKPA 5F cells displayed only faint nuclear fluorescence but intense fluorescence was noted within the cytoplasm, particularly in the perinuclear region. Adriamycin localisation in the DLKPA 5F cells was, therefore, similar to observations in the DLKPA10 cells. The addition of verapamil or cyclosporin A was shown to completely restore nuclear adriamycin accumulation in the DLKPA 2B and DLKPA 5F clones. Intense nuclear fluorescence was observed in both cell variants. While treatment with these agents was also found to enhance nuclear levels of the drug in the DLKPA 6B and the DLKPA 11B, the intensity of fluorescence was significantly less than that observed in the other two clones.

These findings are consistent with the results obtained from quantitative studies, where it was shown that both verapamil and cyclosporin A could not restore cellular levels of adriamycin in the DLKPA 6B and DLKPA 11B clones. Overall, results obtained from adriamycin transport studies on the DLKPA clones suggest that decreased drug accumulation and altered intracellular distribution in the DLKPA 2B and DLKPA 5F is due to overexpression of P-glycoprotein. In contrast, while P-glycoprotein appears to play a major role in mediating resistance in the DLKPA 6B and DLKPA11B clones, further work is required to determine alternative mechanism(s) involved in the verapamil/cyclosporin A independent accumulation defect observed in these clones.

## 4.8 Correlation between drug accumulation and growth inhibition

Although studies have shown a strong correlation between drug accumulation and resistance in some MDR cell lines (Pereira and Garnier-Suillerot, 1994), a number of reports have also illustrated a discrepancy between the amount of drug accumulated at steady state by drug sensitive and highly resistant cells and their degree of resistance to anticancer agents (Beck, 1984; Hu and Chen, 1994). These observations could suggest that, either factors other than drug accumulation may be involved or that they reflect a discrepancy between the short term measurements of drug accumulation and the long term growth inhibition. Studies were carried out to establish if drug accumulation correlated with the level of adriamycin resistance in the SKMES-1/ADR and DLKPA10 cells. The results obtained from studies on the SKMES-1/ADR cells suggested that decreased drug accumulation in this cell line was due to P-glycoprotein expression, while results from studies on the DLKPA10 cells suggested that in addition to overexpression of P-glycoprotein, other mechanisms were also involved in reduced drug accumulation. Consequently, if drug accumulation was not consistent with the level of resistance in the SKMES-1/ADR cells, it would reflect a discrepancy between short term accumulation studies and long term toxicity studies.

The effect of verapamil and cyclosporin A on the toxicity of adriamycin was, therefore, investigated in the SKMES-1/ADR cells, under similar conditions to those employed in the adriamycin accumulation studies. While adriamycin accumulation was measured over a period of four hours, growth inhibition was determined after five days. The results obtained from these studies were difficult to interpret, since treatment with adriamycin or the circumvention agent alone had a toxic effect on the cells. However, a decrease in cell viability of approximately 40% was observed in the SKMES-1/ADR cells following exposure with verapamil or cyclosporin A for four hours. Since the accumulation defect in these cells was shown to be completely reversed by both verapamil and cyclosporin A, lower cell viability was expected. These findings illustrate a discrepancy between short term accumulation and long term toxicity in the SKMES-1/ADR cells. Also, another inconsistency that was noticed in these studies was that, although cyclosporin A was found to be more effective than verapamil at enhancing drug accumulation this was not reflected in the toxicity studies, where both agents were found to exert similar toxicity. The results obtained from studies on the DLKPA10 cell line illustrated a correlation between drug accumulation and drug resistance following treatment with both verapamil and cyclosporin A. Both agents were found to partially restore adriamycin accumulation in the resistant

cells to a level of approximately 50-60% of that observed in the parental cells. When the effect of verapamil and cyclosporin A on adriamycin toxicity was studied under conditions similar to the accumulation studies, a decrease in cell viability of approximately 37% and 47%, respectively was observed. These results are therefore consistent with partial restoration of adriamycin accumulation in the DLKPA10 cells. Cyclosporin A proved to be more effective than verapamil at inhibiting cell growth, which again was consistent with the results obtained from drug accumulation studies. The effect of monensin on adriamycin toxicity was also investigated. However, monensin proved to be extremely toxic to the DLKPA10 cells in long term growth inhibition assays. Subsequently, it was not possible to determine if a correlation existed between drug accumulation and resistance in the DLKPA10 cells.

### 4.9 Alterations in membrane fluidity in MDR cells

The results obtained from drug transport studies in the DLKPA10 cells suggest that the drug accumulation defect in this cell line is predominantly caused by a combination of P-glycoprotein overexpression and the existence of an acidic vesicular traffic system. However, these mechanisms do not fully account for the accumulation defect observed, since the apparent inhibition of both mechanism does not appear to fully restore drug accumulation to a level comparable to the parental cells. Consequently, further studies were carried out in an attempt to identify an alternative mechanism responsible for decreased drug accumulation in the DLKPA10 cells. Several reports have shown that, in addition to alteration in membrane proteins, MDR cell lines can also exhibit several other changes in membrane properties (Alon *et al.*, 1991). A number of studies have demonstrated that lipid structural order is often increased in MDR cells. Ramu *et al.* (1984) demonstrated alterations in the relative amounts of phospholipids in anthracycline resistant variants of the murine leukaemia cell line, P388. While increased amounts of sphingomyelin and triglyceride were detected in the resistant cells, a decrease in phosphatidylcholine levels was observed, relative to the sensitive parental cells. Alterations in the level of phospholipids was also reported in the vinblastine resistant human leukaemia CEM cell line. An increase in the levels of sphinogomyelin and cardiolipin and a decrease in the levels of phosphatidylethanolamine and phosphatidylserine was observed in the cells (Wright *et al.*, 1985). Montaudon *et al.* (1986) showed that MDR C6 cells had increased membrane

fluidity, which could be accounted for by an increase in the content of unsaturated fatty acids. They also demonstrated that culturing resistant cells in polyunsaturated fatty acids resulted in a 2-fold increase in adriamycin sensitivity, with only relatively minor changes in fatty acid composition. These results suggested that drug resistance was very sensitive to changes in membrane lipid composition and/or physical properties. In 1977, Ling *et al.* postulated that a decrease in membrane fluidity might be responsible for the diminished influx of drugs, such as colchicine, in MDR cells. Since alterations in membrane lipid structure has been shown to alter membrane fluidity, altering the lipid composition and/or physical properties of the membrane may enhance drug uptake in MDR cell lines. A number of studies have illustrated an increase in net drug uptake in MDR cells by altering the lipid composition of the growth media. Zjilstra *et al.* (1987) demonstrated that incorporation of docosahexaenoic acid into resistant cells caused an increase in the accumulation of adriamycin and a reversal of cell resistance. Callaghan *et al.* (1993) also reported a significant increase in uptake of vincristine and rhodamine dyes in $CH^RC5$ cells by the addition of membrane rigidifying agents, including the saturated fatty acids, stearic acid and the cholesterol derivative, cholestryl hemisuccinate.

The relationship between membrane physical properties and MDR was investigated in the DLKPA10 cells. In these studies, adriamycin accumulation was determined in the cells following treatment with the amphiphiles, stearic acid and cholestryl hemisuccinate. Both agents spontaneously partition into membranes and may subsequently affect membrane fluidity. The results obtained showed that the addition of stearic acid did not significantly alter the cellular accumulation of adriamycin in either the DLKP or DLKPA10 cells. However, treatment with cholesterol hemisuccinate resulted in a slight enhancement of drug accumulation in both cell lines, which may indicate that this agent was incorporated into the membrane of the DLKPA10 cells and altered fluidity, leading to an increase in drug uptake. This result was unexpected, since toxicity studies had shown that stearic acid was more effective than cholestryl hemisuccinate at decreasing adriamycin resistance in the DLKPA10 cells. The findings would suggest that while stearic acid reduces adriamycin toxicity in the resistant cells, its effect does not appear to be associated with increased drug accumulation. In contrast, cholestryl hemisuccinate appears to slightly alter adriamycin accumulation in the cells, without having any effect on adriamycin toxicity. It is clear from these results that further studies are required to determine the effect of alterations in membrane properties on modulating resistance in the DLKPA10 cells.

## 4.10 Antibodies in the study of MDR in DLKPA10 cells

Given that an alternative mechanism(s) may be involved in mediating decreased drug accumulation in the DLKPA10 cells, additional studies were carried out in an attempt to identify a novel antigen, which could contribute to the resistant phenotype in these cells. Antibodies were, therefore, raised against the DLKPA10 cell line and preliminary characterisation studies were carried out to establish if an MDR related antigen was either overexpressed or down regulated in the cells, relative to the sensitive DLKP cells. A number of monoclonal antibodies, which have proved to be useful tools in the study of MDR, have been generated, including antibodies to P-glycoprotein, topoisomerase I and II, LRP, MRP, glutathione-S-transferase and metallothionein. Several investigators have generated antibodies from mice immunized with whole cell extracts. Lathan *et al.* (1985) produced monoclonal antibodies against the P-glycoprotein of drug resistant chinese hamster ovary CHO cells by immunization with viable CHOC5 cells. Two monoclonal antibodies, MRK16 and MRK17, which also recognise the 170 - 180 kDa P-glycoprotein, were produced by immunization with cellular extracts of the adriamycin resistant human myelogenous leukaemia cell line, K562/ADM (Hamada and Tsuruo, 1986). Mirski and Cole (1989) produced a panel of antibodies following immunization with cellular extracts of the non-P-glycoprotein cell line H69/AR.

A number of immunization procedures have also involved the use of cell membrane extracts to produce antibodies against membrane associated antigens. A number of monoclonal antibodies were generated by immunization with purified plasma membranes, isolated from the colchicine resistant chinese hamster ovary $CH^{R}B30$ cell line and the vincristine resistant human leukaemia $CEM/VBL_{500}$ cell line. This panel of antibodies, which included the commercially available C219 monoclonal antibody, were all specific for P-glycoprotein (Kartner *et al.*, 1985). More recently, Hipfner *et al.* (1994) produced monoclonal antibodies from mice immunized with membrane enriched fractions from the MDR H69/AR cells, which express high levels of the 190 kDa protein, to obtain a probe for immunodetection of MRP. In our studies, an immunization programme was carried out using whole cell extracts of the resistant DLKPA10 cell line, in an attempt to raise antibodies that were not necessarily associated with the plasma membrane. A total of three fusions were performed, which involved the fusion of normal antibody producing B cells, obtained from mice immunized with the DLKPA10 cells, with SP2 myeloma cells. Positive

hybridomas were obtained from two of the fusions. However, when the clones were screened against both the parental DLKP and the DLKPA10 cells by ELISA, only a small number were found to display selective immunoreactivity. The majority of these clones showed stronger immunoreactivity against the DLKP than the DLKPA10 cells. These results were unexpected, since the DLKPA10 cells overexpress a high level of P-glycoprotein. It was anticipated that a number of antibodies to P-glycoprotein would be generated. Although a number of clones displayed immunoreactivity against the DLKP cells, only one hybridoma (D8-8) was selected for further characterisation. The selected hybridoma was grown up as ascitic fluid in Balb/c mice and a suitable working dilution (1:100) was determined by ELISA.

### 4.10.1 Characterisation of the D8-8 antibody

Results obtained from the screening procedure suggested that the D8-8 antibody was generated against an antigen that was either overexpressed in the parental cells or downregulated in the resistant DLKPA10 cells. Consequently, preliminary studies were carried out in an attempt to characterise the D8-8 antibody and to ascertain the role of the antigen in the MDR phenotype observed in the DLKPA10 cells. The supernatants from the D8-8 hybridoma were initially screened against a number of rat monoclonal antibodies to determine the class and subclass of the antibodies produced. These isotyping studies revealed that the antibody was of the IgG class with subclass 1. Western blot analysis was performed on purified membrane extracts from the immunogen, DLKPA10, and the parental DLKP cells. The proteins were subjected to denaturing polyacrylamide gel electrophoresis, transferred to nitrocellulose blots and then probed with the D8-8 antibody to determine the molecular weight and levels of expression of the antigen, bound by the antibody. The results illustrated that the D8-8 antibody preferentially reacted with an antigen of approximately 180 kDa in the DLKP cells under the experimental conditions employed. The results obtained by Western blot analysis were, therefore, consistent with the initial ELISA results, demonstrating strong immunoreactivity of the D8-8 antibody against the DLKP cells. Immunocytochemical studies were also performed with the D8-8 antibody on acetone fixed, cytospin preparations of the DLKP and DLKPA10 cells. While positive immunoreactivity was observed in the majority of the DLKP cells viewed, only faint staining was noted in the DLKPA10 cells. Cytoplasmic staining was visible in the DLKP cells, although cell surface staining was also noted in a large proportion of the cells.

This was particularly evident in areas of contact with neighbouring cells, where intense staining was observed, perhaps implying that the antigen may be involved in cell adhesion or intracellular communication. Since the immunocytochemical studies revealed heterogenous staining of the antibody within the DLKP cell population, additional studies were carried out in three independent clones isolated from the parental cell line (S McBride, PhD thesis). The aim of this work was to establish the extent of preferential binding of the antibody with a particular cell type within the cell population. Positive immunoreactivity was observed with all three clones, although with varying degrees of staining intensity. While two of the clones (DLKP-I and DLKP-SQ) showed similar staining patterns, with positive staining in the majority of cells, only faint staining was observed in the DLKP-M clone. These studies indicate selective binding of the antibody to different cell types within the parental population. Immunocytochemical studies were also preformed on the OAW42 and SKMES-1 variants to investigate if similar staining patterns were observed in these cells. The D8-8 antibody displayed stronger immunoreactivity against the OAW42-S cells, although some staining was also observed in the OAW42-A resistant variant. Studies on the SKMES-1 variants demonstrated positive immunoreactivity in both the SKMES-1 and SKMES-1/ADR cells, although the staining intensity appeared to be slightly greater in the parental SKMES-1 cells. The results would, therefore, suggest that the D8-8 antibody preferentially binds to sites on the sensitive cell lines, an effect which is most clearly seen in the DLKP cells.

Immunofluorescence was carried out on live cells to detect the presence of cell surface antigens. The immunoreactivity of the D8-8 antibody against the DLKP, OAW42-S and SKMES-1, together with their respective resistant variants was investigated. The results obtained illustrated that, while cell surface fluorescence was visible in the DLKP cells, the intensity of surface fluorescence was substantially greater in the resistant DLKPA10 variant. Intense fluorescence was also visible on the surface of the OAW42-S, OAW42-A, SKMES-1 and SKMES-1/ADR cells. However, no significant differences were disguisable in the intensity of fluorescence between the sensitive and related resistant variants in either the OAW42 or SKMES-1 variants. These findings, therefore, indicate that the D8-8 antibody appears to bind strongly to a particular antigen on the surface of each of the cell lines studied, although with the exception of the DLKP parental cells where the intensity was greatly reduced. These results, however, were not consistent with those obtained through the immunocytochemical studies, where a higher level of staining with the antibody was observed in the parental DLKP cells.

Overall, the results would suggest that, while the D8-8 antibody preferentially binds to a cell surface antigen in the DLKPA10 resistant cells, it preferentially binds to an internal antigen in the parental cells. This could imply differential cellular localisation of the antigen in the sensitive and resistant cells. It is also possible, that if the antigen was localised on the cell surface of the DLKPA10 cells it could be destroyed during fixation. Consequently, the antigen would not be detected by immunocytochemical studies having been destroyed during the fixation step of the procedure. In addition, destruction of cell surface antigens may also occur during denaturation. Therefore, the cell surface antigen previously recognised by the D8-8 antibody in the DLKPA10 may subsequently be destroyed during polyacrylamide gel electrophoresis, possibly explaining why the binding of the antibody was not detected by Western blot analysis. The results obtained from studies on the OAW42 and SKMES-1 variants would suggest that the antibody recognizes an antigen that has both intracellular and cell surface localisation in the cells. Although there appears to be no significant difference in cell surface localisation of the antigen between the sensitive and resistant variants, the resistant variants appear to express higher intercellular levels of the antigen. It is clear from these findings that further work is required to determine the role, if any, of this antigen in the MDR cell lines. Since the antigen appears to be externalised to an equal extent in the OAW42 and SKMES-1 sensitive and resistant variants, it mat not be implicated directly with resistance in these cells. It is possible that the antigen may play a role in cell-cell communication or cell-cell adhesion, although this remains to be clarified.

## 4.11 Conclusions and future work

The establishment and characterisation of novel platinum resistant variants of the human lung carcinoma cell line, DLKP, represents the objective of the early work reported in this thesis. In this regard, three carboplatin resistant variants were established, exhibiting resistance to the selecting agent of approximately 4-, 8- and 16-fold. These variants were also found to be cross resistant to cisplatin. Cross resistance to the classical MDR drugs, including adriamycin and vincristine, however, was not displayed. Characterisation studies revealed that the mechanism of resistance in these cells does not appear to be either P-glycoprotein or topoisomerase II mediated. Through further studies, it was also demonstrated that these resistant variants did not exhibit any significant alterations in the activity of the GST enzymes, although this had been implicated in numerous platinum resistant cell lines. Metallothionein expression was, however, found to be increased with the level of expression correlating broadly with the degree of platinum resistance in the respective variants. Two methods of study were applied in determining the extent of metallothionein expression in the platinum resistant DLKP variants. Given that metallothionein expression has been associated with increased resistance to heavy metals, including cadmium, lead and zinc, the cadmium chloride toxicity profile was determined. All three variants were found to exhibit cross resistance to cadmium chloride, exhibiting 2.7-, 9- and 10-fold levels respectively. In addition immunocytochemical studies were carried out to investigate metallothionein expression. Again, positive correlation between platinum resistance and metallothionein expression was detected in all three variants. Although the parental cells were found to exhibit positive immunoreactivity, the extent , as indicated by the intensity of staining, was significantly less than in the established resistant variants. These results therefore, indicate overexpression of metallothionein in the resistant variants and that this overexpression may contribute to the resistant phenotype in the cells. Further work involving Western blot analysis for protein detection could confirm the results obtained from the immunocytochemical studies.

Although metallothionein expression appears to have a role in mediating the resistant phenotype, alternative mechanisms must also be involved, since differences in the level of metallothionein expression, particularly in the DLKPC 25 variant, does not reflect the different levels of platinum resistance in the DLKPC variants. Consequently, additional studies were carried out to establish if resistance in the DLKPC cells resulted from decreased intracellular platinum accumulation. Platinum uptake could not be measured

directly, as a flameless atomic absorption spectrometer was not available for use. However, indirect studies were carried out to determine if alteration of membrane permeability, with subsequent increased platinum uptake would result in increased cytotoxicity in the established DLKPC variants. The results obtained, however, suggested that this was not the case, since the addition of amphotericin B, which has been shown to increase membrane permeability in cisplatin resistant cells, did not significantly alter the toxicity profile of carboplatin in any of the variants. $Na^+K^+$-ATPases have also been shown, through a number of studies, to be central to platinum accumulation in some resistant cells. Accordingly, the toxicity profile of ouabain, a $Na^+K^+$-ATPase inhibitor, in the DLKPC resistant variants was determined. The results obtained demonstrated that the variants exhibited slight cross resistance to ouabain, although ouabain did not significantly alter the cytotoxicity of carboplatin in the cells. These results suggest that alterations in $Na^+K^+$-ATPases may be involved in mediating resistance in the DLKPC variants, although further work involving direct methods of studying platinum accumulation is required before the contribution of $Na^+K^+$-ATPases to the resistant phenotype of the DLKPC variants can be clarified.

The cellular accumulation and subcellular distribution of anticancer drugs in a number of human MDR cell lines represents another focus of this work. The results obtained demonstrated a reduction in drug accumulation in each of the MDR lines studied, relative to their respective parental cells. The addition of verapamil and cyclosporin A, agents known to interact with P-glycoprotein, resulted in complete restoration of cellular drug levels in the MDR cell lines, SKMES-1/ADR, T24A, T24V and the OAW42 resistant clones. These findings, therefore, suggested that the accumulation defect observed in each of these cell lines was P-glycoprotein mediated and additionally that these cell lines exhibited characteristics consistent with the classical MDR phenotype. These results differed, however, from the results obtained for the DLKPA10 cell line. Although cyclosporin A and verapamil enhanced drug accumulation in these cells, the maximum level of drug accumulated was substantially less than that observed in the parental cells. These results could suggest the presence of a mutated form of P-glycoprotein no longer sensitive to verapamil or cyclosporin A treatment, since studies have reported that these agents competitively interact with a common binding site of P-glycoprotein (Foxwell *et al.*, 1989; Tamai and Safa, 1990). Alternatively, the results could suggest that in addition to P-glycoprotein overexpression an alternative mechanism exists causing a decrease in drug

accumulation in the resistant DLKPA10 cells. Consequently, further studies were carried out to identify this non verapamil/cyclosporin A-sensitive accumulation defect in the cells. Metabolic inhibition studies revealed that sodium azide and 2-deoxy-glucose were effective at partially restoring drug accumulation. Complete restoration, however, of cellular drug levels was observed following treatment with antimycin A, indicating an energy dependent non-P-glycoprotein accumulation defect in the resistant DLKPA10 cells. Although numerous studies had reported increased drug accumulation in MDR cell lines when treated with the metabolic inhibitors, sodium azide or 2-deoxy-glucose, literature searches revealed no information on the use of antimycin A in the study of MDR. These findings suggest that antimycin A is substantially more effective than the well documented metabolic inhibitors, sodium azide and 2-deoxy-glucose, in reversing the drug accumulation defect in the resistant cells. This could be explained by the different sites of action attributable to each of these metabolic inhibitors, as they each act at different stages of ATP production. While sodium azide and antimycin A both act by inhibition of the electron transport chain, sodium azide inhibits ATP production at a later stage of the chain and consequently appears less effective at depleting ATP levels in the DLKPA10 cells.

Adriamycin subcellular distribution was studied, with the results demonstrating different localisation of the drug in sensitive and resistant cells. While adriamycin was localised in the nuclei of sensitive cells, the drug distribution appeared to be concentrated predominantly in vesicles within the cytoplasm of resistant cells. While verapamil and cyclosporin A were shown to completely restore adriamycin nuclear fluorescence in the P-glycoprotein cells lines, SKMES-1/ADR, T24A, T24V, OAW42-A1 and OAW42-A, these agents could only partially restore nuclear fluorescence in the DLKPA10 cells. These results were consistent with results obtained from the quantitative studies. Nuclear fluorescence was restored, however, following treatment with antimycin A, thus indicating an energy dependent altered drug distribution pattern in these cells. Pretreatment with the metabolic inhibitors, sodium azide or 2-deoxy-glucose, could only partially restore nuclear levels of the drug in the DLKPA10 cells, further illustrating the effectiveness of antimycin A in depleting cellular levels of ATP. Initially it was thought that nuclear drug exclusion resulted from reduced cellular levels of the drug. However, when the subcellular distribution of adriamycin was studied in the DLKP and DLKPA10 cells at equivalent intracellular concentrations, nuclear exclusion of the drug was still observed in the resistant cells. These results indicate that the altered drug distribution pattern could result from factors other than bulk influx/efflux rates although, given the limitations of the study and the substantially different exposure

times further studies would be required to establish this conclusively.

The observation of intense fluorescence staining in the cytoplasmic regions of resistant cells prompted a number of further studies. These studies were aimed at determining if adriamycin was localised within cellular organelles, including lysosomes and the Golgi apparatus. A number of studies have suggested that an alternative membrane trafficking system, involving either the Golgi apparatus or the lysosomal system, could exist in MDR cells. These could operate to remove drug from the cells. However, the results obtained in our studies suggested that adriamycin was not associated with either the Golgi apparatus or the lysosomes in the DLKPA10 cells, since treatment with the Golgi disrupting agent, brefeldin A or the lysosomotropic agents, chloroquine and methylamine, did not significantly alter the subcellular distribution of the drug. Further studies suggested that adriamycin is localised in acidic vesicles and that an alternative acidic vesicular traffic transport system may play a role in drug exclusion in resistant cells. Depletion of cellular ATP levels, by antimycin A treatment, resulted in disruption of these acidic vesicles, indicating that an active mechanism is required to sustain the drug in the vesicles. Monensin, an agent known to disrupt acidic vesicular traffic, was found to increase the cellular concentration of adriamycin and to redistribute the drug into the nucleus in the DKLKPA10 cells. The mechanism by which monensin acts is unclear, although it is generally believed that it acts by abolishing the $Na^+H^+$ gradient, resulting in an increase in the pH environment and subsequent disruption of the acidic vesicles. However, when the cells were preloaded with adriamycin, it was found, unexpectedly, that although antimycin A caused subcellular drug redistribution, treatment with monensin did not appear to disrupt the cytoplasmic vesicles within time period studied. Accordingly, further work is required to establish the exact mechanism by which monensin acts, although it would appear from the results obtained that it act by inhibiting the sequestration of drug into the vesicles rather than disruption of the vesicles.

The results would, therefore, suggest that in, addition to P-glycoprotein mediated drug efflux an alternative acidic vesicular transport system exists in the DLKPA10 cells. Although vincristine accumulation in the DLKPA10 cells was also increased following treatment with monensin, the extent, unexpectedly, was found to be substantially lower than with adriamycin. Further studies also revealed that while treatment with monensin resulted in a decrease in the cytotoxicity of adriamycin and VP16, no alteration in vincristine toxicity in the DLKPA10 cells was noted. The cause of this apparent drug specific effect

of monensin is unclear, although since both adriamycin and VP16 are topoisomerase II inhibitors, the divergent effect of monensin may be related to topoisomerase inhibition. Substantial further work on VP16 accumulation in the DLKPA10 cells is required to determine the effect of monensin on VP16 accumulation. Specifically, it may be possible that adriamycin (and possibly VP16) are localised in acidic vesicles within the cytoplasm and that disruption of these vesicles by monensin treatment results in an increase in the cellular concentration of the drug. In contrast, since vincristine has a higher molecular weight and a more complex structure, localisation of the drug may not occur within the vesicles. Subsequently, the increase in the cellular concentration of vincristine following treatment with monensin may in fact be mediated by alternative mechanisms, including inhibition of P-glycoprotein or metabolic inhibition.

The results obtained from drug transport studies suggest that the drug accumulation defect in the DLKPA10 cell line is predominantly related to combination of P-glycoprotein overexpression and the existence of an acidic vesicular transport mechanism. However, since the apparent inhibition of both mechanisms does not appear to completely restore drug accumulation, other mechanisms may also be involved. Consequently additional, preliminary, studies were carried out to identify an alternative mechanism responsible for decreased drug accumulation in these cells. In particular results obtained from studies carried out to determine the effect of amphiphiles on adriamycin accumulation in the resistant cells, suggested that membrane fluidity may be decreased in the resistant cells since treatment with cholestryl hemisuccinate slightly increased drug accumulation in the cells. Cholesteryl hemisuccinate and other amphiphiles partition into membranes subsequently affecting membrane fluidity, possibly resulting in increased drug accumulation. Given that membrane fluidity may be decreased in the DLKPA10 cells, detailed studies of the effect of various membrane altering agents on drug accumulation and distribution in the resistant cells is needed to clarify the involvement of alterations in membrane fluidity in decreased drug accumulation in the DLKPA10 resistant cells.

To establish if an antigen associated with decreased drug accumulation was overexpressed in the DLKPA10 cells, antibodies were raised against the resistant cells. It was found that the antibody displaying the highest level of immunoreactivity preferentially bound to the parental cells. This would suggest that expression of the recognised antigen is downregulated in the DLKPA10 cells. However, its role, if any, in mediating resistance in the DLKPA10 cells is unknown. Although, it remains to be clarified, the preliminary

characterisation studies suggest that the antigen may play a role in intercell communication or cell-cell adhesion.

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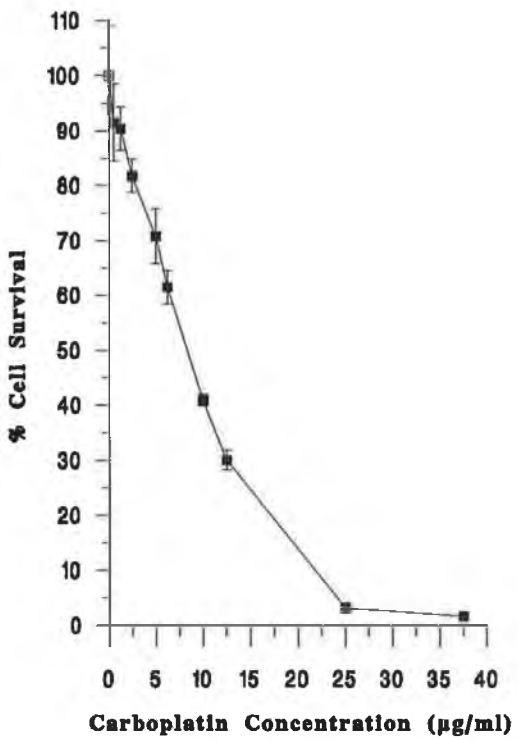
# *6. Appendices*

**Appendix 6.1** **Abbreviations**

| | |
|---|---|
| ADR/AR | Adriamycin |
| ATCC | American Tissue Culture Collection |
| ATP | Adenosine Triphosphate |
| BCA | Bicinchoninic acid |
| BSO | Buthionine sulfoximide |
| COLH | Colchicine |
| C-Pt | Cisplatin |
| CSA | Cyclosporin A |
| DMEM | Dulbeccos Modified Eagles Medium |
| DMSO | Dimethyl sulfoximide |
| DNA | Deoxyribonucleic acid |
| DOX | Doxorubicin (adriamycin) |
| ECACC | European Collection of Animal Cell Culture |
| EDTA | Ethylene diamino tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbant assay |
| FCS | Foetal calf serum |
| FITC | Fluorescein-isocyanate |
| GSH | Glutathione |
| GST | Glutathione-S-transferase |
| HAT | Hypoxanthine, aminopterin, thymidine |
| HCl | Hydrochloric acid |
| HEPES | 4-(2-hydroxethyl)-piperazine ethane sulphonic acid |
| HGPRT | Hypoxanthine-guanine phosphoribosyl transferase |
| HPLC | High performance liquid chromatography |
| HT | Hypoxantine, thymidine |
| $IC_{50}$ | Inhibitory concentration 50% |
| Ig | Immunoglobulin |
| KCl | Potassium chloride |
| kDa | Kilodalton |
| LRP | Lung resistance protein |
| MAB | Monoclonal antibody |
| MDR | Multidrug resistance |
| MEM | Minumum Essential Medium |

| | |
|---|---|
| $MgCl_2$ | Magnesium chloride |
| Mitox/Mx | Mitoxantone |
| mRNA | Messagner RNA |
| MRP | Multidrug resistance associated protein |
| MT | Metallothionein |
| MW | Molecular weight |
| NaCL | Sodium chloride |
| $NaHC0_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| NCTCC | National Cell & Tissue Culture Centre |
| NEAA | Non essential amino acids |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| P-gp | P-glycoprotein |
| PMSF | Phenylmethyl sulfonyl fluoride |
| psi | Pounds per square inch |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| rpm | Revolutions per minute |
| SDS | Sodium dodecyl sulfate |
| TBS | Tris buffered saline |
| TEMED | N,N,N'N'-tetramethyl-ethylenediamine |
| TK | Thymidine kinase |
| Topoisomerase I | DNA Topoisomerase I enzyme |
| Topoisomerase II | DNA Topoisomerase II enzyme |
| Tris | Tris(hydroxymethyl)aminomethane |
| UV | Ultraviolet |
| VCR | Vincristine |
| Ver | Verapamil |
| VM26 | Teniposide |
| VP16 | Etoposide (Vepesid) |
| v/v | Volume to volume ratio |
| w/v | Weight to volume ratio |

## Appendix 6.2 Sample calculation of $IC_{50}$ value




**Sample calculation**

$IC_{50}$ of carboplatin from above graph = 8.5$\mu$g/ml

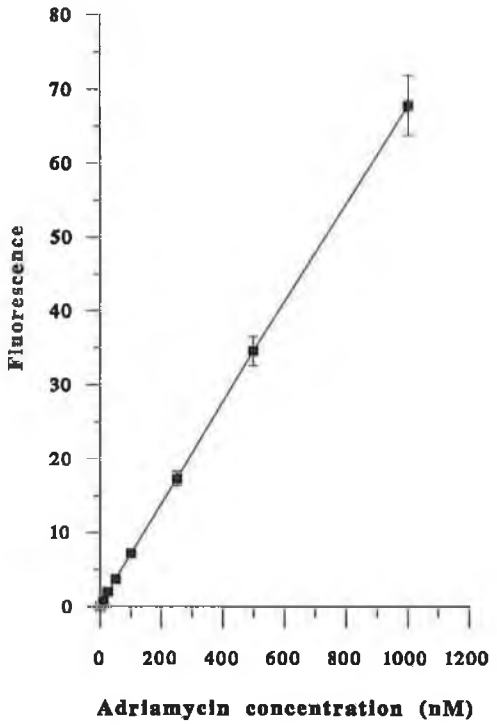
Molecular weight of carboplatin = 375

Calculation = $\frac{8.5 \times 10^6}{375}$

= 22666.7 nM

$IC_{50}$ value = 22.67 $\mu$M

## Appendix 6.3 Sample calculation of subcellular adriamycin concentration




A standard curve was prepared from the fluorescence of known adriamycin concentrations over a range of 0 - 1000 nM. The cellular concentration of adriamycin in each sample was quantitated from the linear standard curve.

**Sample calculation**

Fluorescence value obtained = 28
Adriamycin concentration from above graph = 400nM

400 nmoles/l = 0.4 nmoles/ml

Drug extracted in total of 4mls = 1.6 nmoles/sample
= 1600 pmoles/sample

Total no of cells = $8 \times 10^5$ per well

= 2000 pmoles per $10^6$ cells

= 2 nmole per $10^6$ cells

**Appendix 6.4 Molecular Weights of agents used in these studies**

| | |
|---|---|
| Adriamycin | 580 |
| Vincristine | 824.9 |
| VP16 (etoposide) | 588.6 |
| 5-Fluorouracil | 130.1 |
| Cisplatin | 300 |
| Carboplatin | 375 |
| Verapamil | 454.6 |
| Antimycin A | 548.6 |
| Monensin | 670.9 |
| Nigericin | 725 |
| Brefeldin A | 280.4 |
| Genistein | 270.2 |
| Chloroquine | 319.9 |
| Methylamine | 31.1 |