Investigation of
Multidrug Resistance and Invasion in Human Cancer Cell Lines

A thesis submitted for the degree of Ph D

by

Yizheng Liang B Med

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

National Cell & Tissue Culture Centre
School of Biological Sciences
Dublin City University
Glasnevin
Dublin 9
Ireland

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

Signed: Yizheng Liang **I.D. Number:** 95970631

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Abstract

Through exposure to melphalan or taxol, four novel multidrug resistant variants of the human nasal carcinoma cell line, RPMI-2650 and human lung carcinoma cell line, DLKP, were established *In vitro* toxicity tests revealed that the RPMI-2650 taxol-resistant variant exhibited significant resistance to taxol, vincristine, adriamycin and vinblastine The RPMI-2650 melphalan-resistant variant exhibited significant resistance to melphalan, cadmium chloride, adriamycin, vinblastine and VP-16 The DLKP long term melphalan-selected variant exhibited significant resistance to melphalan, cadmium chloride, cisplatin, adriamycm and vinblastine A similar cross resistance profile was observed in the DLKP pulse selection variant with the exception that this variant displayed no obvious cross resistance to vinblastine Adriamycm distribution studies revealed decreased intracellular adriamycm accumulation in the RPMI-2650 taxol- and melphalan-resistant variants However, intense nuclear fluorescence was observed following treatment with verapamil and cyclosporin A Characterisation studies demonstrated that the resistant phenotype in the RPMI-2650 taxol-resistant variant appeared to be mainly P-glycoprotem mediated Protein and mRNA analysis by Western blotting, immunocytochemistry and RT-PCR demonstrated an overexpression of MRP1, cMOAT (MRP2) and MRP3 m the RPMI-2650 melphalan-resistant variant compared to its parental cell line Sulindac or indomethacin could reverse melphalan toxicity in this variant Western blotting analysis also showed the overexpression of MRP1 in the DLKP melphalan long term and pulse selection cells, suggesting the involvement of MRP family members in mediating melphalan resistance in these variants Significant alterations in GST activity and metallothionein expression in these variants were not observed

Results obtained from invasion assays demonstrated that the RPMI-2650 and DLKP melphalan-resistant variants were more invasive than their respective parental cells and the RPMI-2650 taxol-resistant variant It appears that increased expression of $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, decreased expression of $\alpha_4\beta_1$, stronger adhesion to collagen type IV, laminin, fibronectin and matrigel and expression of MMP-2, MMP-9 and c-myc may all contribute to the high invasiveness of the RPMI-2650 melphalan-resistant variant Increased expression of α_2 and β_1 integrin subunits and overexpression of MMP-2 and MMP-9 may be responsible for the invasiveness of the DLKP melphalan-selected variants Decreased expression of α_2 integrin which may result in decreased attachment to collagen type IV, lack of cytokeratin 18 and no detectable expression of any proteases may be responsible for the non-invasiveness of the RPMI-2650 taxol-resistant variant

These results suggest that exposure to melphalan can result in not only a multidrug resistance phenotype but could also make cancer cells more invasive and metastatic

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1. Introduction

1.1 General introduction

Chemotherapy constitutes the principal therapeutic tool to treat unresectable or disseminated tumours Although there has been significant impact on survival m some malignancies, the majority of human cancers are resistant to chemotherapy at presentation or become resistant after an initial response This suggests that resistance is inherent in a tumour cell or evolves under the selection pressure of drug administration In some cases, tumour cells are not only resistant to a specific drug or group of drugs with structural or functional similarities, but also resistant to a group of functionally and structurally dissimilar drugs This type of resistance is called multidrug resistance (MDR) Mechanisms of MDR include exclusion of drug from the cell, increased detoxification of the drug, alteration in drug targets, failure to activate the drug to its active form, enhanced repair capability of the cell after injury or failure to engage an appropriate response leading to apoptosis in the damaged cells Many of these factors may co-exist in human tumours Thus, a major goal in experimental as well as clinical investigation of MDR is to discover unique methods by which to reverse or circumvent it

Apart from MDR, invasion and metastasis is another major cause of death for cancer patients The complexity of the metastatic process has forced investigators to focus on one step at a time m order to reduce the number of variables Invasion is one of the most critical steps in metastasis Dynamic interactions among cell adhesion molecules, extracellular matrix, proteases, cytoskeletal proteins and signalling molecules contribute to the invasive behaviour of the tumour cells MDR and invasion/metastasis are correlated phenomena Highly multidrug resistant tumour cells can survive chemotherapy and escape to distant sites of the body or highly metastatic cells can escape from the effect of chemotherapy A relationship between these two phenotypes has been demonstrated by two types of experiments firstly, in some cases, invasive/metastatic cells develop drug resistance more readily than non-mvasive/metastatic cells (Cillo *et al*, 1987), secondly, some tumour cells selected for the resistance to chemotherapeutic drugs are more invasive/metastatic relative to non-resistant cells (Lucke-Huhle *et al*, 1994) Mechanisms underlying this relationship are not clear It is supposed that malignant tumours arise as the end result of an accumulation of genetic mutations involving a number of oncogenes and tumour suppressor genes Many of these genes could affect the expression of cell growth, differentiation, drug resistance, invasion and metastasis Hence, an important goal of future research would be to determine whether a

common transcription factor exists that turns on two sets of genes, those responsible for drug resistance, and those responsible for increased cell invasion and metastasis

1.2 Pharmacology of MDR drugs

1.2.1 Taxol (Paclitaxel)

The major source of taxol (paclitaxel) is the bark of a slow-growing yew tree, *taxus brevifolia* Despite the elucidation of its broad activity and its unique structure by Monroe Wall and co-workers in the late 1960s (Wani *et al*, 1971), it is still in a relatively early stage of clinical development In 1979, Horwitz and co-workers discovered that this agent could induce novel biologic effects and had a unique mechanism of action (Schiff *et al*, 1979, 1980) Taxol, similar to etoposide (VP-16), vinblastine and vincristine, is thought to owe its antitumour effects to disruption of mitosis It interferes with chromosome changes during the cell cycle and thus is toxic to proliferating cells Taxol induces a shift in the physiological equilibrium between microtubules and tubulin toward polymerisation and formation of dysfunctional microtubules

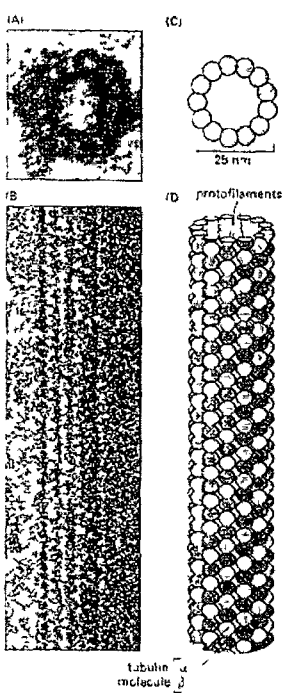


Fig. 1.2.1 A side view of a microtubule with the tubulin molecules aligned into long parallel rows Each of the 13 protofilaments is composed of a series of tubulin molecules (α/β heterodimers) (A) Electron micrograph of a microtubule seen in cross-section (B) Electron micrograph of a side view of a microtubule (C and D) Schematic diagrams of a microtubule

The major component of microtubules is tubulin, a protein containing two non-identical (α and β) 50kD subunits, arranged head-to-tail in linear protofilaments A single microtubule is composed of 13 parallel protofilaments, forming a hollow structure with a "minus" end, which is usually stabilised by attachment to an organising centre, and a "plus" end, at which growth or shrinkage takes place (figure 1 2 1) Individual microtubules can oscillate between polymerisation and depolymerisation and the net status of the microtubule population is very sensitive to factors affecting the equilibrium between free tubulin dimers and assembled polymers In contrast to other known microtubule antagonists, taxol disrupts the equilibrium between free tubulin and microtubules by shifting it in the direction of assembly rather than disassembly As a result, taxol treatment causes both the stabilisation of ordinary cytoplasmic microtubules and the formation of abnormal bundles of microtubules (Schiff *et al*, 1979 and 1980) Competitive binding experiments demonstrated that the taxol binding site is distinct from those used by other agents including colchicine, vincristine, etc (Kumar *et al*, 1981, Schiff *et al*, 1981)

Studies have shown that phosphorylation of bcl-2 at serine residues leads to loss of bcl-2 antiapoptotic function (Haldar *et al*, 1995) It was also found that taxol (1-10μM) induces bcl-2 phosphorylation followed by apoptosis (Haldar *et al*, 1995, 1996) Since taxol inhibits microtubule depolymerisation, it was suggested that microtubule damage may lead to cancer cell apoptosis through a mechanism involving bcl-2 phosphorylation and that this pathway may be important in normal physiological elimination of cells with damaged mitotic apparatus

Resistance to taxol arises through two major mechanisms, *i e* overexpression of P-glycoprotein (Roy *et al*, 1985, Greenberger *et al*, 1987) and mutation of the gene coding for one of the tubulin subunits (Schibler *et al*, 1986) In the latter case it has been hypothesised that, in the absence of drugs, the equilibrium between free tubulin and microtubules is shifted toward disaggregation (Cabral *et al*, 1986), therefore the mutant cells have slightly greater tolerance for taxol-induced stabilisation than that of the parent cells Some mutants were not only resistant to taxol but they also required it for growth, presumably as a result of an extreme shift toward disaggregation

Although taxol is still in a relatively early stage of clinical development, it has been shown to be very active in the treatment of refractory ovarian cancer (Rowinsky *et al*, 1990, McGuire *et al*, 1989) Encouraging results have also been obtained in patients with metastatic breast cancer (Holmes *et al*, 1991, Marty *et al*, 1999) It is hoped that taxol treatment will also be effective in other tumours, including non-small-cell lung cancer and colon cancer

Apart from hypersensitivity, neutropenia is the most common dose-limiting toxicity for taxol Severe mucositis, manifested as ulcerations of the mouth and throat, appears to be a cumulative effect of taxol administration (Rowinsky *et al*, 1990) Taxol causes some reversible neurotoxicities, most often numbness and paresthesia in the hands and feet (Rowinsky *et al*, 1990) A variety of cardiac abnormalities have also been associated with taxol treatment (Rowinsky *et al*, 1991)

Taxol (paclitaxel) and taxotere (docetaxel) are called taxoids Taxotere is a semisynthetic taxoid using a precusor extracted from the European yew tree, *taxus baccata* Taxotere and taxol have similar mechanisms of action while taxotere is about twice as potent as an inhibitor of microtubule depolymerisation (Gelmon *et al*, 1994)

1.2.2 Melphalan

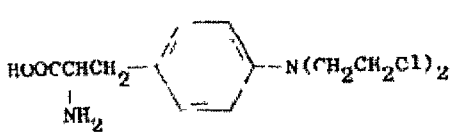


Fig. 1.2.2 The chemical structure of melphalan

Melphalan (L-phenylalamne mustard) is a member of a group of drugs called alkylating agents It was first synthesised in 1954 in an attempt to obtain an anticancer drug that would preferentially localise in a particular tissue Since phenylalanine is a precursor of melanin, it was postulated that phenylalanine mustards might preferentially accumulate in melanomas and thereby produce a selective effect, however the goal of a "site-directed" effect has not yet been achieved (Pratt *et al*, 1994) The alkylating agents are highly reactive compound that can form covalent bonds with a number of nucleophilic groups such as ammo, carboxyl,

sulfhydryl or imidazole moieties in proteins and nucleic acids (Price *et al*, 1975) At neutral or alkaline pH, one of the chlorethyl side chains of melphalan undergoes a cyclisation, releasing chloride ions and forming a highly reactive intermediate which can attack nucleophilic groups One important reaction in the cytotoxic effect of melphalan is the formation of a covalent bond between the drug and the 7-nitrogen group of guanine Since melphalan is a bifunctional alkylating agent (containing two reactive chlorethyl side chains), it can then undergo a similar cyclisation of the second side chain and form a covalent bond with another nucleophilic group

These reactions may result in cross-linking between DNA strands or linkages between bases within the same strand of DNA Strand scission also occurs as a result of endonuclease attack when the cell attempts to repair the segment of DNA containing the drug adduct There is evidence that the cytotoxicity of melphalan at therapeutic levels is mainly due to an inhibition of DNA replication since alkylation of DNA decreases its ability to act as a template for DNA synthesis (Brewer *et al*, 1961, Ruddon *et al*, 1968, Roberts *et al*, 1971)

Although some tumour cells selected for resistance to melphalan have increased excision repair activity (Ewig *et al*, 1977), this mechanism may not be a major factor in the development of clinical resistance to these drugs (Fox, 1984) Two important mechanisms of resistance are increased drug inactivation due to the reaction with cellular thiols and decreased drug uptake Increased drug inactivation is based on elevated cellular levels of glutathione (GSH), a compound that reacts with the cytotoxic electrophilic derivatives of melphalan and detoxifies them The enzyme that catalyses this conjugation is glutathione S-transferase, and increased levels of this enzyme have been observed in model tumour systems (Tew *et al*, 1988) Depletion of GSH with butathionine sulfoximine (BSO), an inhibitor of γ-glutamlycysteine synthetase which catalyses the first step of GSH formation can increase the *in vitro* cytotoxicity of melphalan (Richardson and Sieman, 1992)

It has been shown that tumour cell lines resistant to melphalan take up the drug at a slower rate than that of the drug sensitive parental cells (Goldenberg *et al*, 1984) MRP-mediated ATP-dependent transport was observed for the conjugates monochloro-mono[^{3}H]glutathionyl melphalan (Jedlitschky *et al*, 1996) Resistance mechanisms such as increased excision repair and increased cellular glutathione would be expected to yield cross-resistance among a

variety of DNA cross-linking drugs Resistance due to decreased drug uptake would yield cross resistance only if the same uptake mechanism was shared by different drug classes Tumour cells selected for resistance to one alkylating agent can show cross resistance to another agent and simultaneously respond like the drug–sensitive parent cell line to many other alkylating drugs (Schabel *et al*, 1978) Generally, however, the level of cross-resistance among alkylating agents is low in human tumor cells (Frei *et al*, 1985, 1988) Consequently, high dose alkylating agent combination regimens have been administered to patients with advanced cancers (Antman *et al*, 1987)

High levels of metallothioneins are also associated with resistance to alkylating agents (Kelley, 1988) Metallothioneins are small 6-7kD cysteine-rich proteins that appear to protect cells from the toxicity of heavy metals such as copper, zinc and cadmium The functional significance of metallothioneins may extend beyond their ability to protect cells by binding electrophilic heavy metal ions, since they can be induced by steroids, stress, X-rays, high O_2 tension and alkylating agents Pretreatment of CHO cells with Zn for several hours prior to exposure to melphalan resulted in the induction of considerable resistance to melphalan (Tobey, 1982)

In addition, cells that have an impaired ability to undergo apoptosis would be expected to be resistant to chemotherapeutic agents The overexpression of the bcl-2 oncogene has been shown to inhibit apoptosis in several cell types, and also to produce cells that are less sensitive to alkylating and other cytotoxic agents (Collin, 1992)

Melphalan is generally considered to be cell cycle phase nonspecific It is most cytotoxic to rapidly growing cells (Van Putten *et al*, 1971) Melphalan is used in the treatment of melanoma, multiple myeloma, Ewing's sarcoma, acute leukaemia, malignant lymphoma, colon carcinoma, breast cancer and ovarian cancer (Samuels and Bitran, 1995) The major toxicity of melphalan is bone marrow depression, sterility, amenorrhea and acute nonlymphocytic leukaemia (Greene *et al*, 1986)

1.2.3. Adriamycin

Adriamycin, or doxorubicin, is an anthracycline antibiotic isolated from species of streptomyces It has a characteristic four-ring structure that is linked via a glycosidic bond to

the amino sugar, daunosamine The anthracycline drugs, which are members of noncovalent DNA-binding drugs, all have regions that stack between the paired bases in DNA, forming a tight drug-DNA interaction that is critical for their cytotoxic, mutagenic and carcinogenic effects Adriamycin has also been shown to induce double strand DNA breaks in cultured mammalian cells (Nelson *et al*, 1984) The DNA breaks are caused by the enzyme topoisomerase II This enzyme controls the degree of DNA supercoiling by cleaving and reannealing DNA such that the coil is relaxed by one turn Adriamycin somehow interferes with the DNA strand breakage-reunion reaction of topoisomerase II (Tewey *et al*, 1984) The observation that a direct correlation between topoisomerase II activity and adriamycin resistance exists in several cell lines strongly supports the involvement of topoisomerase II in the cytotoxicity pathway (Glisson *et al*, 1986, Pommier *et al*, 1986, Deffie *et al*, 1989)

The topoisomerase II mechanism is not the only way in which adriamycin cleaves DNA Adriamycin can also precipitate the formation of active oxygen species which cause single-strand breakage Adriamycin contains a hydroxyquinone, which is an iron-chelating structure (Myers *et al*, 1986) The adriamycin-Fe complex forms a complex with DNA that differs from the intercalated drug-DNA complex (Eliot *et al*, 1984) The drug-Fe-DNA complex, like the free drug-Fe complex, catalyses the transfer of electrons from glutathione to oxygen, resulting in the formation of active oxygen species The drug-Fe-DNA complex also generates hydroxyl radicals from hydrogen peroxide, which results in DNA cleavage (Muindi *et al*, 1985) Free radical generation by the drug-Fe-DNA complex is critical for adriamycin-induced cardiotoxicity

The most common resistance mechanism in cells selected for adriamycin resistance *in vitro* is increased drug efflux This is due to the overexpression of a number of proteins including P-glycoprotein, MRP family members and LRP, the multidrug transporters discussed in detail in 1 4, 1 5 and 1 6 respectively Two other mechanisms of resistance to adriamycin have also been reported In a number of cell lines, decreased topoisomerase II activity has been found to correlate with adriamycin resistance (Glisson *et al*, 1986, Pommier *et al*, 1986, Deffie *et al*, 1989), while in other cell lines increased glutathione peroxidase activity has been observed (Sinha *et al*, 1989) Reduced glutathione reacts with drug–generated reactive oxygen compounds such as peroxides (Tew *et al*, 1988) and increased levels of glutathione or glutathione S-transferase have been found in some adriamycin–resistant cell lines (Batist *et al*, 1986) In such cell lines, butathione sulfoximine (BSO), the inhibitor of γ -glutamyl

synthetase, reduces glutathione levels and partially reverses adriamycin resistance (Kramer *et al*, 1987)

Adriamycin has a very broad range of clinical usefulness It is active against carcinomas of the breast, lung (small cell), thyroid, ovary, as well as sarcomas of the bone and soft tissue, pediatric solid tumours and acute leukaemias, lymphomas and myeloma (Hortobagyi, 1997) Adriamycin treatment frequently causes nausea and vomiting, and patients may experience anorexia and diarrhoea Myelosuppression is dose related and occurs in 60% to 80% of patients (O'Bryan *et al*, 1977) Adriamycin also produces unique cardiotoxic reactions in both adults and children (Lenaz *et al*, 1976, Gilladoga *et al*, 1976) Two types of reactions are observed early, transient electrocardiographic changes and a delayed progressive cardiomyopathy (Lenaz *et al*, 1976) which presents as a severe congestive heart failure Efforts to reduce cardiotoxicity focused on iron chelators The EDTA analogue, ICRF-187, appears to be able to decrease adriamycin-induced cardiac toxicity (Doroshow, 1991)

1.2.4. Vinca alkaloids

While working with extracts of the periwinkle plant (*Vinca rose Linn*), Noble and coworkers (1958) observed granulocytopenia and bone-marrow suppression in rats These observations led to the purification of an active alkaloid Johnson *et al* (1963) demonstrated activity of certain alkaloidal fractions against acute lymphocytic neoplasm in mice Fractionation of these extracts yielded two active alkaloids vinblastine and vincristine

The vinca alkaloids are cell-cycle specific agents, and similar to other drugs such as colchicine and podophyllotoxin, they bind specifically to free tubulin dimers Although these agents do not share the same binding site, their interactions with tubulin have the common effect of disrupting the balance between microtubule polymerisation and depolymerisation This results in the net dissolution of microtubules, destruction of the mitotic spindle, and arrest of cells in metaphase (Margulis, 1973, Wilson *et al*, 1975, Mandelbaum-Shavit *et al*, 1976) In the absence of an intact mitotic spindle, the chromosomes may disperse throughout the cytoplasm or may occur in unusual groupings, such as balls or stars The inability to segregate chromosomes correctly during mitosis ultimately leads to cell death The binding of vinca alkaloids to tubulin is kinetically complex (Na *et al*, 1986) and depends on many factors, including ionic strength (Singer *et al*, 1988), magnesium-ion concentration (Na *et al*, 1986), interaction with nontubulin proteins (Donoso *et al*, 1979) and nucleotide

concentration (Bowman *et al*, 1988)

Acquired resistance to vinca alkaloids usually results from overexpression of P-glycoprotein, MRP1 or cMOAT It can be overcome *in vitro* by incubating the cells with compounds that compete with vinca alkaloids for binding to P-glycoprotem or MRP and studies with one such agent, verapamil, indicate that this strategy may also be practical *in vivo* (Tsuruo *et al*, 1983, Akiyama *et al*, 1988, Dalton *et al*, 1989) Resistance can also be due to alterations in tubulin structure In some instances these mutations affect binding of the drug to tubulin (Ling *et al*, 1979) Many other mutants have been isolated, in which tubulin has been altered so as to affect its intrinsic ability to form microtubules (Cabral *et al*, 1986) These mutants have several common characteristics, including relatively low levels of resistance (two- to three-fold), cross-resistance to other microtubule-assembly inhibitors, and collateral sensitivity to microtubule-stabilising agents (e g taxol)

The minor differences in the structure of vinca alkaloid molecules result in notable differences in both the toxicity and antitumour spectra of vincristine and vinblastine Vincristine, in combination with prednisone, is widely used in combination chemotherapy for induction of remission of acute leukaemias in adults and children due to its rapidity of action and its lesser tendency for myelosuppressive action Complete remissions are obtained in 80-90% of such patients (Rowinsky *et al*, 1991, Willemze *et al*, 1980, Ortega *et al*, 1977) Similar success is achieved in treating Hodgkin's lymphoma (Bonadonna *et al*, 1986) and aggressive non-Hodgkin's lymphoma (Longo *et al*, 1991) with vincristine-containing combinations Vincristine is also included in combinations for treating various solid tumours, such as small-cell lung carcinoma and breast carcinoma in adults and Wilm's tumor, neuroblastoma, rhabdomyosarcoma and Ewing's sarcoma in children (Rowinsky *et al*, 1991, Johnson *et al*, 1987) The most important clinical use of vinblastine is with bleomycin and cisplatin in the treatment of metastatic testicular tumours Vinblastine is also an important drug in the treatment of Hodgkin's lymphoma, where it has been used instead of vincristine, providing similar antitumour activity with less neurotoxicity (Sutcliffe *et al*, 1978, Diggs *et al*, 1977) Vinblastine is effective against non-Hodgkin's lymphoma (Palmier *et al*, 1990) and has produced responses in patients who had developed resistance to vincristine, indicating that cross-resistance does not always occur between these two drugs (Jackson *et al*, 1987)

The clinical toxicity of vincristine is mostly neurological, the most common symptom of which is a depressed Achilles tendon reflex (Sandler *et al*, 1969) Other toxicities include cranial nerve palsies, transient cortical blindness, confusion, delirium and depression (Byrd *et al*, 1981) Unlike the great majority of antineoplastic agents, vincristine does not cause significant myelosuppression Myelosuppression (primarily neutropenia) is the major toxicity caused by vinblastine (Rowinsky *et al*, 1991) The neurotoxic symptoms caused by vinblastine are similar to (although less common and less severe than) those resulting from vincristine administration

1.2.5. Etoposide (VP-16)

Etoposide is a semisynthetic derivative of podophyllotoxin, a microtubule inhibitor found in extracts of the mandrake plant Although it was initially thought that etoposide might also inhibit microtubule function, further investigation indicated that the enzyme topoisomerase II (Topo II) (discussed in detail in 1 5 2) was the major target of this drug in eukaryotic cells (Topo II can temporarilly break DNA strands and reseal these breaks, resulting in the DNA been sufficiently untangled and relaxed to allow transcription and replication) (Ross *et al*, 1984)

Topoisomerase II first interacts with DNA by reversibly forming a noncovalent complex At this stage no DNA strand breaks have been made and this initial complex is termed "noncleavable" The enzyme then cut both DNA strands, forming a covalent bond between its protein and one of the newly formed 5'- phosphate ends of the DNA This complex is called "cleavable" The cleavable complex is either rapidly converted back to the noncleavable complex or the breaks are resealed and the enzyme dissociates from the DNA Inhibitors such as etoposide stabilise the cleavable complex, preventing it from going back to the non-cleavable complex Although stabilisation of the cleavable complex persists while the etoposide is present, removal of the drug results in a rapid return of apparently normal topoisomerase function (Long *et al*, 1985)

A phenotype has been identified in which cells that are resistant to etoposide retain normal sensitivity to vinca alkaloids and normal drug-transport characteristics (Danks *et al*, 1987) This phenotype appears to arise from the expression of forms of topoisomerase II with altered catalytic activities (Sullivan *et al*, 1989, Glisson *et al*, 1986) Another form of resistance has been traced to a mutation that resulted in underexpression of topoisomerase II protein with

otherwise normal catalytic properties (Deffie *et al*, 1989)

Overexpression of P-glycoprotein or MRP is another mechanism of resistance to etoposide which results in decreased intracellular drug accumulation (Sinha *et al*, 1988) due to reduced influx of drugs (Politi *et al*, 1989) It has also been observed that etoposide treatment prevents activation of protein kinase, $p34^{cdc2}$ (Lock *et al*, 1990) This kinase, which ordinarily becomes activated at the end of the G_2 phase of the cell cycle, is thought to have a critical role in allowing cells to begin mitosis It is therefore possible that the arrest of cells in the G_2 phase of the cell cycle observed after treatment with etoposide is due to interference with $p34^{cdc2}$ function

Etoposide has the most significant impact in the treatment of testicular cancers in which it was found to be effective in patients whose tumours were resistant to prior treatment (Loehrer *et al*, 1991, Newlands *et al*, 1977) Small-cell lung cancer is another disease in which etoposide plays an important therapeutic role (Johnson *et al*, 1991) The major toxicity attributable to etoposide is myelosuppression Mucositis can be dose-limiting at higher doses in which marrow is rescued by transplantation (Herzig, 1991) Rapid drug infusions occasionally cause hypotension (Cohen *et al*, 1977)

1.2.6. Cisplatin (cis-diammmedichloroplatinum II)

The potential of cisplatin as an antitumour agent was recognised through the observation made by Rosenberg *et al* (1965) that certain metal compounds inhibited bacterial division Their study had been designed to explore the possible effects of an electric field on the growth of *E Coli*, but they noted that the bacteria ceased dividing and grew in the form of long filaments

It became clear that cell division was being inhibited by an electrolysis product of the platinum electrode Rosenberg and his colleagues tested the antitumour activity of several platinum compounds and demonstrated that cisplatin was effective against leukaemia in mice (Rosenberg *et al*, 1969) Since all the trans-compounds tested have been ineffective, the cis-configuration appears to be required When cisplatin is dissolved in water, chloride ions are displaced, allowing the formation of the reactive forms of the compound Both cis- and trans-isomers exchange chloride ions for such nucleophilic group as RS^-, $R\text{-}S\text{-}CH_3$, $R\text{-}NH_2$ etc to

form links that can be very stable There is considerable evidence that DNA is the principal target for the cytotoxic action of cisplatin (Pinto *et al*, 1985) Regardless of whether it is purified DNA, intact cells, or tumour-bearing patients that are exposed to cisplatin, the principal coordinate is an intrastrand cross-link formed by binding of the drug to two neighbouring guanines Intrastrand cross-linkage accounts for inhibition of DNA replication which leads to cell death In addition to DNA-DNA cross-links, DNA-protein cross-linking also occurs

No dominant mechanism of resistance to cisplatin has been identified Often resistant cells have an increased capacity to repair mtrastrand adducts (Fox, 1984), but in many studies the increase in repair capacity has not been sufficient to explain the extent of resistance (Eastman *et al*, 1988) Cisplatin reacts readily with compounds containing SH groups Thus, resistance in some cell lines has been found to correlate with increased glutathione content (Behrens *et al*, 1987) Another thiol-containing modifier of drug response is the metal–binding protein metallothionein It has been found that tumours from cells with a high level of metallothionein have a decreased therapeutic response to cisplatin in comparison to tumours from the parent cells which have a low metallothionein levels (Endresen *et al*, 1984) Resistance to cisplatin has also been shown to be associated with decreased intracellular accumulation which might be caused by MRP family members (see section 1 4)

Cisplatin is an effective component of combination drug protocols used to treat a variety of human malignancies, most notably tumours of the testis, ovary, head and neck, lung and bladder (Einhorn, 1986, Ozols *et al*, 1991) The major delayed toxic effects of cisplatin are nephrotoxicity, peripheral neuropathy, with nephrotoxicity and ototoxicity often being dose-limiting Cisplatin produces a dose-dependent ototoxicity that may be manifested by tinnitus or hearing loss (Piel *et al*, 1974) Patients receiving cisplatin should have audiometric testing, particularly if they are being treated with other potentially ototoxic drugs

1.2.7. 5-Fluorouracil

5-Fluorouracil (5-FU) was first synthesised in 1957 and is a member of the antimetabolite group of drugs Its major action is through inhibition of nucleotide synthesis and through incorporation into nucleic acids (Pratt *et al*, 1994)

5-FU is structurally similar to thymine and is the most important pyrimidine antagonist 5-FU can be metabolised to FdUMP (5-fluorodeoxyuridine monophosphate) which inhibits thymidylate synthetase and consequently DNA synthesis 5-FU can also be metabolised to FUTP (fluorouridine triphosphate) which can incorporate into various types of RNA

As is the case with all antimetabolites, alterations in the enzymatic pathways by which the administered drug is converted to active metabolites can influence its actions The quantity (Washtien *et al*, 1982, Berger *et al*, 1988) and quality (Berger *et al*, 1988) of the target enzyme are critical determinants of the extent of inhibition of thymidylate synthesis It has also been recognised that the availability of the reduced folate cofactor has a profound impact on the effectiveness of thymidylate synthetase inhibition by 5-FU (Ullman *et al*, 1978) This knowledge has been applied to the design of clinical treatment protocols and the increased efficacy of 5-FU treatment by concurrent administration of a reduced folate represents a significant advance in the use of this drug (Germ *et al*, 1987)

However, if thymidylate synthetase inhibition was the only important mechanism in 5-FU-induced cytotoxicity, then exogenously supplied thymidine would always protect cells from 5-FU However this has failed to act as a universal antidote to 5-FU It is therefore probable that 5-FU's primary cytotoxic mechanism is not the same in all cell lines, and that this heterogeneity is the basis for the wide variability of response to 5-FU treatment observed *in vivo*

Table 1 2 7 represents the mechanisms of resistance to 5-FU

1	increased level of expression of thymidylate synthetase
2	altered bindmg affinity of thymidylate synthetase for FdUMP
3	decreased incorporation of 5-FU into RNA
4	decreased incorporation of 5-FU into DNA
5	decreased pools of the reduced folate
6	increased level of expression of dUTPas

Table 1.2.7 Mechanisms of resistance to 5-FU (Chu *et al*, 1994)

5-FU is used to treat several types of solid tumours, including colorectal, breast, head and neck, gastric and pancreatic cancers (Germ, 1990) In addition to having antitumour activity on its own, 5-FU interacts with radiotherapy to produce radiosensitisation Nausea, vomiting, anorexia, stomatitis and diarrhoea can occur during systemic therapy with 5-FU with the frequency of side effects varying with the treatment schedule employed Stomatitis and diarrhoea are the most common dose-limiting toxicities when 5-FU is administered by continuous infusion

1.3 P-glycoprotein (Pgp)

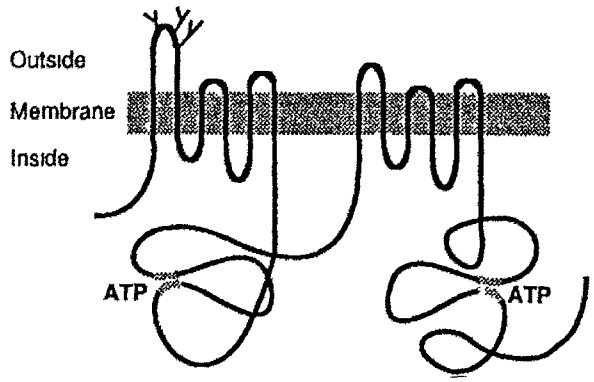


Fig. 1.3 Predicated structure of P-glycoprotein with cluster of glycosylation sites in the first external domain and nucleotide binding folds (Croop, 1993)

P-glycoprotein is the best characterised MDR marker Juliano and Ling (1976) first identified a 170kD membrane glycoprotein overexpressed in multidrug resistant cell lines The length of Pgp varies from 1276 to 1281 amino acids predicting a molecular weight of approximately 140kD Approximately 10-15kD of the molecular weight is accounted for by N-linked glycosylation (Greenberger *et al*, 1988) Examination of the amino acid sequence identifies a peptide which traverses the plasma membrane 12 times A short, highly charged cytoplasmic domain precedes three membrane loops which are followed by a large cytoplasmic domain which binds to ATPase A cluster of potential N-linked glycosylation sites are located in the first external loop The large cytoplasmic domain is followed by three additional membrane loops and another large cytoplasmic domain which can also bind to ATPase Experimental evidence indicates that Pgp binds and hydrolyses ATP (Ambudkar *et al*, 1992) which is required for the energy supply for drug transport Posttranslational modification of Pgp

through phosphorylation has been demonstrated for many years (Carlsen *et al*, 1977, Center, 1983, Fine *et al*, 1988, Ma *et al*, 1991) Exposure of cells to substrates for Pgp including chemotherapeutic drugs and chemosensitisers produces hyper-phosphorylation of the molecule (Hamada *et al*, 1987) Pgp can be phosphorylated by a variety of serine/threonine kinases such as protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and calmodulin-dependent protein kinase II PKC has received most attention It has been found that PKC is often increased in MDR cells compared to the parental cells (Aquino *et al*, 1988, Posada *et al*, 1989, Anderson *et al*, 1991), but not in all MDR cell lines (Palyyoor *et al*, 1987) Some data suggested that the effect of phosphorylation of Pgp was to increase the velocity of transport and not the affinity of the transporter for its substrates (Hait *et al*, 1993)

Two distinct human genomic clones were isolated by cross hybridisation with probes from the amplified hamster domain (Rominson *et al*, 1986) One of these clones, human mdr1, identified amplified DNA sequences and a 4 5Kb mRNA overexpressed in MDR cell lines Similarly, using an independently derived hamster mdr probe, a novel set of cDNAs with high homology to human mdr1 were isolated from a human liver cDNA library and designated mdr3 (Van der Bliek *et al*, 1987, 1988) Cross hybridisation and sequence comparisons indicate that the cDNAs isolated from human, murine and hamster libraries are members of a multigene family which encodes the P-glycoprotein (Gros *et al*, 1986, Ueda *et al*, 1986) Comparisons of intron sequences (Ng *et al*, 1989), untranslated sequences (Endicott *et al*, 1987, Hsu *et al*, 1989) and coding regions (Hsu *et al*, 1989, Endicott *et al*, 1991) have identified three classes of mammalian P-glycoprotein as indicated in table 1 3 P-glycoprotein multigene families are clustered in tandem on chromosome 7 in humans (Lincke *et al*, 1991)

Class	I	II	III
Human	mdr1		mdr3
Mouse	mdr3	mdr1	mdr2
Hamster	pgp1	pgp2	pgp3

Table 1.3 Classification and nomenclature of mammalian P-glycoproteins

Substrates for Pgp include adriamycin, daunorubicin, mitoxantrone, colchicine, vinblastine, vincristine, taxol, etoposide, temposide, actinomycin D, verapamil and rhodamine 123 Resistance patterns associated with Pgp overexpression have been described in a vast number of MDR cell lines The typical pattern includes cross-resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids, colchicine, taxol and actinomycin D

The compounds which interact with Pgp are usually small lipophilic cations (Beck, 1990) and are likely to enter the cell by diffusion Pgp may recognise its substrates while they are still present within the lipid bilayer and remove them directly from the plasma membrane Thus, Pgp which acts as an energy-dependent drug efflux pump might itself be responsible for both a decreased influx and increased efflux of drugs

The cellular and tissue distribution of Pgp has been widely studied Immunohistochemical stainmgs have shown high levels of Pgp in human adrenal cortex, kidney and placenta (Sugawara *et al*, 1988a, 1988b) Pgp has also been localised to the apical biliary surface of hepatocytes, the columnar epithelial cells of colon and jejunum (Thiebaut *et al*, 1987) and the apical brush border membrane surface of epithelial cells from renal proximal tubule cells (Lieberman *et al*, 1989) The endothelial cells of human capillary blood vessels at the blood-brain barrier and blood-testis barrier also express Pgp (Cordon-Cardo *et al*, 1989, Thiebaut *et al*, 1989) The expression of Pgp in these tissues suggests either a physiological role in secreting substances from these tissues, such as bilirubin (Gosland *et al*, 1993) or steroid hormones (Ueda *et al*, 1992), or a protective role by excluding potentially toxic xenobiotics or mutagenic chemicals (Ferguson and Baguley, 1993)

A major study by Goldstein *et al* (1989) analysing clinical samples demonstrated elevated Pgp expression in untreated tumours derived from tissues known to normally express Pgp, such as colon cancer, renal cell carcinoma, hepatoma and adrenocortical carcinoma Many other untreated tumours from tissues which do not normally express Pgp had low or undetectable levels of mdr1 mRNA, such as breast cancer, non-small cell lung cancer, gastric cancer, carcinoma of prostate and chronic myelogenous leukaemia

Chan *et al* (1991) conducted a retrospective analysis of tumour biopsies from 67 children with neuroblastoma using immunohistochemical staining for Pgp This study showed a strong association between Pgp expression and lack of chemotherapeutic response and poor

survival Analysis of samples from 30 children with rhabdomyosarcoma showed that while the presence or absence of Pgp expression did not predict for clinical responsiveness since nearly all patients responded, it proved a significant predictor of relapse and overall survival (Chan *et al*, 1990) Therefore, the expression of Pgp was prognostic of the success or failure of therapy for these two types of childhood malignancies Ronmson and co-workers used PCR technique to retrospectively analyze the correlation between low levels of mdr1 mRNA and response to chemotherapy in 100 ovarian and small cell lung cancer patients (Holzmayer *et al*, 1992) A highly significant correlation was found between the absence of PCR detectable gene expression and responsiveness to chemotherapy

Bradley *et al* (1994) studied the expression of Pgp during stepwise progression to rat liver cancer Their results showed that Pgp expression was higher in the large hyperplastic nodules and in hepatocellular carcinoma than in the normal liver These findings suggested that Pgp might be associated with a more progressed malignant phenotype in liver carcinogenesis

1.4 MRP family members

In humans, six members of the multidrug resistance protein (MRP) family, designated MRP1-MRP6, have been identified They are membrane proteins mediatmg the adenosine triphosphate (ATP)-dependent transport of anionic conjugates and amphiphilic anions, such as the bilirubin glucuronosides

1.4.1 MRP1

MRP1 is a 1531-amino acid integral membrane phosphoprotein with a molecular weight of 190kD that is encoded by a 6 5-kilobase mRNA (Cole *et al*, 1992) MRP1 was cloned originally from a multidrug resistant small cell lung cancer cell line H69AR (Cole *et al*, 1992) and has subsequently been found to be overexpressed in a number of drug-selected cell lines derived from a variety of different tumour types (Slovak *et al*, 1995, Zaman *et al*, 1994, Barrand *et al*, 1993, Schneider *et al*, 1995) Computer-assisted analysis of MRP mRNA suggested that it encodes a member of the ABC superfamily of transmembrane transporters The amino acid sequence identity between Pgp and MRP1 is very low (approximately 15%) and restricted to the generally conserved nucleotide binding domains (NBDs) (Cole *et al*, 1992) MRP1 contains two NBDs, each preceded by a multi-spanning

transmembrane region The protein has also been shown to be glycosylated and phosphorylated, primarily on serine residues (Almquist *et al*, 1995, Ma *et al*, 1995) Unmodified MRP1 polypeptide has a molecular weight of 170kD and is processed into a mature 190kD form by the addition of N-linked complex oligosaccharides (Almquist *et al*, 1995) The protein kinases responsible for the phosphorylation of MRP1 have not been identified, although preliminary inhibitor studies have suggested that PKC may be involved (Ma *et al*, 1995) Chromosomal localisation of mrp1 gene is at 16p13 1(Cole *et al*, 1992)

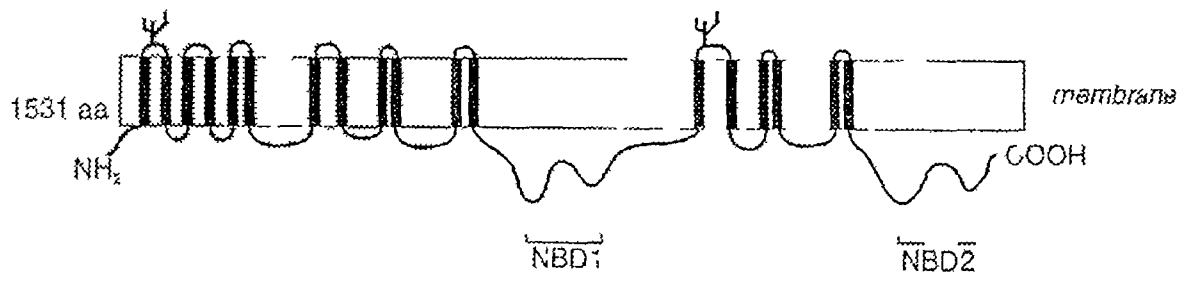


Fig. 1.4.1 A predicted structure of MRP1 Each half of MRP1 consists of several membrane-spanning segments, followed by a NBD (nucleotide binding domain) (Lautier *et al*, 1996)

The pattern of resistance in MRP1 overexpressing cell lines typically includes cross-resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids but not anti-metabolites In contrast to Pgp overexpressing cells, MRP1-transfected HeLa cells are resistant to arsenite, but not to mitoxantrone (Cole *et al*, 1994) The resistance profile of murine NIH 3T3 cells transfected with human MRP1 is similar to that of transfected HeLa cells (Breuninger *et al*, 1995) MRP1-transfected human SW1573 lung cancer cells also have a similar profile except that they are relatively more resistant to colchicine than the anthracycline and are not resistant to arsemte (Zaman *et al*, 1994, 1995) Although it is possible that these differences may be attributed to the cell type used for transfection, they may also be explained by differences in the levels of MRP1 in the various transfectants

drug	Pgp	MRP1	cMOAT(MRP2)	MRP3
Adriamycin	+	+	+	+
Vincristine	+	+	+	+
Vinblastine	+	controversial	+	?
VP-16	+	+	–	+
Taxol	+	–	–	–
Melphalan	–	+	?	?
Cisplatin	–	+	+	+
Cadmium Chloride	–	+	?	?
5-FU	–	–	–	–

Table 1.4.1.1 Chemotherapeutic agents known to be the substrates of Pgp or MRP family members

MRP1 overexpressing cells selected in VP-16, vincristine, epirubicin, and novobiocin have been described (Loe *et al.*, 1996b), as well as cell lines expressing both Pgp and MRP1 (Slapak *et al.*, 1994; Brock *et al.*, 1995; Hasegawa *et al.*, 1995). What determines whether MRP1 or Pgp will be overexpressed is not known. It has been suggested that overexpression of MRP1 may confer initial low levels of resistance, while overexpression of Pgp may appear later, in response to higher drug concentrations. During subsequent selection in higher drug concentrations, expression of MRP1 may decrease or remain constant and overexpression of Pgp may emerge. However, this does not occur in all cases (Lautier *et al.*, 1996).

MRP1 transfectants have been uniformly found to display a decrease in drug accumulation (Cole *et al.*, 1994; Breuninger *et al.*, 1995; Zaman *et al.*, 1994). MRP1 has been reported to be located predominantly in the plasma membrane of several cell lines including MRP transfected HeLa and SW1573 cells, as well as the drug selected cell lines H69AR, COR-L23R and GLC4/ADR (Almquist *et al.*, 1995; Flens *et al.*, 1994; Barrand *et al.*, 1995). The localisation of MRP1 in the plasma membrane supports a role for this protein as an efflux pump. However, in HL60/ADR cells and certain other cells, MRP1 has been reported to be located mainly in the endoplasmic reticulum or associated with cytoplasmic structures,

possibly the Golgi apparatus (Flens *et al*, 1994, 1996, Marquardt *et al*, 1992, Krishnamachary *et al*, 1993) Whether the localisation of MRP1 in all of these intracellular compartments contributes to the drug resistance phenotype is unknown

There is now considerable evidence that MRP1 can function as a high affinity transporter of cysteinyl leukotrienes, most notably the GSH-conjugate LTC_4 (Leier *et al*, 1994, Muller *et al*, 1994, Loe *et al*, 1996a) This arachidonic acid derivative and its metabolites, LTD_4 and LTE_4, are active components of the "slow reacting substance of anaphylaxis" They are involved in the control of vascular permeability and smooth muscle contraction and have been implicated in the pathogenesis of asthma (Hay *et al*, 1995) Several other potential substrates for MRP1 have also been identified by direct transport studies, among them are glutathione disulfide (oxidised glutathione) and steroid glucuromdes such as 17β-estradiol 17-(β-D glucuronide) (Leier *et al*, 1996, Jedhtschky *et al*, 1996) Many organic anions have been shown to act as competitive inhibitors of either LTC_4 or 17β-estradiol 17-(β-D glucuronide) transport These include a wide range of anionic conjugates of bile salts and steroid hormones (Leier *et al*, 1996, Jedlitschky *et al*, 1996, Loe *et al*, 1996) The broad substrate specificity of MRP1 for organic anions prompted the suggestion that MRP1 may be the multispecific organic anion transporter (MOAT) In rodent liver, cMOAT (canalicular multispecific organic anion transporter) mediates the biliary excretion of endogenous and exogenous compounds following their modification by phase II conjugating enzymes to form amphiphilic anions Despite certain common pharmacologic features of MRP1- and MOAT-mediated transport, evidence indicated that they are different proteins due to the cloning of a novel cMOAT from rat liver (Paulusma *et al*, 1996)

Although MRP1 has been shown to reduce cellular accumulation of some drugs to which it confers resistance, such as daunorubicin, VP-16 and vincristine, it has not been possible to demonstrate direct transport of these compounds or other unmodified chemotherapeutic drugs by MRP1-enriched membrane vesicles (Muller *et al*, 1994, Loe *et al*, 1996a, Jedlitschky *et al*, 1996) These chemotherapeutic agents have also been found to be very poor inhibitors of LTC4 transport (Muller *et al*, 1994, Loe *et al*, 1996a) These observations, combined with the demonstrated ability of MRP1 to transport GSH-conjugate LTC_4, led to the suggestion that MRP1 reduced drug accumulation, not by transporting unmodified drugs directly, but by effluxing drugs after their conjugation to GSH However, GS-conjugates of daunorubicin or

vincristine are not detectable in the culture medium of MRP1-overexpressing cells exposed to either of these two drugs (Zaman *et al*, 1995) Alternative possibilities currently under investigation are that MRP1 can co-transport GSH and drug without conjugate formation or that GSH facilitates drug transport by an as yet undefined mechanism

MRP1 expression has been detected in a wide range of non-selected human tumour cell lines, including those derived from small cell and non-small cell lung, colon, gastric, breast, ovarian carcinomas, neuroblastoma, thyroid and glioma (Loe *et al*, 1996, Nooter *et al*, 1995, Kruh *et al*, 1995) Expression of MRP1 has also been detected in tumour samples from patients with chronic and acute leukaemias

Verapamil and cyclosporin A, which are well characterised inhibitors of Pgp, usually show effect on drug sensitivity and transport in drug selected or MRP1-transfected cells (see section 4 4) Compounds reported to reverse MRP1-associated resistance to a significant degree in at least one model system include dihydropyridine nicardipine and NIK 250 (Cole *et al*, 1989, Abe *et al*, 1995), difloxacin (Gollapudi *et al*, 1995), the bisindolymaleimide GF109203X (Gekeler *et al*, 1995a), MK571 (Gekeler *et al*, 1995b) and the cyclosporin analogue PSC 833 (Barrand *et al*, 1993) Sensitisation by these compounds is often dependent on the drug and type of cells tested

The observation that membrane vesicles from MRP overexpressing cells could transport the glutathione conjugate LTC4 suggested that intracellular GSH levels might be involved in MRP1-associated drug resistance The levels of GSH in transfected and drug-selected MRP1 cells relative to their sensitive parental cells vary considerably (Zaman *et al*, 1995, Versantvoott *et al*, 1995a, Cole *et al*, 1990) The ability of the γ-glutamylcysteine synthetase inhibitor BSO (which results in depletion of GSH) to circumvent MRP1-mediated MDR varies greatly as well (Zaman *et al*, 1995, Gekeler *et al*, 1995b, Cole *et al*, 1990, Schneider *et al*, 1995, Davey *et al*, 1995) The basis of this variability is not understood

1.4.2 cMOAT (MRP2)

An isoform of MRP1, with a similar substrate specificity and a distinct sequence, has been cloned and localised predominantly to the hepatocyte canalicular membrane (Buchler *et al*, 1996, Paulusma *et al*, 1996, Keppler and Kartenbeck, 1996, Keppler and Konig, 1997) This

isoform has been termed cMOAT (canalicular multispecific organic anion transporter) or MRP2 cMOAT mediates the ATP-dependent transport of glutathione S-conjugates and glucuronides from the liver into bile (Keppler and Konig, 1997, Jedlitschky *et al*, 1997) and across the apical membrane of kidney proximal tubules (Schaub *et al*, 1997)

Human cMOAT is composed of 1545 amino acids Comparison between human MRP1 and cMOAT indicates an overall amino acid identity of 49%, where the highest degree of identity is in the carboxyl-terminal domain and in nucleotide-binding domains (Buchler *et al*, 1996) The molecular weight of unglycosylated cMOAT is 174kD (Taniguchi *et al*, 1996) It is N-glycosylated in its mature form and has 190kD molecular weight Chromosomal localisation of cmoat gene is at 10q24 In polarised epithelial cells, such as hepatocytes, cMOAT is strictly locahsed to the apical domains of hepatocytes (Buchler *et al*, 1996) and proximal tubule epithelial cells of the kidney (Schaub *et al*, 1997)

The substrate specificity of cMOAT has been studied most extensively in inside-out hepatocyte canalicular membrane vesicles from normal rat liver in comparison with membrane vesicles from mutant rats lacking cMOAT (Ishikawa *et al*, 1990, Buchler *et al*, 1996) cMOAT has a similar spectrum of substrates as MRP1 including LTC_4, LTD_4, 17β-glucuronosyl estradiol, oxidized glutathione (GSSG) and monoglucuronosyl bilirubin Substrate specificity differences between MRP1 and cMOAT are indicated by a higher affinity and transport rate of MRP1 for LTC_4 and by the preferential transport of monoglucuronosyl bilirubin and bisglucuronosyl bilirubin by cMOAT (Jedlitschky *et al*, 1997)

The human Dubin-Johnson syndrome is a rare autosomal recessive liver disorder Patients have impaired hepatobiliary transport of bile salt organic anions (reviewed by Roy Chowdhury *et al*, 1994) The absence of cMOAT is the molecular basis of this hereditary disease (Buchler *et al*, 1996, Kartenbeck *et al*, 1996) A highly similar phenotype has been described for a mutant Wistar rat strain, the transport deficient (TR^-) rat, which is defective in cMOAT These mutant animals lack the hepatobiliary excretion of many organic anions including bilirubin-glucuronide, cysteinyl-leukotrienes and some divalent bile salt conjugates (Paulusma *et al*, 1996)

Overexpression of cMOAT has been linked with resistance to vinblastine (Evers *et al*, 1998), cisplatin, vincristine, adriamycin and camptothecin derivatives, but not to etoposide, 5-FU and mitomycin C (Koike *et al*, 1997, Taniguchi *et al*, 1996)

1.4.3 MRP3

In addition to MRP1 and cMOAT (MRP2), other MRP homologues encoding GS-X pumps might be present in the human genome, considering that there are at least four MRP homologues expressed in the soil nematode *caenorhabditis elegans* (Broeks *et al*, 1996) Boguski *et al* (1996) therefore searched the expressed sequence tag (EST) library for putative human MRP homologues and found three MRP homologues which were designated MRP3, MRP4 and MRP5 in humans

Like the GS-X pumps MRP1 and cMOAT, MRP3 is also an organic anion and multidrug transporter Of all the MRP family members, MRP3 is closest to MRP1 (58% ammo acid identity) (Kool *et al*, 1999) In Madin-Darby canine kidney II cells, MRP3 is located on the basolateral membrane and mediates organic anion S-(2,4-dinitrophenyl-)glutathione transport (Kool *et al*, 1999) MRP3 is also localised to the basolateral membrane of human hepatocytes (Konig *et al*, 1999) It is composed of 1527 amino acids and encoded by 4581 base pairs of cDNA The MRP3 gene is located on chromosome 17 (Kool *et al*, 1997) To date, MRP3 has been found to be expressed in the liver, colon, pancreas, kidney (Konig *et al*, 1999), intestine and adrenal gland (Kool *et al*, 1999)

Overexpression of MRP3 has been demonstrated in several adriamycin-resistant sublines of the SW1573/S1 human non-small-cell lung cancer cell line and also in the cisplatm-resistant HCT8/DDP colon carcinoma cell line (Kool *et al*, 1997) In ovarian carcinoma cells (2008), MRP3 overexpression results in low-level resistance to the epipodophyllotoxins etoposide and teniposide and high-level resistance to methotrexate (Kool *et al*, 1999) Studies have shown that the MRP3-overexpressing cell line 2008 and Madin-Darby canine kidney II cell line do not show increased glutathione export or decreased levels of intracellular glutathione, in contrast to cells overexpressing MRP1 or cMOAT (Kool *et al*, 1999)

1.4.4 MRP4-6

Not much is known about the substrate specificity of MRP4, MRP5 or MRP6 MRP4 and MRP5 genes are located on chromosome 13 and 3 respectively (Kool *et al*, 1997) In a panel of human cell lines derived from various tissues and their resistant sublines (selected with either adriamycin, cisplatin, tetraplatin or cadmium chloride), MRP4 was expressed at very low levels in the parental cells and no overexpression of MRP4 was detected in resistant sublmes In contrast, MRP5 was found to be highly expressed in all the parental cell lines, with the most significant mcrease observed in the lung adenocarcinoma cell line MOR/P and ovarian carcinoma cell line 2008 However, similar to the results obtained with MRP4, no significant MRP5 overexpression was noted in the resistant cells A slight increase of MRP5 RNA was detected in three cisplatin-resistant cell lines, T24 DDP10 (bladder carcmoma cell lme), HCT8/DDP (colon carcmoma cell line) and KCP-4 (epidermoid carcinoma cell lme) (Kool *et al*, 1997) MRP4 is also reported to be expressed at a low level in a number of tissue types whereas MRP5 was detected in substantial amounts in every tissue tested, with relatively high expression in skeletal muscle and brain (Kool *et al*, 1997) The function of MRP4 and MRP5 remains unknown It seems that MRP4 may not be correlated to multidrug resistance

Overexpression of the MRP6 gene in MDR cells is invariably associated with the amplification of the adjacent MRP1 gene and MRP6 probably does not contribute to resistance (Kool *et al*, 1999)

1.5 Alternative mechanisms of MDR

1.5.1 Lung resistance-related protein (LRP)

Lung resistance-related protein (LRP) is a 110kD protein which was identified in the adriamycin-resistant non-small cell lung cancer cell line SW1573/2R120 m 1993 (Scheper *et al*, 1993) Since its identification, LRP has been found to be overexpressed in a large number of MDR cell lines indicating that LRP overexpression is a frequent feature in non-Pgp mediated MDR LRP overexpressing MDR cell lines include the small cell lung cancer cell line GLC4/ADR, the fibrosarcoma cell lme HT1080/DR4, the breast cancer cell lines MCF7/Mitox and MCF7/MR and the myeloma cell lme 8226/MR40 (Scheper *et al*, 1993) Up-regulation of LRP has been observed early during the process of drug selection in various

cell lines This observation suggests that the LRP-associated mechanism might be involved in low or moderate levels of drug resistance (Izquierdo *et al*, 1995)

Comparative sequence analysis indicated that LRP shares 57% and 87 7% ammo acid identity with the major vault proteins from *Dictyostelium Discoideum* (Kedersha *et al*, 1993) and *Rattus Norvigecus* (Kickhoefer *et al*, 1994) respectively Thus, LRP was identified as the human major vault protein (Scheffer *et al*, 1995) which is the component of cellular organelles termed vaults (Rome *et al*, 1991, Kedersha *et al*, 1986)

Vaults were first identified by negative staining and transmission electron microscopy in 1986 (Kedersha *et al*, 1986) The term "vaults" was chosen by Rome *et al* (1991) to describe the morphology of the particles which consist of multiple arches similar to those from cathedral vaults Vaults are complex ribonucleoprotein particles which, in the rat, are composed of a major vault protein of 104kD (accounting for > 70% of the mass of the particle), three minor proteins of 210, 192 and 54 kD and a small RNA molecule (Rome *et al*, 1991, Kedersha *et al*, 1986, 1991) The vault particle has 2-fold symmetry and each half can be opened into a flower-like structure which contains eight petals surrounding a central ring (Kedersha *et al*, 1991) To date, the function of vaults is unknown Most vaults are present in the cytoplasm but a small fraction of vaults are localised to the nuclear membrane and nuclear pore complexes (Chugani *et al*, 1993) where the vaults were hypothesised to constitute the central plugs of the nuclear pore complex so that they might protect the nucleus from nuclear toxins Transfection of only the LRP gene itself, coding for one human major vault protein, has failed to confer MDR (Scheffer *et al*, 1995) This finding indicates that the complete vault particle is required to carry out functional activity

The concomitant overexpression of LRP and MRP appears to be a frequent event in non-Pgp MDR cell lines such as the SW1573/2R120 and GLC4/ADR cell lines The LRP gene has been localised to the short arm of chromosome 16 (16p11 2), which is proximal to the MRP1 gene (16p13 1) This raised the possibility that the LRP gene was simply co-amplified with the MRP1 gene However, this theory was eliminated when amplification of the MRP gene, but not of the LRP gene, was found in SW1573/2R120 and GLC4/ADR cells (Slovak *et al*, 1995) Furthermore, in HT1080/DR4 cells, both LRP and MRP1 genes were amplified m the homogeneously stained region (hsr), but only the MRP1 genes were contained within hsr (Slovak *et al*, 1995) These results indicated that although both the MRP1 and LRP genes

map to the short arm of chromosome 16, they are rarely co-amplified and are not normally located within the same amplicon

LRP and Pgp are rarely simultaneously overexpressed in drug-selected MDR cells such as non-small lung cancer SW1573/2R160, the ovarian carcinoma A2780AD and the myeloma 8226/Dox6 and 8226/Dox40 cell lines which are Pgp-positive but LRP-negative (Scheper *et al*, 1993) LRP overexpression has also been reported in Pgp/MRP-negative MDR cell lines such as the mitoxantrone selected MCF7/MR cell line (Futscher *et al*, 1994)

High LRP expression has been shown in epithelia of the bronchus, digestive tract, keratinocytes, adrenal cortex and macrophages (Izquierdo *et al*, 1995) Relatively high expression has also been found in proximal tubules of the kidney, transitional urothelium and ductal pancreatic cells (Izquierdo *et al*, 1995) Studies have also shown that colon, renal, pancreatic and endometrial cancers express high levels of LRP whereas low levels of LRP were noted in germ cell tumours, Wilm's tumours, rhabdomyosarcoma, Ewing's sarcoma and acute myeloid leukaemia (Izquierdo *et al*, 1995) A report on the expression of LRP in 42 lung cancer specimens showed LRP positivity in 83% of the squamous cell carcinomas, 59% of adenocarcinomas and in 36% of large cell undifferentiated carcinomas In contrast, only 5% of the small cell lung cancers expressed LRP (Dingemans *et al*, 1995)

1.5.2 Topoisomerase II

Among the cytotoxic drugs currently used, a number are active on specific nuclear enzymes called topoisomerases The function of topoisomerase is to regulate the DNA topology, transposition, repartition, recombination, replication, transcription and other DNA processes (Liu, 1983, Kafiani *et al*, 1986, Zijlstra *et al*, 1990) Two types of topoisomerases exist one capable of introducing single-stranded breaks (Topo I), the other capable of producing double-stranded breaks (Topo II) in the DNA molecules (Wang, 1985, D'arpa, 1989)

Two distinct isoforms of Topo II designated topoisomerase α and β with molecular weights of 170 and 180 kD respectively have been isolated (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 regard to cell proliferation The

level of topoisomerase II α 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 (Heck *et al*, 1988) In contrast, the level of topoisomerase II β does not appear to vary greatly throughout the cell cycle (Woessner *et al*, 1991) Topoisomerase II α 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 (Drake *et al*, 1987)

The mechanism of action of topoisomerase II is as follows the recognition and binding of the topoisomerase enzyme to DNA, the cleavage of the first double strand of the DNA, the passage of a second double strand of DNA through the break, the religation of the strand-cleaved DNA, and the ATP-dependent turnover of the topoisomerase II (Osheroff *et al*, 1986) Topoisomerase II is the target for anthracyclines, epipodophyllotoxins, quinolones, coumarins and acridines, all examples of topoisomerase II inhibitors Topoisomerase II inhibitors are thought to kill cells by binding to the topoisomerase II enzyme which then binds to DNA forming a complex which halts the catalytic activity of topoisomerase II prior to the religation of the cleaved DNA intermediate (Robinson and Osheroff, 1990) The stabilised intermediate complexes form barriers to DNA fork progression and thus prevent DNA replication (Liu, 1989)

Two mechanisms of multiple drug resistance are associated with topoisomerase II In classical multidrug resistance, inhibitors are actively effluxed from cells by P-glycoprotein In atypical multidrug resistance, topoisomerase II is either reduced or mutated to a form that does not interact with the inhibitor Therefore any treatment that increases the number of active topoisomerase II molecules per cell could partially circumvent this type of resistance Several types of agonist interactions at cell surfaces have been reported to induce transient increases in the cellular activities of topoisomerase II These include arginine, vasopressin, thrombin, leukotriene D_4 and tumour necrosis factor (Nambi *et al*, 1989, Mattern *et al*, 1990 and 1991, Utsugi *et al*, 1990) These activations are likely to be related to signal transduction pathways that result in phosphorylation and activation of topoisomerases e g by activated protein kinase C or cyclic nucleotide-dependent kinases

Modulation of topoisomerase II may involve phosphorylation of the enzyme It has been shown that topoisomerase II is phosphorylated at specific serine residues, resulting in an increase in enzyme activity (Fry and Hickson, 1993) 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) On the other hand, the activity of topoisomerase I and topoisomerase II is regulated in a way that maintains cellular homeostasis with respect to higher order DNA structure *i e* the amount of change in DNA tertiary structure that is catalysed by topoisomerase I in a given cell is balanced by the amount of change m DNA tertiary structure catalysed by topoisomerase II (Gellert *et al*, 1982) Consequently a deficit in topoisomerase II activity might be partially offset by an increase in topoisomerase I activity This suggests that combination treatment with topoisomerase I and topoisomerase II inhibitors may have the potential to prevent topoisomerase-mediated multidrug resistance

1.5.3 Glutathione and Glutathione S-transferase

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, catalysed 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

The glutathione S-transferases (GST) are a family of multifunctional cellular enzymes first discovered in the early 1960s (Booth *et al*, 1961) It has long been known that GST enzymes are an integral part of the phase I (oxidation)/ phase II (conjugation) systems that metabolise 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 catalyse the conjugation of glutathione, through the sulphur atom of its cysteine residue, to various electrophiles, forming stable secretory metabolites (Jakoby, 1978) GSTs have been localised in human kidney, lung, brain, intestine, skeletal muscle and adrenal glands (Tsuchida and Sata, 1992) There are three main classes of cytosolic GST π, α and μ,

their classification being based on the enzymes N-terminal amino acid sequence, 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

MCF-7 human breast cancer cells have been found to be a good model to examine the effects of GST on drug resistance since the parental cell line has remarkably low levels of GST activity (Moscow *et al*, 1989) In a number of clones of MCF-7 transfected with individual GST cDNAs, the increased levels of GST isozyme failed to confer significant levels of resistance to any of the anticancer drugs tested Thus, it seemed that increased activity of GST itself is not sufficient for conferring MDR

A number of other studies have shown that GST is often expressed at remarkably high levels in many types of tumours when compared to GST levels in normal tissues of origin (Shea *et al*, 1988, Moscow *et al*, 1989, Peter *et al*, 1990 and 1992, Moorghen *et al*, 1991) This is particularly true of gastrointestinal malignancies (Peters *et al*, 1990 and 1992, Howie *et al*, 1989) Studies relating GST expression to clinical outcome in leukaemia have suggested that GST isozyme expression may be a prognostic marker in leukaemia (Tidefelt *et al*, 1992) In breast cancer, the idea that GST expression might be a useful prognostic marker arose from the observation that GST expression is inversely correlated with estrogen receptor status (Moscow *et al*, 1988, Howie *et al*, 1989)

Overall, there is no consistent or compelling evidence that GSH and GST augment resistance in multidrug resistant cell lines

1.5.4 Metallothionein (MT)

The discovery of MT (Margoshes and Vallee, 1957) resulted from the search for a biological role for cadmium (Cd) Metallothioneins are a family of ubiquitous metal binding proteins The mammalian MTs have a molecular weight of 6000-7000 daltons, usually containing 61 or 62 amino acids including 20 cysteines The cysteinyl thiolate side chains permit MTs to bind a wide spectrum of heavy metals, most notably Zn, Cd, Cu, Pt and Hg In mammals, MTs bind zinc predominantly, but zinc can be readily displaced by copper and cadmium Humans have at least 16 MT genes clustered on chromosome 16 (West *et al*, 1990) Four classes of MTs have been found MTI and II are expressed at all stages of development in many cell types of most organs, MT III is expressed predominantly in neurons but also in glia

and male reproductive organs (Masters *et al*, 1994, Uchida *et al*, 1991, Moffatt *et al*, 1998) and MT IV is expressed in differentiating stratified squamous epithelial cells (Quaife *et al*, 1994)

The physiological functions of metallothioneins include heavy metal detoxification, regulation of intracellular Zn, Cu and Cd levels, free radical scavenging, protection from ionizing radiation and control of growth and differentiation Induction of MTs has been widely studied both *in vivo* and in cell culture Intracellular MTs content can be increased by heavy metals, alkylating agents, stress, infection, interleukin-1, interferon, estrogens, progesterone, glucocorticoids, glucagon, etc (Lazo and Basu, 1991)

MTs serve as reservoirs for essential metals while preventing metal toxicity MT can donate metal to metal-free metalloprotein (apo MT or thionein) Thus the fully metal-complexed MT serves as a metal donor, and thionein acts as a chelating agent, most notably is in the case of Zn Micro-injection of apoMT into living cells has been shown to be capable of removing zinc from zinc finger DNA binding proteins, such as SP-1 and transciption factor IIIA (Zeng *et al*, 1991a, 1991b) Apo MT also has the potential to remove zinc from p53 and inactivate it similarly to other zinc chelators (Hainaut *et al*, 1993, 1996, Oren *et al*, 1996) This suggests that persistent apo MT overexpression in tumour cells, if present, may promote their accelerated growth and increased survival through induction of a p53-null state The recently observed capacity of MT-specific anti-sense oligonucleotides to cause both cell growth arrest and induce apoptosis in ductal breast carcinoma cells supports this hypothesis (Mageed *et al*, 1996)

Heavy metal detoxification and rendering resistance to some electrophilic drugs are the most conclusively supported roles for MTs The first hne of defence against influx of heavy metals appears to be rapid metal efflux Induction of MT and sequestration of these metals by MT provides a secondary line of defence Various cell lines that cannot synthesise any MT are sensitive to cadmium toxicity, whereas cells that express excess amounts of MT are resistant to this metal Selection for cadmium resistance with mammalian cells resulted in up to 80-fold amplification of the entire MT locus (Durnam and Palmiter, 1987)

There is now considerable evidence that MT has some role in determining the responsiveness of cells to electrophilic drugs which can react with the cysteines of MT Studies indicate that cells with an increase in metallothionein levels are more resistant to some anticancer drugs (See Table 1 4 6)

Agent	Reference
Cisplatin	Andrews *et al*, 1987
Melphalan	Yu *et al*, 1995
Chlorambucil	Endresen *et al*, 1983
Adriamycin	Webber et al, 1988
Bleomycin	Kondo *et al*, 1995
Cytarabine	Kondo *et al*, 1995
Cyclophosphamide	Satoh *et al*, 1994
Predmmustme	Endresen *et al*, 1984
Mitomycin C	Lohrer *et al*, 1989

Table 1 4 6 Anticancer drug resistance and metallothionein overexpression

Direct evidence that MT is important in resistance to electrophilic drugs was obtained by DNA transfection studies in which the human MT II gene was introduced into mouse cells on a bovine papilloma virus vector The resulting transfectants were resistant to cisplatin, chlorambucil and melphalan but not to 5-FU or vincristine (Kelley *et al*, 1988) The effect of the loss of MT expression on the cytotoxicity of anticancer agents using transgenic mice with targeted disruptions of MT I and II genes (MT -/-) has been examined MT -/- cells expressed no detectable MT compared to wild type cells, and showed enhanced sensitivity to cisplatin, melphalan, bleomycin and cytarabine (Kondo *et al*, 1995)

1.6 MDR modulators

A major goal in experimental as well as clinical investigation of drug resistance is to discover unique methods by which to reserve or circumvent it The first report of the pharmacological reversal of MDR was from Tsuruo and his colleagues (1981) It was demonstrated that the calcium channel blocker, verapamil, and the calmodulin antagonist, trifluoperazine, greatly

potentiated the antiproliferative activity of vincristine and produced an increased cellular accumulation of vincristine in an MDR murine leukaemia cell line *in vitro* and *in vivo*. Since this original observation, many compounds have been shown to antagonize MDR and are termed "chemosensitisers" or "resistance modifiers". These compounds alter the drug accumulation defect in MDR cells, usually without completely reversing it, and cause little or no potentiation of drug cytotoxicity in sensitive cells.

The chemosensitisers described to date are grouped into six broad categories: (1) calcium channel blockers, (2) cyclosporins, (3) calmodulin antagonists, (4) noncytotoxic anthracycline and vinca alkaloid analogues, (5) steroids and hormonal analogues, (6) miscellaneous hydrophobic, cationic compounds. Although these compounds share only broad structural similarities, all are extremely lipophilic, and many are heterocyclic, positively charged substances.

1.6.1 Verapamil

Tsuruo's group initially analysed the effects of the calcium channel blocker verapamil in a vincristine resistant murine leukaemia cell line P388 (Tsuruo *et al.*, 1981). It was shown that verapamil reversed approximately 30-fold resistance to vincristine and 7-fold resistance to vinblastine in these P388/VCR cells. Incubation of P388/VCR cells with verapamil for 5 hours also caused a 10-fold increase in the accumulation of [^{3}H]-vincristine. Since verapamil was shown not to alter vincristine binding to tubulin, the target of vinca alkaloids, it was concluded that the chemosensitising effect of verapamil was due to alterations in drug accumulation. These studies also demonstrated the effect of verapamil in a MDR human acute myelogenous leukaemia (AML) cell line K562/VCR and adriamycin resistant P388/ADM cell line (Tsuruo *et al.*, 1981).

Since these early observations, many investigators have demonstrated the chemosensitising activity of verapamil in various MDR cell lines. The sensitivity to cytotoxic drugs of most sensitive cells from which these MDR cell lines were derived was not significantly affected by verapamil.

The effect of verapamil on cross-resistance to chemotherapeutic drugs other than those used for initial selection has also been investigated. In some studies, verapamil produced as great an effect on cross-resistance to drugs as on primary resistance to the selecting agent. In a

human uterine sarcoma cell line, MES-SA selected for 100-fold resistance to adriamycin, 6μM verapamil caused a 7-fold sensitisation to adriamycin and a similar magnitude of reversal of cross-resistance to daunomycin, actinomycin D and mitoxantrone (Harker *et al.*, 1986; Sikic *et al.*, 1989). In other studies, verapamil has been found to be more effective in reversing resistance to the selecting agents than to the other cross resistant drugs. For example, 5μM verapamil caused a 13-fold reversal of 200-fold adriamycin resistance in the MCF-7 Adr^R cells, but resulted in only a 4-fold change in the 400-fold cross-resistance to colchicine (Ford *et al.*, 1990). In contrast, verapamil has also been shown to have a greater effect on cross-resistance than on primary resistance to selecting drugs in some cell lines. Fojo *et al.* (1985) demonstrated that 20μM verapamil completely reversed the 20- to 70-fold cross-resistance to adriamycin, vinblastine and vincristine in colchicine selected KB human epidermal carcinoma cells, while causing only a 60-fold reduction in the 220-fold resistance to colchicine.

These findings are of interest with respect to both the mechanism and pharmacology of the chemosensitising activity of verapamil and in defining the mechanisms by which Pgp transports structurally distinct molecules. It is unclear why some cross-resistance is refractory to modulation by verapamil. One possibility is that mutations in the mdr1 gene (Choi *et al.*, 1988) or posttranslational modifications of Pgp (Hait and Aftab, 1992) may cause chemosensitisers for the putative drug binding sites. Alternatively, multiple drug binding sites within Pgp could explain these phenomena. Other studies performed in several different MRP1-overexpressing cell lines including small cell lung cancer cell line H69AR, fibrosarcoma cell line HT-1080/DR4 and large cell lung cancer cells COR-L23R showed that verapamil was effective as a chemosensitiser (Germann *et al.*, 1997; Barrand *et al.*, 1993; Cole *et al.*, 1989), suggesting that the mechanisms by which verapamil circumvents MRP1-mediated drug resistance may be distinct from those in Pgp-mediated drug resistance.

Kessel and Wilberding (1985) studied the effect of verapamil on the cellular kinetics of anthracycline in P388/ADR cells. Drug influx was not altered by verapamil, consistent with a diffusional model of anthracycline inward transport while daunomycin efflux from P388/ADR cells was inhibited by varying degrees by 2 to 20μM verapamil. It has been clearly shown that verapamil inhibits the Pgp associated, energy-dependent drug efflux in the MDR cells. A number of investigators have shown that photoactivated verapamil analogues

bind to Pgp and that verapamil inhibits the binding of many chemotherapeutic drugs as well as other chemosensitisers to Pgp (Akiyama *et al*, 1988, Beck *et al*, 1988, Cornwell *et al*, 1987, Safa, 1988a, 1988b, 1987)

1.6.2 Cyclosporin A

The immunosuppressive drug, cyclosporin A, a hydrophobic cyclic peptide of 11 amino acids, was first investigated for potential anti-MDR activity in part due to reports of its calmodulin binding properties (Colombam *et al*, 1985), though it was later shown that cyclosporin A does not specifically inhibit calmodulin-mediated processes (Hait *et al*, 1987)

Slater *et al* (1986) first studied the effect of cyclosporin A in MDR cells and found that it caused a 3- to 4-fold potentiation of daunomycin toxicity in Ehrlich ascites cells that had a very low level of resistance (2-fold) to daunomycin, as well as causing a 2-fold potentiation of daunomycin cytotoxicity in sensitive Ehrlich ascites cells Since then, several groups have shown that cyclosporin A reverses resistance and cross-resistance in MDR cell lines (Hait *et al*, 1989, Twentyman, 1988, 1987) Cyclosporin A enhanced the sensitivity to adriamycin in MDR P388/DOX cells, but had no effect in the P388 cells, although a small increase in adriamycin accumulation was seen in both cell lines (Hait *et al*, 1989) Chambers *et al* (1989) demonstrated an 11-fold enhancement of adriamycin toxicity in sensitive Chinese hamster ovary AuxB1 cells and a 62-fold enhancement in colchicine-resistant MDR CH^RC5 cells caused by cyclosporin A However, when the effect of cyclosporin A on drug accumulation was studied, it was found that cyclosporin A increased adriamycin accumulation in the sensitive cells, but had no effect on drug accumulation in the MDR cell line It has also been found that 0 5-3μM cyclosporin A partially reversed the accumulation defect of daunomycm in another Chinese hamster ovary MDR cell line, CH^RB3, but did not affect daunomycin accumulation in sensitive AuxB1 cells (Silbermann *et al*, 1989) These findings suggest that the mechanism of action of cyclosporin A as a chemosensitiser may be more complex than verapamil It may not be due only to modification of Pgp mediated drug transport, in accord with its known activity as an inhibitor of many important cellular enzymes In fact, cyclosporin A is the inhibitor of protein kinase C (PKC) which can phosphorylate Pgp and thus increase the velocity of drug transport of Pgp (Aftab *et al*, 1991)

Tamai and Safa (1991) reported kinetic analyses of the effect of cyclosporin A on drug accumulation 0 5μM cyclosporin A inhibited the photoaffinity labelling of Pgp by a derivative of vinblastine by approximately 50% This study suggests that cyclosporin A reverses MDR at least in part by interacting with Pgp and inhibiting outward transport of cytotoxic drugs However, excess verapamil was incapable of inhibiting cyclosporin A transport, indicating either that cyclosporin A does not directly interact with Pgp or that these chemosensitisers bind at different sites to Pgp

1.7 The cytoskeleton

The cytoskeleton is a complex network of protein filaments that extends throughout the cytoplasm of eucaryotic cells Unlike a skeleton made of bone, the cytoskeleton is a highly dynamic structure that reorganises continuously The cytoskeleton allows cells to adopt a variety of shapes and to carry out coordinated and directed movements It also provides the machinery for intracellular movements, such as the transport of organelles and the segregation of chromosomes at mitosis The cytoskeleton is absent from bacteria, and it may have been a crucial factor in the evolution of eucaryotic cells

The diverse activities of the cytoskeleton depend on three types of protein filaments, micro filaments, microtubules, and intermediate filaments Each type of filament is formed from a different protein subunit actin for microfilament, tubulin for microtubules, and a family of related fibrous proteins, such as cytokeratin or vimentin, for intermediate filaments

1.7.1 Microtubules

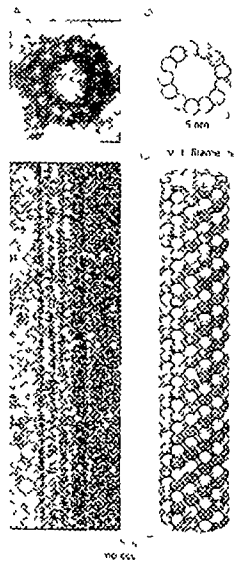


Fig. 1.7.1 A side view of a microtubule with the tubulin molecules aligned into long parallel rows Each of the 13 protofilaments is composed of a series of tubulin molecules (α/β heterodimers)

Microtubules are formed from molecules of tubulin, each of which is a heterodimer consisting of two closely related and tightly linked globular polypeptides called α-tubulin and β-tubulin (Amos *et al* , 1979) Although tubulin is present in virtually all eucaryotic cells, the most abundant source for biochemical studies is the vertebrate brain Extraction procedures yield 10 to 20% of the total soluble protein as tubulm in brain, reflecting the unusually high density of microtubules in the elongated processes of nerve cells (Wade *et al* , 1993) A microtubule is a cylindrical structure in which the tubulin heterodimers are packed around a central core The structure is built from 13 linear protofilaments, each composed of alternating α–and β-tubuhn subunits and bundled in parallel to form a cylinder (Amos *et al* , 1979) Tubulin molecules are diverse In mammals there are at least six forms of α-tubulin and a similar number for β-tubulin, each encoded by a different gene (Sullivan, 1988) The different forms of tubulin are very similar, and will generally copolymerise into mixed microtubules in test tubes The microtubule is a polar structure If purified tubulin molecules are allowed to polymerise for a short time at the ends of fragments of stable microtubules, one end can be seen to elongate at three times the rate of the other The fast growing end is thereby defined as the plus end and the other as the minus end (Bergen *et al* , 1980)

The mitotic spindle, which forms after the cytoplasmic microtubules disassemble at the onset of mitosis, is the target for a variety of specific antimitotic drugs that act by interfering with

the exchange of tubulin subunits between the microtubules and the free tubulin pool (Inoue, 1981) One such antimitotic drug is colchicine (Salmon *et al*, 1984) Each molecule of colchicine binds tightly to one tubulin molecule and prevents its polymerisation However, it cannot bind to tubulin once the tubulin has polymerised into a microtubule Exposure of a dividing cell to colchicine causes the rapid disappearance of the mitotic spindle which kills many dividing cells The effects of vinblastine and vincristine are similar to those of colchicine Taxol, however, has the opposite effect by causing much of the free tubulin to assemble into microtubules and then stabilises them The stabilisation of microtubules by taxol arrests dividing cells in mitosis

The microtubules in the cytoplasm of interphase cells in culture can be visualised by staining the cells with fluorescent anti-tubulin antibodies following fixation The microtubules are seen in greatest density around the nucleus and radiate out into the cell periphery in fine lace-like threads The centrosome is the major microtubule-organising center in almost all animal cells (Glover *et al*, 1993) In interphase, it is typically located to one side of the nucleus, close to the outer surface of the nuclear envelope Embedded in the centrosome is a pair of cylindrical structures called centrioles arranged at right angles to each other in an L-shaped configuration The centrosome duplicates and splits into two equal parts during mterphase, each half containing a duplicated centriole pair These two daughter centrosomes move to opposite sides of the nucleus when mitosis begins, and form the two poles of the mitotic spindle (Mazia, 1984) The centrosome contains a number of centrosome-specific proteins, including a special minor form of tubulin, γ-tubulin, which may interact with the normal α/β tubulin dimer to help nucleate microtubules (Joshi *et al*, 1992)

Individual microtubules grow toward the cell periphery at a constant rate for some period after which they suddenly shrink rapidly back toward the centrosome They may shrink partially and then recommence growing, or they may disappear completely, to be replaced by a different microtubule (Sammak *et al*, 1988) This behaviour, called dynamic instability, plays a major role in positioning microtubules in the cell and provides an organising principle for cell morphogenesis (Mitchison *et al*, 1991) The dynamic instability of microtubules requires an input of energy which is generated from the hydrolysis of GTP GTP binds to the β-tubulin subunit of the heterodimeric tubulin molecule, and when a tubulin molecule adds to the end of a microtubule, this GTP molecule is hydrolysed to GDP (Erickson *et al*, 1992)

Tubulin subunits can be covalently modified after they polymerise. These modifications are the acetylation of α-tubulin on a particular lysine and the removal of the tyrosine residue from the carboxyl terminus of α-tubulin (Greer *et al.*, 1989). Acetylation and detyrosination occur only on microtubules and not on free tubulin molecules. Thus the longer the time that has elapsed since a particular microtubule has polymerised, the higher will be the fraction of its subunits that are acetylated and detyrosinated. Acetylation and detyrosination can be detected by specific antibodies, and they provide a useful indication of the stability of microtubules in cells.

1.7.2 The intermediate filaments

The intermediate filaments are clearly distinguishable from microfilaments and mocrotubules by their diameter size. The diameters of intermediate filaments, microfilaments and microtubules are 7-14nm, 5nm and 20-25nm respectively. Intermediate filaments are "insoluble" in high salt buffers, and refold spontaneously and rapidly *in vitro* and in near-physiological buffers from the denatured state to α–helical coiled-coils. These coiled-coils then self-assemble to 2-3nm double coiled-coils, to long protofilaments of 2-3nm diameter and finally into intermediate filaments that are essentially indistinguishable from those formed in the living cell (Traub, 1985; Steinert *et al.*, 1988).

Of the three major types of cytoskeletal proteins in eukaryotic cells - actin filaments, intermediate filaments and microtubules, intermediate filaments are the most complex. There are approximately 50 different intermediate filament proteins which can be further subdivided into six groups (see table 1.7.2).

Type I	Acidic keratins
Type II	Neutral and basic keratins
Type III	Vimentin, desmin, peripherin and glial fibrillary acidic protein (GFAP)
Type IV	neurofilaments
Type V	Nuclear lamins
Type VI	Nestin (which is found in melanoma and melanocyte)

Table 1.7.2 Classification of intermediate filament proteins

The intermediate filament structures in different cell types are not formed by the same protein but by different members of a large multigene family, the "intermediate filament proteins" Members of this family are differentially expressed in patterns specific for a given cell type or pathway of differentiation (Moll *et al*, 1982, Osborn *et al*, 1983, Fuchs *et al*, 1987) Consequently, the specific cellular intermediate filament protein complement is characteristic of that cell type

In human cells and tissues, for example, the products of approximately 50 different intermediate filament protein genes have been identified The largest and most complex class of intermediate filament proteins are the cytokeratins, the hallmarks of epithelial differentiation The various mtermediate filament protems differ considerably m molecular size and electric charge, but have a common basic molecular arrangement The central element is a core segment, a "rod" of 309-331 amino acids This is characterised by a predominance of amino acids favouring α-hehcal conformation and also a certain arrangement of units of hydrophobic amino acids that is known for its tendency to assume a two-chain coiled α-helical configuration (Crewther *et al*, 1983, Conway *et al*, 1990) In contrast, the domains flanking the rod, *i e* the N-terminal "head" and the C-terminal "tail", do not exhibit a constitutive α-helical coiled-coil forming character and vary greatly in size and amino acid sequence (Bader *et al*, 1988, Stasiak *et al*, 1989)

The rod domain has been relatively well conserved in the diverse mtermediate filament proteins during evolution, not only in size and structural character, but also in terms of amino acid sequence homology Studies have indicated that an intact rod domain is essential for intermediate filament formation (Lu *et al*, 1990, Raats *et al*, 1990) Deletions of rod segments or even certain point mutations in the rod result in the appearance of aberrant structures Rod portions alone, however, are not capable of assembly into intermediate filaments (Geisler *et al*, 1982) In contrast, the tail domain has been shown to be dispensable for mtermediate filament assembly under a wide range of conditions (Bader *et al*, 1988, Stasiak *et al*, 1989)

The general patterns of differential expression of the genes encodmg the various intermediate filament proteins during embryogenesis as well as in adult vertebrates, and in certain pathogenic processes, including tumour formation and metastasis, have been established

(Traub, 1985, Steinert *et al*, 1988, Fuchs *et al*, 1987) As a result of the relatively high degree of maintenance of the expression patterns specific for certain cell types during malignant transformation, antibodies to the various intermediate filament proteins have become valuable tools for immunohistochemical "cell typing" in tumor diagnosis, including cases of metastatic tumours in which the site of origin of the primary tumour is unknown (Osborn *et al*, 1989) In certain cell types, particularly during embryogenesis and in tumours, additional intermediate filament protein genes can be expressed, often spontaneously which are not part of the "normal" complement of the specific cell type (Gould *et al*, 1990a) The function of intermediate filaments, in general, or in any specific cell, is still unclear No distinct cellular functions can be ascribed to them Several cultured cell lines and tumours have been found to lack intermediate filament proteins and immunoprecipitation of intermediate filament by antibodies injected into living cells has resulted in non-detectable damage or functional disturbance in the cells (Klymkowsky *et al*, 1995)

1.7.2.1. Cytokeratins

The human genome contains at least 20 different genes encoding epithelial cytokeratin polypeptides, in addition to another ten cytokeratin polypeptides characteristic of hair- and nail-forming cells (Stemert *et al*, 1988, Moll *et al*, 1990) The individual cytokeratin polypeptides differ remarkably in molecular weight They can be divided into two subfamilies, the more acidic type I and the more basic type II cytokeratins (Markl *et al*, 1989) The two subfamilies, however, share only 30% amino acid sequence identity in their best conserved domain, the α–helical "rod" (Fuch *et al*, 1987, Steinert *et al*, 1988) The head and the tail domains differ markedly between the various cytokeratin polypeptides, even of the same subfamily

The subunit of cytokeratin filament is a tetramer, *i e*, a double coiled-coil comprising two polypeptides of each type I and type II (Steinert *et al*, 1988, Quinlan *et al*, 1984) Many of the cytokeratin bundles are anchored at desmosomes, resulting in higher order arrays of corresponding cytokeratin fibril patterns in adjacent cells and hence throughout the tissue (Franke *et al*, 1981, 1982) Cytokeratin 8 and keratin 18 are the first intermediate filament proteins to be expressed during embryogenesis (Oshima *et al*, 1983) Both ecto-and endoderm are characterised by typical polarised epithelial cells with extended arrays of intermediate filaments containing cytokeratins 8 and 18 that are mostly attached to

desmosomes (Jackson *et al* , 1980, Oshima *et al* , 1983) Subsequently, cytokeratin synthesis ceases in some cell types, mostly nonepithelial tissues, but is maintained in the various epithelia with specific patterns of expression For example, there are two types of epitheha simple and stratified Simple epithelia are distinguishable in their cytokeratin pattern (cytokeratin 7, 8, 17, 18, 19 and 20) from stratified epithelia (cytokeratin 1, 5, 6, 10, 11, 14-16) (Moll *et al* , 1983)

The observation that cytokeratin synthesis is generally maintained in malignantly transformed cells, even in morphologically altered ones that have lost their epithelial structure, has resulted in cytokeratin antibodies been valuable tools in the diagnosis of epithelium derived tumours, notably carcinomas (Moll *et al* , 1982, Osborn *et al* , 1983) In conjunction with desmosomal proteins, cytokeratins provide the best biochemical markers of epithelial character and hence for the identification of carcinomas and their metastases (Moll *et al* , 1982, Osborn *et al* , 1983)

1.7.2.2. Vimentin

Vimentin (MW 54kD) is the intermediate filament protein typical of fibroblasts, osteocytes, chondrocytes, melanocytes, Langerhans' cells of the skin and endothelial cells and most but not all lymphomas and leukaemias It is a substrate for certain protein kinases with phosphorylation restricted to the head domain (Evans, 1988, Inagaki *et al* , 1988, Chou, 1989) In cultured cells at interphase, vimentin runs from the cytoplasm near the nucleus to the plasma membrane After treatment with colchicine to depolymerise microtubules, vimentin forms coils near the nucleus Thus *in vivo* intermediate filaments and microtubules may interact Draberova and Draber (1993) have identified a 210kD microtubule-associated protein that might play a role in linking vimentin filaments to microtubules Vimentin is a major cellular phosphoprotein and its level of phosphorylation changes during the cell cycle (Lai *et al* , 1993) It was proposed that vimentm acts as a "phosphate sink" buffering the cell against the effects of "excess kinase activity" If this were a real function of vimentin, its effects on cellular physiology could be quite subtle

Most muscle sarcomas coexpress desmin and vimentin Keratin and vimentin coexpression in epithelial cell lines is also common Carcinomas of some tissues have been found to co-express keratin and vimentm, e g kidney and thyroid However, studies have shown that some carcinomas only express vimentm, e g breast, while other carcinomas only express

keratin, e g gastrointestinal tract Vimentin coexpression in node negative breast carcinomas may be associated with a poorer prognosis (Domagala *et al*, 1990) Thompson *et al* (1992) reported that lack of estrogen receptor (ER) and the presence of vimentin (VIM) was associated with poor prognosis in human breast cancer In a panel of breast cancer cell lines, ER^+/VIM^- (MCF-7, T47D, ZR-75-1) and ER^-/VIM^- (MDA-MB-468, SK-Br-3) cell lines were found to be non-invasive while ER^-/VIM^+ (BT549, MDA-MB-231, MDA-MB-435, MDA-MB-436, Hs 578T) lines were shown to be invasive This suggests that human breast cancer progression results first in the loss of ER, and subsequently in VIM acquisition, the latter being associated with increased matastatic potential through enhanced invasiveness Vimentin expression may provide useful insights into the mechanisms of breast cancer progression

1.8 The Extracellular Matrix

The extracellular matrix (ECM) is a complex ordered aggregate composed of a number of different macromolecules whose structural integrity and functional composition are important in maintaining normal tissue architecture, in development and in tissue specific function Four major classes of macromolecules - the collagens, proteoglycans, structural glycoproteins and elastin collectively comprise the ECM of animal cells (Zern and Reid, 1993) To date, two major families of cell surface receptors - the integrins (Kramer *et al*, 1993) and the syndecans (Rapraeger, 1993) have been identified as mediating the influence of the ECM on cells

The ECM profoundly influences the cellular phenotype such as control of growth, differentiation, development and metabolic response of cells The importance of growth factors, cytokines, hormones, vitamins and cell-to-cell contact as regulators of the cellular phenotype has been established Thus, two important concepts have emerged firstly, growth factors, cytokines, vitamins, hormones and cell-to-cell contact exert their effect through regulation of extracellular matrix production (Esposito and Zern, 1993) Secondly, many biological changes attributed to the effects of growth factors, cytokines, hormones, vitamins and cell-to-cell contact are similar to those of the ECM (Kleinman *et al*, 1993)

To reconcile these observations, a paradigm has emerged (Kleinman *et al*, 1993) This paradigm states that four major types of interactions (growth factors/cytokines, hormones/vitamins, cell-to-cell contacts, and the ECM) regulate the growth, shape, state of differentiation, development and biochemical response of the cell In addition, each type of interaction results in altered ECM expression (Labat-Robert *et al*, 1990, Sage and Bornstein, 1991) - a phenomenon termed "mutual reciprocity" (Sage and Bornstein, 1991) This complexity is further illustrated by the findings documenting that many growth factors and cytokines are specifically bound by matrix components and hence are resident in the ECM (Vlodavsky *et al*, 1993) and that the extracellular matrix can modulate the expression of receptors for growth factors (Marx *et al*, 1993) Thus, it appears that no factor influencing the biology of the animal cell does so without affecting extracellular matrix biosynthesis

In the past decade, cloning of the extracellular matrix genes and establishment of their DNA sequences have led to the acquisition of a large body of evidence documenting mutations in these genes as underlying numerous inherited connective tissue diseases Mutations in the type I collagen genes cause osteogenesis imperfecta (Byers, 1993) The Alport syndrome, a heritable kidney disorder, has been connected with mutations in the collagen α_5(IV) gene (Zhou *et al*, 1991) Macular corneal dystrophy (MCD) is an inherited human disease resulting from a failure to synthesise keratan sulfate (Hassell *et al*, 1980) Two animal models carrying mutations resulting in a defective aggrecan core protein have been described nanomelia in the chicken (Argraves *et al*, 1981) and cartilage matrix deficiency in the mouse (Kimata *et al*, 1981) Both of these mutations result m shortened long bones, as well as other abnormalities of cartilagenous tissue In contrast to the collagen and proteoglycan families, with one exception, no inherited diseases resulting from mutations in the structural glycoprotein genes have been described This may reflect the importance of the majority of the molecules in this family in embryonic development (George *et al*, 1993)

Changes in the ECM have also come to be recognised as critical components in the initiation and progression of a variety of acquired diseases Among these are atherosclerosis (Badimon *et al*, 1993), liver fibrosis (Gressner, 1991), glomerulonephritis and glomerulosclerosis (Sterzel *et al*, 1992), pulmonary fibrosis (Khalil and Greenberg, 1991) and the secondary consequences of diabetes (Ziyadeh *et al*, 1989) Extracellular matrix components have also been implicated as the autoimmune antigens in several diseases including Goodpasture

syndrome (an autoimmune disease characterised by rapid progressive glomerulonephritis and pulmonary haemorrhage). Finally and most importantly, changes in extracellular matrix synthesis, deposition, metabolism and matrix receptors are important components in cancer development and metastasis (Stetler-Stevenson *et al.*, 1993). Clearly, as we understand more of the chemistry and function of ECM, more pathogenesis of these diseases will be elucidated.

1.8.1 Basement membrane and Matrigel

Basement membrane is a highly specialised extracellular matrix. It appears as an amorphous sheet-like structure that is positioned either between a cell layer and a thick collagenous stroma, as exemplified by the dermal-epidermal junction of human skin or between two layers of cells, such as the renal glomerular basement membrane. Basement membranes not only underlie epithelial and endothelial cells, but also surround muscle, fat, and the entire nervous system. The major structural components of basement membranes are type IV collagen, laminin, entactin and heparan sulfate proteoglycan (a polymer consisting of alternating glucosamine and glucuronic/iduronic acid units).

The importance of basement membranes in development and adult tissue function has been inferred from a number of observations. For example, cells migrate along basement membranes during development. Basement membranes are required for the polarisation of cells in both the embryo and the adult, and they also serve as substrates for cell adhesion and migration during wound healing and nerve regeneration (Streuli *et al.*, 1991; Paulsson, 1992; Ekblom, 1993; Kleinman and Schnaper, 1993). Basement membrane components promote cell adhesion via integrins and various studies have shown that cells are more differentiated when in contact with basement membrane. Basement membranes also serve as depositories of growth factors and may thereby modulate access to, and activity of growth factors (Haralson, 1993).

The importance of basement membrane in adult tissue function has been directly demonstrated by genetic diseases caused by mutations in the genes for structural besement membrane components. In junctional epidermolysis bullosa (Epstein, 1992), patients lack a laminin variant normally present in the skin basement membrane. Patients with Alport syndrome (which is a progressive renal disease characterised by hematuria and hearing loss) lack a collagen type IV variant in the kidney (Barker *et al.*, 1990). More recently, defects in

the major laminin variant in muscle, merosin, has been shown to be correlated with muscular dystrophies in man and animals (Sunada *et al*, 1994)

A reconstituted basement membrane, isolated from the EHS (Engelbreth-Holm-Swarm) tumour termed "Matrigel" has been the breakthrough in the use of basement membrane (Kleinman *et al*, 1986) Matrigel contains all basement membrane components and is a solution at 4°C, but when the temperature is raised to 24-37°C, the components interact with each other and within 1 hour they form a polymerised gel The gel can be used as a substratum for cells or alternatively, the cells can be mixed at 4°C with matrigel and then either plated onto cell culture dishes or injected into animals When tumour cells are mixed with matrigel and injected into mice, tumours will rapidly grow

A critical step in metastasis is the invasion of basement membrane In the past, studies on invasion had been carried out using isolated basement membranes, particularly from the amnion (Hendrix *et al*, 1989) Since the use of matrigel, matrigel invasion assays have been the most widely accepted system for analysis of tumour cell invasion (Albini *et al*, 1987) This *in vitro* invasion was found to correspond to relative metastatic potential *in vivo* for a wide variety of cells Endothelial cells, the key cellular element in tumour neovascularisation, are able to breach their own basement membrane during angiogenesis, a necessary event in metastasis Induction of endothelial cell invasion by tumour cell products and its inhibition have also been studied in matrigel invasion assays (Thompson *et al*, 1991)

1.8.2 Major components of basement membrane and the ECM

Investigations have documented that four major classes of macromolecules - the collagens, proteoglycans, structural glycoproteins and elastin comprise the ECM of animal cells (Piez and Reddi, 1984) Proteoglycans consist of a protein core and one or more glycosaminoglycan side chains covalently bound to the core protein Members of structural glycoproteins are heterogeneous in size, structure and tissue distribution such as fibronectin and laminin The elastic fibre, which confers flexibility and distensibility to all verbrate tissue, is a complex of elastin with several non-elastic glycoprotein termed fibrillar components

1.8.2.1 Type IV Collagen

Collagen is the most abundant protein in the animal kingdom, representing approximately one-third of all protein in tissue (Van der Rest and Garrone, 1991) Collagens are composed of either three identical or similar α chains characterised by the repeating -Gly-X-Y- sequence where X and Y can be any amino acid There is a glycine residue at every third ammo acid which allows the assembly of three chains into a triple-helical structure At present, 18 different types of collagens with more than 31 genetically distinct chains have been identified (Miller and Gay, 1992) These collagens can form a variety of different structures in the extracellular space and thus are divided into several classes (1) fibril-forming collagens, (2) sheet-forming collagens, (3) filament-forming collagens and (4) anchoring fibril forming collagens

Kefalides (1971) was the first person to recognise that basement membranes contain a unique collagen which was designated type IV collagen Type IV collagen is classified as a sheet-forming collagen At present, six chains, α_1(IV) to α_6(IV) are known The major form of collagen IV which is present in all basement membranes is a heterotrimer consisting of two α_1(IV) and one α_2(IV) chains The genes for these chains are mapped very closely on human chromosome 13 (Griffin *et al*, 1987) A similar gene organisation is found in a pair of genes for α_3(IV) and α_4(IV) chains while the α_5(IV) and α_6(IV) chains are mapped on human chromosomes 2 and X respectively (Hostikka *et al*, 1990) Mutations have been identified in the α_5(IV) gene of patients with X-linked form of Alport syndrome which is a progressive renal disease characterised by hematuria and hearing loss (Barker *et al*, 1990) The C-terminal globular domain of the α_3(IV) and α_4(IV) chains have been identified as target regions for antibodies from patients with Goodpasture syndrome, an autoimmune disease characterised by rapid progressive glomerulonephritis and pulmonary haemorrhage (Saus *et al*, 1988)

Collagen IV not only provides a biomechanically stable scaffold into which the other constituents of basement membranes are incorporated, but it also plays an important role in the interaction of basement membranes with cells The cell-binding site of the human $[\alpha_1(IV)]_2\alpha_2(IV)$ molecule is located about 100nm away from the N-terminus of collagen IV This area contains the recognition site for the two integrin receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Vandenberg *et al*, 1991) The proteolytic attack of the collagen IV specific gelatinase A -

MMP-2 occurs in the area where the $\alpha_2\beta_1$ recognition site is destroyed whereas the recognition site of $\alpha_1\beta_1$ remains intact.

1.8.2.2 Laminin

Laminin (LN) is the major structural glycoprotein of basement membrane. Laminin was first purified in 1979 (Timpl *et al.*, 1979) and was initially identified as a product of a cultured mouse carcinoma line. Subsequently, this structural glycoprotein was isolated from the mouse EHS sarcoma which produces significant quantities of basement membrane.

Laminin exists as a cruciform-like structure formed by three chains: α, β, γ. It is now recognised that there is a family of laminins since eight genetically distinct laminin chains (α_1, α_2, α_3, β_1, β_2, β_3, γ_1, γ_2) and seven different assembly forms (laminin-1 to -7) are known so far. The most extensively characterised is EHS laminin which is termed laminin-1 ($\alpha_1\beta_1\gamma_1$). The α chain (400kD) is called the "long arm", having three globular domains at their amino terminus which are separated by EGF-like repeats. It also has a coiled domain and a large globular domain at the carboxy terminus containing five globules. The β and γ chains are called the "short arms", containing only two globular domains and two EGF repeats at each amino terminus. Disulfide bonds link the chains near the carboxy terminus while the amino termini remain free, giving laminin its classic cruciform structure (Figure 1.8.2.2).

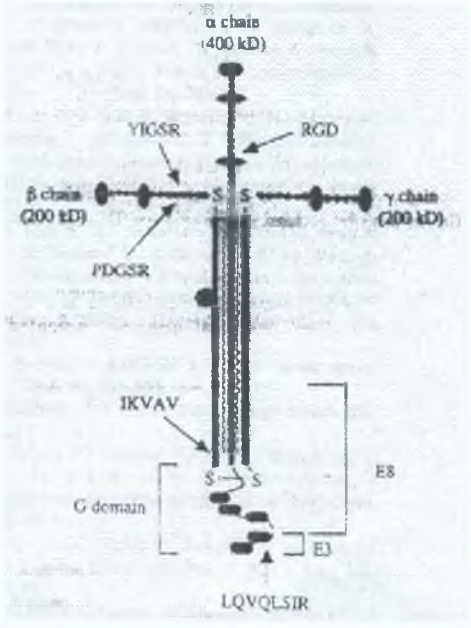


Fig. 1.8.2.2 Schematic model of laminin. The location of several active sites defined by synthetic peptides is designated by the arrows. The globular domain is defined by bracket (Timpl and Brown, 1994).

Laminin can bind many components of the basement membrane, including collagen IV, perlecan and entactin, as well as binding to itself It is likely to play a role in organising and possibly initiating the formation of the basement membrane This hypothesis is supported by the fact that in the developing embryo, laminin is the first extracellular matrix molecule to be synthesised (Malinda and Kleinman, 1996) The adhesion of both normal and malignant cells to laminin was the first activity shown for laminin (Martin and Timpl, 1987) It is now well established that cell binding occurs via a variety of cellular receptors including integrins and several less-well-characterised non-integrin receptors (Kramer *et al*, 1993) At least six different integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_v\beta_3$) have been identified to bind to laminins The $\alpha_6\beta_1$ integrin seems to be the major receptor for laminin-1 (Sonnenberg *et al*, 1990) A similar specificity was found for the $\alpha_7\beta_1$ integrin, but shows a more restricted expression (Kramer *et al*, 1993) The $\alpha_3\beta_1$ integrin shows preference for laminin-5 (Delwel *et al*, 1994), while the collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to laminin-1 (Kramer *et al*, 1993) but not to lammin-2 or -4

Laminin also promotes the malignant phenotype Cells cultured on laminin *in vitro* form more tumours *in vivo* than cells not cultured on laminin In addition, cells selected by adhesion to laminin *in vitro* also form more tumours *in vivo* than the control cells Furthermore, co-injection of laminin with tumour cells yields increased numbers of lung colonies (Barsky *et al*, 1994) In addition, Topley *et al* (1993) also found that by injecting a number of cell lines with purified laminin, the growth of subcutaneous tumours in nude mice can be promoted Several laminin-derived synthetic peptides have been found to influence the growth and metastasis of tumours (Yamada, 1991) A sequence on the β_1 chain YIGSR (tyr-ile-gly-ser-arg), reduces tumours growth and experimental lung metastasis (Saiki *et al*, 1989) Laminin also increases the secretion of collagenase IV that is involved in tumour spread A sequence on the α chain (ile-lys-val-ala-val) has been found to promote protease activity, angiogenesis and the growth and metastases of tumours Laminin secretion by a fibrosarcoma cell line has been correlated with its metastatic potential (Varani *et al*, 1983, Malinoff *et al*, 1984) Many types of central and peripheral neuronal cells also respond to laminin (Nurcombe, 1992) Laminin can promote nerve regeneration *in vivo* and has been found to increase the survival of nerve grafts in the brain

These biological activities, as well as the ability of laminin to promote cell migration, growth and differentiation, suggest that laminin may have an important role in wound repair and cancer metastasis

1.8.2.3 Fibronectin

Fibronectin (FN) is a large glycoprotein and an important component of the extracellular matrix It is a product of most mesenchymal and epithelial cells and can be purified from plasma, tissue and cultured cells Due to the relatively high content of fibronectin in soluble disulphide-linked dimer form, plasma is the most convenient source

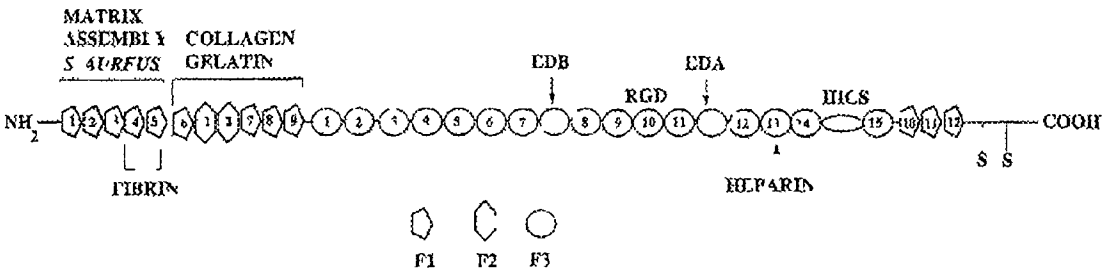


Fig. 1.8.2.3 The modular structure of fibronectin showing the position of F_1, F_2 and F_3 modules, and alternatively spliced ED-A, ED-B and III-CS regions Binding sites are also shown (Potts and Campbell, 1996)

Fibronectin is a mosaic protein composed almost entirely of three different types of protein module (F_1, F_2 and F_3) which were first identified in fibronectin but have since been found in a variety of other proteins It has long been recognised that the binding sites for a wide range of molecules have been associated with various fragments of fibronectin Fibronectins isolated from different sources contain chains that differ in size, indicating a number of different splicing variants - a concept proven by the sequencing of cDNA clones To date, the family of fibronectins contains at least eight splice variants The alternatively spliced regions are termed ED-A, ED-B and III-CS (Figure 1 8 2 3)

Fibronectin binds to a number of biological macromolecules including heparin, collagen, fibrin and cell surface receptors For most cells, adhesion to fibronectin is mediated by the central cell-binding domain of fibronectin through a (Gly)-Arg-Gly-Asp-(Ser) (RGD) sequence and a second, distinct region Pro-His-Ser-Arg-Asn (PHSRN) sequence that acts

synergistically with the RGD sequence. Another cell adhesive region is located near the carboxy-terminus in the alternatively spliced IIICS module. The critical minimal sequences for this region are Leu-Asp-Val (LDV) and Arg-Glu-Asp-Val (REDV) which function in an additive rather than synergistic fashion.

Many of the β_1 integrin family can bind fibronectin including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$. Many integrins, including the $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and the $\alpha_{IIb}\beta_3$ recognise the RGD site in adhesive proteins. The fibronectin-specific integrin, $\alpha_5\beta_1$, is the major fibronectin receptor on most cells. The $\alpha_5\beta_1$ integrin interacts with the central cell adhesive region of fibronectin and requires both the RGD and synergy sites for maximal binding (Akiyama *et al.*, 1995). The major platelet integrin $\alpha_{IIb}\beta_3$ also recognizes a similar synergy site in fibronectin for mediating platelet interactions with fibronectin (Bowditch *et al.*, 1994). The $\alpha_4\beta_1$ integrin is the receptor for the IIICS region of fibronectin. It binds to both the CS1(LDV) and CS5(REDV) sequences of the IIICS region (Massia and Hubbell, 1992).

Fibronectin may play an important role in cell transformation, cell migration, invasion and metastasis. It was found that the level of cell-surface fibronectin was greatly reduced in transformed cells (Hynes, 1976). A panel of antibodies that bind to the α_5, α_4 and β_1 integrin subunits can inhibit cell migration on fibronectin and cell invasion (Yamada *et al.*, 1990; Qian, 1994).

Numerous studies have been carried out using antibodies to the various integrin subunits. An antibody that generally inhibits all β_1 integrin-mediated cell adhesion was found to inhibit cell migration, probably by inhibiting cell adhesion to a level below the threshold required for traction (Akiyama *et al.*, 1989). In contrast, an antibody that selectively inhibits α_5-mediated adhesion was found to actually increase cell migration, possibly by inhibiting the formation of receptor clusters which could then result in less cell adhesion (Akiyama *et al.*, 1989). An anti-β_1 integrin antibody was shown to be a very effective inhibitor of invasion in both HT-1080 fibrosarcoma cells and MDA-MB-231 breast carcinoma cells (Yamada *et al.*, 1990; Newton et al., 1995). An anti-α_5 antibody was found to have no significant effect on invasion in the HT-1080 cells and only a small effect on invasion of MDA-MB-231 cells (Yamada *et al.*, 1990; Newton *et al.*, 1995). Both anti-β_1 and anti-α_5 antibodies were found to inhibit experimental metastasis resulting from intravenous injection of MDA-MB-231 breast

carcinoma cells into athymic nude mice (Newton *et al*, 1995)

1.9 Invasion and Metastasis

By the time of diagnosis, a high proportion of patients have clinically detectable metastasis It is assumed that dissemination is impossible until invasion has occurred For breast cancer, the period of transition from hyperproliferative but non-invasive disease to invasive cancer is estimated to average 6 years (Spratt *et al*, 1986) The time period after the invasive carcinoma grows to reach the minimum threshold size of detection (0 25cm in diameter), to establishment of the first metastasis, can be less than a year (Spratt *et al*, 1986) Thus, transition of preinvasive carcinoma to invasion provides a much larger window for intervention compared to the conventional goal which focuses on established invasive carcinoma

1.9.1 Invasion

Invasion is the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers Invasion is not simply due to growth pressure but involves additional genetic deregulation over and above those molecular events that cause uncontrolled proliferation At the biochemical level, the mechanism of invasion used by tumour cells, may parallel, or be similar to, that used by nonmalignant cells which traverse tissue boundaries under normal physiological conditions Examples of physiological invasion are smooth muscle cell migration from the tunica media (which contains smooth muscle fibers and elastic and collagenous tissues) to the intima (which is composed of the endothelial cell layer) of blood vessels, angiogenesis, embryogenesis and morphogenesis, nerve growth cone extention and homing, and trophoblast implantation In contrast to malignant invasion, physiological invasion is tightly regulated and ceases when the stimulus is removed Invading tumour cells appear to have lost the control mechanisms which prevent normal cells from invading neighbouring tissue at inappropriate times and places Thus, the fundamental difference between normal and malignant cells is regulation The difference must lie in the proteins that start, stop, or maintain the invasion program at times and places that are inappropriate for nonmalignant cells A major goal, therefore, is to understand what signals and signal transduction pathways are perpetually activated or unrestrained in malignant invasion

Liotta (1977) has proposed a three-step theory of invasion the first step is tumour cell attachment via cell surface receptors which specifically bind to components of the matrix The anchored tumour cell next secretes hydrolytic enzymes (or induces host cells to secret enzymes) which can locally degrade the matrix The third step is tumour cell locomotion into the region of the matrix modified by proteolysis Thus, regulation of molecular events necessary for invasion requires spatial and temporal coordination and cyclic on-off processes at the level of the individual cell

For cells migrating within a three dimensional extracellular matrix, such as penetrating a basement membrane, protrusion of a cyclindrical pseudopod is the first step, prior to translocation of the whole cell body (Condeehs, 1993) A number of observations support the central role of cytoskeleton-driven pseudopodia as organs of motility and invasion Pseudopodia aggregate or concentrate cell surface degradative enzymes and adhesion receptors (Guirguis *et al* , 1987) A balance must switch from proteolysis to adhesion in order for the advancing pseudopod to grip the matrix and pull the cell forward General unregulated proteolysis alone cannot be responsible for the entire invasion cascade When the cell moves into the zone of lysis, adhesion is required and proteolysis must be shut down At the rear of the cell, dissociation from adjacent cells, and detachment from previous attachment sites are necessary to release the cell

The basement membrane and interstitial stroma also play an important regulatory role in invasion They serve as a storage depot as well for latent proteinase, cytokines and growth factors (including angiogenesis factors) which can be activated or released by the invading cells The critical pathological turning point is the initiation of local invasion leading to the dissemination of tumour cells Tumour-induced neovascularisation occurs in parallel with the invasion and provides a vascular entry necessary for dissemination A positive correlation between tumour aggressiveness and protease levels has been documented for all four classes of proteases including serine, aspartyl, cysteinyl and matrix metalloproteinase (MMP) It is now well established that one way the mtegrity of the basement membrane can be regulated is by the balance between metalloproteinases and their inhibitors (Talhouk *et al* , 1992)

The molecular characterisation of invasion has led to the identification of two categories of checkpoints that constitute intervention targets The first category includes cell surface and secreted proteins such as adhesion receptors, degradative enzymes and their inhibitors, and motility-stimulating cytokines The second checkpoint category includes regulatory proteins and pathways such as calcium-mediated signalling, G-protein activation and tyrosine phosphorylation events As for the first category, anti-invasion agents have been divided into two groups blocking agents and suppressing agents Blocking agents act as a barrier to prevent cancer-producing substances from reaching or reacting with cellular target sites Suppressing agents prevent the evolution of the neoplastic process Some suppressing agents stimulate differentiation while others reverse the consequences of activated oncogenes and suppressor genes

Altered signalling pathways have been associated with the process of invasion and metastasis A prime example is the introduction of activated c-Ha-ras into primary rat embryo fibroblast cells (Pozzatti *et al*, 1986) This transfection resulted in development of a malignant and invasive phenotype characterised by production of MMP-9 (see section 1 10) and marked pulmonary metastasis after tail vein inoculation of transfected cells Similar effects have been observed with expression of other oncogenes, such as Her-2/neu (Yu *et al*, 1993) The oncosuppressor gene Rb-1, has been demonstrated to be able to inhibit proliferation and invasion of tumour cells (Valente *et al*, 1996) However, p53, has been found to be a weaker inhibitor of invasion (Li *et al*, 1996)

1.9.2 Metastasis

Metastasis is the spread of cancer from a primary tumour to distant sites of the body and is a defining feature of cancer Most deaths from cancer are due to metastases that are resistant to conventional therapies Current therapies fail to eradicate metastasis for three major reasons Firstly, when initially diagnosed, most tumours are well advanced and metastasis has occurred Secondly, specific organ environment can modify the response of a metastatic tumour cell to systemic therapy and alter the efficiency of anticancer agents The third reason and the greatest obstacle to the success of therapy, is the heterogeneous composition of tumours, where highly metastatic cells can escape from the effect of therapeutic agents

The major steps in the formation of a metastasis are as follows escape of cells from the primary tumour, intravasation (entry of cells into the lymphatic or blood circulation), survival and transport in the circulation, arrest in distant organs, extravasation (escape of cells from the circulation), and growth of cells to form secondary tumours in the new organ environment Angiogenesis, the recruitment of new blood vessels, is required for primary and metastatic tumours to grow beyond minimal size Evasion of immune destruction is necessary at various steps throughout the process The formation of detectable metastases can be prevented by interruption at any one or more of these steps The outcome of the process is dependent on both the intrinsic properties of the tumour cells and the response of the host

Metastasis is known to be an inefficient process Large numbers of cells can be shed into the circulation from a primary tumour but only a small fraction of the cells will succeed in forming metastases Results from a series of experiments using intravital videomicroscopy (IVVM), a technique which permits direct observation of the microcirculation *in vivo*, suggest that early steps in metastasis, including destruction of cells in circulation and extravasation, contribute less to metastatic inefficiency than previously assumed Rather, regulation of growth of individual extravasated cells in target tissue appears to be the rate limiting step (Chambers *et al* , 1995)

Some metastases will arise on the basis of circulatory anatomy The lungs are a common site of metastasis Similarly, metastases from the colon often arise in the liver as the liver receives direct drainage from the large intestine Metastases also arise in other organs due to "Seed and soil" theory In 1889, Paget concluded that metastasis occurred only when certain favoured tumour cells (the "seed") had a special affinity for certain specific organs (the "soil") The formation of metastases required the interaction of the right cells with the compatible organ environment Metastasis favours the survival and growth of a few subpopulations of cells that pre-exist within the parent neoplasm (Fidler and Kripke, 1977) Thus, metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells (Talmadge *et al* , 1982)

Organ microenvironment can influence the biology of cancer growth and metastasis in several different ways For example, human colon carcinoma (HCC) cells implanted subcutaneously (ectopic site) into nude mice produce low levels of secreted type IV collagenase, whereas the same cells growing in the wall of the colon (orthotopic site) produce

high levels of Type IV collagenase (Nakajima *et al*, 1990) Similarly, transplanted human renal cell carcinoma cells (HRCC) rarely metastasise after implantation into the subcutis in the nude mice regardless of the degree of malignancy in the patient (Fidler, 1986) whereas the highest incidence of metastasis was produced by the same cell line growing m the kidney (Naito *et al*, 1986) Organ-specific fibroblasts directly influence the production of collagenase type IV by HCC (Fabra *et al*, 1992) or HRCC (Gohji *et al*, 1993) cells This conclusion is based on results obtained from co-culturing HCC (Fabra *et al*, 1992) or HRCC (Gohji *et al*, 1994) with mouse fibroblasts isolated from ectopic or orthotopic tissues The inhibition of collagenase type IV production by skin fibroblasts was shown to correlate with production of interferon beta (IFN β) (Fabra *et al*, 1992, Gohji *et al*, 1994)

Metastasis is the final stage in tumour progression from a normal cell to a fully malignant cell in some cases The best developed example is the characterisation of molecular progression in colon cancer, in which specific changes (e g loss of tumour-suppressor genes and mutation of oncogenes) are preferentially associated with specific stages of progression (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996) However, the final stage in tumour progression to a metastatic phenotype has eluded characterisation at a molecular genetic level in colon or other cancers Transfection with a variety of oncogenes (e g ras and src) has been shown to produce metastatic cells (Chambers and Tuck, 1993) For example, metastatic H-ras transfected NIH-3T3 cells had increased levels of a variety of gene products including proteinase and adhesive proteins (Tuck *et al*, 1991a and 1991b) Loss of tumour-suppressor gene function also has been implicated in the conversion to metastatic ability in specific tumour types, e g nm23 (MacDonald *et al*, 1995), KAI1 (Dong *et al*, 1995), KiSS-1 (Lee *et al*, 1996), although none is likely to be universally implicated in all tumour types It appears more likely that regulation of expression of genes that contribute functionally to metastasis can occur in a tissue-specific manner Whatever gene is involved, all the required traits have sufficient proteolytic capacity to complete all the steps in metastasis Thus, MMPs and their inhibitors have been repeatedly implicated in this context

1.10 Matrix metalloproteinase

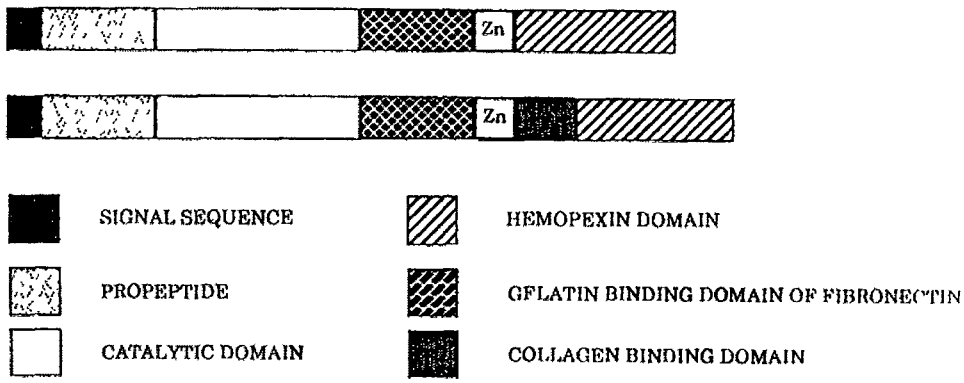


Fig. 1.10 Domain structure of MMP-2 and MMP-9 (top MMP-2, bottom MMP-9)(McDonnell and Fingleton, 1993)

The matrix metalloproteinases (MMPs) are a family of at least fifteen secreted or membrane-bound zinc endopeptidases capable of degrading all of the components of the extracellular matrix In 1962, interstitial collagenase, responsible for the resorption of tadpole tails (Gross and Lapiere, 1962), was the first MMP to be described and purified Most of these enzymes are secreted by a variety of connective tissue cells, includmg fibroblasts, osteoblasts, chondrocytes and endothelial cells, as well as inflammatory cells such as macrophages, neutrophils and lymphocytes

Comparison of the amino acid sequences of human MMPs has revealed a high degree of homology between these enzymes The minimal structure comprises a signal peptide, a propeptide domain and a catalytic domain The protein that actually has this structure is the smallest member of the MMP family, matrilysin (MMP-7) The signal peptide domain directs the MMP to the endoplasmic reticulum The propeptide domain is involved in the maintenance of enzyme latency and is removed during enzyme activation (Van Wart *et al*, 1990) The catalytic domain is the zinc-binding domain With the exception of MMP-7, all MMPs have a C-terminal domain that has homology with both haemopexin and vitronectin (Hunt *et al*, 1987) MMPs can be classified into 4 groups on the basis of sequence homology and substrate specificity the type I collagenases, the type IV collagenases, stromelysm and membrane-type metalloproteinases (MT-MMP)

The expression of MMPs is very highly regulated and occurs at many different levels Regulation occurs at the transcriptional level by a variety of growth factors, cytokines and oncogenes and at the translational level by the latency of the secreted enzymes as well as by the presence of naturally occurring inhibitors The latter includes general plasma proteinase inhibitors such as α_2-macroglobulin, as well as more specific inhibitors such as the tissue inhibitors of metalloproteinases, TIMP-1 to -4 Numerous studies indicate that members of the MMP family have the ability to activate one another and serine proteinases have been shown to activate MMP-1 and MMP-9 via plasmin (Sang *et al* , 1995, Crabbe *et al* , 1994)

MMPs have an important role in processes such as wound healing, pregnancy and parturition, bone resorption and mammary involution However, the main interest in the MMPs relates to their role in certain diseases in which breakdown of the extracellular matrix is a key feature Such diseases include rheumatoid arthritis, osteoarthritis, bronchiectasis, Crohn's disease, liver cirrhosis, etc and most importantly, cancer invasion, metastasis and angiogenesis Evidence that MMPs play a functional role in metastasis came originally from experiments with genetically manipulated levels of TIMP-1 Schultz *et al* (1988) first showed that an intraperitoneal injection of recombinant TIMP-1 reduced lung colonisation of intravenously injected B16F10 melanoma cells Furthermore, a reduction in TIMP-1 levels by antisense RNA in mouse fibroblasts resulted in the formation of metastatic tumours in nude mice (Khokha *et al* , 1989) These results provide strong support for a role for MMPs in the establishment of metastatic lesions

MMPs are not the only enzymes which can degrade ECM There are four main classes of proteases involved in proteolytic degradation of the extracellular matrix (Shi *et al* , 1993) (1) serine proteases, e g plasminogen activators, (2) cysteine proteases, e g cathepsin B and cathepsm L, (3) aspartyl proteases, e g cathepsin D and (4) MMPs All of these proteinases interact in a complex manner that ultimately leads to the process of ECM degradation

There is a large body of evidence which shows that MMP expression is increased in cancer tissue relative to corresponding noncancerous tissue The increase in stromal production of certain MMPs in carcinomas is likely to be the result of the release of factors by cancer cells In support of this hypothesis, Nole *et al* (1993, 1994) demonstrated that conditioned medium taken from cultures of a breast carcinoma cell line, MDA-MB231, increased the expression

of interstitial collagenase and gelatinase A in cultures of human skin and breast fibroblasts. Since then, a variety of growth factors, including EGF, TGF, PDGF, have been shown to regulate MMP expression. The results indicate that cancer cells have the ability to increase their proteolytic activity without increasing their own production and secretion of proteases, and they can concentrate and activate proteases in the pericellular space. This provides them with routes of escape against attempts to control the production and secretion of proteases in cancer cells.

1.10.1 MMP2 and MMP9

From early work of Liotta *et al.* (1977, 1980) and Tryggvason *et al.* (1984), interest was focused on type IV collagenase, the enzyme responsible for degradation of type IV collagen. The enzymes responsible for this activity are now recognised to be either MMP-2 (gelatinase A, 72kD type IV collagenase) or MMP-9 (gelatinase B, 92kD type IV collagenase). They are distinct from other members of the MMP family in that they possess a unique region immediately adjacent to the metal-binding domain that is homologous to the gelatin-binding domain of fibronectin. MMP-2 and MMP-9 differ from other MMPs by their ability to interact, as latent proenzymes, with the endogenous TIMPs (TIMP-1 and TIMP-2). The MMP-2 proenzyme forms a complex with TIMP-2, while the MMP-9 proenzyme binds with TIMP-1 (Stetler-Stevenson *et al.*, 1989; Goldberg *et al.*, 1989). TIMPs are not known to bind other latent MMPs, but will inhibit all MMPs once these enzymes are activated. In addition, MMP-2, also thought to be activated by MT1-MMP (Cao *et al.*, 1995), can activate MMP-9 proenzyme to an active form.

Inhibition of endogenous MMP-2 activity in human melanoma cells results in enhanced cellular adhesion. This indicates that MMP-2, in addition to contributing to proteolysis of ECM components, also lyses cell surface components that mediate attachment of tumour cells to the ECM. Thus, MMP-2 may function to modulate cell attachment and facilitate cell migration and invasion (Ray *et al.*, 1995). It has also been shown that factors which increase MMP-2 expression, e.g. IL-8, also increase invasion and metastasis (Luca *et al.*, 1997).

Correlative evidence for the involvement of MMP-2 and MMP-9 in the invasive phenotype is abundant (Monteagudo *et al.*, 1990; Ura *et al.*, 1989; Levy *et al.*, 1991; Schultz *et al.*, 1988). In one study on a series of bronchial epithelial cell lines containing various oncogenes (Ura *et al.*, 1989), it was also found that invasiveness and metastatic capacity were positively

correlated with expression of the MMP-2 gene Induction of the malignant phenotype using the c-Ha-ras oncogene has been shown to enhance expression of MMP-2 and MMP-9 (Ura *et al*, 1989) Most invasive colonic, gastric, ovarian and thyroid adenocarcinomas and desmoplastic stroma of breast lesions have been shown to be immunoreactive for MMP-2, whereas benign proliferative disorders of the breast, colon, gastric mucosa and benign ovarian cysts show decreased or negative staining for this enzyme (Monteagudo *et al*, 1990, Levy *et al*, 1991) For example, in surgical specimens from patients with colorectal carcinoma, an antibody to MMP-2 was used to compare the staining of malignant tissue with adjacent normal tissue Duke's stage was highly correlated with the percentage of cells staining positively for MMP-2 (Levy *et al*, 1991) The ratio of activated to latent MMP2 was significantly higher in malignant versus benign breast lesions and a higher proportion of activated enzyme was related to increasing tumour grade (Davies *et al*, 1993a, 1993b) However, the expression of MMP-2 failed to predict relapse or survival in the patients with node-negative breast cancers (Daidone *et al*, 1991)

Although overexpression of MMP-2 is more often observed in many tumours, MMP-9 also appears to play a critical role in tumour invasion Increased MMP-9 expression has been demonstrated in squamous and basal carcinoma of the skin (Pyke *et al*, 1992), breast (Davies *et al*, 1993a), colon (Davies *et al*, 1993b) and bladder cancer

1.10.2 TIMP

One of the naturally occurring inhibitors of proteases is a large (750kD) protein, α_2-macroglobulin (α_2M) produced by the liver and detectable in normal serum α_2M is a non-specific inhibitor of all four classes of proteases The tissue inhibitors of metalloproteinases (TIMPs) are proteins that specifically inhibit the MMPs To date, four members of the TIMP family (TIMP1-4) have been identified They are all low-molecular-weight (all around 25kD) secreted proteins that bind to the active form of MMPs, inhibiting their enzymatic activity MMP-2 and MMP-9 are unique among other MMPs in that latent forms of these proteinases can form complexes with TIMP-2 and TIMP-1, respectively (Grignon *et al*, 1996) Evidence that MMPs play an important role in invasion and metastasis came originally from experiments with genetically manipulated levels of TIMP-1 Schultz *et al* (1988) first showed that an intraperitoneal injection of TIMP-1 reduced lung metastasis of intravenously injected B16F10 melanoma cells A reduction in TIMP-1 levels by antisense RNA in mouse

fibroblasts resulted in the formation of metastatic tumours in nude mice (Khokha *et al*, 1989) An important point that must be made, however, is that the simplistic expectation that malignant tumours would have increased MMP expression accompanied by decreased TIMP expression is often not met In several cases, malignant tumours have been shown to have increased rather than decreased TIMP levels (Grigon *et al*, 1996) These studies showed a complex relationship between MMPs, TIMPs and cancer

Koop *et al* (1994) reported that when cancer cells were assessed using IVVM (intravital videomicroscopy), the expectation was that the reduced metastatic ability of the TIMP-1 overexpressing cells would manifest itself in defective extravasation However, both TIMP-1 overexpressing cells and control cells were found to extravasate with identical kinetics The reduced metastatic ability of the TIMP-1-overexpressing cells was manifested at 3 days after injection, when the morphology of micrometastatic colonies was strikingly different from that of the control cells The TIMP-1-overexpressing cells lacked adhesive contacts to other tumour cells and vessels, and had abundant stroma between the cells TIMP-2-expressing cells also demonstrated a reduction in growth rate and a differentiated morphology (Montgomery *et al*, 1994) Thus, it appears that both TIMP-1 and TIMP-2 can have growth inhibitory effects

The effects of TIMPs on the growth of tumours is further complicated by the observation that TIMP-1 and TIMP-2 also display growth-promoting activity for a variety of cell types, thus demonstrating that TIMPs are bifunctional molecules (Bertaux *et al*, 1991, Nemeth *et al*, 1996, Hayakawa *et al*, 1994) There are several examples in which TIMPs either have no effect or enhance tumour growth and/or metastasis, effects contrary to that expected from antimetalloproteinase activity (Soloway *et al*, 1996, Sun *et al*, 1996) It is possible that the growth-promoting effects of TIMPs are cell type specific, manifested only in cells which have an appropriate receptor for the TIMP domam containing the growth-promoting activity Alternatively, factors such as the relative concentrations of specific TIMPs and/or MMPs and the extracellular environment may all affect how a tumour cell responds to alterations in TIMP expression

1.11 Cell Adhesion Molecules

Cell adhesion contributes to all morphogenetic events and is also linked to many other key processes including the control of cell division, cell differentiation and apoptosis Although not covered in this study, cell adhesion also occurs in bacteria and plays an essential role during the interaction between pathogen and host Cell adhesion can be mediated by numerous surface glycoproteins and glycoconjugates that can be grouped into families The most extensively studied families are integrins, immunoglobulins, selectins and cadherins

1.11.1 Integrin

Integrins are a family of cell surface proteins that mediate cell-extracellular matrix adhesion and cell-cell adhesion The name "integrin" signifies the role of these proteins in integrating the intracellular cytoskeleton with the extracellular matrix components, such as fibronectin, laminin, collagen, fibrm(ogen), entactin, tenascin and vitronectin

Subunits		Ligands
β_1	α_1	Collagens, laminin
	α_2	Collagens, laminin
	α_3	Fibronectin, laminin, collagens
	α_4	Fibronectin, VCAM-1
	α_5	Fibronectin
	α_6	Laminin
	α_7	Laminin
	α_8	?
	α_v	Vitronectin, flbronectin
β_2	α_L	ICAM-1, ICAM-2
	α_M	Fibronectin, ICAM-1
	α_X	Fibrinogen
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	α_V	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen
β_4	α_6	Laminin
β_5	α_V	Vitronectin
β_6	α_V	Fibronectin
β_7	α_4	Fibronectin, VCAM-1
	α_{IEL}	?
β_8	α_V	?

Table 1.11.1 The integrin receptor family

Integrins are transmembrane glycoproteins composed of non-covalently linked α and β subunits. There are currently 15 known α subunits and 8 β subunits, assembling into more than 20 different integrin receptors. In general, the combination of particular α and β

subunits determines the ligand specificity of the integrin complex (Table 1 11 1) Specific peptide regions within extracellular matrix proteins have been identified as integrin-binding sites The best described of these regions is the arginine-glycine-aspartic acid (RGD) sequence found in fibronectin, fibronogen, vitronectin, collagen and laminin (Ruoslahti *et al*, 1987) Peptides containing this sequence have the ability to inhibit cell adhesion to certain extracellular proteins

Integrins have been found in all types of tissues Some integrins are cell type-specific For example, $\alpha_{IIb}\beta_3$, is expressed exclusively in platelets and megakaryocytes (Ginsberg *et al*, 1988) $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are expressed only in leukocytes (Springer, 1990) The $\alpha_6\beta_4$ integrin is specific for epithelial cells (Kajiji *et al*, 1989)

Two binding modes have been identified for integrins cell-ECM, through binding of integrin and ECM components, cell-cell, through binding of integrin and cell membrane protein Upon ligand binding, integrins are aggregated in transmembrane complexes known as focal contacts, which are enriched in specific cytoskeletal proteins including talin, vinculin, α-actinin and actin (Burridge *et al*, 1988) The organisation of integrin-associated cytoskeleton and thus the ability of integrins to bind ECM ligands is regulated by Rho and Ras family members (Guan, 1997) Coordinate regulation of integrin binding affinity and cytoskeleton dynamics is of fundamental importance not only to cell adhesion, but to the overall cellular architecture, cell motility and to integrin-related signaling events It has recently been found that integrins and other adhesion receptors have a vital role in signal transduction processes (Daly *et al*, 1998) Integrin-mediated signaling can be roughly divided into two categories The first is "direct signalling", in which ligation and clustering of integrins is the only extracellular stimulus Adhesion to ECM proteins can activate cytoplasmic tyrosine kinases (e g focal adhesion kinase (FAK)), and serine/threonine kinases (such as those in the mitogen-activated protein kinase cascade), induce ionic transients and stimulate lipid metabolism (e g phosphatidylinositol-4,5-bisphosphate (PIP_2) synthesis) The second category of integrin signaling is "collaborative signaling" in which integrin-mediated cell adhesion modulates signalling events initiated through other types of receptors, particularly receptors that are activated by growth factors Also, the bidirectional transfer of information can be mediated from the outside to the inside of the cell, and from the inside to the outside of the cell, which has been termed "outside-in" and "inside-out" signalling respectively

Occupation of integrins at focal adhesion sites results in tyrosine phosphorylation of focal adhesion kinase (FAK) and regulates actin cytoskeleton reorganisation. This is termed "outside-in signalling". "Inside-out signalling" refers to changes in the activation state of integrins *via* intracellular signalling. Cells can vary their adhesive properties by modulating the affinity of integrins through "inside-out" mechanisms acting on the cytoplasmic domains of the integrin subunits (Martin-Bermudo *et al.*, 1998).

Integrin-mediated cell adhesion impacts on two key aspects of growth regulation. Firstly, integrin mediated adhesion can influence the activity of the cell-cycle machinery, consisting of various cyclin-dependent kinase (CDK) complexes. Secondly, integrin-mediated anchorage is also a key regulator of apoptosis (Frisch and Ruoslahti, 1997). Integrins also have been found to play a role in platelet aggregation, immune functions, tissue repair and tumour invasion and metastasis (Daly *et al.*, 1998).

The pattern of integrin expression in tumour cells *in situ* as well as in cultured cells appears to be complex and dependent on the individual tumour type. Each tumour type or cell line displays a great deal of heterogeneity in integrin expression. It is difficult to reconcile all the information concerning the role of integrins in tumour growth and metastasis. In some cases, there is a general down-regulation of integrin subunits in epithelial tumours compared to their normal tissue counterparts.

1.11.1.1 β_1 integrin subunit

Members of the β_1 subfamily have been correlated with tumour cell invasiveness. Several factors have made it difficult to draw definitive conclusions regarding the role of individual β_1 integrins in tumour pathogenesis. These include the presence of different integrins which have overlapping binding capabilities, the fact that cells usually express more than one integrin, and especially the heterogeneity within the tumour (Hynes, 1992).

It has been demonstrated that monoclonal antibodies to β_1 integrin blocked tumour cell migration through basement membrane *in vitro* (Yamada, 1990). The disruption of both alleles of the β_1 integrin gene in the highly metastatic murine T-cell lymphoma cell line resulted in no alteration in the *in vivo* growth capacity of the cells, but a significant reduction in their metastatic capacity (Stroeken *et al.*, 1998). All these data indicate that β_1 integrin is

essential for invasion and metastasis

1.11.1.2 $\alpha_2\beta_1$ integrin

The role of $\alpha_2\beta_1$ integrin as a collagen and laminin receptor, initially on platelets and subsequently on other cell types, e g endothelial cells, has been clearly established (Santoro and Zutter, 1995) The $\alpha_2\beta_1$ binding specificity may be modulated by cell-type specific factors (Kirchhofer *et al*, 1990) For example, the $\alpha_2\beta_1$ integrin from endothelial cells can bind laminin while $\alpha_2\beta_1$ on platelet cannot, but both can bind to collagen

In addition to its expression on fibroblasts and endothelial cells, high level of $\alpha_2\beta_1$ expression has been observed on numerous epithelial cells This observation prompted an investigation of $\alpha_2\beta_1$ integrin expression in malignancy using breast cancer as a model (Zutter *et al*, 1990) The results showed that $\alpha_2\beta_1$ was highly expressed in the epithelium of ducts and ductules of normal breast tissue Markedly reduced or undetectable $\alpha_2\beta_1$ expression was seen in poorly differentiated adenocarcinomas (Zutter *et al*, 1990, Koukoulis *et al*, 1991, Pignatelli *et al*, 1991, 1992) Studies of other adenocarcinoma (*i e* colon, prostate, lung, pancreas and skin) have yielded similar findings regarding $\alpha_2\beta_1$ expression (Koretz *et al*, 1991, Stallmach *et al*, 1992, Hall *et al*, 1991, Bonkoff *et al*, 1993, Stamp and Pignatelli, 1991) A review of existing literature reveals that decreased expression of $\alpha_2\beta_1$ is the most common change in integrin expression m epithelial malignancies, especially in breast tissue (Varner and Cheresh, 1996, Zutter and Santoro, 1998) *In vitro* studies showed that diminished $\alpha_2\beta_1$ integrin expression contributes to motility and the invasive behaviour of tumour cells (Zutter *et al*, 1995) whereas reexpression of the $\alpha_2\beta_1$ integrin in a poorly differentiated, invasive breast carcinoma cell line greatly diminishes its malignant potential (Keely, 1995) Overexpression of Erb-B2 in mammary carcinoma cells may lead to reduced α_2 integrin gene expression (Ye *et al*, 1996) It is suggested that $\alpha_2\beta_1$ may function as a tumour suppressor for breast and other epithelial malignancies (Zutter and Santoro, 1998)

The role of $\alpha_2\beta_1$ integrin m cells of epithelial origin appears to differ from α_2 integrin subunit expression m cells of mesenchymal origin Chan *et al* (1991) overexpressed the $\alpha_2\beta_1$ integrin in a rhabdomyosarcoma (RD) cell line (a skeletal muscle tumour), which did not express the $\alpha_2\beta_1$ mtegrm The resulting cells were no more tumorigenic than the parental cell line, but

were more metastatic Similarly, primary nonmetastatic melanomas expressed $\alpha_2\beta_1$ mtegrin at low levels, however, the melanoma cell lines that were highly metastatic *in vivo* expressed high levels of the $\alpha_2\beta_1$ mtegrin (Mortarini *et al*, 1991) These studies suggest that the function of the $\alpha_2\beta_1$ integrin may be cell type-dependent

1.11.1.3 $\alpha_3\beta_1$ integrin

The α_3 subunit forms a heterodimer complex with mtegrin β_1 subunit, this heterodimer is also called very late antigen -3 (VLA-3) $\alpha_3\beta_1$ is the only integrin which can bind to collagen, laminin and fibronectin It has been demonstrated that $\alpha_3\beta_1$ can bind directly to the collagen type IV α_1(IV) 531-543 sequence (Miles *et al*, 1995) Studies have shown that $\alpha_2\beta_1$ and $\alpha_1\beta_1$ are high affinity receptors for type IV collagen while $\alpha_3\beta_1$ is a low-affinity receptor (Kuhn and Eble, 1994) It is believed that binding to type IV collagen is initiated via the $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrins, and then $\alpha_3\beta_1$ integrin is recruited to sites on the substrate

Antibodies against $\alpha_3\beta_1$ stimulate the expression of MMP-2 (Chintala *et al*, 1996, Kubota *et al*, 1997) and MMP-9 (Larjava *et al*, 1993) Antibodies to the α_3 subunit can also inhibit melanoma cell motility on type IV collagen (Melchiori *et al*, 1995)

$\alpha_3\beta_1$ is widely distributed not only in normal tissues, but also in various types of tumours Some studies have suggested that reduced expression may be involved in local and metastatic tumour growth It has been reported that highly invasive cell subpopulations derived from the human prostate carcinoma line PC-3 expressed lower level of $\alpha_3\beta_1$ (Dedhar *et al*, 1993) The human colon carcinoma cell line SW620, derived from a metastatic lesion and with higher metastatic potential than the parental line SW480, does not express α_3 which is present in SW480 cells (Sampson-Johannes *et al*, 1996) Roussel *et al* (1994) showed that in comparison to normal pneumocytes, lung adenocarcinoma cells exhibit a significant reduction of $\alpha_3\beta_1$ Reduced expression of $\alpha_3\beta_1$ has also been shown in breast cancer cells (Gui *et al*, 1995) However, other studies have demonstrated that $\alpha_3\beta_1$ expression increases during tumour progression in cutaneous malignant melanoma (Natali *et al*, 1993)

1.11.1.4 $\alpha_4\beta_1$ integrin

The integrin α_4 subunit is noncovalently associated with either β_1 or β_7 chains The ligand binding specificities of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins partially overlap The vascular cell adhesion molecule-1 (VCAM-1) and fibronectin are recognised by both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins with similar efficiency, while the mucosal vascular adhesion molecule-1 (MAdCAM-1) is a preferential ligand for integrin $\alpha_4\beta_7$ (Berlin *et al*, 1993) Studies have shown that expression of $\alpha_4\beta_1$ in CHO B_2 Chinese hamster ovary cells enabled the cells to adhere and migrate in response to fibronectin, but not to assemble a fibronectin matrix (Wu *et al*, 1997)

Expression of α_4 integrins has been demonstrated in many different human tumours and cell lines It has been shown that α_4 integrins are absent from cultured melanocytes but are detected on different cell lines derived from metastatic melanomas (Albelda *et al*, 1990) Highly invasive melanomas express α_4 integrins more frequently than melanomas with low metastatic capacity (Aldelba *et al*, 1990) Similarly, moderate levels of $\alpha_4\beta_1$ integrins were found on primary sarcomas, while α_4 integrins were highly expressed by metastatic sarcoma cells (Pavonen *et al*, 1994) In animal models, it has been demonstrated that the metastatic capacity of melanoma and sarcoma cells was enhanced through $\alpha_4\beta_1$-VCAM-1 interactions since VCAM-1 positive endothelial cells can retain the $\alpha_4\beta_1$ positive cancer cells after i v injection of cancer cells into the blood vessels (Garofalo *et al*, 1995, Mattila *et al*, 1992)

In contrast to intravenous injection of cancer cells, subcutaneous injection mimics the early step of metastasis, beginning with cell growth at a primary tumour site Under these conditions, B16 melanoma sublines with high expression of $\alpha_4\beta_1$ showed decreased ability to form tumour colonies in the lung Moreover, transfection of highly metastatic B16 melanoma cells with the α_4 integrin subunit cDNA induced the low metastatic phenotype (Qian *et al*, 1994) These observations led to the conclusion that homophilic adhesion mediated by α_4 integrins prevents the detachment of melanoma cells from the primary tumour thereby inhibiting invasiveness and subsequent metastasis formation Inhibition of matrix metalloproteinase could provide an alternative explanation for the reduced metastasis formation of $\alpha_4\beta_1$ integrin-positive B16 melanoma cells after subcutaneous injection (Qian *et al*, 1994)

The results obtained to date suggest that the effects mediated by α_4 integrin are dependant on the stage of tumour progression at which α_4 integrin is induced For efficient ligand binding of integrins, mere expression on the cell surface is not sufficient It is essential that integrins are converted to an active state to bind their ligand (Hynes, 1992) For α_4 integrin, several pathways of cellular activation have been identified that lead to a rapid and transient increase in integrin activity Importantly, these mechanisms do not affect the cell surface density of α_4 integrin, but appear to operate either by modulating conformation dependent integrin affinity for ligand or cytoskeleton dependent cell surface clustering of integrin (Hynes, 1992)

1.11.1.5 $\alpha_5\beta_1$ integrin

Integrin $\alpha_5\beta_1$, a fibronectin-specific integrin, binds to the central RGD/PHSRN site of fibronectin whereas the $\alpha_4\beta_1$ integrin binds to the IIICS site $\alpha_5\beta_1$ has been implicated in the regulation of gene expression, cell growth and tumorigenicity Transfection of human α_5 and β_1 cDNA into transformed Chinese hamster ovary (CHO) cells resulted in the cells depositing more fibronectin in their ECM, migrating less than parental cells and showing reduced ability to grow These cells were also nontumourigenic when injected subcutaneously into nude mice (Giancotti and Ruoslahti, 1990) This study indicates that the $\alpha_5\beta_1$ integrin is an important determinant in the fibronectin assembly process as well as in cell growth and tumourigenicity Conversely, loss of $\alpha_5\beta_1$ expression in CHO cells increased tumorigemcity (Schreiner *et al*, 1991) A variant of K562 erythroleukemia cells selected for increased ability to attach to fibronectin showed a 5-fold up-regulation of $\alpha_5\beta_1$ and displayed significantly reduced growth as well as reduced turmorigenicity (Symington, 1990) Overexpression of $\alpha_5\beta_1$ in human colon carcinoma HT29 cells has been shown to induce the transcription of growth arrest-specific gene1 (GAS-1) and block the transcription of immediate early genes C-fos, C-jun and jun-B (Varner *et al*, 1995)

It has been suggested that $\alpha_5\beta_1$ is the fibronectin receptor of stationary cells (Giancotti and Ruoslahti, 1990) Locomotory cells may use $\alpha_3\beta_1$ or $\alpha_v\beta_1$ to interact with fibronectin and to migrate on it, while interaction through the $\alpha_5\beta_1$ would reduce migration This hypothesis would explain why adhesion peptides and integrin antibodies of broad specificity inhibit cell migration (Boucaut *et al*, 1984, Bronner-Fraser, 1985) whereas anti-α_5 subunit antibodies can facilitate it (Akiyama *et al*, 1989)

The $\alpha_5\beta_1$ integrin may play a role in apoptosis It was found that cells attached through the $\alpha_5\beta_1$ integrin survived in serum-free cultures (Zhang *et al*, 1995) This response is associated with an elevated expression of the anti-apoptosis protein Bcl-2

1.11.1.6 $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins

Sonnenberg *et al* (1988) reported that the integrin complex $\alpha_6\beta_1$ functions as a laminin receptor on platelets Later it was found that $\alpha_6\beta_1$ was widely expressed in most cell types At least nine different integrins can function as laminin receptors, a number that probably reflects the diversity and specificity of laminin-mediated phenomena The best studied integrins that function as laminin receptors are $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$

It was reported that inhibiting $\alpha_6\beta_1$ function or its expression can reduce the invasive and metastatic ability of several types of tumour cells (Lin *et al*, 1993, Rabinovitz *et al*, 1995, Ruiz *et al*, 1993, Shaw *et al*, 1996) For example, the metastatic potential of human melanoma cells injected into nude mice was diminished by using anti-α_6 antibodies (Ruiz *et al*, 1993) Neoplastic transformed cells resulted in increased expression of α_6 integrin (Dedhar *et al*, 1990) Histopathologically, however, the picture is varied Pancreatic carcinoma (Weinel *et al*, 1992) and head and neck squamous cell carcinomas (Van Waes and Carey, 1992) have been shown to have increased α_6 staining Breast carcinomas that preserve α_6 integrin expression are associated with reduced survival (Friedrichs *et al*, 1995) In contrast, other studies have shown decreased α_6 expression in human breast (Gould *et al*, 1990) and renal carcinomas (Korhonen *et al*, 1992)

The $\alpha_6\beta_4$ integrin was identified initially by Falcioni *et al* (1986) This integrin is only expressed on epithelial cells and the β_4 is the only integrin subunit whose cytoplasmic domain is unusually large $\alpha_6\beta_4$ integrin has been implicated in the formation and maintenance of hemidesmosome, a multiprotein complex that provides the cells with a strong and stable anchor to the basement membrane However, a general correlation between the type or degree of malignancy and β_4 integrin expression cannot be established Falcioni *et al* (1994) found that high clinical stage of colon carcinoinas express high amounts of $\alpha_6\beta_4$ and this expression correlates with the degree of tumour aggressiveness (Serini *et al*, 1996)

whereas normal thyroid cells do not express $\alpha_6\beta_4$ In contrast, other tumours, such as breast, prostate and basal cell carcinomas exhibit decreased β_4 integrin expression (Gui *et al*, 1995, Rabinovitz *et al*, 1995, Korman and Hrabovsky, 1993) A common observation in many of these studies is that the localisation of the β_4 integrin in invasive and metastatic carcinoma cells is diffuse in comparison with its polarised, basal localisation in normal epithelia

It was observed that the expression of the $\alpha_6\beta_4$ integrin in RKO cells, a β_4-deficient rectal carcinoma cell line, significantly increased the ability of these cells to invade matrigel and collagen (Chao *et al*, 1996) Expression of the $\alpha_6\beta_4$ in a human breast carcinoma cell line MDA-MB-435 which lacks integrins also results in a dramatic increase in invasion A related aspect of $\alpha_6\beta_4$ involvement is that this integrin can promote the formation of pseudopodia because it has been noted that expression of $\alpha_6\beta_4$ in RKO cells induces the formation of pseudopodia (Chao *et al*, 1996) $\alpha_6\beta_4$ could also induce the release of specific proteases such as MMPs although the direct evidence is lacking Another mechanism used by $\alpha_6\beta_4$ to facilitate invasion is by altering other adhesion receptors This is supported by the observation that expression of $\alpha_6\beta_4$ in RKO cells altered their invasion on collagen I which is not a ligand for $\alpha_6\beta_4$ (Chao *et al*, 1996)

$\alpha_6\beta_4$ has also been shown to be expressed in capillary "sprouts" and the possibility that it may be involved in angiogenesis has been raised (Enenstein and Kramer, 1994) The $\alpha_6\beta_4$ integrin may also have a role in apoptosis Expression of the β_4 cytoplasmic domain in cells has been shown to activate P21 (an inhibitor of cyclin dependent kinases) and induce growth arrest (Clarke *et al*, 1995) In addition, expression of β_4 cDNA in a bladder carcinoma cell line results in a loss of survival (Kim *et al*, 1997)

1.11.2 E-cadherin

The cadherins are a family of transmembrane glycoproteins that mediate cell-cell adhesion through Ca^{2+}-dependent mechanisms (Takeichi, 1988) Several subclasses of cadherins have been described, E-(epithelial), N-(neural) and P-(placental) cadherins Cadherins appear to function by connecting cells to each other by homophilic interactions, in which they bind selectively to identical cadherin types (Nose *et al*, 1990) In epithelial cells, E-cadherm, also known as uvomorulin and L-CAM, represents a key molecule in the establishment and

stabilisation of cellular junctions The cytoplasmic domain of E-cadherin is complexed with either β-catenin or γ-catenin (plakoglobin) β-catenin and γ-catemn bind directly to α-catenin, giving rise to two distinct cadherin-catenin complexes α-catenin is thought to link cadherin to actin filaments E-cadherin also plays a role in one of the earliest events in mammalian development known as compaction, which separates the 8-celled embryo into trophoblast and inner cell mass

Eidelman *et al* (1989) demonstrated a down-regulation of E-cadherm in poorly differentiated tumours compared to the corresponding normal tissues They studied 60 tumours of varying origins for their expression of E-cadherm and could not identify a single case of epithelial malignancy lacking E-cadherin, although they did note a decrease of E-cadherin staining in the more undifferentiated tumours In colorectal cancer, for example, some independent studies (Van der Wuff *et al*, 1992, Kinsella *et al*, 1993, Dorudi *et al*, 1993) have shown that expression of E-cadherm is inversely correlated with tumour differentiation Similarly, in breast and gastric cancer, loss of E-cadherin expression has been found in high grade tumours with a poor prognosis (Oka *et al*, 1993, Mayer *et al*, 1993) In contrast, Shimoyama *et al* (1989) stained 44 lung carcinomas using antibodies directed against E-cadherin They found that the staining intensity of E-cadherm varied considerably from case to case, but when present, staining was always restricted to cell-cell borders No clear down regulation of E-cadherin in the primary tumours or in metastases was noted

In vitro studies have clearly demonstrated that loss of E-cadherin is a critical step in the acquisition of an invasive and undifferentiated phenotype Non-transformed Madin-Darby canine kidney epithelial cells (MDCK) (Mareel *et al*, 1992) as well as a well-differentiated colon carcinoma cell line (Pignatelli *et al*, 1992) acquire a poorly differentiated phenotype when intercellular adhesion is specifically inhibited by anti-E-cadherin antibodies Transfection of mouse E-cadherin cDNA into a poorly differentiated human breast carcinoma cell line resulted in increased intercellular cohesion which was associated with inhibition of the invasive phenotype *in vitro* (Frixen *et al*, 1991) Similar results have been obtained by transfecting the same plasmid into a poorly differentiated colon carcinoma cell line (Liu *et al*, 1992)

In general, good experimental evidence exists to support the hypothesis that loss of E-cadherin expression or function is associated with increased invasiveness in transformed epithelial cells Unfortunately, these observations have not been consistently confirmed in immunohistochemical analysis of human tumours which indicates that unknown host factors could play important roles in the heterogeneous nature of E-cadherin expression

Aims of thesis

The initial aim of this thesis was to establish and characterise novel taxol-resistant and melphalan-resistant variants of the human nasal carcinoma cell line RPMI-2650 in order to investigate the mechanisms of multidrug resistance which is the major factor limiting the effect of chemotherapy Studies of drug-resistance mechanisms *in vitro* have been complicated by the fact that many human tumour cell lines already express high levels of resistance-associated proteins such as Pgp and MRP1 The purpose of this project was to identify a very sensitive cell line, preferably expressing low levels of active Pgp and/or MRP1, and then to select for resistant variants by exposure to a drug believed to be pumped by Pgp but not to any great extent by MRP1, such as taxol and to an MRP1 but non-Pgp substrate, such as melphalan It was also proposed to attempt to co-select the RPMI-2650 cells with taxol and melphalan, to determme if a novel mechanism exists which would confer cross-resistance to both drugs (obviously neither MPR1 nor Pgp alone can achieve this)

The dramatic morphological changes observed in the RPMI-2650 taxol- and melphalan-resistant variants during the study lead to the investigation of a number of properties of these variants including cell adhesion to extracellular matrix, cell adhesion molecules, cell invasion and motility Possible mechanisms of invasive behaviour in these variants were then further investigated

To clarify whether melphalan can increase invasiveness only in RPMI-2650 cell line or if it can have a similar effect in other cell lines, melphalan-resistant variants of the human lung carcinoma cell line, DLKP were established by both continuous exposure and pulse selection methods Preliminary investigation into possible mechanisms underlying their invasiveness was undertaken

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 0.22μm 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 Sterilisation

All glassware used in cell culture, such as media bottles, roller bottles etc. were sterilised by autoclaving. All items were autoclaved before use at 121°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 sterilised using the above conditions. Filter sterilisation was employed for unstable reagents, including cell culture media and protein solutions. Sterile disposable 0.22μm filters (Millex-GV, SLGV025BS) were used for small volumes. Larger volumes, such as culture media, were filter sterilised through a micro-culture cell 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 II laminar flow cabinet. All media were prepared from a 10X stock of liquid or powder concentrates and

made up in batches of 5 litres 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 liters 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 four basal media used in these studies The medium was then filter sterilised through a 0 2 μm pore size micro-culture capsule bell filter (Gelman Sciences 12158) into 500ml sterile bottles and aliquots from each bottle were removed for sterility tests The bottles were labelled and stored at 4°C and used only after the sterility tests proved negative

Medium	Catalogue no	Volume H_2O (UHP)	Volume 10X concentrate	Volume 1M HEPES	Volume 7 5% $NaHCO_3$
DMEM	D5648 (Sigma)	4 7L	5L concentrate	100mls	45mls
MEM	21430-020 (Gibco)	4 3L	500mls	100mls	45mls
Hams F12	N6760 (Sigma)	4 7L	5L concentrate	100mls	45mls
RPMI 1640	R6504 (Sigma)	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 80mls 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 100mls $NaHCO_3$ was prepared by dissolving 7 5g in 100mls 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 100mls of L-gluthmine was aliquoted and stored at –20°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 MEM

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 5-10 samples of serum were screened on a number of cell lines and suitable foetal calf serum was chosen 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 long term at -20°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 A 10ml aliquot of medium from each bottle was removed and tested for both bacterial and fungal contamination 1ml samples were inoculated m tryptone soya broth (Oxoid CM129) which supports the growth of fungus and aerobic bacteria and thioglycollate broth (Oxoid CM173) which supports the growth of anaerobic and aerobic bacteria All sterility tests were incubated for 14 days to test for bacterial and fungal 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 with tryptone soya agar at 37°C for three days prior to use

2.2 Cell Culture

2.2.1 Cell lines used in studies

All cell culture work was performed in class II vertical down-flow recirculating laminar flow cabinets (Holten Cytoguard) The cell lines used throught this work are outlined in table 2 1 The cell lines used were anchorage dependent The cells were grown in 25cm^2 (Costar 3055) and 75cm^2 (Costar 3375) tissue culture treated unvented flasks All cell lines were incubated at 37°C and were fed every two to three days

Cell Line	Source	Description	Basal Medium
RPMI-2650	ATCC	Human nasal squamous carcinoma cell line	MEM
A-549	ATCC	Human lung squamous carcinoma cell line	MEM
SKLU-1	ATCC	Human lung squamous carcinoma cell line	MEM
BT-20	ATCC	Human breast carcinoma cell line	MEM
MX-1	DKFZ, Germany	Human breast carcinoma cell line	RPMI-1650
HT-1080	ATCC	Human fibrosarcoma cell line	MEM
NIH-3T3	ATCC	Murine fibroblast	MEM (with new born calf serum)
COR-L23S	Peter Twentyman's lab, Medical research Centre, UK	Human large cell lung cancer cell line	MEM
COR-L23R	same as COR-L23S	The adramycin-resistant variant of COR-L23S	MEM
DLKP	NCTCC	Human lung carcinoma cell line	DMEM + Hams F12
DLKP-A	Same as DLKP	An adriamycin-resistant variant of DLKP	DMEM + Hams F12
DLKP-C	Same as DLKP	The carboplatin-resistant variant of DLKP	DMEM + Hams F12

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 were incubated at 37°C with 5ml of trysin/EDTA 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 trysin / 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

Cells required in large numbers were cultivated in roller bottles Approximately 100ml of growth medium was allowed to equilibrate in a roller bottle at 37°C after which a single cell suspension of approximately 2x10^7 cells was added The roller bottle was incubated at 0 25rpm overnight and then the rotor speed was increased to 0 50 rpm The cells were allowed to grow to 80% confluency and were fed when necessary

2.2.3. Cell counting

An aliquot of the single cell suspension was removed for counting and mixed in a ratio of 5 1 with trypan blue dye (Gibco 043-0525OH) The suspension was left for approximately 5 minutes 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 calculated with the dilution factor taken into account and the final number was multiplied by 10^4 to give the number of cells per ml Non viable cells stained blue with the trypan blue dye while the viable cells remained unstained

2.2.4 Cell freezing

All the cell lines used throughout this work were stored long term at -196°C in a liquid nitrogen tank 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 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 operator 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

2.2.5 Cell thawing

The vial of cells to be thawed was removed from the liquid phase in the liquid nitrogen tank 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°C and transferred to a sterile universal container containing 5ml of complete medium The cells were pelleted by centrifugation at 1000 rpm for 5 minutes The medium was decanted and the pellet resuspended in 5ml of complete medium The cell viability was determined and the cell suspension was then transferred to tissue culture flasks and incubated for 24 hours The following day 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 contammation, 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 at least 3 passages in drug free and antibiotic free medium, and after this time the spent 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°C 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) to methanol (BDH 10158)) The cells were then fixed for 10 minutes with Carnoys solution which was kept at -20°C for at least 30 minutes before use After 10 minutes, Carnoys solution was removed and coverslips were air dried The cells were then stained in 2ml of Hoechst (Sigma B-2883) working stock (50ng/ml in PBS) 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 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

150mls of mycoplasma agar (Oxoid CM 401) and mycoplasma broth (Oxoid CM 403) were prepared Each was supplemented with 50mls serum, 25mls yeast extract (15% w/v) and 25mls stock solution (12 5g dextrose and 2 5g L-arginine HCl in 250ml ultrapure water) All of these substances were either autoclaved or filter sterilised 0 5 ml of the growth medium from the cell lines was incubated in 5 mls of mycoplasma broth for 5-7 days at 37°C and then a streak from the broth was inoculated onto 10mls of the solidified agar The agar plates were incubated in a CO_2 gaspak system for 3-4 weeks, on day 7 and 21, the plates were exammed 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°C or at –20°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	Storage	Disposal
Adriamycin	4°C in darkness	Inactivated with 1% hyperchloride solution and disposed with excess water
Vincristine Vinblastine	4°C in darkness	Inactivated by autoclaving and disposed with excess water
VP16	Room temperature	Incineration
Taxol	Room temperature	Incineration
Melphalan	Room temperature in darkness	Incineration
Cisplatin	Room temperature in darkness	Incineration
5-Fluorouracil	Room temperature in darkness	Inactivated with 5M NaOH and disposed with excess water
Cadmium Chloride	Room temperature in darkness	Incineration

Table 2.4. Storage and disposal of cytotoxic drugs

2.5 Adaptation of MDR variants

Throughout the course of this study, a number of MDR variants were developed by selecting the sensitive parental cell lines, RPMI-2650 and DLKP with taxol or melphalan.

2.5.1 Continuous selection

Cells were grown to 70-80% confluency in 75cm^2 flasks and then exposed to an initial concentration of selecting agent which was equivalent to 80% kill when calculated using a 96 well plate miniature toxicity assay (see section 2.6). The cells were grown at this concentration of drug until they appeared healthy and had reached approximately 70% confluency, after which time the concentration of drug to which the cells were exposed was increased approximately 1.5 folds. This process was continued over a number of months until desired concentration was reached. Toxicity assays were carried out to monitor any changes in drug sensitivity when compared with the untreated parental cells.

2.5.2 Pulse selection

Cells were grown to 70-80% confluency in 75cm^2 flasks. The cells were then exposed to 3X the initial drug concentration for continuous selection for 4 hours, after which time the drug was removed and the cells rinsed twice with fresh media. The cells were then grown in drug free media at 37°C for 6 days (refed with fresh media every 2-3 days). This was repeated for 10 "pulses" after which time sensitivity to the selecting drug was monitored using miniaturised toxicity assay (see section 2.6).

2.6 Miniaturised in vitro toxicity assays

The toxicity profile of the cell lines used throughout this work was determined using miniaturised toxicity assays by an acid phosphatase procedure (Martin and Clynes, 1991). The assays were carried out when the cells grown in a 75cm^2 flask reached 80% confluency and were fed in complete medium the previous day.

2.6.1 Toxicity assays - 96 well plates

The cells were plated in 96 well plates (Costar 3596) at a concentration of 2x10^3 cells/well in 100µl of complete medium. The first lane of each plate was used as a cell blank to which 100µl 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°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 100µl of the drug dilution was added to each well in replicates of eight and 100µl 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 5-7 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 100µl of the assay substrate (10mM P-nitrophenyl phosphate (Sigma 104) in 0 1M sodium acetate (Sigma S-2889), 0 1% Triton X-100 (Sigma X-100)) was added to each well 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°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 50µl of 1N NaOH which also stopped the enzymatic reaction

2.6.2 Circumvention assays - 96 well plates

The cells were plated in 96 well plates at a concentration of $2x10^3$ cells per well in 100µl of medium 100µl 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°C in 5% CO_2 to allow the cells to attach A series of 4X drug dilution were prepared and 50µl was added to the wells in replicates of eight A 4X stock of the circumvention agent was also prepared and 50µl 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 mcubated at 37°C in 5% CO_2 for 5-7 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

2.7 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 cytoskeletal proteins and immunocytochemistry was performed on fixed cells to detect the presence of both intracellular and membrane associated antigens.

2.7.1 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 resuspended in complete medium. An aliquot of the cell suspension was removed and the cells counted using a haemocytometer. 10^5 cells/ml were added to each well of a sterile multiwell slides. The multiwell slide was then placed in a sterile petri dish and incubated overnight at 37^oC in 5% CO_2 to allow the cells to attach. The following day, the multiwell slides were washed with PBS and fixed in ice-cold acetone for 2 minutes. The slides were then left to air dry for 15 minutes. The cells were then incubated with the primary antibody for 30 minutes at 4^0C followed by washing three times with PBS. 50µl of the secondary antibody (FITC-labelled antimouse IgG (Boehringer Mannheim 821462) diluted to 1:50 in PBS) was added to each well and the cells were incubated for a further 30 minutes at 4^0C in darkness since the FITC-labelled antibodies were light sensitive. After the incubation period the cells were washed three times with PBS. The cells were then mounted in vectashield (Vector Lab. Inc. H-1000) and sealed with nail varnish. The cells were viewed for fluorescence under ultraviolet illumination using a Nikon microscope equipped with a mercury lamp.

2.7.2 Immunocytochemistry

2.7.2.1 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 (Sigma P-1379) 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 10µl 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

(Huang *et al.*, 1983). The slides were air dried overnight and stored at room temperature until required.

The cells required for the cytospins were grown in $75cm^2$ flask until they had reached approximately 80% confluency. The trysinisation procedure was carried out and a single cell suspension of $1x10^5$ cells per ml was prepared. Two 100µl aliquots of the cell suspension were 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°C until required.

2.7.2.2 Immunocytochemistry

All immunocytochemical studies on cytospins of cell lines were performed following the method of Hsu *et al.* (1981) using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC). The cytospins were removed from -20°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 ice-cold acetone (BDH 10003) for appropriate time periods. 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 serum from species which donated secondary antibody, such as normal rabbit serum (DAKO X0902) for 30 minutes at room temperature, after which time the excess serum was tapped off and the optimally diluted primary antibody was applied. Slides were then placed in a humidified atmosphere and incubated at room temperature for 2 hours or at 4°C over night. Following primary antibody incubation, slides were washed 3 times in 15 minutes in 1xTBS, 0.1% (v/v) Tween 20. The cells were then incubated with a biotinylated secondary antibody for 30 minutes at room temperature and then washed thoroughly three times with TBS/0.1% Tween-20. 50µl 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 50µl 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 (Sigma HHS-1S) for 15 seconds, differentiated in 1% (v/v) hydrochloric acid in methanol for 15 seconds and differentiated in Scotts tap water for 10 seconds, rinsing in water between each step A series of dehydration steps in alcohol (70%, 90% and 100% methanol) were carried out and slides cleared in xylene (BDH 305756G) (2 changes with 5 minutes each) before the cells were mounted in DPX (BDH 36029) and viewed under a Nikon microscope when dry

2.7.2.2.1 P-glycoprotein

The presence of P-glycoprotein in a number of cell lines was investigated using a monoclonal mouse MDR-1 antibody (BRI, BR-002) (Moran *et al*, 1997) The cells were incubated with a 1 40 dilution of the primary antibody for 2 hours at room temperature The cells were then washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated rabbit anti-mouse IgG antibody (1 300) (DAKO, E354) for 30 minutes

2.7.2.2.2 cMOAT (MRP2)

The presence of cMOAT (MRP2) was detected using the monoclonal mouse antibody cMOAT 2III-6 (a gift from Prof Rik Scheper, Holland) The cells were incubated with a 1 50 dilution of the primary antibody at 4°C overnight The cells were then washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated rabbit anti-mouse IgG antibody (DAKO E354) for 30 minutes at room temperature

2.7.2.2.3 Lung resistance protein (LRP)

The presence of LRP was detected using a mouse monoclonal antibody to LRP (LRP-56) (TCS, ZIM1001) The cells were incubated with a 1 20 dilution of the primary antibody for 3 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 30 minutes at room temperature

2.7.2.2.4 Topoisomerase II α

The presence of Topo II α in a number of cell lines was mvestigated using a monoclonal mouse Topo II α antibody (BRI, BR-003) After fixation in acetone, the slide was placed for 3 minutes in Triton solution (0 1% Triton-X-100 in 1X TBS) which was prepared and stored at 4°C for at least 24 hours before use The cells were then incubated at room temperature for 2

hours with a 1 50 dilution of the Topo II α antibody After this incubation period, the slide was washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated rabbit anti-mouse IgG antibody (DAKO E354) (1 300) for 30 minutes at room temperature

2.7.2.2.5 Glutathione S-transferase α, μ and π

The presence of GST α, μ and π in a number of cell lines was investigated using rabbit GST α (NOVO Lab NCL-GST alpha), GST μ (NOVO Lab NCL-GSTmuM2) and GST π (DAKO A3600) antibodies The cells were incubated at room temperature for 2 hours with a 1 100 dilution of the GST α antibody, or a 1 200 dilution of the GST μ antibody, or a 1 200 dilution of the GST π antibody After this incubation period, the slides were washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated goat anti-rabbit antibody (1 500) (DAKO E0432) for 30 minutes at room temperature

2.7.2.2.6 Metallothionein

The presence of metallothionein in a number of cell lines was investigated using a monoclonal mouse anti-metallothionein antibody (DAKO MTM 0639) The cells were incubated at 4°C for 16 hours with a 1 20 dilution of the primary antibody After this incubation period, the slides were washed thoroughly with TBS/0 1% tween-20 and the secondary antibody added for 30 minutes 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

2.7.2.2.7 Integrins

The presence of integrins in a number of cell lines was investigated using a range of mouse integrin antibodies (Serotec) The cells were incubated at room temperature for 2 hours with a 1 50 dilution (for integrin subunits α_2, α_3, α_4, α_5, α_6, β_1 and β_4) or 1 100 dilution (for integrin subunit α_1) of the primary antibody After this incubation period, the slides were washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated rabbit anti-mouse antibody (DAKO E354) (1 300) for 30 minutes at room temperature

2.7.2.2.8 E-cadherin

The presence of E-cadherin was detected using a mouse monoclonal antibody to E-cadherin (R&D BTA1) The cells were incubated with a 1 200 dilution of the primary antibody for 2 hours at room temperature The cells were then washed thoroughly with TBS/0 1% Tween-20 and incubated with the secondary biotinylated rabbit anti-mouse IgG antibody (1 300) (DAKO E354) for 30 minutes at room temperature

2.8 Protein analysis by Western blotting

2.8.1 Whole cell extract preparation

The cells used in the preparation of whole cell extracts were grown in $75cm^2$ flask 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 The cell pellet was then either suspended in 5ml of lysis buffer containing PBS and 1X proteinase inhibitor and sonicated (Labsonic U) until lysis occurred as determined microscopically or the cell pellet was lysed in 500µls M-PER™ mammalian protein extraction reagent (Pierce 78501) containing 1X proteinase inhibitor

2.8.2 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^{2+} (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 2mls of the working reagent was added to each tube 0 1ml of diluent was used as a blank The test tubes were then incubated at 60°C 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

2.8.3 Seperation of protein by SDS-Polyacrylamide gel electrophoresis (PAGE)

Prior to Western blotting, cell proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970) 7 5% polyacrylamide gels were used in this work to separate proteins present in the cellular extracts The gels were prepared as shown in table 2 8 3

Gel Component	Resolving gel (7 5%)	Stacking gel (5%)
30%Acrylamide/Bis-acrylamide stock (Sigma A-3574)	3 3ml	0 8ml
ultrapure H_2O	8 3ml	3 6ml
3M Tris-HCl pH 8 8 (BDH 44409 2E)	1 88ml	——
2M Tris-HCl pH 6 8 (BDH 44410 2T)	——	0 31ml
10% SDS (Sigma L-4509)	150µl	50µl
10% Ammonium Persulphate (Sigma A-1433)	60µl	17µl
TEMED (Sigma T-8133)	10µl	5µl

Table 2.8.3. Preparation of resolving and stacking gels for electrophoresis

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 10µg/10µl 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

2.8.4 Western blotting

Western blotting was performed by the method of Towbin *et al* (1979) Following SDS-polyacrylamide gel electrophoresis (PAGE), the gels were equilibrated for 15 minutes in 1X transfer buffer (Sigma T-4904) The gels were then aligned onto PVDF membrane (Boehringer Mannheim 1722026) which had been equilibrated in the same transfer buffer 7-8 sheets of whatman 3mm filter paper were soaked in transfer buffer and then placed underneath the PVDF membrane and also on top of the gel The transfer of protein from the acrylamide gel to the PVDF membrane was carried out using a Bio-Rad semi-dry blotting system for approximately 20 minutes at 15 volts and a maximum current of 345 mA Following protein transfer, the PVDF membranes were placed m blocking buffer which consisted of 5% non-fat dried milk (Marvel skimmed milk) in TBS (20mM Tris, 500mM NaCl pH 7 6) for at least 2 hours The PVDF membranes were then rinsed with TBS prior to the addition of the primary antibody at 4°C overnight The PVDF membrane was then washed three times in TBS containing 0 5% Tween-20 (Sigma P1379) The membrane was exposed to the secondary antibody (horse radish peroxidase (HRP)-conjugated, diluted in TBS containing 0 1% Tween-20) for 1 5 hours at room temperature The membrane was again washed three times in TBS and was developed as outlined in section 2 8 5

Primary antibody	dilution	Secondary antibody	dilution
MDR-1(BRI, BR-002)	1 50	Goat anti-mouse (DAKO P0447)	1 2000
MRP1 (TCS-ZUC201)	1 80	Rabbit anti-rat (DAKO P0450)	1 2000
Integrin β_1(Chemicon AB1937)	1 10000	Goat anti-rabbit (DAKO P0448)	1 2000

Table 2.8.4 Antibodies used in Western blotting

2.8.5 Development of Western blots

Immunoblots were developed using an Enhanced Chemiluminescence kit (Amersham, RPN2109) which facilitated the detection of bound peroxidase-conjugated secondary antibody Following the final wash, the PVDF membranes were subjected to ECL A layer of parafilm was flattened over a glass plate and the PVDF membrane was placed gently on the plate A volume of 5ml of a 50 50 mixture (solution A solution B) of ECL regents was used to cover the PVDF membrane The ECL reagent mixture was completely removed after a period of one minute and the membrane wrapped in cling film All excess air bubbles were removed The PVDF membrane was then exposed to autoradiographic film (Kodak X-OMATS) for various times (from 1 to 15 minutes depending on the signal) The exposed antoradiographic film was developed in developer (Kodak LX24) until the bands on the film were visible The film was then washed in water for 15 seconds and transferred to a fixative (Kodak FX-40) for 2 minutes Finally, the film was washed with water for 5-10 minutes and left to dry at room temperature

2.9 Subcellular distribution of adriamycin

The subcellular localisation of adriamycin in a number of cell lines was investigated by fluorescence microscopy The cells studied were grown on glass coverslips and then viewed under ultraviolet illumination (Chauffert *et al*, 1984)

2.9.1 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 1ml of the cell suspension was added to each of the petri dishes which were then incubated overnight at 37°C in 5% CO_2 to allow attachment of the cells to the sterile coverslips The following day the medium was carefully removed from the petri dishes and the cells incubated with 1ml of medium containing 10μM adriamycin for 4 hours

After this time, the drug containing medium was decanted and the cells were washed twice with ice-cold PBS The covershps 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 occurred very rapidly

30μg/ml Verapamil (Sigma V-4629) or 10 μg/ml Cyclosporm A (Sandoz) were added to 1ml medium (containing 10μM adriamycin) with which the cells were incubated to determine if they were effective at altering the accumulation and/or subcellular distribution of adriamycm in various cell lines

2.10 Determination of glutathione S-transferase activity

Glutathione S-transferase activity was determined using cytosolic extracts of the cells 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 340 nm (Evans *et al*, 1987)

2.10.1 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 2ml 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 7800 rpm for 10 minutes at 4^0C The supernatant was collected and centrifuged at 38,000 rpm for 1 hour at 4°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

2.10.2 Assay for glutathione S-transferase

A 30mM stock of glutathione (Sigma G-4251), a 30mM stock of dinitrochlorobenezene (BDH 10076) in ethanol and a potassium phosphate buffer (100mM KH_2PO_4 and 100mM HK_2PO_4) were prepared. 2 cuvettes containing 0.1ml of the glutathione stock, 0.1ml of the dinitrochlorobenzene stock and 2.2ml of potassium phosphate buffer were placed in the constant temperature chamber of a dual beam spectrophotometer (Shimadzu UV 160A). 0.6ml of the cytosolic extract was added to the sample cuvette and 0.6ml 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 protein level of each sample determined by BCA assay as outlined in section 2.8.2.

2.11 Time lapse videomicroscopy

Time-lapse videomicroscopy was carried out on a Nikon Diaphot inverted microscope (Micron optical, Bray, Ireland) equipped with phase-contrast optics, linked to a Mitsubishi CCD-100 colour video camera. Images were recorded in S-VHS onto a Mitsubishi HS-S5600 video recorder with time lapse capabilities. Recording speed was set at 3.22 sec/field (480 hour mode), which at normal playback speed resulted in an acceleration factor of 160. The temperature of the culture vessel was controlled by a Linkam Colo2 warm stage controller. This controller was adjusted to keep the culture medium inside the vessel at 37°C, as measured using a TB3301 probe. All time-lapse video equipment was obtained from laboratory instruments (Ashbourne, Ireland).

Cells were seeded at $1x10^5$cells/ml one day before the recording was started. Cell motility was monitored by locating the initial position of the cell and the final position of the same cell after certain time periods.

2.12 Extracellular matrix adherence assays

2.12.1 Reconstitution of ECM proteins

Adhesion assay was performed using the method of Torimura *et al.* (1999). Collagen Type IV (Sigma C-5533), fibronectin (Sigma F-2006) and laminin (Sigma L-2020) were reconstituted in PBS to a stock concentration of 500μg/ml. Stocks were aliquoted into sterile eppendorfs. Fibronectin and collagen stocks were stored at -20°C while laminin stocks were stored at -80°C. Matrigel gel (Sigma E1270) was aliquoted and stored at -20°C until use. Matrigel

undergoes thermally activated polymerisation when brought to 20-40°C to form a reconstituted basement membrane

2.12.2 Coating of plates

Each of the ECM proteins, collagen, fibronectin and laminin, was diluted to 25µg/ml while Matrigel was diluted to 1mg/ml with PBS 250µl aliquots were placed into wells of a 24-well plate The plates were tapped gently to ensure that the base of each well was completely covered with solution The plates were then incubated at 4°C overnight The ECM solutions were then removed from the wells and the wells rinsed twice with sterile PBS 0 5ml of a sterile 0 1% BSA/PBS solution was dispensed into each well to reduce non-specific binding The plates were incubated at 37°C for 20 minutes and then rinsed twice again with PBS

2.12.3 Adhesion assay

Cells were set up in 75cm^2 flasks and then harvested and resuspended in serum free DMEM medium The cells were then plated at a concentration of 2 5x10^4 cells per well in triplicate and incubated at 37°C for 60 minutes Control wells were those which had been coated but contain no cells After 60 minutes, the medium was removed from the wells and rinsed gently with PBS The cells were then stained with 0 5ml of 0 25% crystal violet dye for 10 minutes The plates were then rinsed and allowed to dry The dye was eluted with 200µl of 33% glacial acetic acid and 100µl aliquots were transferred to a 96-well plate and the absorbance was read on an ELISA reader at 570 and 620nm (Torimura *et al*, 1999)

2.12.4 Adhesion blockade assay

After the coating of the plate as in section 2 12 2, cells were harvested and resuspended in serum free DMEM medium at a concentration of 4x10^4 cells per ml The cells were incubated with 10µg/ml of a range of anti-mtegrin antibodies (Serotec) for 30 minutes at 4°C Control samples were cells incubated at 4°C without addition of antibody The cells were then plated into the wells at a concentration of 2x10^4 cells per well and incubated for 30 minutes at 37°C After 30 minutes, the medium was removed from the wells and rinsed gently with PBS The cells were then stained as in section 2 12 3

2.13 Invasion assay

Invasion assay was performed using the method of Albini (1998) Matrigel (Sigma E-1270) (11mg/ml) was diluted to 1mg/ml in serum free DMEM 100µl of 1mg/ml Matrigel was placed into each insert (Falcon 3097) (8 0 µm pore size, 24 well format) which stands on 24-well plate (Costar) The inserts and the plate were incubated overnight at 4°C The following day, the cells were harvested and resuspended in DMEM containing 5% FCS at a concentration of $1x10^6$ cells/ml The inserts were washed with serum free DMEM, then 100µl of the suspension cells were added to each insert and 250µl of DMEM containing 5% FCS was added to the well underneath the insert Cells were incubated at 37°C for 24 hours After this time period, the inner side of the insert was wiped with a wet swab while the outer side of the insert was stained with 0 25% crystal violet for 10 minutes and then rinsed and allowed to dry The inserts were then viewed under the microscope

2.14 Motility assay

The procedure for carrying out motility assays was identical to the procedure used for invasion assay with the exception that the inserts were not coated with matrigel (section 2 13) (Genda *et al*, 1999)

2.15 Zymography

Zymography was used to assess the level of proteolytic activity of different proteinases The choice of substrate incorporated into the resolving gel depends on substrate specificity to the species of enzymes detected (Johansson *et al*, 1986) Gelatin is a substrate for matrix metalloproteinases (MMPs), serine and cysteine proteinases However, gelatin zymography is not suitable for the assay of MMPs with weak or absent gelatinase activity This is the case of the stromelysin 1 and 2 (MMP-3 and MMP-10, respectively) and matrilysin (MMP-7) for which casein zymograpgy is more suitable

The gel was prepared by incorporating gelatin within the polymerised acrylamide matrix 10% acrylamide gels were used The amount for one gel is given below

Gel component	Resolving gel (10%)	Stacking gel
30% Acrylamide/Bis-acrylamide (Sigma A-3574)	3 3ml	0 5ml
3mg/ml Gelatin (Sigma G-8150)	2 5ml	——
Ultrapure water	1 7ml	2ml
3M Tris-HCl, PH 8 8 (BDH 44409 2L)	2 5ml	——
2M Tris-HCl, PH 6 8 (BDH 44410 2T)	——	0 8ml
10% Ammonium Persulphate (Sigma A-1433)	33µl	33µl
TEMED (Sigma T-8133)	5µl	5µl

Table 2.15 Preparation of resolving and stacking gel for zymography

Cells were grown in petri dishes (Greiner) When the cells were 80% confluent, they were rinsed twice with sterile PBS followed by 4-hour incubation with serum-free medium The cells were then grown in fresh serum-free medium for another 24-72 hours After the time period, supernatants were collected as samples Samples were mixed 3 1 with 4 X sample buffer (10% glycerol, 0 25 M Tris-HCl, pH 6 8, 0 1% (w/v) bromophenol blue) and loaded onto the gel The gels were run at 30 mA per gel in running buffer (0 025M Tris, 0 19M glycine, 0 1% SDS) until the dye front reached the bottom of the gel Following electrophoresis, the gels were soaked in 2 5% Triton-X-100 with gentle shaking for 30 minutes at room temperature The gels were then rinsed in substrate buffer (50mM Tris-HCl, pH8 0, 5mM $CaCl_2$) and then incubated for 24 hours in substrate buffer at 37°C The gels were then stained with Coomassie blue (2 5mg/ml) for 2 hours by shaking and destained in destain water (50ml acetic acid, 150ml isopropanol, 300 ml distilled water) until clear bands were visible The gels were then scanned by scanner

Inhibitors or enhancers of proteinases were added to 2 5% Triton-X-100 and substrate buffer to identify the different classes of proteinases (Table 2 15 a)

Inhibitor/enhancer	Enzyme inhibited/enhanced	Concentration
EDTA (inhibitor)	MMPs	30mM
PMSF (inhibitor)	Serine proteinases	1mM
Cysteine (Enhancer)	Cysteine proteinases	10mM

Table 2.15.a Inhibitors or enhancers of different classes of proteinases used in gelatin-zymography

2.16 Reverse transcriptase - Polymerase chain reaction (RT-PCR)

2.16.1 RNA extraction

Total RNA was isolated from all cell lines using standard method (O'Driscoll *et al*, 1993) and quantified spectrophotometrically at 260nm and 280nm (Ausubel *et al*, 1991) For all procedures using RNA, most glassware, solutions and plastics were treated with 0 1% diethyl pyrocarbonate (DEPC) (Sigma D-5758) before use

RNA was extracted from the cells as follows

Cells were harvested from the 75cm^2 flask at exponential phase The pellet was washed once with PBS and then lysed using 1ml TRI Reagent (Sigma T-9424) (This product, a mixture of guanidine thiocyanate and phenol in a mono-phase solution, effectively dissolves DNA, RNA and protein in samples) The samples were allowed to stand for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes 0 2ml of chloroform was then added per ml of TRI Reagent used and the sample was shaken vigorously for 15 seconds and allowed to stand for 15 minutes at room temperature The sample was then centrifuged at 13000 rpm for 15 minutes at 4°C This step separated the mixture into 3 phases, the RNA was contained in the colourless upper aqueous layer This layer was then transferred to a new eppendorf and 0 5ml of isopropanol was added The sample was mixed and allowed to stand at room temperature for 10 minutes before being centrifuged at 13000 rpm for 10 minutes at 4°C The RNA formed a precipitate at the bottom of the tube The supernatant was removed and the pellet was washed with 1ml of 75% ethanol and centrifuged at 13000 rpm for 5 minutes at 4°C The supernatant was removed and the pellet was briefly allowed to air-dry 20µl of DEPC water was then added to the RNA to resuspend the pellet

The concentration of RNA was calculated by determining its OD at 260nm and 280nm and using the following formula

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

The purity of the RNA extraction was calculated by determining its OD at 260nm and 280nm An OD_{260nm} OD_{280nm} ratio is indicative of pure RNA Only those samples with ratios between 1 7 and 2 1 were used

2.16.2 Reverse Transcriptase Reaction of RNA isolated from cell lines

Reverse transcriptase reactions were carried out in laminar flow cabinets

cDNA was formed using the following procedure

1µl Ohgo (dT) primers (1µg/µl) (Promega C1101)

1µl total RNA (1µg/µl) (see 2 16 1)

3µl water

were mixed in a 0 5ml eppendorf (Eppendorf, 0030 121 023), heated to 70°C for 10 minutes and then chilled on ice To this, the following were added

4µl 5X buffer (250 mM-Tris/HCl pH 8 3, 375mM-KCl and 15mM-$MgCl_2$) (Gibco 510-8025 SA)

2µl DTT (100mM) (Gibco 510-8025 SA)

1µl RNasin (40U/µl) (Promega N2511)

1µl dNTPs (10mM each of dATP, dCTP, dGTP and dTTP)

6µl water

1µl Molony murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/µl) (Gibco, 510-8025 SA)

The solutions were mixed and the mixture was incubated at 37°C for an hour to allow the MMLV-RT enzyme catalyse the formation of cDNA on the mRNA template The enzyme was then inactivated and the RNA and cDNA strands separated by heating to 95°C for 2 minutes The cDNA was used immediately in the PCR reaction or stored at –20°C until required for analysis

2.16.3 Polymerase Chain Reaction

Each PCR eppendorf tube contained the following

24 5µl water

5µl 10X buffer (100mM-Tris/HCl, pH 9 0, 50mM-KCl, 1% Triton X-100) (Promega N1862)

3µl 25mM-$MgCl_2$ (Promega N1862)

8µl dNTPs (1 25mM each of dATP, dCTP, dGTP and dTTP) (Promega U1240)

1µl each of first and second strand target primers (250ng/ml)

1µl each of first and second strand control primers (250ng/ml) (β-actin)

0 5µl of 5U/µl Taq DNA polymerase enzyme (Promega N1862)

5 µl cDNA

The cDNA was amplified by PCR using the following program

95°C for 1 5 min (denature double-stranded DNA)

30 cycles 95°C for 1 5 min (denature double-stranded DNA)

55°C for 1 min (anneal primers to cDNA)

72°C for 3 min (extend)

72°C for 7 min (extend)

The reaction tubes were stored at 4°C until analysed by gel electrophoresis

2.16.4 Electrophoresis of PCR products

2 5% agarose (Promega V3122) gel was prepared in TBE buffer (5 4g Tris, 2 75g boric acid, 2ml 0 5M-EDTA pH 8 0 in 500ml water) and melted in a microwave oven After allowing to cool, 0 003% of a 10mg/ml ethidium bromide (Sigma E8751) solution was added to the gel which was then poured into an electrophoresis apparatus (BioRad) Combs were placed in the gel to form wells and the gel was allowed to set

10µl loading buffer (50% glycerol, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue, 1mM EDTA) was added to each 50µl PCR sample and 20µl was run on the gel at 80-90mV for approximately 2 hours using TBE as running buffer The ϕ174 DNA/Hae III marker (Promega G1761) was run simultaneously as size reference When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a transilluminator and photographed by polaroid camera

Gene	Primer sequence	Length of product amplified	Optimum anealing temp (°C)
mdr1	FWD GTT CAA ACT TCT GCT CCT GA	157bp	54
	REV CCC ATC ATT GCA ATA GCA GG		
mrp1	FWD GTACATTAACATGATCTGGTC	202bp	54
	REV CGTTCATCAGCTTGATCCGAT		
cmoat (mrp2)	FWD CTGCCTCTTCAGAATCTTAG	241bp	54
	REV CCCAAGTTGCAGGCTGGCC		
mrp3	FWD GATACGCTCGCCACAGTCC	262bp	62
	REV CAGTTGGCCGTGATGTGGCTG		
mrp4	FWD CCA TTG AAG ATC TTC CTG G	239bp	42
	REV GGT GTT CAA TCT GTG TGC		
mrp5	FWD GGA TAA CTT CTC AGT GGG	381bp	49
	REV GGA ATG GCA ATG CTC TAA AG		
mmp-2	FWD TGA CAT CAA GGG CAT TTC AGG AGC	180bp	54
	REV GTC CGC CAA ATG AAC GGT CCT TG		
mmp-9	FWD GGTCCCCCCACTGCTGGCCCTTCTACGGCC	640bp	54
	REV GTCCTCAGGGCACTGCAGGATGTCATAGGT		
timp-1	FWD TGCACCTGTGTCCCACCCCACCCACAGACG	551bp	54
	REV GGCTATCTGGGACCGCAGGGACTGCCAGGT		
α_2 *integrin*	FWD TGG GGT GCA AAC AGA CAA GG	541bp	54
subunit	REV GTA GGT CTG CTG GTT CAG C		
β_1 *integrin*	FWD GCG AAG GCA TCC CTG AAA GT	663bp	54
subunit	REV GGA CAC AGG ATC AGG TTG GA		
c-Ha-ras		129bp	60
c-myc		336bp	60
β-actin (large)	FWD GAAATCGTGCGTGACATTAAGGAGAAGCT		
	REV TCA GGA GGA GCA ATG ATC TTG A	383bp	
β-actin (small)	FWD TGG ACA TCC GCA AAG ACC TGT AC		
	REV TCA GGA GGA GCA ATG ATC TTG A	142bp	

Table 2.16 Sequence of primers used for RT-PCR

3. Results

3.1 Toxicity assays of chemotherapeutic drugs in the SKLU-1, BT-20, RPMI-2650, MX-1 and A-549 cell lines

3.1.1 General toxicity assays

In order to determine if certain types of carcinomas (*i e* lung or breast) display similar sensitivity to a range of chemotherapeutic drugs, toxicity assays were carried out in a number of cell lines using various chemotherapeutic drugs The cell lines used were the lung cell lines SKLU-1 (squamous epithelial cell line) and A-549 (adenocarcinoma cell line), the breast cell lines BT-20 and MX-1 and the nasal epithelial carcinoma cell line, RPMI-2650 The chemotherapeutic drugs used were adriamycin, vincristine, vinblastine, VP-16, taxol, melphalan, cisplatin and 5-FU

Table 3 1 1 represents the IC_{50} values obtained for each chemotherapeutic agent tested The cell lines showed significantly different resistance profiles for these drugs RPMI-2650 is the most sensitive cell line to most of the agents It was hundreds or even in some cases thousands of times more sensitive than the A-549 or MX-1 cells The SKLU-1 and BT-20 cells generally showed similar drug resistance profiles Although the SKLU-1 and BT-20 cells displayed some resistance to most of the agents tested, both cell lines are more sensitive than the A-549 and MX-1 cells In contrast, A-549 and MX-1 showed very high IC_{50} values Both cell lines are more resistant to the group of drugs tested In conclusion, lung cancer and breast cancer cell lines do not display similar sensitivities to the range of chemotherapeutic drugs tested The resistance profile appears to be dependent on the particular cell line studied rather than on the site of origin of the tumour

IC_{50} (nM)	SKLU-1	BT-20	RPMI-2650	A-549	MX-1
Adriamycin	38 ± 1	35 ± 5	18 5 ± 3 5	991 ± 23	5117 ± 2130
Vincristine	2 8 ± 0 2	2 ± 0 1	2 4 ± 0 4	145 ± 42	1071 ± 353
Vinblastine	1 4 ± 0 1	1 ± 0 2	1 78 ± 0 12	94 ± 11	3323 ± 932
VP-16	63 ± 10	64 ± 13	23 ± 3	1706 ± 347	166 ± 60
Taxol	14 ± 1	6 ± 1	2 3 ± 0 9	15 ± 0 8	336 ± 110
Melphalan	6085 ± 2522	11468±1158	582 ± 10	48952±9221	6129 ± 2209
5-FU	9071 ± 1305	4295 ± 313	1624 ± 164	5612 ± 109	4728 ± 163
Cisplatin	1924 ± 12	542 ± 150	1494 ± 39	10887±1777	942 ± 12

Table 3.1.1 IC_{50} values of a group of chemotherapeutic drugs for SKLU-1, BT-20, RPMI-2650, A-549 and BT-20 cell lines

3.1.2 Heavy metal drug toxicity studies in A-549, SKLU-1 and MX-1

3.1.2.1 Toxicity assays with cisplatin and cadmium chloride

It was noted from the results shown in section 3 1 that the cell lines A-549 and SKLU-1 were resistant to cisplatin, with A-549 exhibiting the higher level of resistance to this drug These results also illustrated that the human breast cell line MX-1 was very sensitive to cisplatin Since cisplatin is a heavy metal drug, sensitivities to another heavy metal drug, cadmium chloride, were studied in these three cell lines The results indicated that the trends of sensitivities to cadmium chloride were consistent with those to cisplatin

IC_{50} (nM)	A-549	SKLU-1	MX-1
Cisplatin	10887 ± 1777	1924 ± 12	942 ± 12
Cadmium chloride	36957	2820	655

Table 3.1.2 IC_{50} of cisplatin and cadmium chloride in the A-549, SKLU-1 and MX-1 cell lines

3.1.2.2 Immunocytochemical detection of metallothionein

Metallothioneins are a family of the proteins which are related to heavy metal drug resistance in cells Immunocytochemical detection of metallothionein was carried out in the A-549, SKLU-1 and MX-1cell lines No difference of the expresssion of metallothionein was observed between A-549, SKLU-1 and MX-1 This suggests that some other factor might be involved in the different resistance levels to the cadmiun chloride and cisplatm in these three cell lines

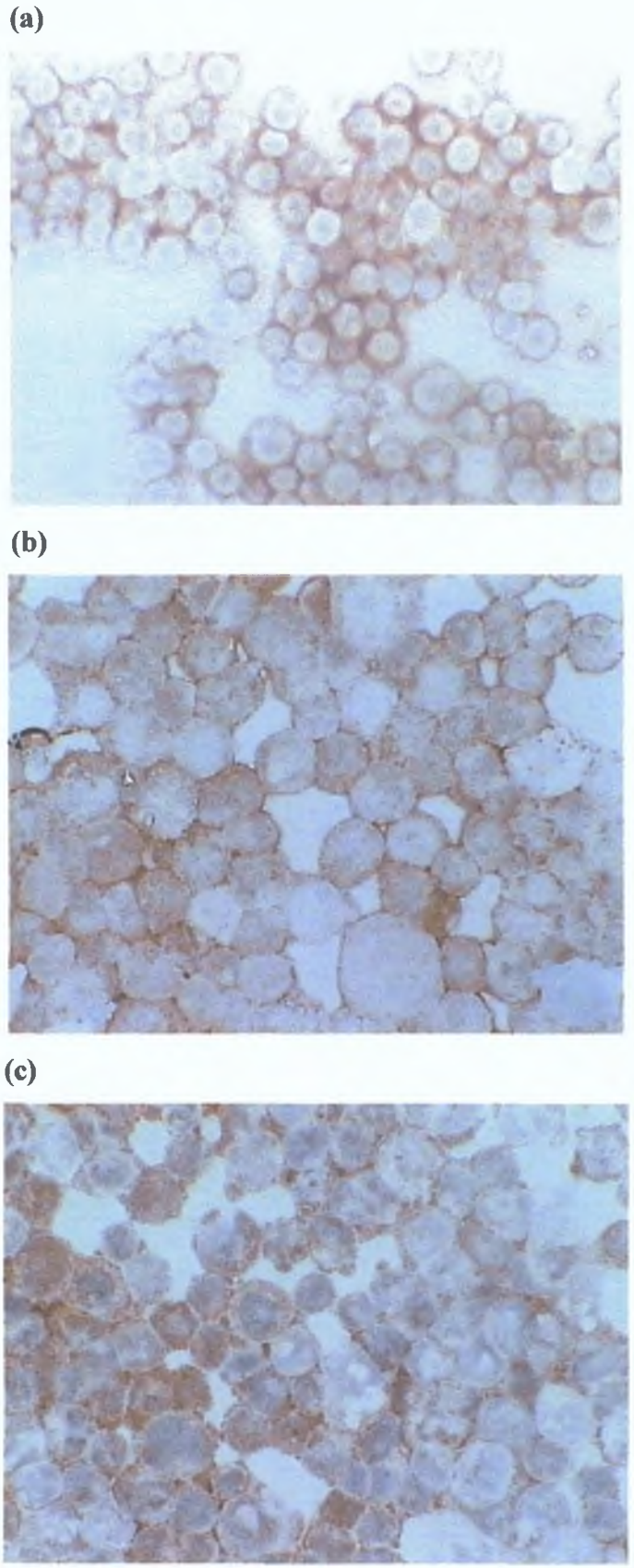


Fig. 3.1.2 Immunocytochemical staining for metallothionein (40x) in the (a) A-549 cell line (b) SKLU-1 cell line (c) MX-1 cell line

3.2 Establishment of novel MDR variants of RPMI-2650 and DLKP cell lines

The RPMI-2650 cell line was established in 1962 by G E Moore and A A Sandberg from the pleural effusion of a patient with an extensive malignant tumour of the nasal septum The tumour was diagnosed as an anaplastic squamous cell carcinoma

As described in section 3 1, RPMI-2650 is very sensitive to a number of chemotherapeutic drugs Consequently, attempts were made to produce multidrug resistant variants of this cell line The cells were cultured in increasing concentrations of drug until MDR variants were established Toxicity assays were conducted in order to determine the level of resistance of the MDR variant relative to the parental cells

A poorly differentiated squamous lung cancer cell line, DLKP, was established from a lymph node metastasis biopsy by bronchoscopy in this centre (Law *et al*, 1992) Toxicity assays indicated that DLKP was very sensitive to adriamycin, vincristine, VP-16 and cisplatin (Clynes *et al*, 1992) Melphalan-resistant variants of the DLKP cell line were established by both continuous exposure and pulse selection methods

3.2.1 Establishment of the RPMI-2650 MDR variants

3.2.1.1 Establishment of the RPMI-2650 taxol-resistant variant

The RPMI-2650 cells were initially exposed to 4ng/ml taxol However, after a few days, the cells appeared to have stopped proliferation and after approximately one week, very few viable cells were visible The cells were then cultured in drug free medium until the cells were approximately 60% confluent After this time the cells were again exposed to 4ng/ml of the drug After approximately 1 month, the concentration of drug was increased to 20ng/ml Cells were adjusted to growth in 80ng/ml after a further month and a final concentration of 200ng/ml was achieved within another one and a half months Cells were frozen and stored at the stages when they were adjusted to growth in 80ng/ml and 160 ng/ml taxol

3.2.1.2 Establishment of the RPMI-2650 melphalan-resistant variant

The RPMI-2650 cells were initially exposed to 1 25μg/ml melphalan and adjusted to growth in 1 7μg/ml after approximately 2 months, to 5μg/ml after another two months and to a final concentration of 6 5μg/ml after a further 4 months exposure During the selection period, the cytoplasm was elongated like the axon of the nerve cell Cells became spindle-shaped after the selection was finished

3.2.1.3 Establishment of the RPMI-2650 taxol and melphalan co-selection variant

The cells were initially exposed to 10ng/ml taxol and 1μg/ml melphalan It was observed that this concentration was very toxic to the cells The selection procedure was initiated on 2 further occasions at lower drug concentrations (6ng/ml taxol and 1μg/ml melphalan, 4ng/ml taxol and 1μg/ml melphalan), however, these combinations also proved to be extremely toxic to the RPMI-2650 cells These observations indicate that the combination of taxol and melphalan was lethal to the RPMI-2650 cells It is possible that the cells could not find an alternative way to survive the combination of toxicity effects of taxol and melphalan This suggests the usefulness of co-administration of taxol and melphalan in cancer treatment

3.2.2 Establishment of the novel MDR variants of the DLKP cell line

3.2.2.1 Establishment of the DLKP melphalan-resistant variant by long term selection

The DLKP cells were initially exposed to 2μg/ml melphalan and adjusted to growth in 4μg/ml after approximately 2 months Due to the poor proliferation, the cells were then grown in drug-free medium for a month After this time, the cells were exposed to 5μg/ml melphalan for a month and to a final concentration of 6 5μg/ml within a further 2 months During the selection procedure, the cells seemed to become larger and slightly more elongated than the parental cells (see section 3 2 3 2)

3.2.2.2 Establishment of the DLKP melphalan-resistant variant by pulse selection

The DLKP cells were exposed to 6μg/ml melphalan for four hours per week for 1 week The selection for the following week was suspended due to the poor growth of the cells The selection process was resumed as soon as the cells began to proliferate After ten pulses, the melphalan-resistant variant was established by checking the melphalan resistance fold by toxicity assay During the selection procedure, the cells seemed to be slightly more elongated than the parental cells (see section 3 2 3 2)

3.2.3 Morphology of the RPMI-2650, DLKP and their MDR variants

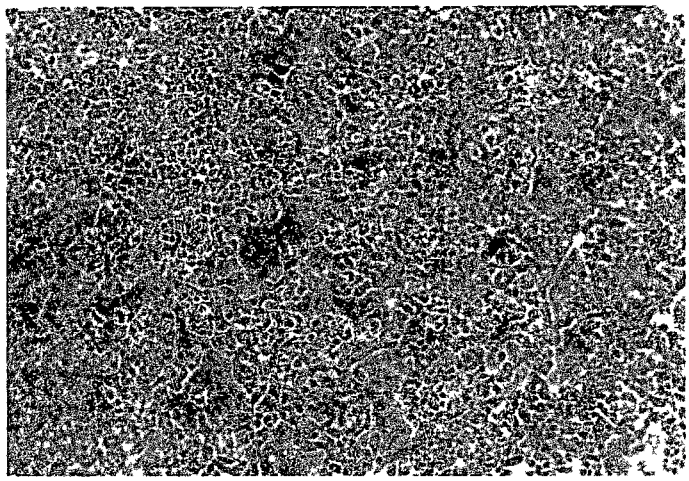
3.2.3.1 Morphology of the RPMI-2650 and its MDR variants

During the selection procedure with taxol or melphalan, the morphology of the RPMI-2650 cells changed dramatically Figure 3 2 3 1 illustrates the morphology of the parental RPMI-2650 cells and the two resistant variants The RPMI-2650 cells are very small and grow in dense clusters which fuse to form a thick layer of cells The RPMI-2650 taxol-resistant cells are larger and rounder than their parental cells, but they still grow in dense clusters The RPMI-2650 melphalan-resistant cells are spindle-shaped, more elongated than their parental cells and do not grow in clusters

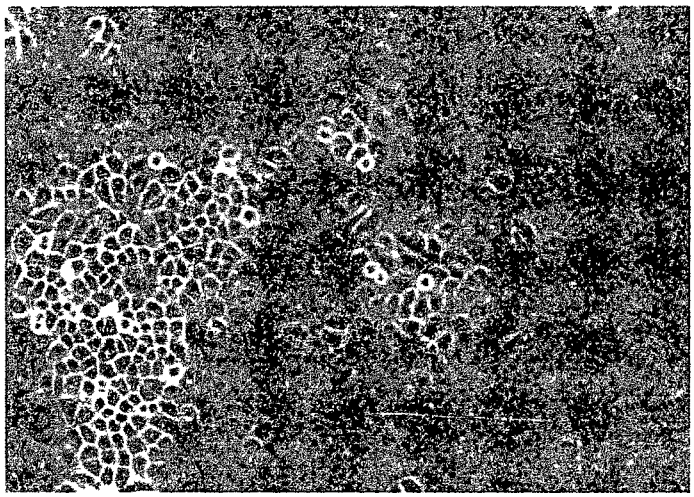
3.2.3.2 Morphology of the DLKP and its MDR variants

During the selection procedure with melphalan, both the DLKP long term melphlan-selection variant and the DLKP melphalan-pulse selection variant became slightly more elongated than the parental cell line, especially the long term selection line (Figure 3 2 3 2)

(a)



(b)



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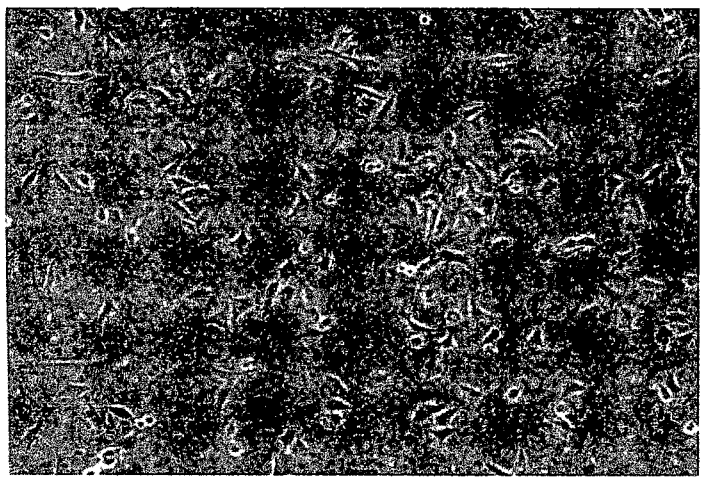
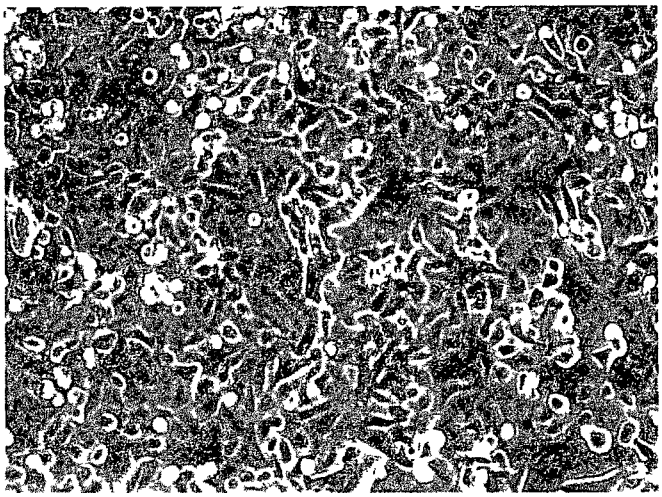


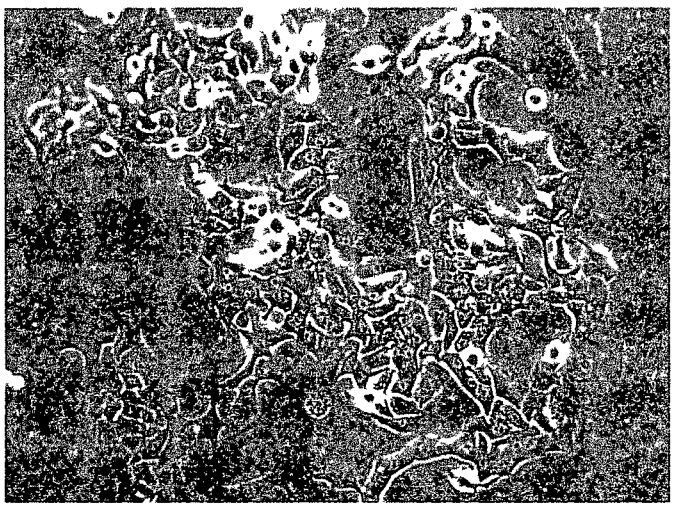
Fig. 3.2.3.1 The morphology of the RPMI-2650 and its MDR variants (10x)

(a) RPMI-2650 parental cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



(b)



(c)

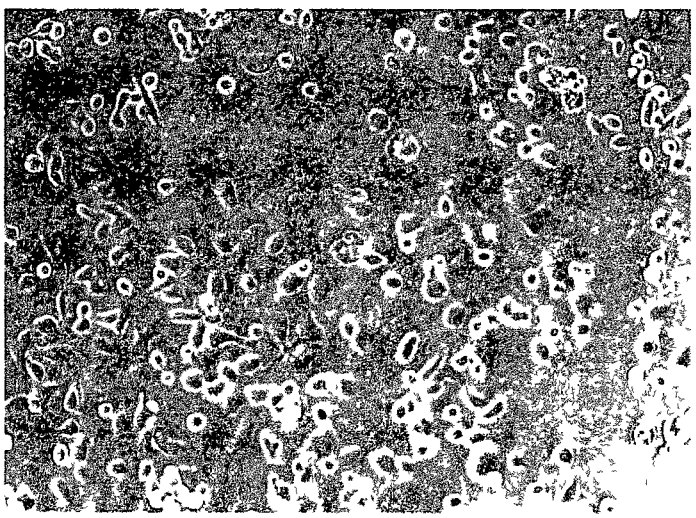


Fig. 3.2.3.2 Morphology of the DLKP cell line and its MDR variants (20x) (a) DLKP (b) DLKP long term melphalan-selected variant (c) DLKP melphalan-pulse selected variant

3.2.4 Cross resistance profiles of the MDR variants of RPMI-2650 and DLKP cell lines

When cells are repeatedly treated with a chemotherapeutic drug, the drug resistant variant which is eventually produced usually not only displays resistance to this selective drug, but also to different groups of drugs

To investigate the resistance profiles of the MDR variants of RPMI-2650 and DLKP cell lines, toxicity assays were conducted as described in section 2 6 2 The chemotherapeutic agents included the selective drug, taxol or melphalan, adriamycin, vincristine, vinblastine, VP-16, cisplatin, cadmium chloride and 5-FU

3.2.4.1 Cross resistance profile of the RPMI-2650 taxol-resistant variant

Table 3 2 4 1 a represents the IC_{50} values of each of the chemotherapeutic agents for the parental RPMI-2650 cell line and the taxol-selected variant The results demonstrate that the taxol-resistant variant exhibited cross resistance to all the chemotherapeutic drugs tested with the exception of the alkylating agent cisplatin In particular, the taxol-resistant cells appeared to be highly resistant to the classical MDR drugs such as adraimycin, vincristine, vinblastine and VP-16, all of which are substrates of P-glycoprotein

IC_{50} (nM)	RPMI-2650 parental cells	RPMI-2650 taxol sele Cells
Adriamycin	18 5 ± 3 5	4030 ± 350
Vincristine	2 4 ± 0 4	1427 ± 377
Vinblastine	1 78 ± 0 12	168 ± 18
VP-16	23 ± 3	464 ± 53
Taxol	2 3 ± 0 9	519 ± 18
Melphalan	582 ± 10	2621 ± 164
5-FU	1624 ± 164	5898 ± 98
Cisplatin	1494 ± 39	1600 ± 267
Cadmium Chloride	709 ± 68	5891 ± 465

Table 3.2.4.1.a IC_{50} values for RPMI-2650 and the RPMI-2650 taxol-resistant variant (Toxicity assays were repeated at least three times for each drug in each cell line)

Drug	Fold resistance
Adriamycin	218
Vincristine	595
Vinblastine	94
VP-16	20
Taxol	226
Melphalan	4 5
5-FU	3 6
Cisplatin	1 1
Cadmium Chloride	8 3

Table 3.2.4.1.b Fold resistance of the RPMI-2650 taxol-resistant variant relative to the parental RPMI-2650 cell line

3.2.4.2 Cross resistance profile of the RPMI-2650 melphalan-resistant variant

The sensitivity of the RPMI-2650 melphalan-resistant variant to the same range of chemotherapeutic agents was also determined Table 3 2 4 2 a represents the IC_{50} values of each of the agents for the parental RPMI-2650 cell line and the RPMI-2650 melphalan-resistant variant The results showed that the RPMI-2650 melphalan-resistant variant was 11 times more resistant to melphalan than the parental cells This variant showed highest cross resistance to cadmium chloride The variant also exhibited cross-resistance to adriamycin, vinblastine, VP-16 and to a lesser extent to vincristine and cisplatin compared to its parental cell line No significant cross resistance was observed with taxol and 5-FU

IC_{50} (nM)	RPMI-2650 parental cells	RPMI-2650 mel sele cells
Adriamycin	18 5 ± 3 5	425 ± 70
Vincristine	2 4 ± 0 4	15 ± 2 4
Vinblastine	1 78 ± 0 12	42 ± 3 4
VP-16	23 ± 3	587 ± 60
Taxol	2 3 ± 0 9	3 85 ± 1 05
Melphalan	582 ± 10	6135 ± 172
5-FU	1624 ± 164	1784 ± 16
Cisplatin	1494 ± 39	3874 ± 125
Cadmium Chloride	405 ± 102	22038 ± 218

Table 3.2.4.2.a IC_{50} values for RPMI-2650 and RPMI-2650 melphalan-resistant variant (Toxicity assays were repeated at least three times for each drug in each cell line)

Drug	Fold resistance
Adriamycin	23
Vincristine	6
Vinblastine	24
VP-16	26
Taxol	1 7
Melphalan	11
5-FU	1 1
Cisplatin	2 6
Cadmium Chloride	54

Table 3.2.4.2.b Fold resistance of the RPMI-2650 melphalan-resistant variant relative to the parental RPMI-2650 cell line

3.2.4.3 Cross resistance profile of the DLKP long term melphalan-selected variant

Table 3 2 4 3 a represents the IC_{50} values of adriamycin, vinblastine, taxol, melphalan, cisplatin and cadmium chloride for the DLKP cell line and its long term melphalan-selected variant The results showed that the DLKP long term-selected variant was 3 3 times more resistant to melphalan than the DLKP cells This variant exhibited high cross-resistance to cadmium chloride and cisplatin, and to a much lesser extent to adriamycin and vinblastine No significant cross resistance was observed with taxol

3.2.4.4 Cross resistance profile of the DLKP melphalan-pulse selected variant

Table 3 2 4 4 a represents the IC_{50} values of the same range of drugs for the DLKP melphalan-pulse selected variant The results showed that the variant was 2 1 times more resistant to melphalan than the parental cells The variant also exhibited cross resistance to adriamycin and cisplatin, and to a greater extent to cadmium chloride No significant cross resistance was observed with taxol or vinblastine

IC_{50} (nM)	DLKP parental cells	DLKP long term melphalan-selected cells
Adriamycin	44 8 ± 6 8	82 8 ± 7 7
Vinblastine	1 ± 0 14	1 9 ± 0 16
Taxol	0 84 ± 0 23	1 1 ± 0 14
Melphalan	2834 ± 69 5	9220 ± 400
Cisplatin	940 ± 36 7	7615 ± 307
Cadmium Chloride	1800 ± 25 6	17592 ± 1735

Table 3.2.4.3.a IC_{50} values for the DLKP and DLKP long term melphalan-selected cells (Toxicity assays were repeated twice for each drug in each cell line)

Drug	Fold resistance
Adriamycin	1 8
Vinblastine	1 9
Taxol	1 3
Melphalan	3 3
Cisplatin	8 1
Cadmium chloride	9 8

Table 3.2.4.3.b Fold resistance of the DLKP long term melphalan-selected variant relative to the DLKP parental cell line

IC_{50} (nM)	DLKP parental cells	DLKP melphalan-pulse selected cells
Adriamycin	44 8 ± 6 8	67 2 ± 8 6
Vinblastine	1 ± 0 14	1 2 ± 0 19
Taxol	0 84 ± 0 23	1 ± 0 21
Melphalan	2834 ± 69 5	5951 ± 87
Cisplatin	940 ± 36 7	2933 ± 25
Cadmium Chloride	1800 ± 25 6	13092 ± 1472

Table 3.2.4.4.a IC_{50} values for the DLKP and DLKP melphalan-pulse selected variant (Toxicity assays were repeated twice for each drug in each cell line)

Drug	Fold resistance
Adriamycin	1 5
Vinblastine	1 2
Taxol	1 2
Melphalan	2 1
Cisplatin	3 1
Cadmium Chloride	7 3

Table 3.2.4.4.b Fold resistance of the DLKP melphalan-pulse selected variant relative to the DLKP parental cell line

3.2.5 Doubling time of the RPMI-2650 cell line and its MDR variants

The rate of cell growth was determined in the RPMI-2650 parental cells and the RPMI-2650 MDR variants over a time period of 7 days as described in section 2 2 3 The results obtained are presented in table 3 2 4 No significant difference in doubling time between the parental and resistant cells was observed

Cell line	Doubling time (hours)
RPMI-2650 parental cell line	30
RPMI-2650 taxol resistant variant	34 3
RPMI-2650 melphalan resistant variant	31 6

Table 3.2.5 Doubling time of the RPMI-2650 cell line and its MDR variants

3.3 Protein analysis of MDR markers in the RPMI-2650 and DLKP MDR variants

P-glycoprotein and MRP are very important MDR markers Both of these markers are glycoproteins mainly located at the cell membrane Protein analysis of these two markers by Western blotting was carried out in the RPMI-2650 and DLKP parental cell lines and their MDR variants as described in section 2 8

3.3.1 Western blotting of P-glycoprotein

3.3.1.1 In the RPMI-2650 cell line and its MDR variants

DLKP-A, an adriamycin-selected variant of a human lung epithelial carcinoma cell line, DLKP (Law *et al*, 1992), was used in these studies as a positive control This cell line, DLKP-A, has previously been shown to overexpress P-glycoprotein (Clynes *et al*, 1992) The anti-P-glycoprotein monoclonal antibody, BRI MAB MDR-1 (clone 6/1c) (BR-002) was used to detect P-glycoprotein

Figure 3 3 1 illustrates the results obtained for each of the cell lines A P-glycoprotem band at a molecular weight of approximately 170kD is visible in all cell line with the DLKP-A showing the strongest immunoreactivity indicating high Pgp expression The RPMI-2650 taxol-resistant variant also shows strong immunoreactivity while weaker bands can be seen with the parental line and the melphalan-resistant variant

3.3.1.2 In the DLKP cell line and its MDR variants

No bands at a molecular weight of approximately 170kD are visible in the DLKP cell line and its MDR variants.

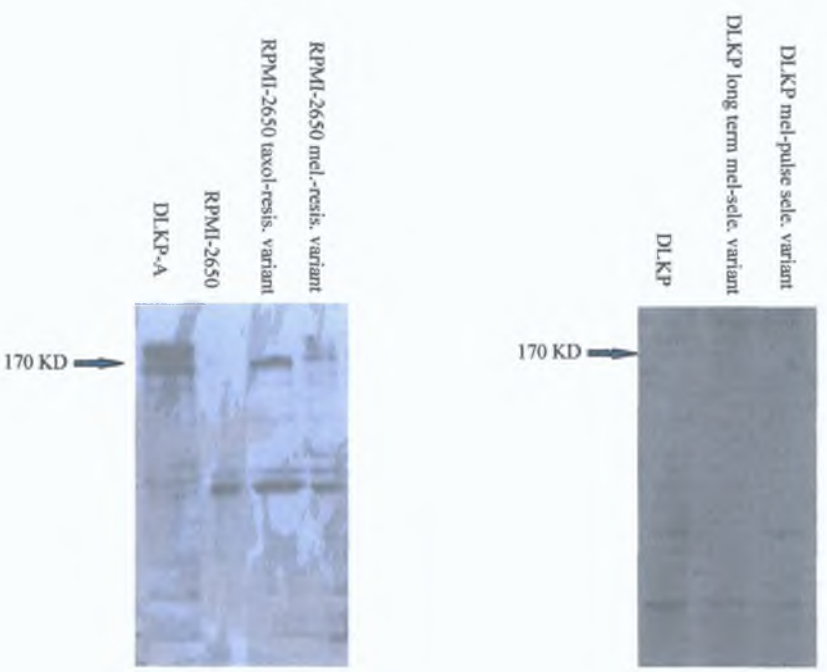


Fig. 3.3.1 Western blot of P-glycoprotein in the RPMI-2650 and DLKP cell lines and their MDR variants. DLKP-A served as positive control.

3.3.2 Western blotting of MRP1

3.3.2.1 In the RPMI-2650 cell line and its MDR variants

The human lung adenocarcinoma cell line, A-549, was used as a positive control to analyse the expression of MRP1 in the RPMI-2650 and its MDR variants An anti-MRP monoclonal antibody was used to detect MRP1 as described in section 2 8 Figure 3 3 2 illustrates a band in the RPMI-2650 parental cell line and its melphalan-resistant variant at a molecular weight of approximately 190kD corresponding to MRP1 Stronger immunoreactivity is observed in the RPMI-2650 melphalan-resistant variant than in the parental cell line, indicating that MRP1 is overexpressed in this cell line A very weak band was visible in the taxol-resistant variant

3.3.2.2 In the DLKP cell line and its MDR variants

Figure 3 3 2 illustrates a band in the DLKP parental cell line and its melphalan-resistant variants at a molecular weight of approximately 190kD corresponding to MRP1 Stronger immunoreactivity was observed in the DLKP long term and pulse selection variants than in the parental cell line, indicating that MRP1 was overexpressed in these variants

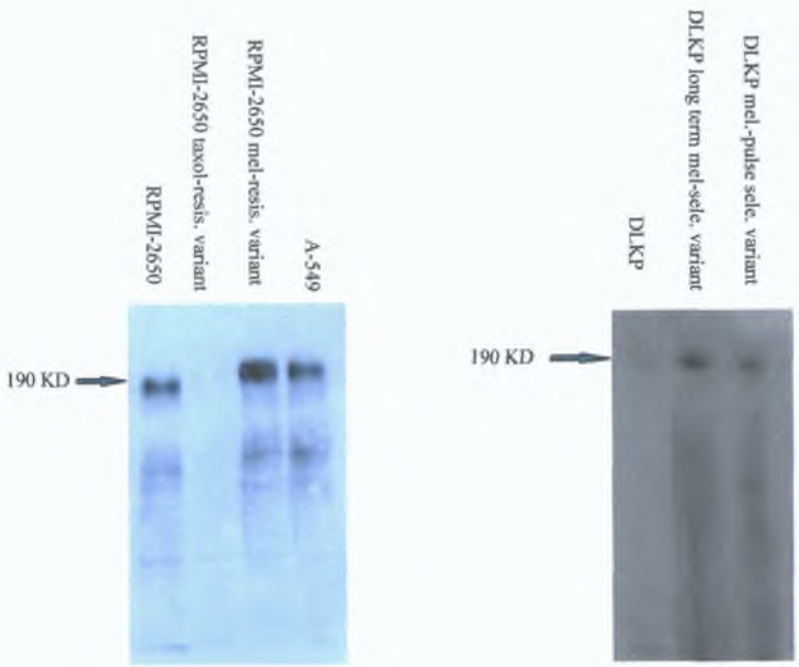


Fig. 3.3.2 Western blot of MRP1 in the RPMI-2650 and DLKP cell lines and their MDR variants. A-549 served as positive control.

3.3.3 Western blotting of cMOAT (MRP2)

A-549 was used again as positive control to analyse the expression of cMOAT (MRP2) in the RPMI-2650 and its resistant variants Figure 3 3 3 illustrates a strong band in the RPMI-2650 melphalan-resistant variant at a molecular weight of approximately 190kD corresponding to cMOAT No band was observed in the DLKP and RPMI-2650 parental lines and the RPMI-2650 taxol-resistant variant (figure 3 3 3) This work was carried out by Lisa Connolly

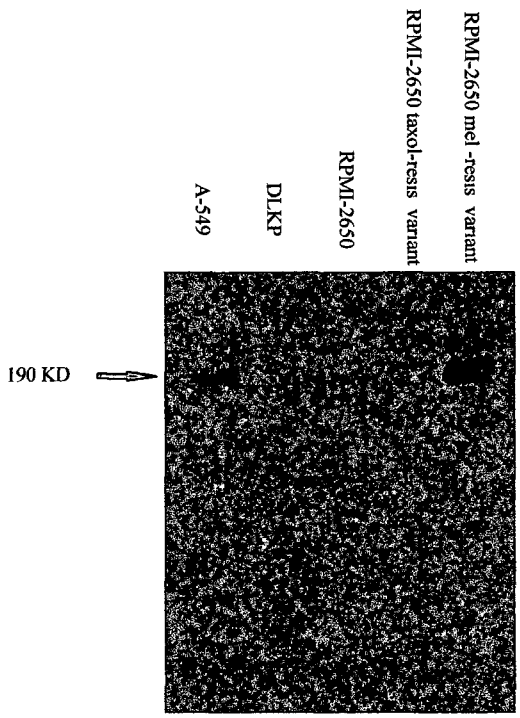


Fig. 3.3.3 Western blot of cMOAT (MRP2) in the RPMI-2650 cell line and its MDR variants (carried out by Lisa Connolly) A-549 served as positive control

3.4 Immunological studies in the RPMI-2650 MDR variants

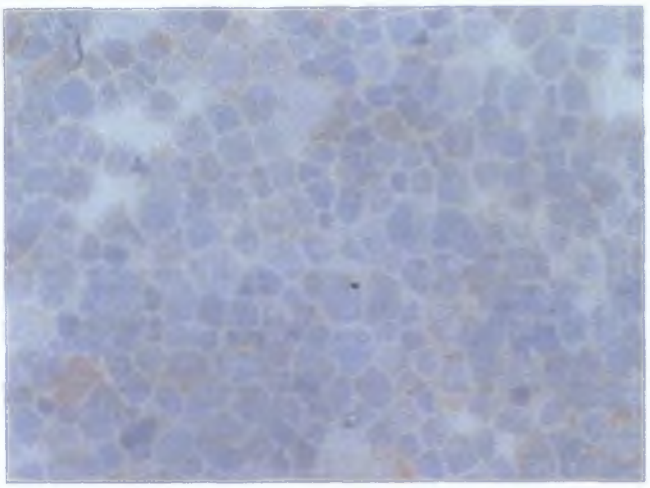
3.4.1 Immunocytochemical detection of MDR markers

Immunocytochemistry, as described in section 2 7 2, was carried out to detect the presence of a range of MDR markers in the RPMI-2650 parental cell line and its MDR variants

3.4.1.1 P-glycoprotein

The presence of P-glycoprotein was investigated in the parental RPMI-2650 cell line and its MDR variants, using the BRI MDR-1 (Clone 6/1C) monoclonal antibody as described in section 2 7 2 2 1 Figure 3 4 1 1 illustrates that the RPMI-2650 cell line and its MDR variants were all stained with P-glycoprotein antibody around the cell membrane However, more intense staining is noted in the RPMI-2650 taxol-resistant variant

(a)



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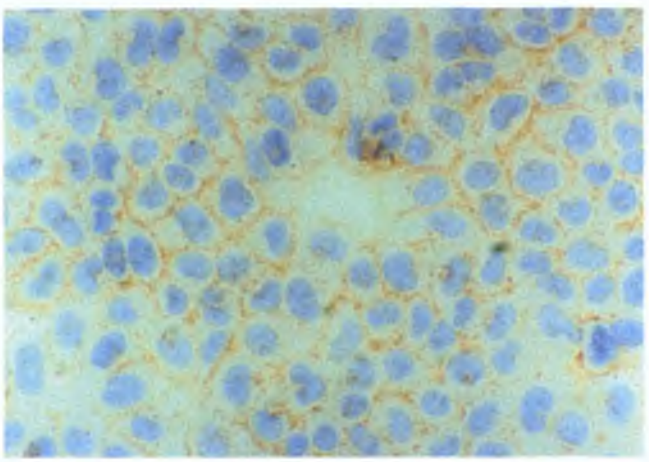


(c)

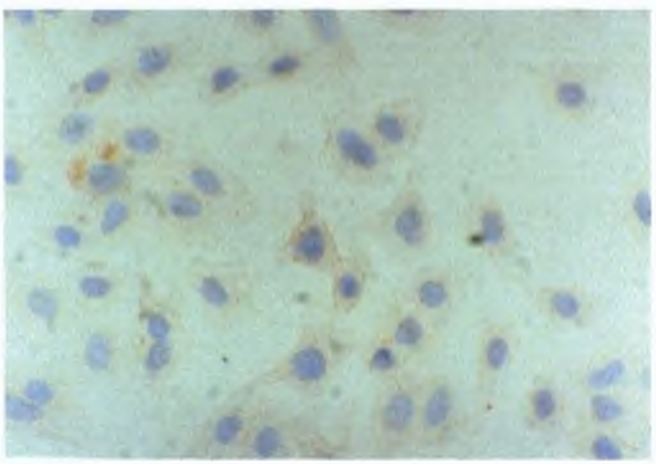
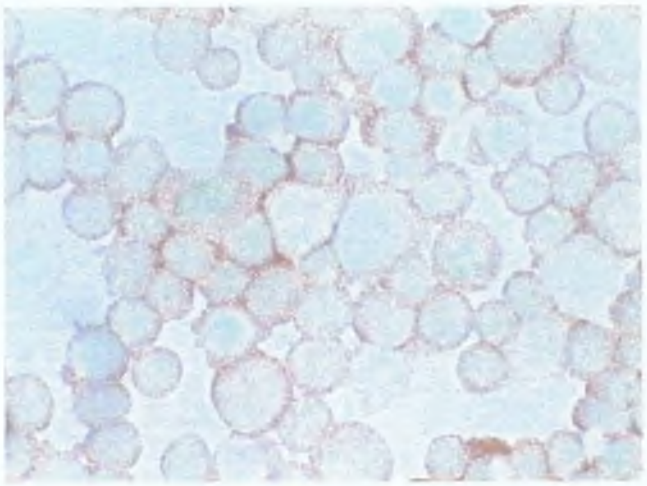


Fig. 3.4.1.1 Immunocytochemical staining of Pgp (40x) in the (a) RPMI-2650 parental cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.4.1.2 cMOAT

The presence of cMOAT, also called MRP2, was investigated in the RPMI-2650 cell line and the RPMI-2650 melphalan-resistant variant using cMOAT 2III-6 antibody as described in section 2 7 2 2 2 DLKPC which is the cisplatin-selected variant of DLKP was used as the positive control Faint staining was observed in the RPMI-2650 cell line while strong staining was observed in the RPMI-2650 melphalan-resistant variant (figure 3 4 1 2)

(a)



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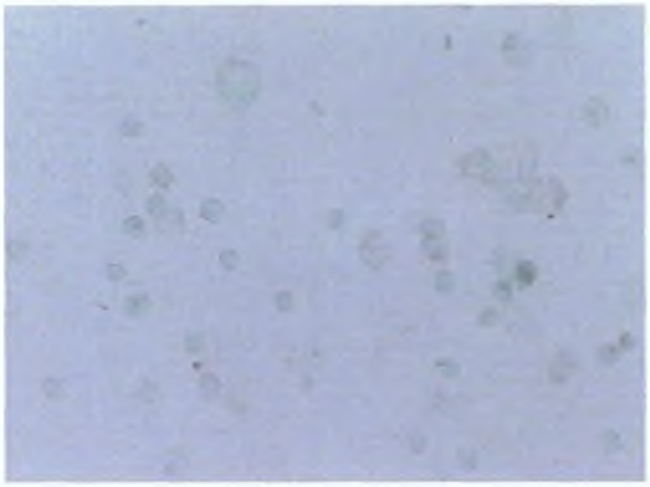


(c)

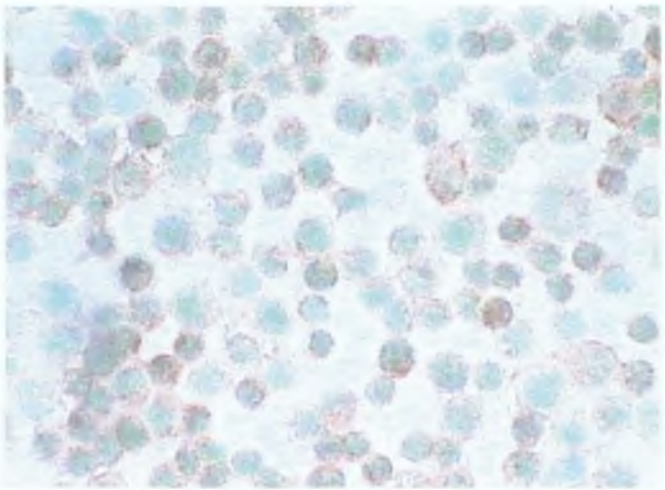
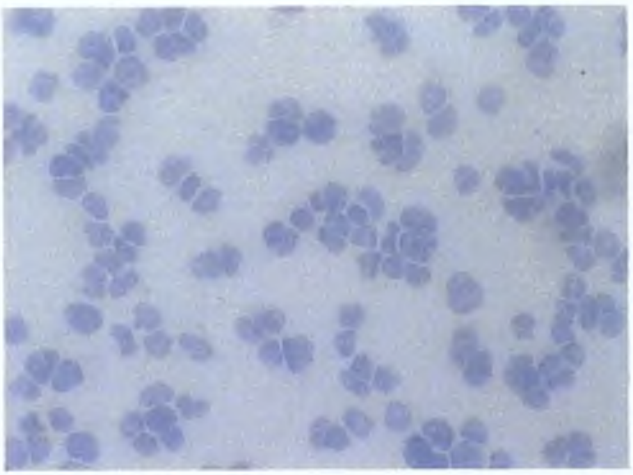


Fig. 3.4.1.2 Immunocytochemical staining of cMOAT (40x) in the (a) DLKP-C (b) RPMI-2650 (c) RPMI-2650 melphalan-resistant variant

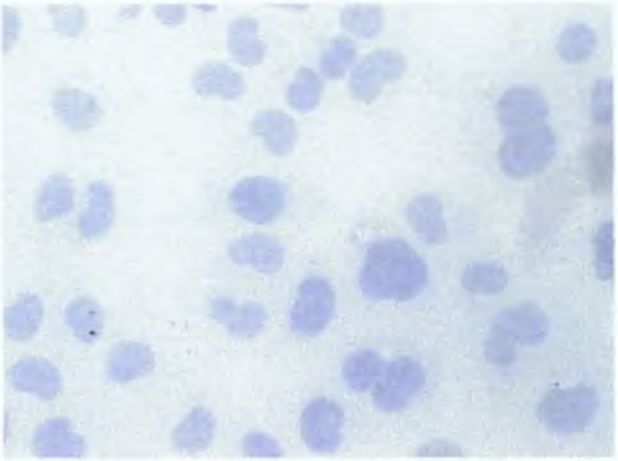
3.4.1.3 LRP

LRP was found to be involved in the mechanism of MDR in 1996 and later was identified as the major protein of vault LRP has been shown to be localised predominantly in the cytoplasmic regions of the cells The presence of LRP was investigated using the mouse monoclonal antibody LRP-56 as described in section 2 7 2 2 3 LRP expression was not observed in the RPMI-2650 parental cell line and its taxol-resistant and melphalan-resistant variants as shown in figure 3 4 1 3

(a)



(b)



(C)

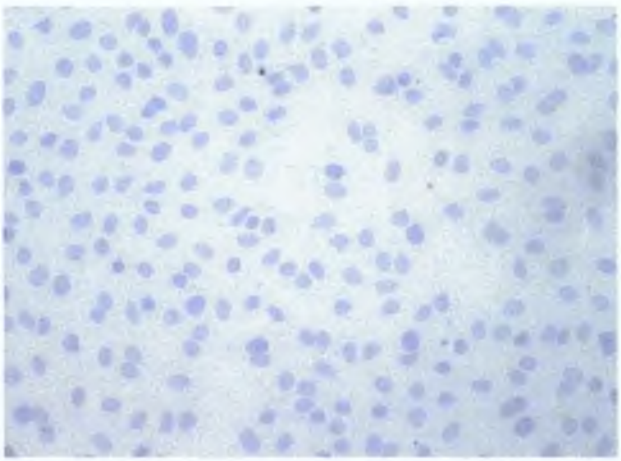


Fig. 3.4.1.3 Immunocytochemical staining of LRP in the RPMI-2650 and its MDR variants (a) RPMI-2650 (40x) (b) RPMI-2650 taxol-resistant variant (40x) (c) RPMI-2650 melphalan-resistant variant (10x)

3.4.1.4 GST α, μ, π

Since alteration in the level of GST has been shown to be associated with resistance, the expression of the three main classes of GST, α, μ, π was investigated in the RPMI-2650 parental and resistant cells Rabbit polyclonal anti-GST α, μ and π antibodies were used to detect the presence of the GSTs in these cell lines as described in section 2 7 2 2 5 No staining for GST α and GST μ was noted in the RPMI-2650 parental cell line and its MDR variants as shown in figure 3 4 1 4 (pictures were not shown for GST μ since they were similar to those of GST α) Expression of GST π was observed in the cytoplasm of all the cell lines as shown in figure 3 4 1 4 a However, no significant difference in the level of staining was noted between these cell lines

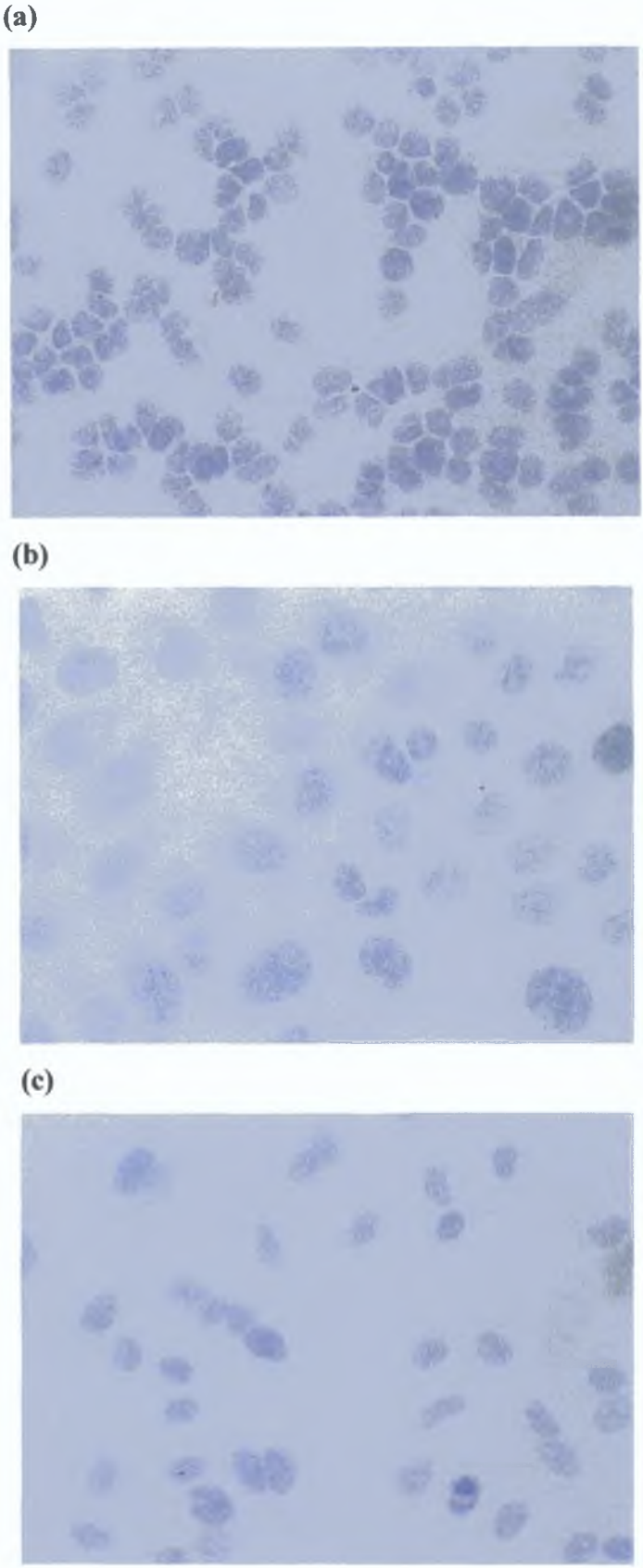


Fig. 3.4.1.4 Immunocytochemical staining of GST α in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

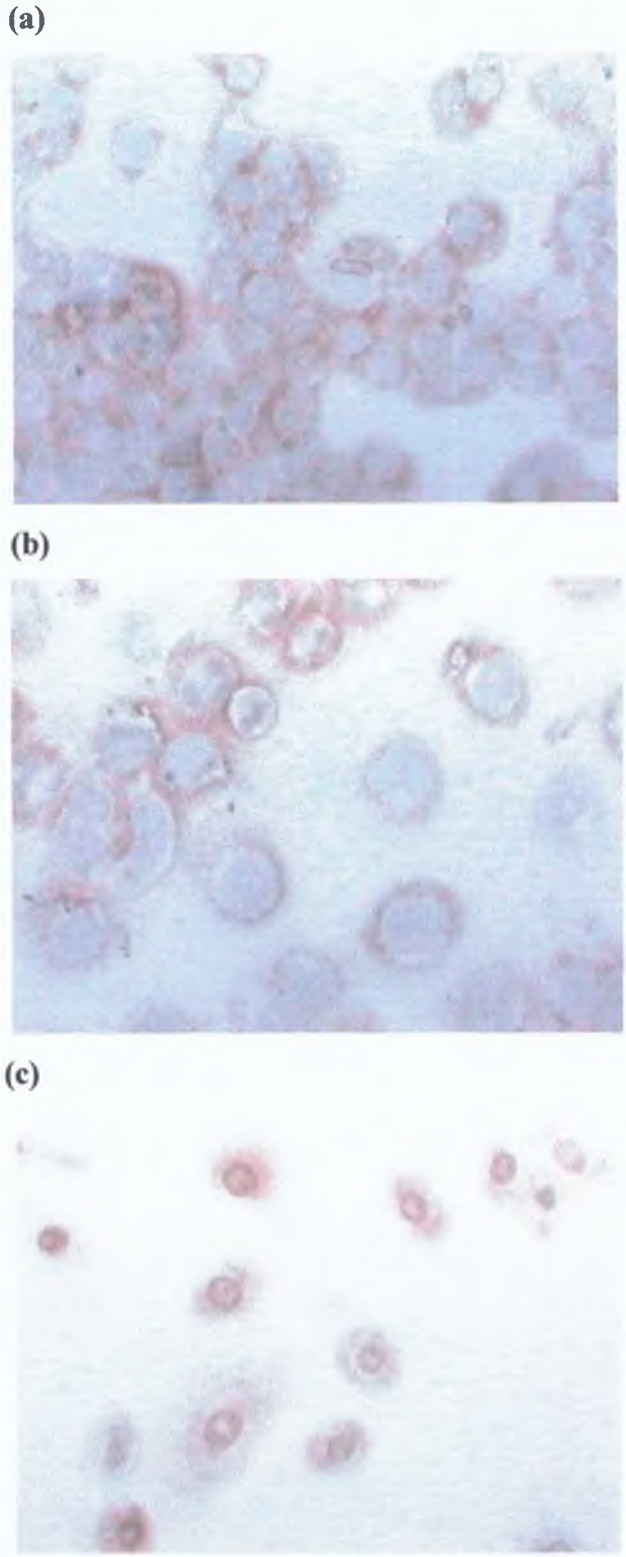
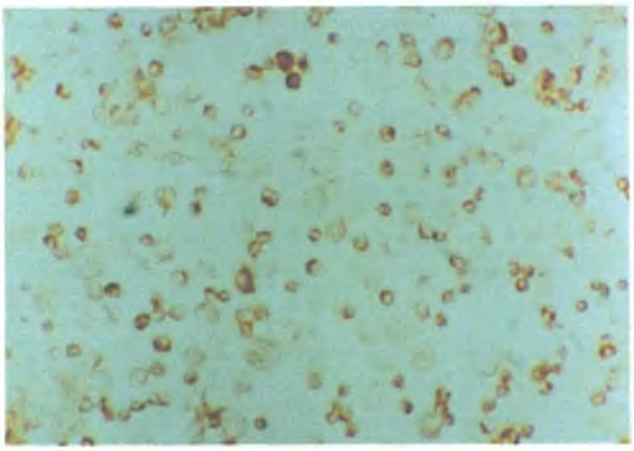


Fig. 3.4.1.4.a Immunocytochemical staining for GST π in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

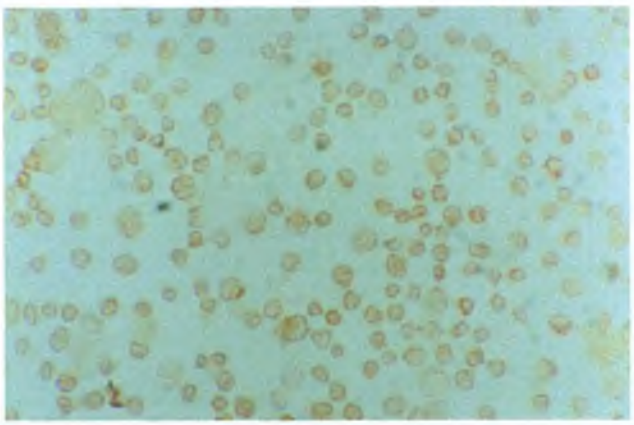
3.4.1.5 Topoisomerase II α

The expression of the enzyme, topoisomerase II α, was also studied in the RPMI-2650 parental and resistant cells using a mouse topoisomerase II α monoclonal antibody as described in section 2.7.2.2.4. The results obtained demonstrated the presence of topoisomerase II α in the nuclei of each of the cell lines (figure 3.4.1.5). However, no significant difference in the level of topoisomerase expression between the cell lines was observed.

(a)



(b)



(c)

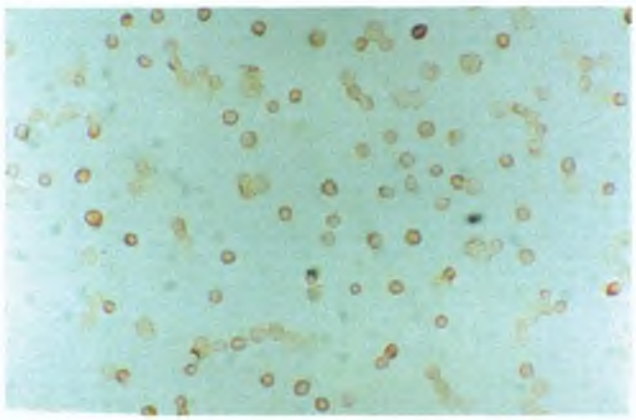


Fig. 3.4.1.5.a Immunocytochemical staining of Topo II α in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

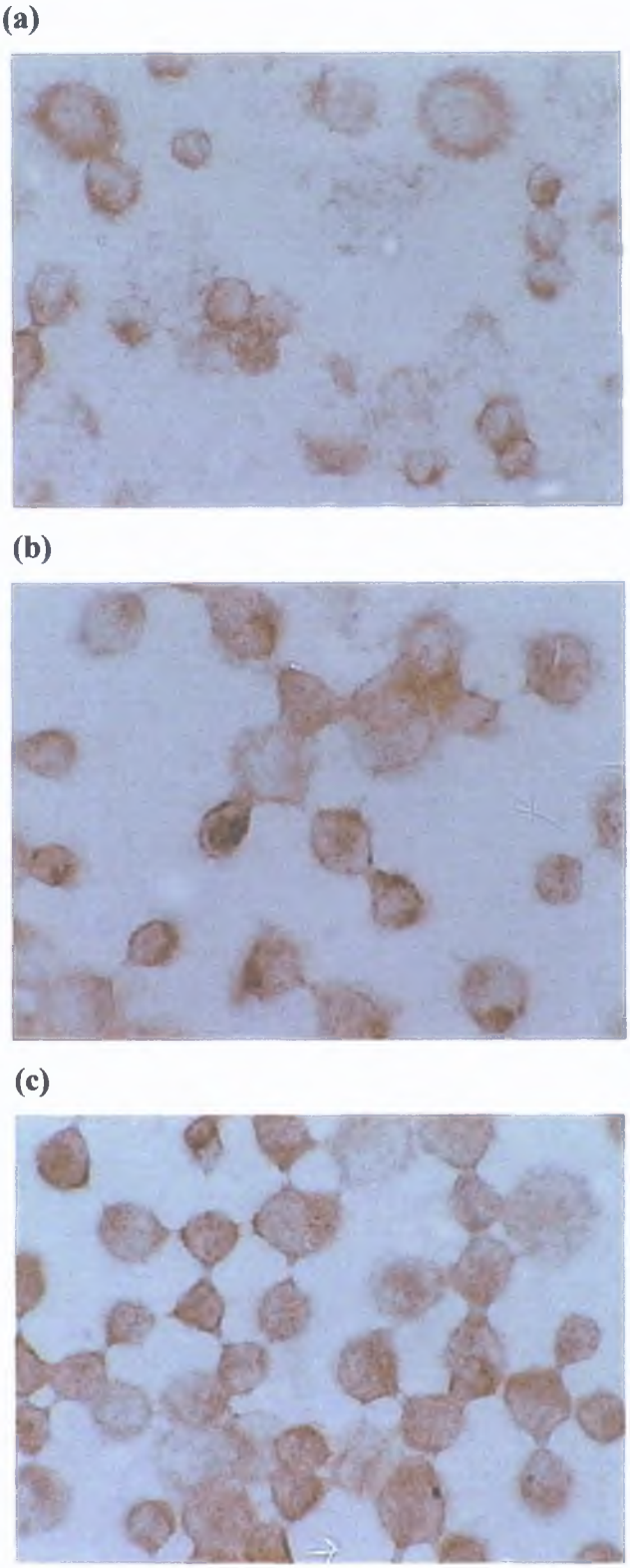
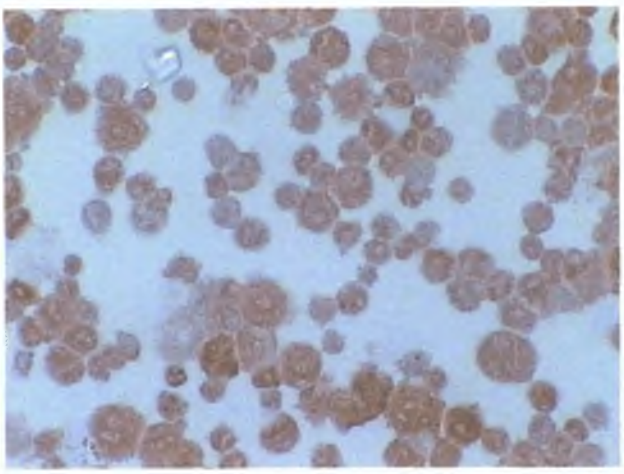


Fig. 3.4.1.5.b Immunocytochemical staining of Topo II α in the RPMI-2650 cell line and its MDR variants (100x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

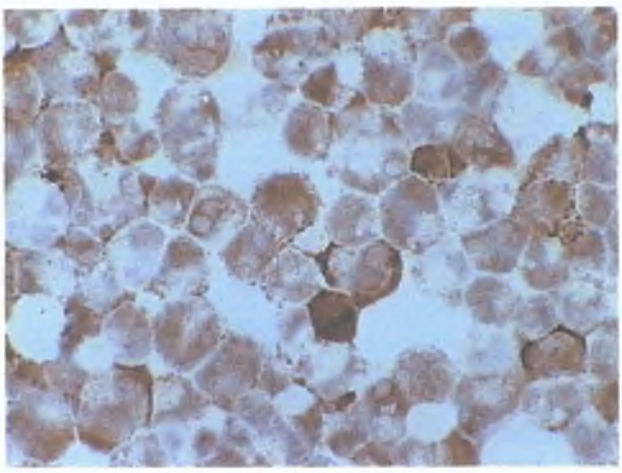
3.4.1.6 Metallothionein

Metallothionein is related to resistance to heavy metal drug and alkylating agents The presence of metallothionein was investigated using the mouse monoclonal metallothionein antibody as described in section 2 7 2 2 6 Expression of metallothionein was observed in the cytoplasm of the RPMI-2650 parental cell line, its taxol- and melphalan-resistant variants However, no significant difference in the level of metallothionein expression between the cell lines was observed (figure 3 4 1 6)

(a)



(b)



(c)

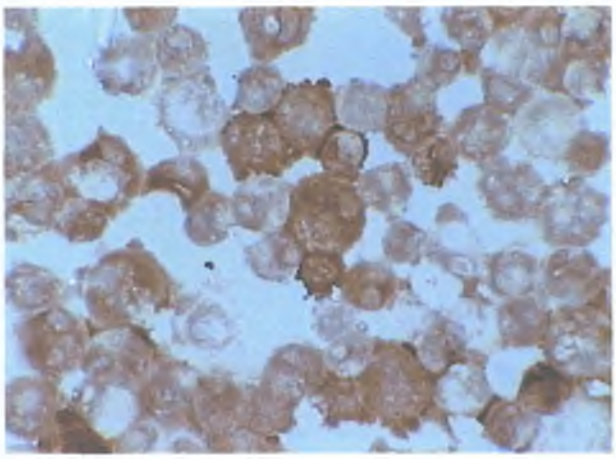


Fig. 3.4.1.6 Immunocytochemical staining for metallothionein in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.4.2 Indirect immunofluorescence studies in the RPMI-2650 MDR variants

Resistance to taxol has been attributed to altered α or β tubulin subunits in some taxol-resistant cell lines Intermediate filaments such as cytokeratin and vimentm have also been found to contribute to the resistance phenotype of epithelial cells Thus, immunofluorescence studies on tubulin, cytokeratin and vimentin were carried out in the RPMI-2650 cell line and its MDR variants as described in section 2 7 1

3.4.2.1 α-tubulin

An anti-α-tubulin monoclonal antibody was used as the primary antibody to detect the presence of α-tubulin RPMI-2650 parental cell line, its taxol- and melphalan-resistant variants all showed cytoplasmic fluorescence as shown in figure 3 4 2 1 However, more intense fluorescence was noted in the RPMI-2650 cell line

3.4.2.2 β-tubulin

An anti-β-tubulm monoclonal antibody was used as the primary antibody to detect the presence of β-tubulin The level of fluorescence in the RPMI-2650 parental cell line and its taxol- and melphalan-resistant variants is similar as shown in figure 3 4 2 2

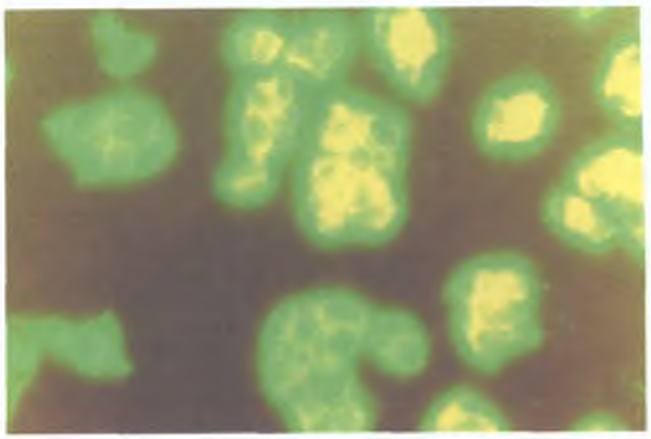
3.4.2.3 Cytokeratin 18

A cytokeratin 18 monoclonal antibody was used as the primary antibody to detect the presence of cytokeratin 18 Figure 3 4 2 3 illustrates that the RPMI-2650 parental cell line and the RPMI-2650 melphalan-resistant variant show very faint cytoplasmic fluorescence for cytokeratin 18 while little or no immunofluorescent staining for cytokeratin 18 was observed in the taxol-resistant variant

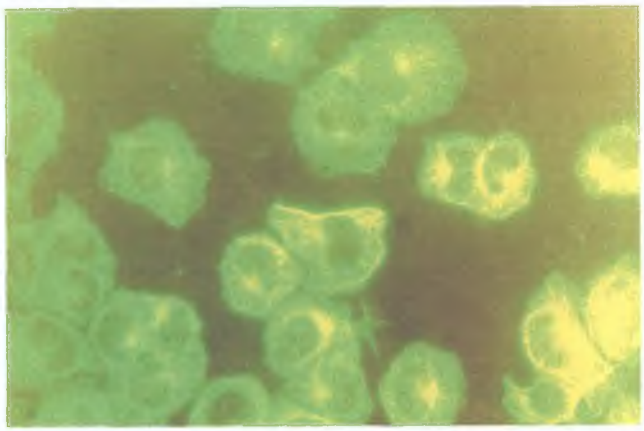
3.4.2.4 Vimentm

A mouse monoclonal anti-vimentin antibody was used as the primary antibody to detect the presence of vimentin The RPMI-2650 parental cell line and its MDR variants showed similar fluorescence level of vimentm as shown in figure 3 4 2 4

(a)



(b)



(c)

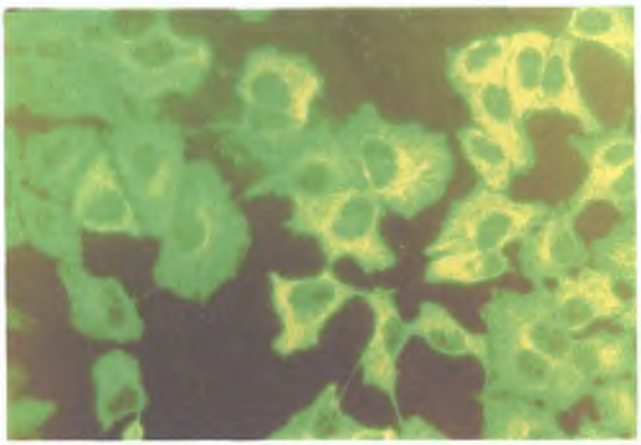
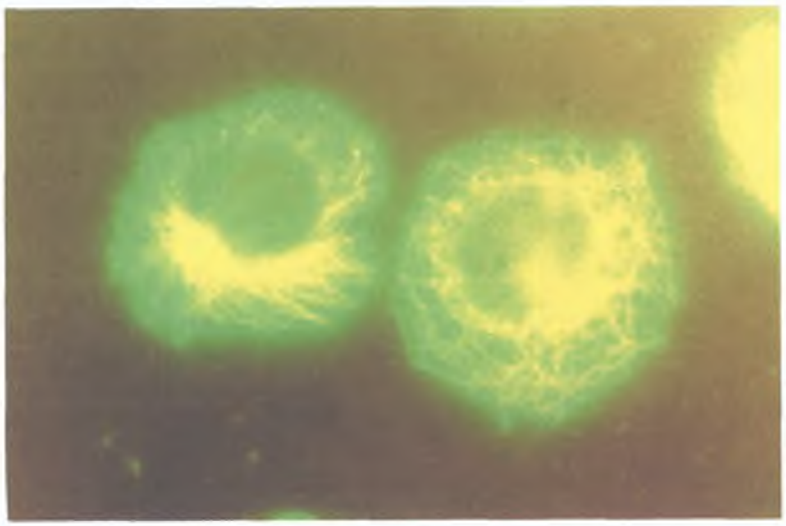


Fig. 3.4.2.1 Immunofluorescence of α-tubulin in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (b) RPMI-2650 melphalan-resistant variant

(a)



(b)

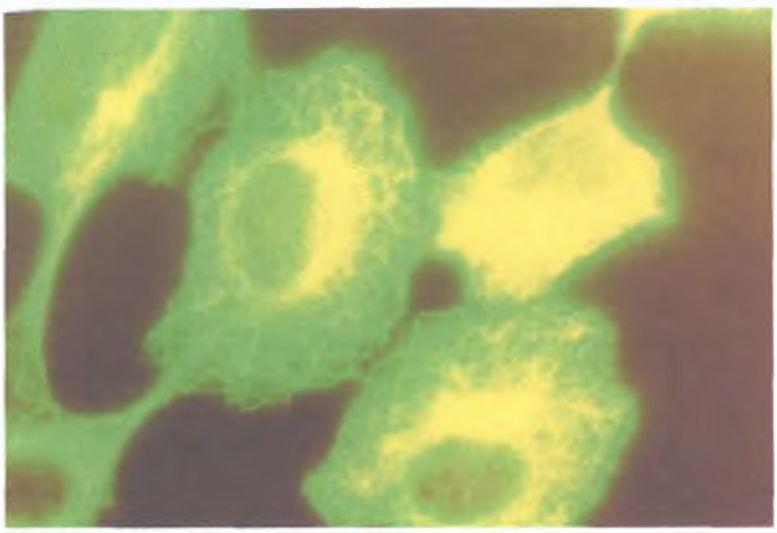


Fig. 3.4.2.1.a Immunofluorescence of α-tubulin (100x) in the (a) RPMI-2650 taxol-resistant variant (b) RPMI-2650 melphalan-resistant variant

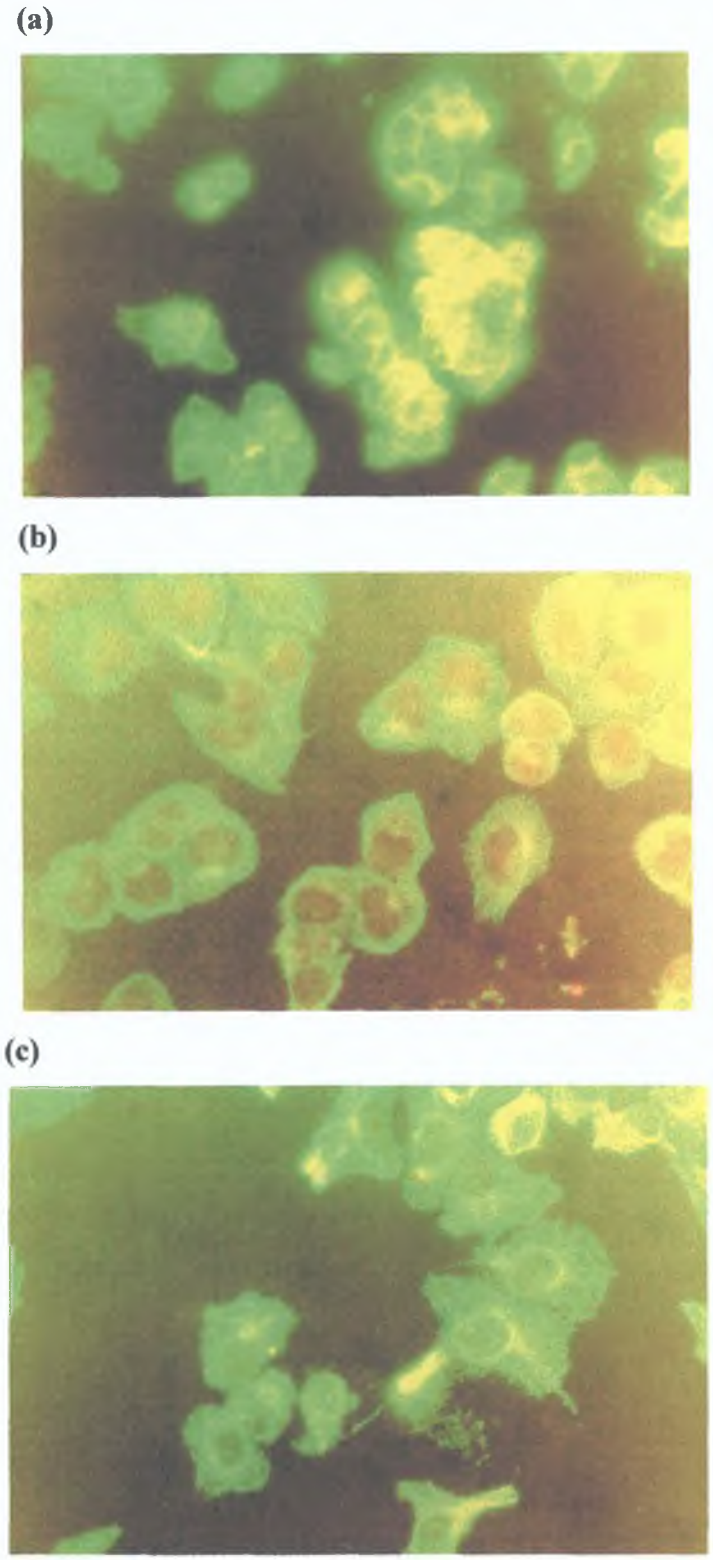
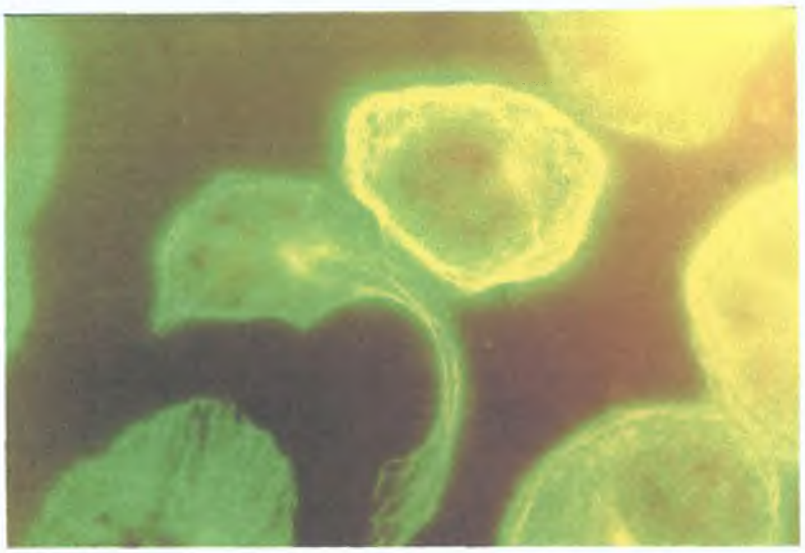


Fig. 3.4.2.2. Immunofluorescence of β-tubulin in the RPMI-2650 and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



(b)

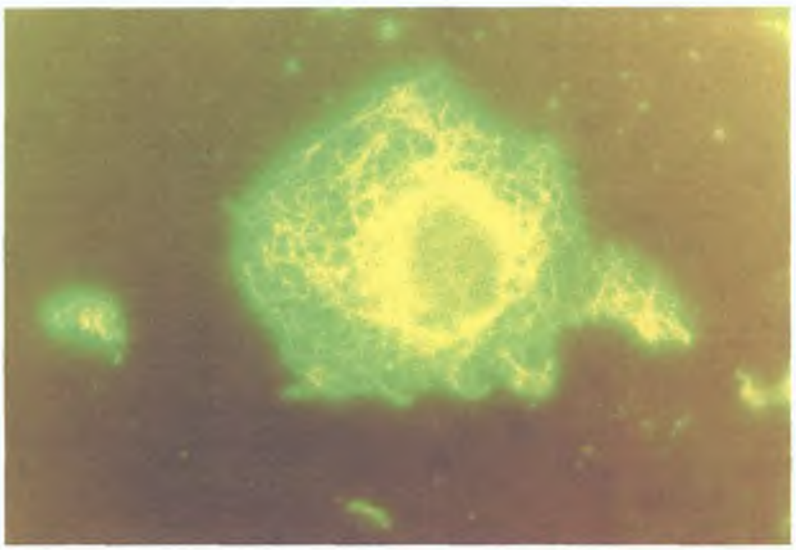


Fig. 3.4.2.2.a Immunofluorescence of β-tubulin (100x) in the (a) RPMI-2650 taxol-resistant variant (b) RPMI-2650 melphalan-resistant variant

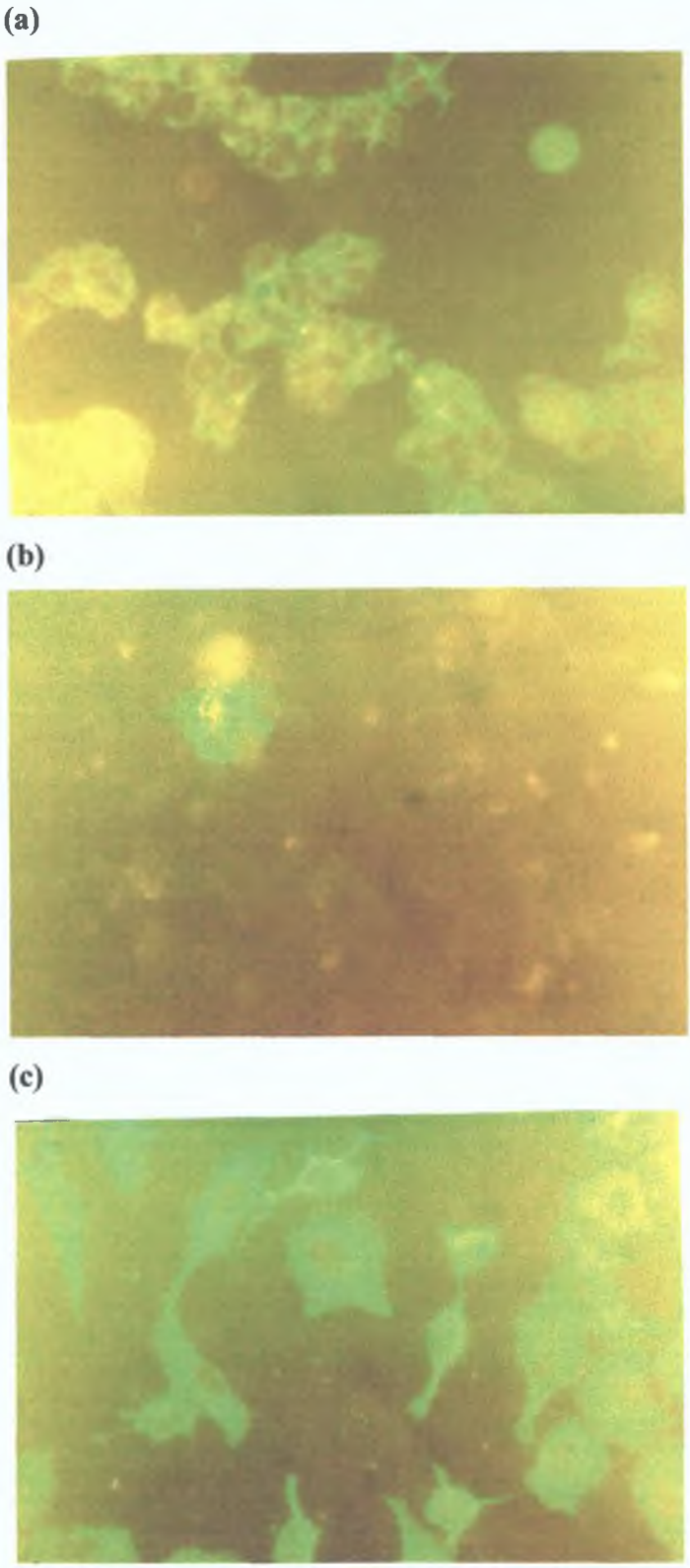
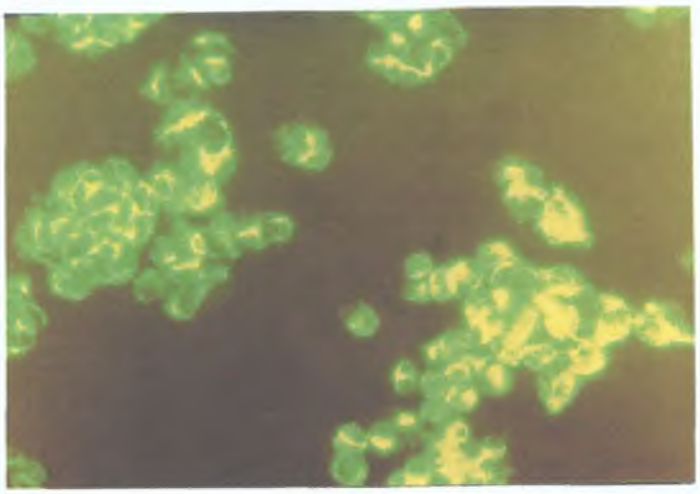
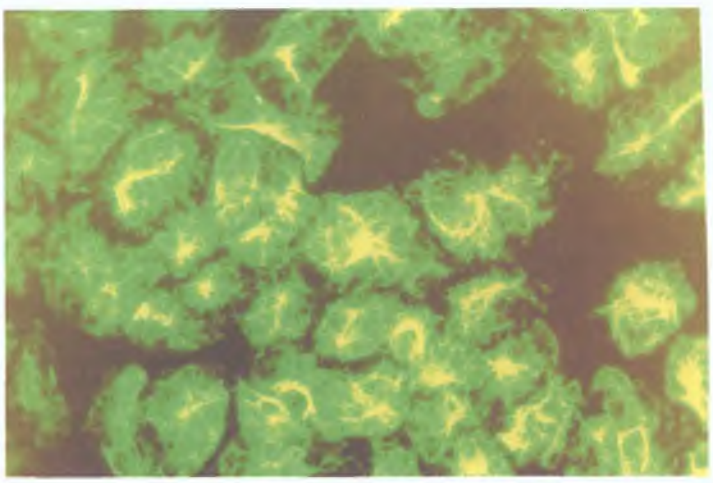


Fig. 3.4.2.3 Immunofluorescence of cytokeratin 18 in the RPMI-2650 and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



(b)



(c)

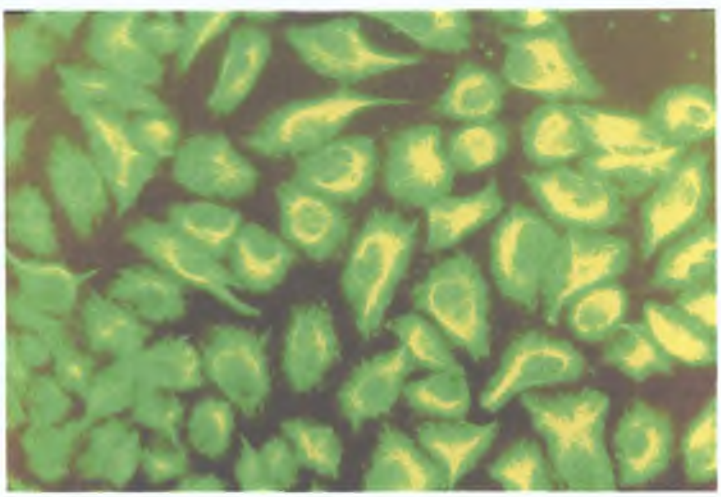


Fig. 3.4.2.4 Immunofluorescence of vimentin in the RPMI-2650 and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.5 RT-PCR of MDR markers in the RPMI-2650, *DLKP* cell lines and their MDR variants

To detect the expression of MDR markers at the RNA level, RT-PCR was carried out as described in section 2 16 Since MRP3, MRP4 and MRP5 are very new members of the MRP family and there are no commercially available antibodies for them, RT-PCR can serve as a guide to the expression of mrp3, mrp4 and mrp5

3.5.1 mdr-1

3.5.1.1 mdr-1 expression in the RPMI-2650 cell line and its MDR variants

RT-PCR analysis showed that mdr-1 expression was undetectable in the RPMI-2650 parental cell line while the strongest band was observed in the taxol-resistant variant The mdr-1 was slightly overexpressed in the melphalan-resistant variant compared to its parental cell line

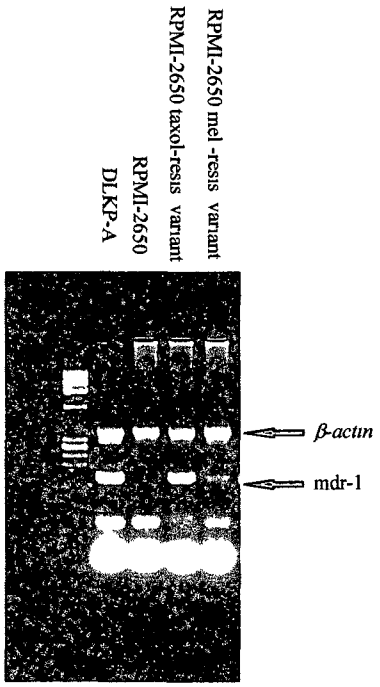


Fig. 3.5.1.1 RT-PCR analysis of mdr-1 expression in the RPMI-2650 cell line, the RPMI-2650 taxol-resistant variant and melphalan-resistant variant DLKP-A served as the positive control (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.1.2 mdr-1 expression in the DLKP and DLKP long term melphalan-selection variant

RT-PCR analysis showed that mdr-1 expression was undetectable in the DLKP cell line and its long term melphalan-selection variant (figure 3 5 1 2)

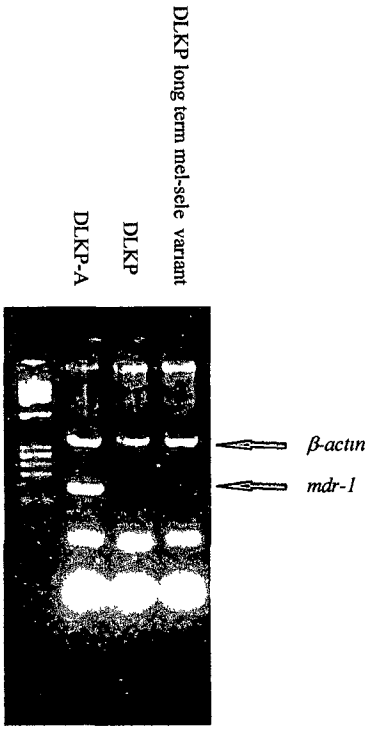


Fig. 3.5.1.2 RT-PCR analysis of mdr-1 expression in DLKP and the DLKP long term melphalan-selection variant, DLKP-A served as positive control (RT-PCR analysis was repeated twice RNA extraction was performed once)

3.5.2 mrp1

3.5.2.1 mrp1 expression in the RPMI-2650 and its MDR variants

RT-PCR analysis illustrated that the RPMI-2650 melphalan-resistant variant expressed a slightly stronger mrp1 band than its parental cell line while a very weak band was observed in the taxol-resistant variant

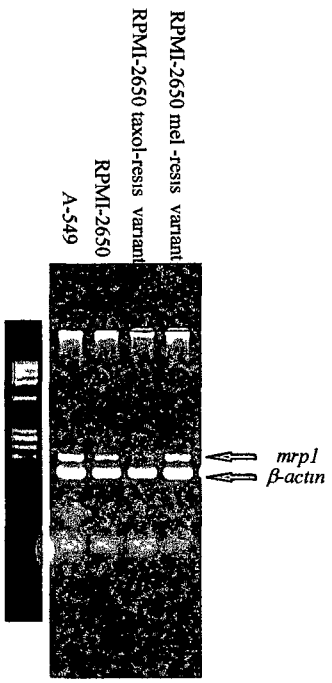


Fig. 3.5.2.1 RT-PCR analysis of mrp1 expression in the RPMI-2650 cell line, the RPMI-2650 taxol-resistant variant and melphalan-resistant variant A-549 served as the positive control (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.2.2 mrp1 expression in the DLKP and DLKP long term melphalan-selection variant

RT-PCR analysis demonstrated that the DLKP long term melphalan-selection variant expressed a slightly stronger mrp1 band than the DLKP cell line (it was clearer in the original photo)

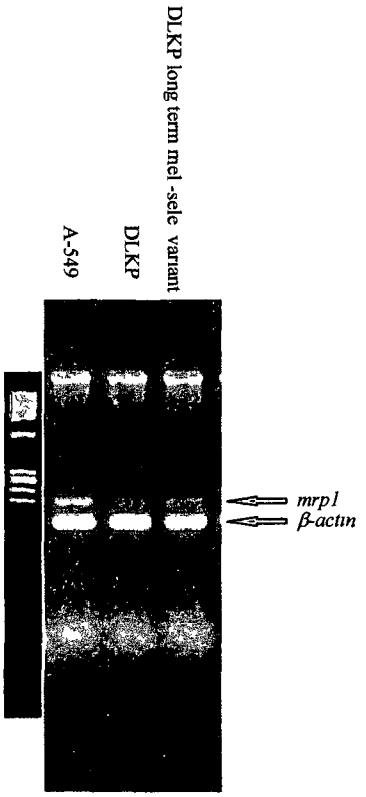


Fig. 3.5.2.2 RT-PCR analysis of mrp1 expression in the DLKP and DLKP long term melphalan-selection variant, A-549 served as positive control

3.5.3 cMOAT (mrp2)

3.5.3.1 cMOAT mRNA expression in the RPMI-2650 and its MDR variants

No detectable expression for cMOAT (MRP2) mRNA was observed in the RPMI-2650 parental cell line and its taxol-resistant variant by RT-PCR analysis However, the RPMI-2650 melphalan- resistant variant showed a strong band for cmoat

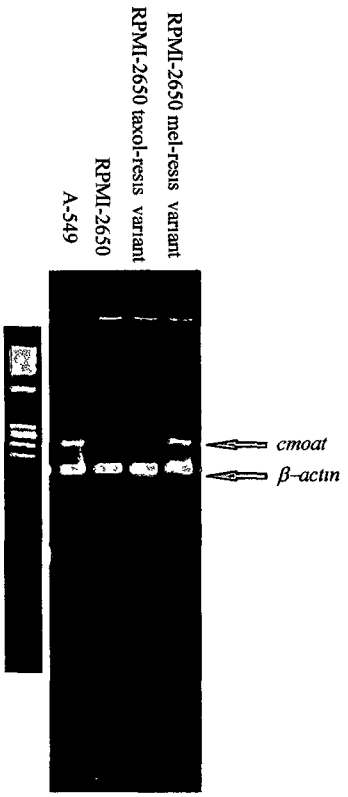


Fig. 3.5.3.1 RT-PCR analysis of cMOAT(MRP2) mRNA expression in the RPMI-2650 cell line, the RPMI-2650 taxol-resistant and melphalan-resistant variants A-549 served as the positive control (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.3.2 cMOAT mRNA expression in the DLKP and DLKP long term melphalan-selection variant

No detectable expression for cMOAT (MRP2) mRNA was observed in either the DLKP or the DLKP long term melphalan-selection cell lines (figure 3 5 3 2)

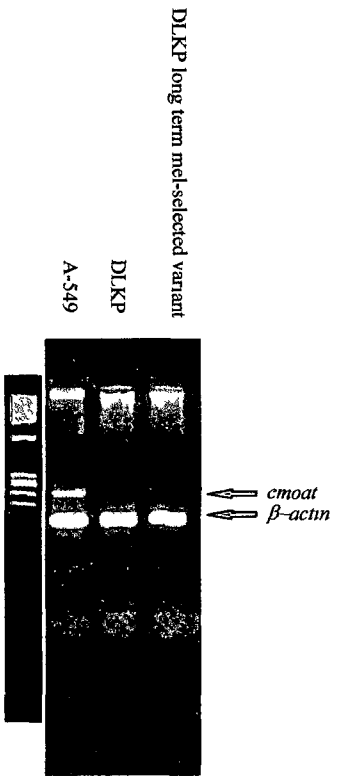


Fig. 3.5.3.2 RT-PCR analysis of cMOAT(MRP2) mRNA expression in the DLKP and DLKP long term melphalan-selection variant, A-549 served as positive control (The RT-PCR analysis was repeated twice RNA extraction was performed once)

3.5.4 mrp3

A very weak band of mrp3 was observed in the RPMI-2650 parental cell line by RT-PCR The expression of mrp3 was undetectable in the taxol-resistant variant while there was an overexpression of mrp3 in the melphalan-resistant variant compared to its parental cell line

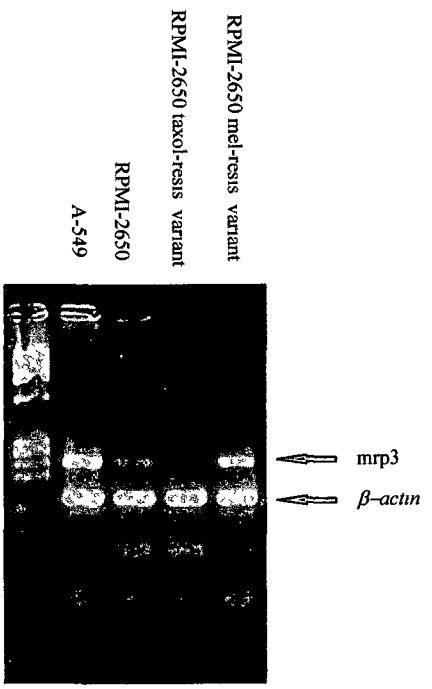


Fig. 3.5.4 RT-PCR analysis of mrp3 expression in the RPMI-2650 parental cell line, RPMI-2650 taxol-resistant variant and melphalan-resistant variant A-549 served as the positive control (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.5.mrp4

The expression of mrp4 was undetectable in the A-549, RPMI-2650 and its taxol-resistant variant and melphalan-resistant variant by RT-PCR analysis

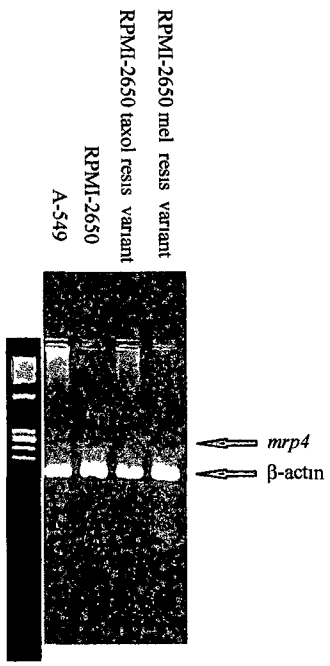


Fig. 3.5.5 RT-PCR analysis of mrp4 expression in the RPMI-2650 parental cell line, its taxol- and melphalan-resistant variants (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.6 mrp5

The expression of mrp5 was undetectable in the A-549, RPMI-2650 and its taxol- and melphalan-resistant variants by RT-PCR analysis

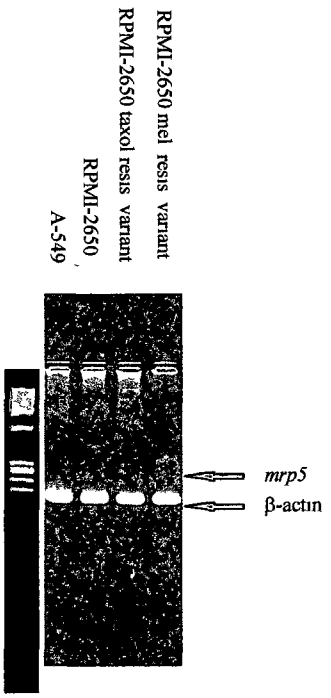
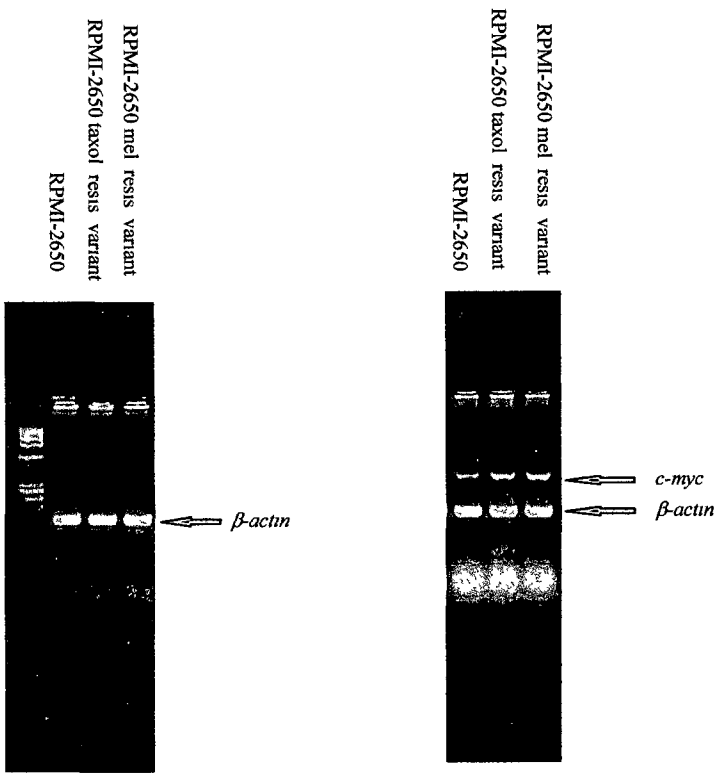


Fig. 3.5.6 RT-PCR analysis of mrp5 expression in the RPMI-2650 parental cell line, its taxol- and melphalan-resistant variants (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.5.7 c-Ha-ras and c-myc

RT-PCR analysis showed that expression of c-Ha-ras was undetectable in the RPMI-2650 cell line and its taxol- and melphalan-resistant variants whereas c-myc was overexpressed in the taxol- and melphalan-resistant variants compared to their parental cell line



(a) (b)

Fig. 3.5.7 RT-PCR analysis of (a) c-Ha-ras (b) c-myc in the RPMI-2650 cell line and its taxol-resistant variant and melphalan-resistant variant (The marker for c-myc is the same as c-Ha-ras) (The RT-PCR analysis was repeated twice RNA extraction was performed once)

3.6 Adriamycin distribution studies in the RPMI-2650 MDR variants

3.6.1 Subcellular distribution of adriamycin in the RPMI-2650 MDR variants

Adriamycin is a fluorescent chemotherapeutic drug which exerts its cytotoxic effect mainly in the nuclei of the cells It can be effluxed from the cells by P-glycoprotem and MRP The localisation of adriamycin in RPMI-2650, and in its taxol-resistant and melphalan- resistant variants was investigated by fluorescent microscopy as described in section 2 9 Figure 3 6 1 illustrates the fluorescence pattern observed in these three cell lines following four hours incubation with adriamycin (10 μM) The results obtained showed intense nuclear fluorescence in the RPMI-2650 cells indicating the localisation of adriamycin within the nuclei of these cells A similar fluorescence pattern was observed in the RPMI-2650 melphalan-resistant variant although the intensity was less than that observed in the parental cell line In contrast, faint nuclear fluorescence was observed in the taxol-resistant variant However, the fluorescence patterns demonstrated the localisation of adriamycin in the cytoplasm of these cells

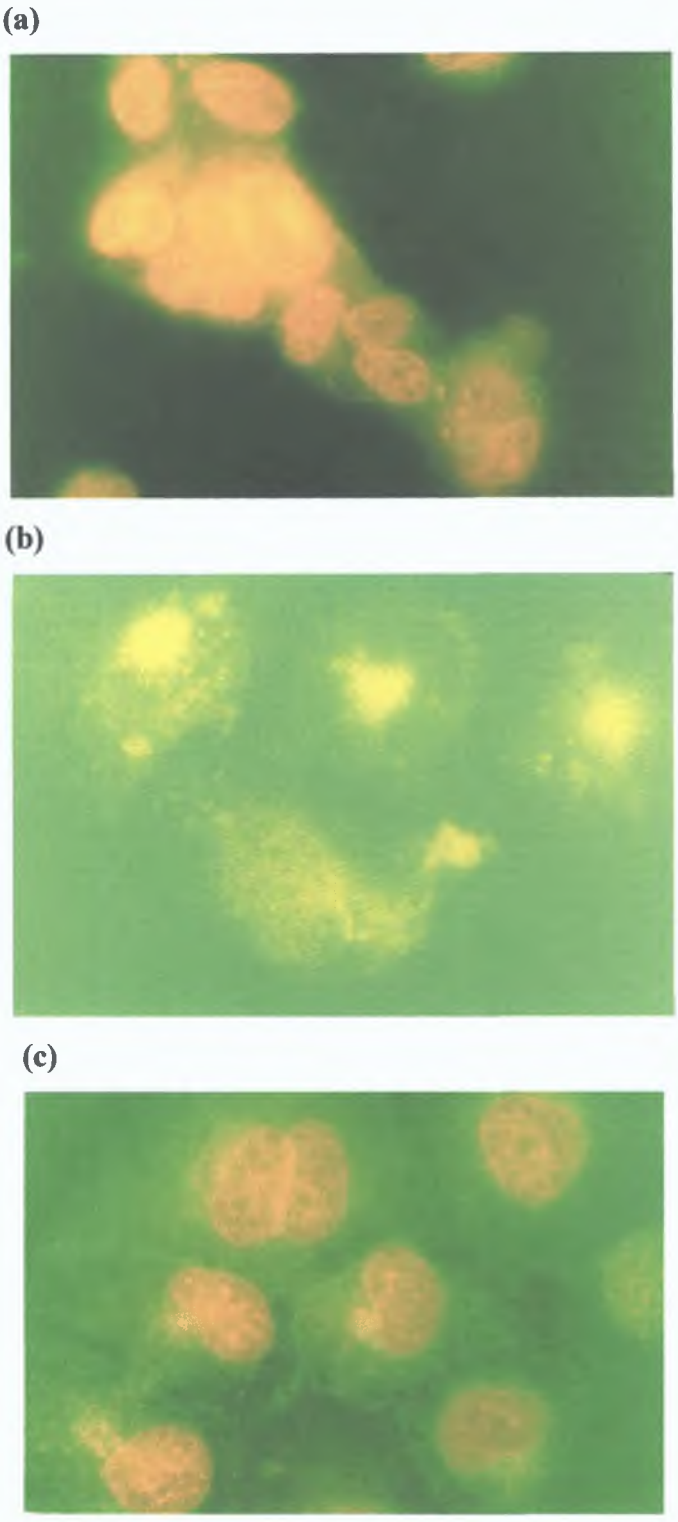


Fig. 3.6.1 Adriamycin localisation in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.6.2 Effect of verapamil on adriamycin distribution

The addition of verapamil (30μg/ml) which is a MDR modulator caused enhancement of nuclear fluorescence in the RPMI-2650 taxol-resistant variant and melphalan-resistant variant No significant difference was observed in the RPMI-2650 parental cells The results indicate that verapamil can increase the concentration of adriamycin within the nucleus and thus circumvent drug resistance in the RPMI-2650 taxol- and melphalan-resistant variants (Figure 3 6 2)

3.6.3 Effect of cyclosporin A on adriamycin distribution

Cyclosporin A is also a MDR circumvention agent The addition of cyclosporin A (10 μg/ml) also greatly enhanced adriamycin nuclear fluorescence in the RPMI-2650 taxol-resistant and melphalan-resistant variants Cyclosporin A resulted in a very slight increase in the intensity of nuclear fluorescence in the RPMI-2650 parental cell line (Figure 3 6 3)

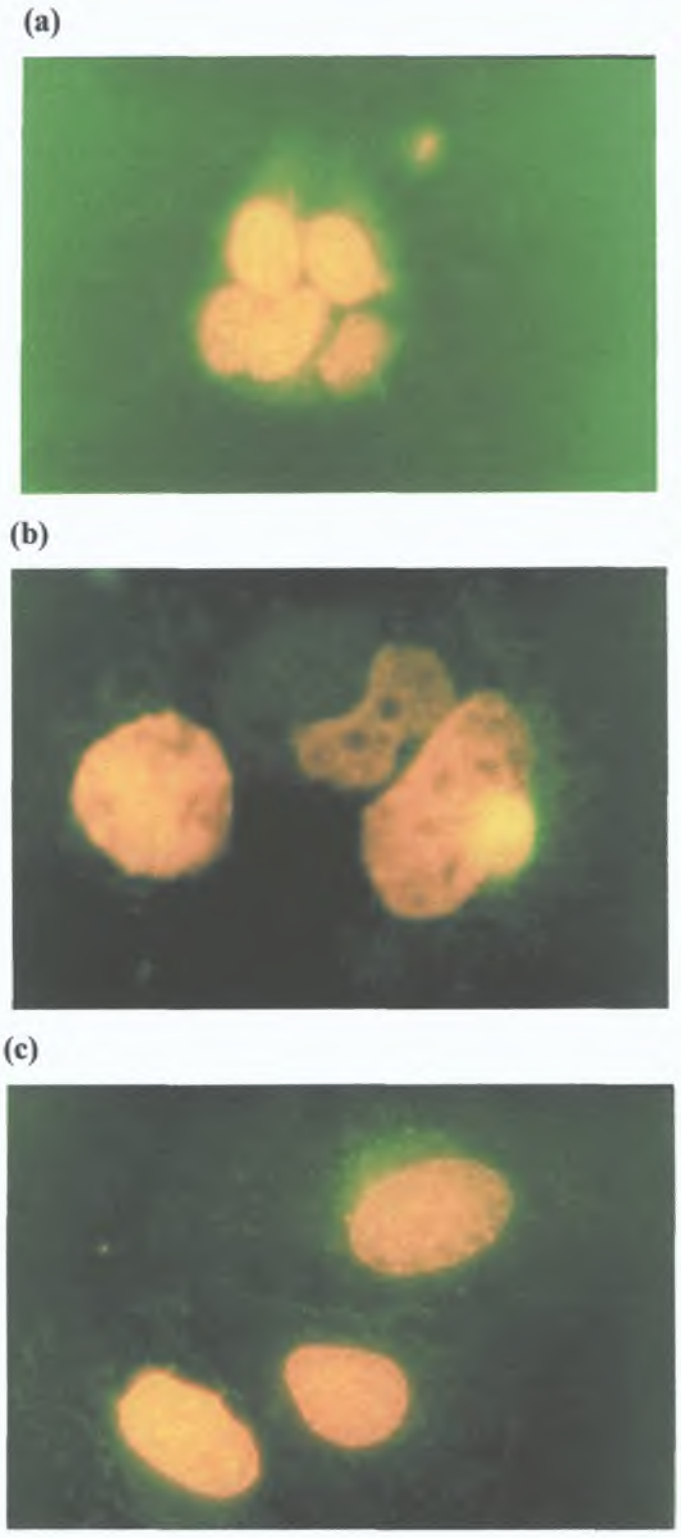


Fig. 3.6.2 Adriamycin localisation after incubation with verapamil in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

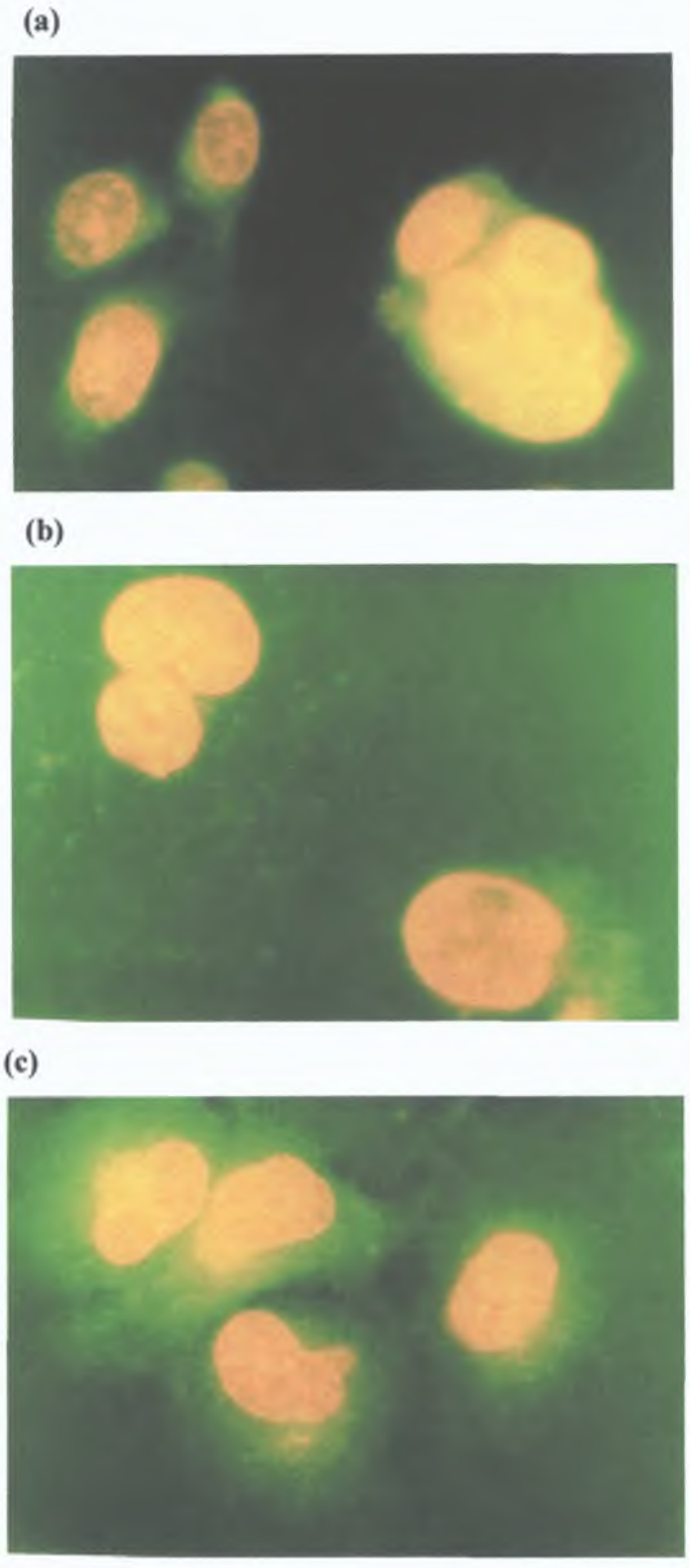


Fig. 3.6.3 Adriamycin localisation after incubation with cyclosporin A in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.7 Determination of glutathione S-transferase activity in the RPMI-2650 MDR variants

Alteration in the activity of glutathione S-transferase was investigated in the RPMI-2650 parental cell line and its MDR variants using cytosolic cellular extracts, prepared as described in section 2 10 A slight increase in GST activity was observed in the RPMI-2650 melphalan-resistant and RPMI-2650 taxol-resistant variants when compared to the parental cells

Cell line	GST activity
RPMI-2650 parental cell line	0 55 Δabs/min/mg protein
RPMI-2650 taxol-resistant variant	0 62 Δabs/min/mg protein
RPMI-2650 melphalan-resistant variant	0 7 Δabs/min/mg protein

Table 3.7 Glutathione S-transferase activity in the RPMI-2650 parental cell line and its taxol-resistant and melphalan-resistant variants

3.8 Circumvention studies

3.8.1 Circumvention studies with verapamil and cyclosporin A

Verapamil and cyclosporin A are both MDR modulators. To investigate whether these two agents can circumvent drug resistance in the RPMI-2650 parental cell line and its MDR variants, toxicity assays were carried out. As presented in table 3.8, the addition of verapamil resulted in a decrease in the IC_{50} of adriamycin in the RPMI-2650 taxol-resistant and melphalan-resistant variants with the greatest decrease observed in the melphalan-resistant variant. The addition of cyclosporin A also resulted in a decrease in the IC_{50} of adriamycin in the RPMI-2650 taxol-resistant and melphalan-resistant variants, especially in the taxol-resistant variant. A slight decrease of IC_{50} of adriamycin in the RPMI-2650 parental cells by both verapamil and cyclosporin A was also observed. DLKP-A, which is a P-glycoprotein overexpressing, adriamycin-selected variant of DLKP and COR-L23R, which is a MRP overexpressing, adriamycin-selected variant of COR-L23S served as positive controls.

3.8.2 Circumvention studies with sunlindac and indomethacin

Sulindac and indomethacin are both nonsteroidal antiinflammatory (NSAID) compounds which were found to circumvent adriamycin toxicity in some MRP-overexpressed cell lines (Duffy *et al.*, 1998). To test whether these two compounds could circumvent drug toxicity in the novel MDR variants established in this study, toxicity assays in RPMI-2650, DLKP cell lines and their MDR variants were first carried out to determine the concentration which could be used in the circumvention studies. The results showed that indomethacin was about 2-fold more toxic than sulindac. The DLKP MDR variants seemed to be more resistant to sulindac than the DLKP cells. No significant difference in toxicity for these two compounds was observed between RPMI-2650 and its melphalan-resistant variant. Preliminary circumvention study demonstrated that sulindac and indomethacin can circumvent melphalan toxicity in the MRP-overexpressing RPMI-2650 melphalan-resistant variant, but not in the parental cell line. (This circumvention study was only carried out once. The results will be confirmed by future work.)

IC_{50} (nM)	ADR	ADR+Verapamil (0 5μg/ml)	ADR+Verapamil (1μg/ml)
COR-L23R	2097 ± 364	1067 ± 159	938 ± 146
DLKPA	5750 ± 275	1196 ± 124*	441 ± 157*
RPMI-2650	110 ± 37	74 ± 28	59 ± 18
RPMI-2650 taxol-resistant variant	5750 ± 224	4508 ± 263	2171 ± 195
RPMI-2650 melphalan-resistant variant	690 ± 138	83 ± 24	48 ± 15

(*The concentrations of verapamil used in the DLKP-A cell line were 1 5μg/ml and 3 μg/ml respectively)

IC_{50} (nM)	ADR	ADR+Cyclosporin A (0 5μg/ml)	ADR+Cyclosporin A (1 μg/ml)
COR-L23R	2097 ± 364	1058 ± 143	1435 ± 129
DLKPA	5750 ± 275	/	1582 ± 167*
RPMI-2650	110 ± 37	64 ± 21	77 ± 19
RPMI-2650 taxol-resistant variant	5750 ± 224	377 ± 129	497 ± 118
RPMI-2650 melphalan-resistant variant	690 ± 138	322 ± 98	304 ± 125

(*The concentration of cyclosporin A used in the DLKP-A cell line was 5μg/ml)

Table 3.8.1 Circumvention studies with verapamil and cyclosporin A in the RPMI-2650 cell line and its taxol- and melphalan-resistant variants

IC_{50} (μg/ml)	Sulindac	Indomethacin
DLKP	34 3 ± 2 5	31 8 ± 2 4
DLKP melphalan-pulse selected variant	68 3 ± 5 7	25 1 ± 4 2
DLKP long term melphalan-selection variant	77 2 ± 9 6	32 8 ± 5 3
RPMI-2650	71 4 ± 8 9	34 3 ± 6 2
RPMI-2650 melphalan-resistant variant	58 6 ± 2 7	40 5 ± 2 1

Table 3.8.2.a IC_{50} values for DLKP and RPMI-2650 cell lines and their MDR variants

IC_{50} (nM)	Melphalan	Melphalan+Sulindac (10μg/ml)	Melphalan + Indomethacin (5μg/ml)
RPMI-2650	852	917	786
RPMI-2650 melphalan-resistant variant	5998	3670	3014

Table 3.8.2.b Circumvention studies with sulindac and indomethacin in the RPMI-2650 cell line and its melphalan-resistant variant

3.9 Western blotting of β_1 integrin in the RPMI-2650 variants

Integrins are a widely expressed family of cell surface adhesion receptors. All integrins are $\alpha\beta$ heterodimers. The β_1 integrin subunit can make heterodimer with α_1, α_2, α_3, α_4, α_5, α_6, etc. To confirm the expression of β_1 integrin in the RPMI-2650 parental cell line and its MDR variants, Western blotting was carried out as described in section 2.8.

The results in figure 3.9 showed that both the RPMI-2650 taxol-resistant and melphalan-resistant variants had stronger β_1 integrin bands than the parental cells. DLKP-A, which is a lung carcinoma cell line, was used as the positive control.

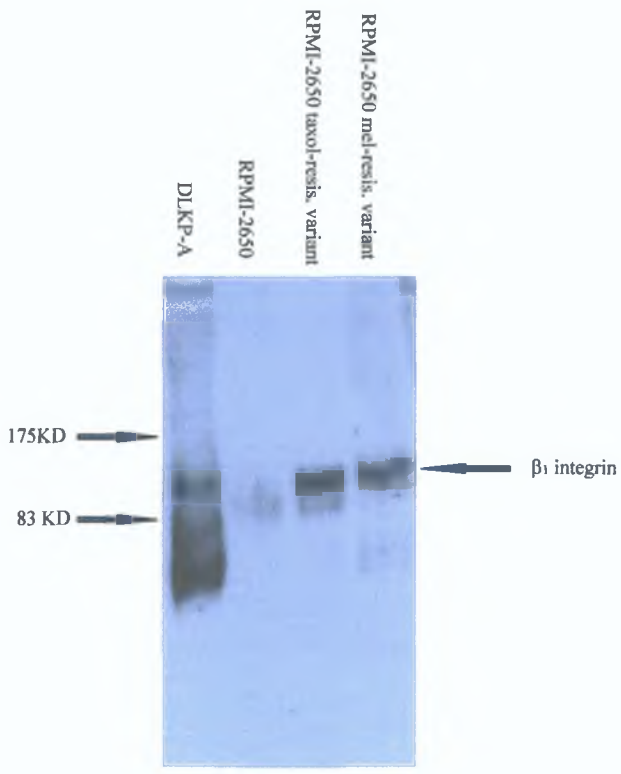


Fig. 3.9 Western blotting of β_1 integrin subunit in the RPMI-2650 parental cell line and its MDR variants. DLKP-A served as positive control.

3.10 Immunocytochemical detection of integrins and E-cadherin

To detect the presence of α_1, α_2, α_3, α_4, α_5, α_6, β_1 integrin subunits and E-cadherin in the RPMI-2650 and DLKP parental cell lines and their MDR variants, immunocytochemistry was carried out as described in section 2 7 2

3.10.1 α_1 integrin subunit

A mouse monoclonal anti-α_1-integrin antibody was used to detect α_1 integrin As shown in figure 3 10 1, no cell membrane staining for α_1 integrin was observed in the RPMI-2650 cell line Weak staining for the α_1 subunit was visible in the taxol-resistant and melphalan-resistant variants

3.10.2 α_2 integrin subunit

A mouse monoclonal anti-α_2-integrin antibody was used to detect the presence of the α_2 integrin subunit as described in section 2 7 2 2 7 There is quite uniform staining in the RPMI-2650 parental cell line as shown in 3 10 2 a More intense staining of α_2 mtegrin was observed in the RPMI-2650 melphalan-resistant variant compared to its parental cell line Interestingly, in the RPMI-2650 taxol-resistant variant, α_2 mtegrin staining seemed to concentrate on certain subclones of the cells, the rest of the cells did not have any staining (Figure 3 10 2 b) To determine whether this phenomenon exists in other MDR cell lines, expression of α_2 was investigated in the MDR cell lines DLKP-A, DLKP melphalan-long term and pulse selection variants and their sensitive parental line DLKP As shown in figure 3 10 2 c, the staining for α_2 integrin concentrated on certain subclones in both DLKP and DLKPA cell lines, this effect was most evident in the DLKP-A cells Strong uniform staining was noted in the DLKP long term melphalan-selection variant whereas very weak staining was observed in the pulse selection cells

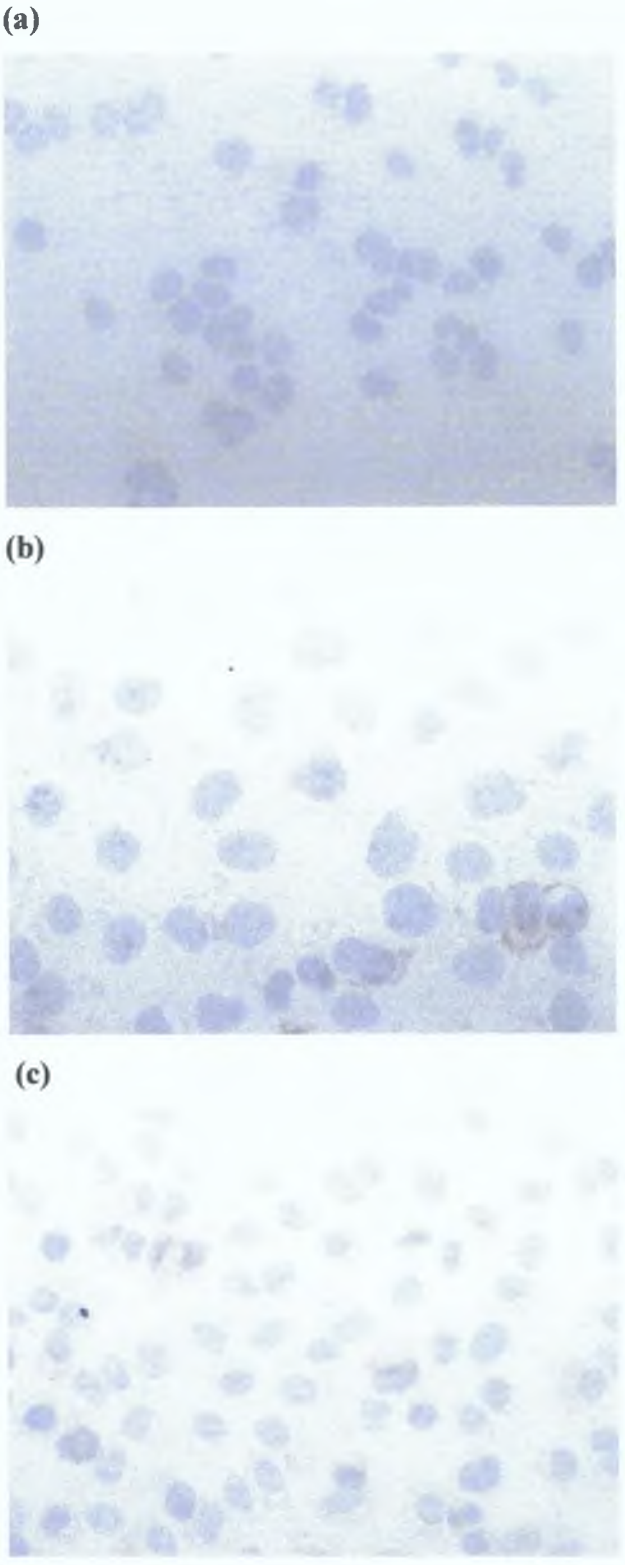
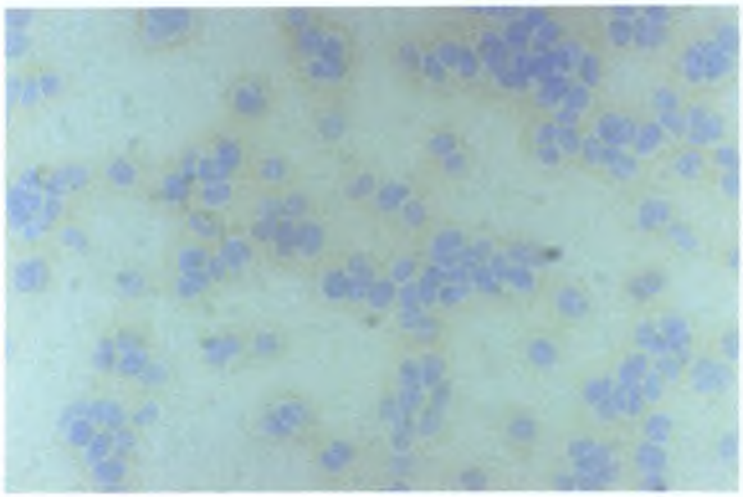


Fig. 3.10.1 Immunocytochemical staining for integrin α_1 subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



(b)

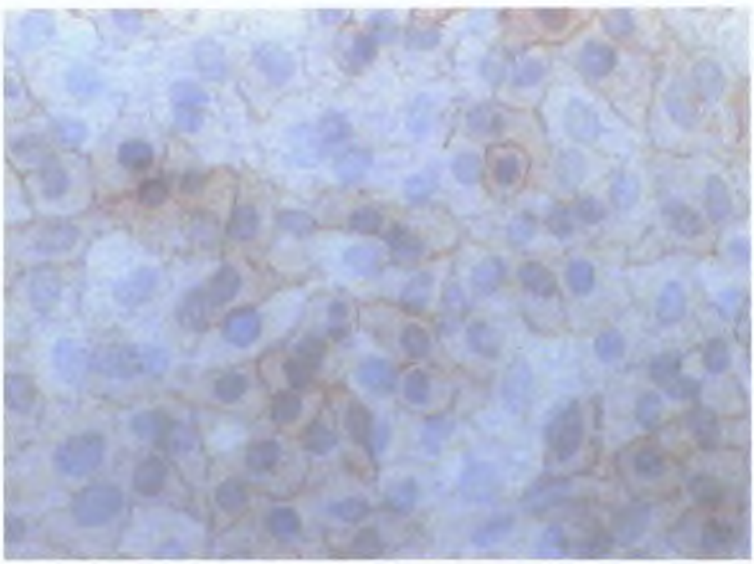
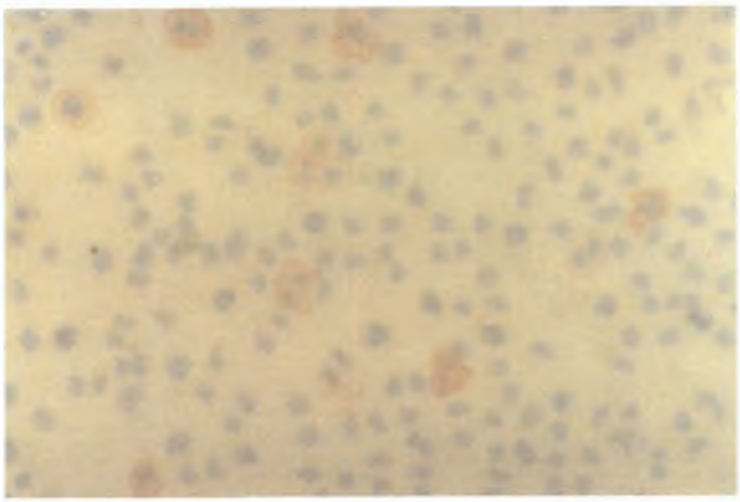


Fig. 3.10.2.a Immunocytochemical staining for integrin α_2 subunit (40x) in the (a) RPMI-2650 cell line (b) RPMI-2650 melphalan-resistant variant

(a)



(b)

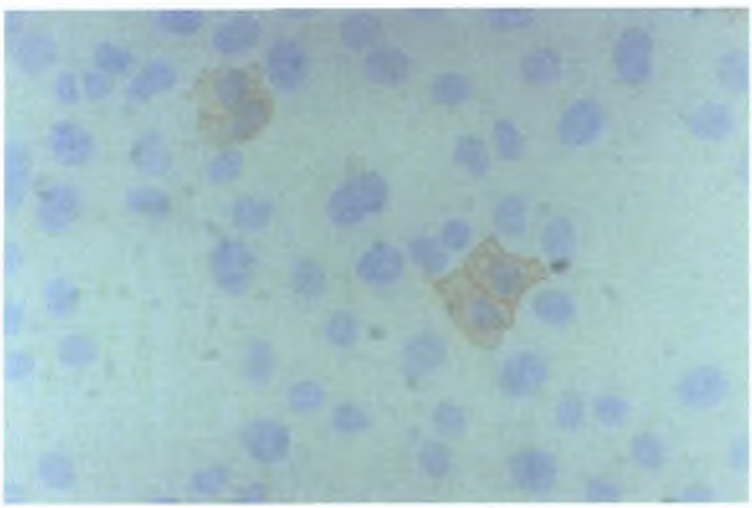
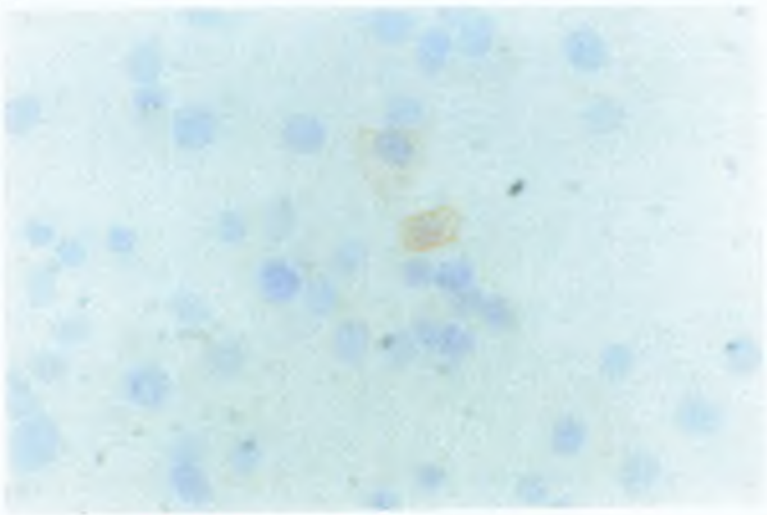
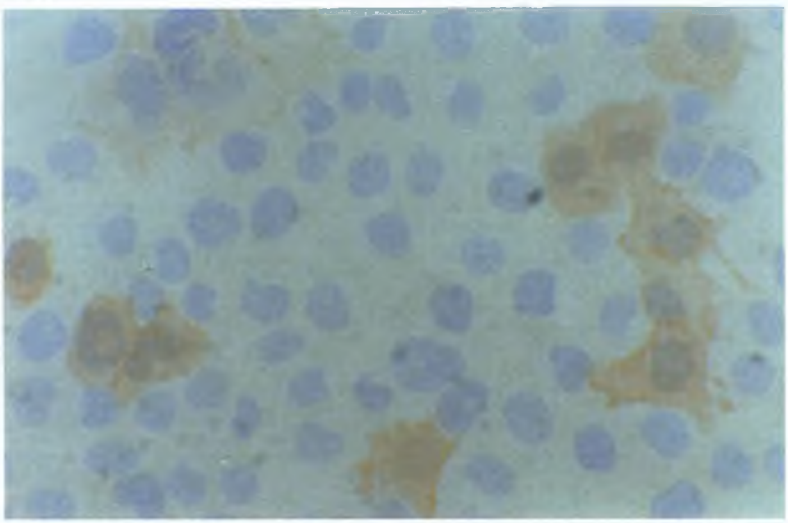


Fig. 3.10.2.b Immunocytochemical staining for integrin α_2 subunit in the RPMI-2650 taxol-resistant variant (a) 20x (b) 40x

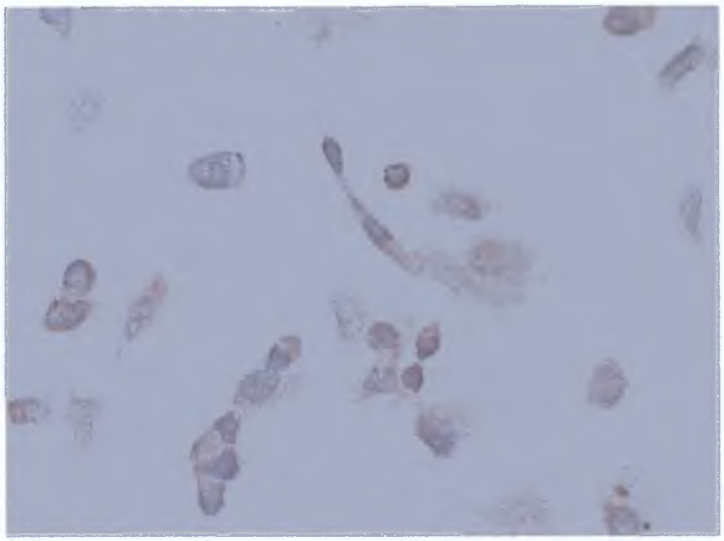
(a)



(b)



(c)



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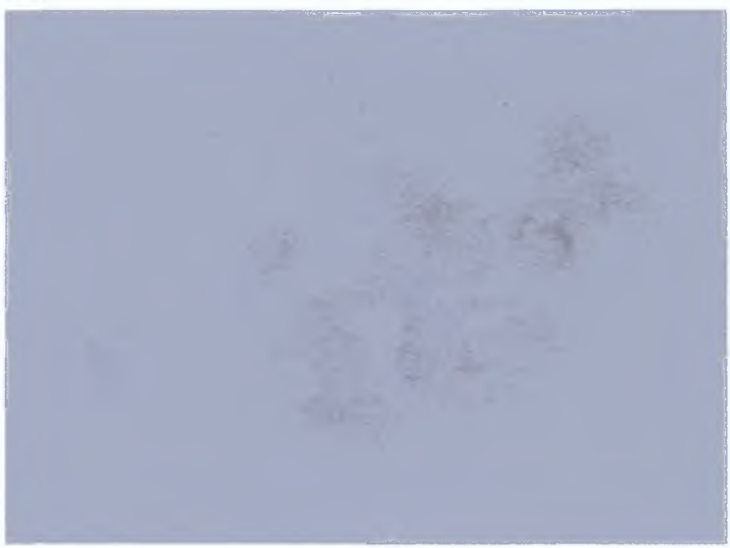


Fig. 3.10.2.c Immunocytochemical staining for integrin α_2 subunit in the DLKP and its MDR variants (40x) (a) DLKP (b) DLKP-A (c) DLKP long term melphalan-selection variant (d) DLKP melphalan-pulse selection variant

3.10.3 α_3 integrin subunit

A mouse monoclonal anti-α_3-integrin antibody was used to detect α_3 integrin subunit as described in section 2.7.2.2.7. As shown in figure 3.10.3, there is weak staining for α_3 integrin in the RPMI-2650 parental cell line, its taxol-resistant and melphalan-resistant variants. No significant difference in the level of staining of α_3 integrin was observed in all these cell lines (figure 3.10.3).

3.10.4 α_4 integrin subunit

A mouse monoclonal anti-α_4-integrin antibody was used to detect α_4 integrin subunit as described in section 2.7.2.2.7. The results obtained demonstrated strong staining for α_4 integrin in the RPMI-2650 parental cell line, but no significant staining in its taxol-resistant and melpahaln-resistant variants. No staining was observed in DLKP and its MDR variants (figure 3.10.4.1 and 3.10.4.2).

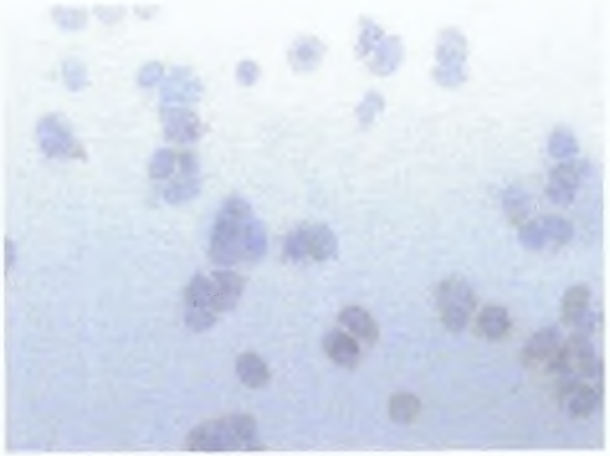
3.10.5 α_5 integrin subunit

A mouse monoclonal anti-α_5-integrin antibody was used to detect α_5 integrin subunit as described in section 2.7.2.2.7. As shown in figure 3.10.5, there is weak staining for α_5 integrin in the RPMI-2650 parental cell line and its taxol-resistant variant. The staining in the melphalan-resistant variant appears to be more intense than in the other two cell lines.

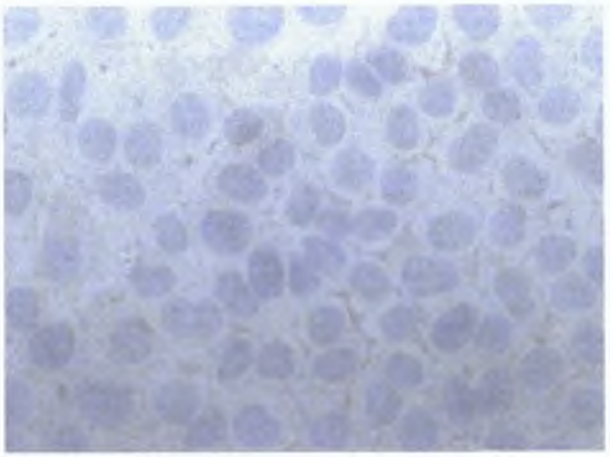
3.10.6 α_6 integrin subunit

A mouse monoclonal anti-α_6-integrin antibody was used to detect α_6 integrin subunit as described in section 2.7.2.2.7. The results obtained show weak staining for α_6 integrin in the RPMI-2650 parental cell line and its taxol-resistant variant. The staining in the melphalan-resistant variant is more intense than in the other two cell lines (figure 3.10.6).

(a)



(b)



(c)

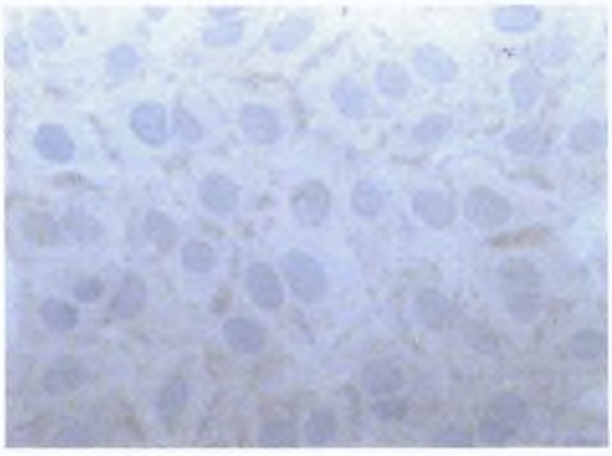
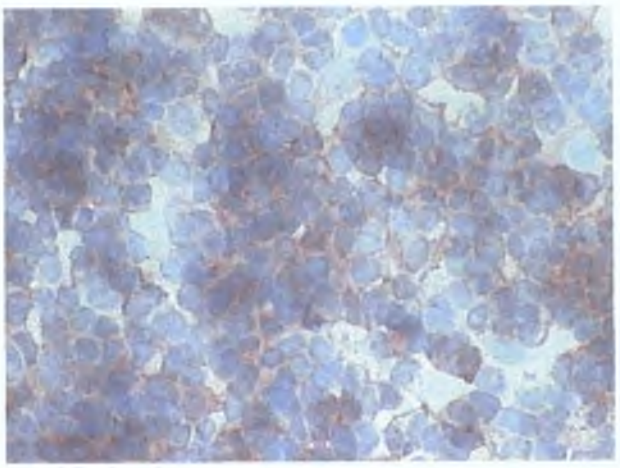
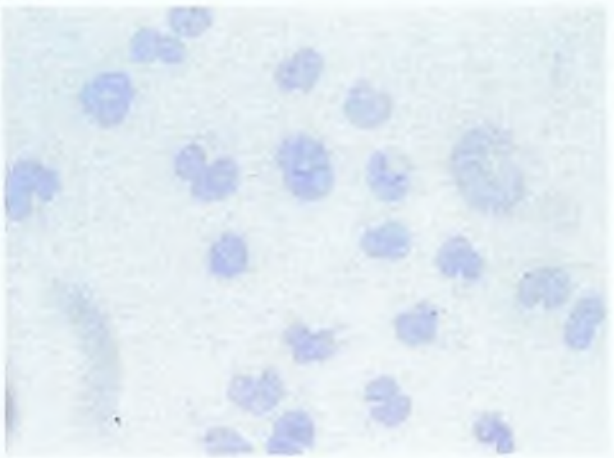


Fig. 3.10.3 Immunocytochemical staining for α_3 integrin subunit in the RPMI-2650 and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



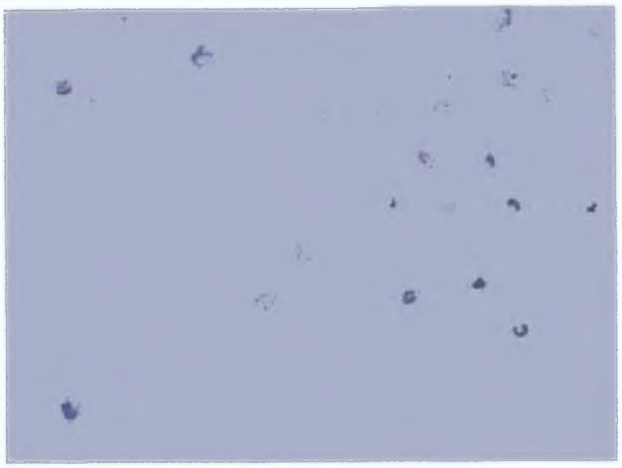
(b)



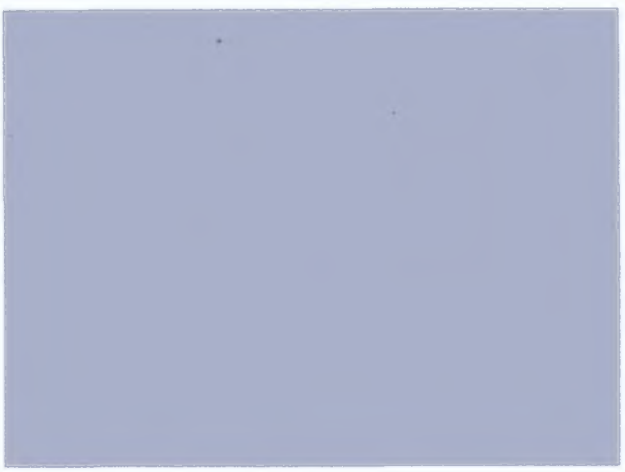
(c)

Fig. 3.10.4.1 Immunocytochemical staining for α_4 integrin subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



(b)



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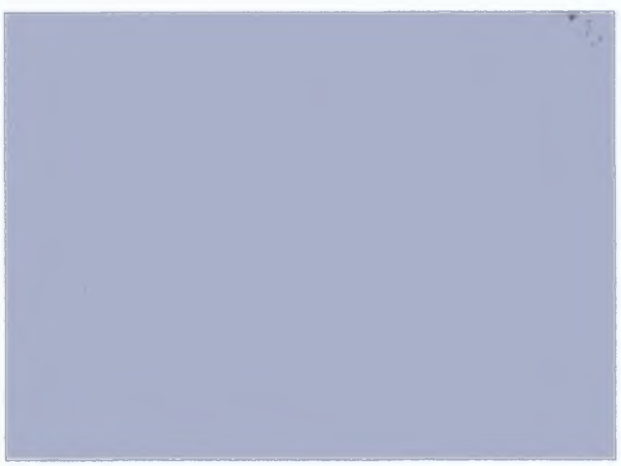
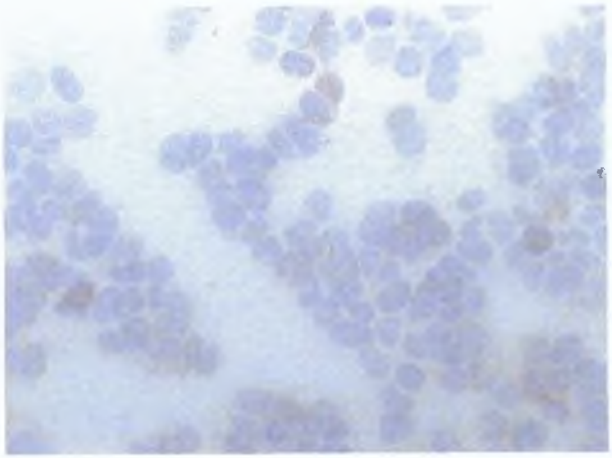


Fig. 3.10.4.2 Immunocytochemical staining for α_4 integrin subunit in the DLKP cell line and its MDR variants (a) DLKP cell line (b) DLKP long term melphalan-selection variant (c) DLKP melphalan-pulse selection variant

(a)



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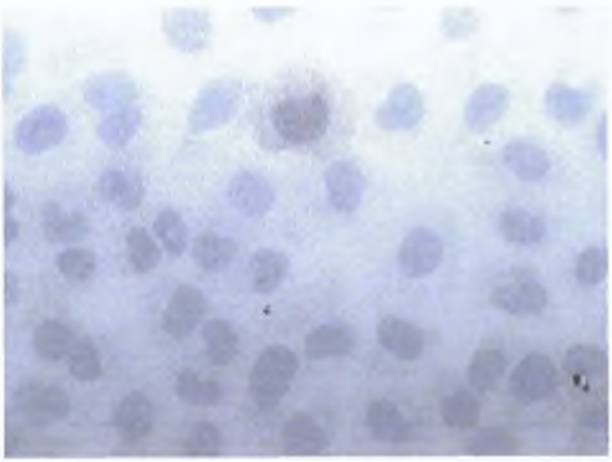


(c)

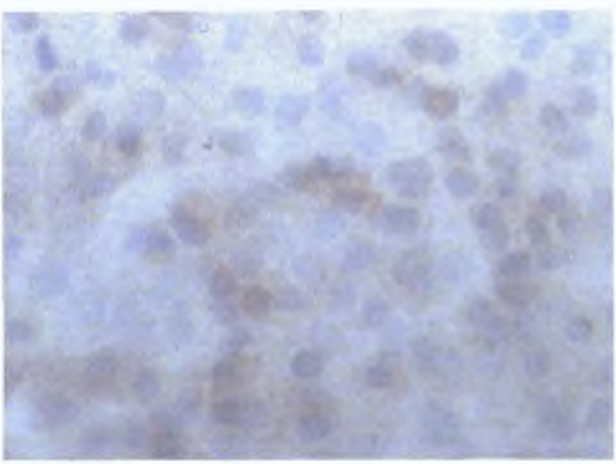
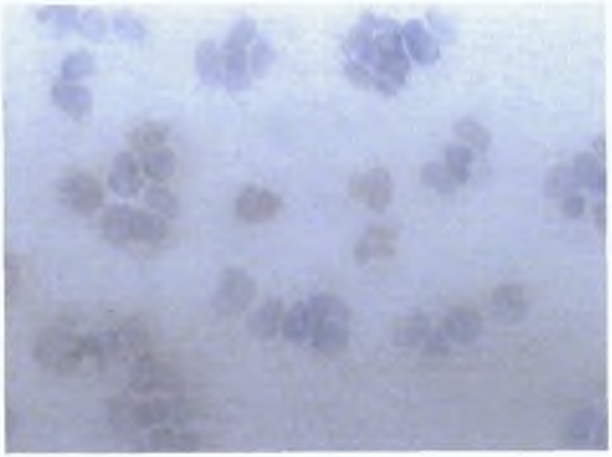


Fig. 3.10.5 Immunocytochemical staining for α_5 integrin subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



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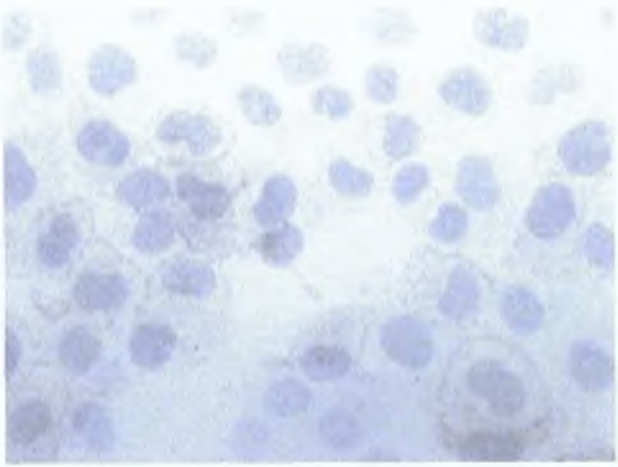

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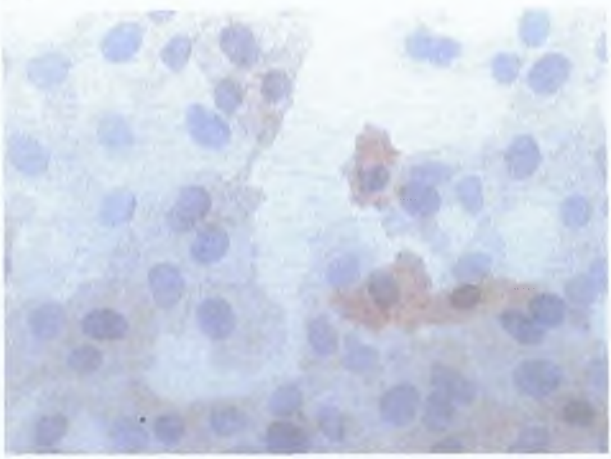


Fig. 3.10.6 Immunocytochemical staining for α_6 integrin subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

3.10.7 β_1 integrin subunit

A mouse monoclonal anti-β_1-integrin antibody was used to detect the presence of β_1 integrin subunit as described in section 2 7 2 2 7 As shown in figure 3 10 7 a, there is staining for β_1 integrin in the RPMI-2650 parental cells The results obtained indicate a significantly higher level of expression of β_1 integrin in the RPMI-2650 taxol-resistant and melphalan-resistant variants, especially in the melphalan-resistant variant The results also show a significantly higher level of β_1 integrin expression in the DLKP-A and DLKP long term melphalan-selected cell lines than in the DLKP and its pulse selection cell lines (figure 3 10 7 b)

3.10.8 β_4 integrin subunit

β_4 integrin subunit forms heterodimer with α_6 $\alpha_6\beta_4$ is expressed exclusively in epithelial cells To detect the presence of β_4 integrin, a mouse monoclonal anti-β_4-integrin antibody was used as described in section 2 7 2 2 7 As shown in figure 3 10 8, the RPMI-2650 melphalan-resistant variant expressed β_4 integrin whereas the RPMI-2650, its taxol-resistant variant, DLKP and its MDR variants did not

3.10.9 E-cadherm

E-cadherm is the major cell-cell adhesion molecule Immunocytochemical detection of E-cadherin in RPMI-2650 and its MDR variants was carried out as described in section 2 7 2 2 8 by using a mouse monoclonal anti-E-cadherin antibody MCF-7, a breast carcinoma cell line, was used as the positive control Trace level of staining for E-cadherin was observed in the RPMI-2650 parental cell line However, no staining was observed in the taxol-resistant and melphalan-resistant variants as shown in figure 3 10 9

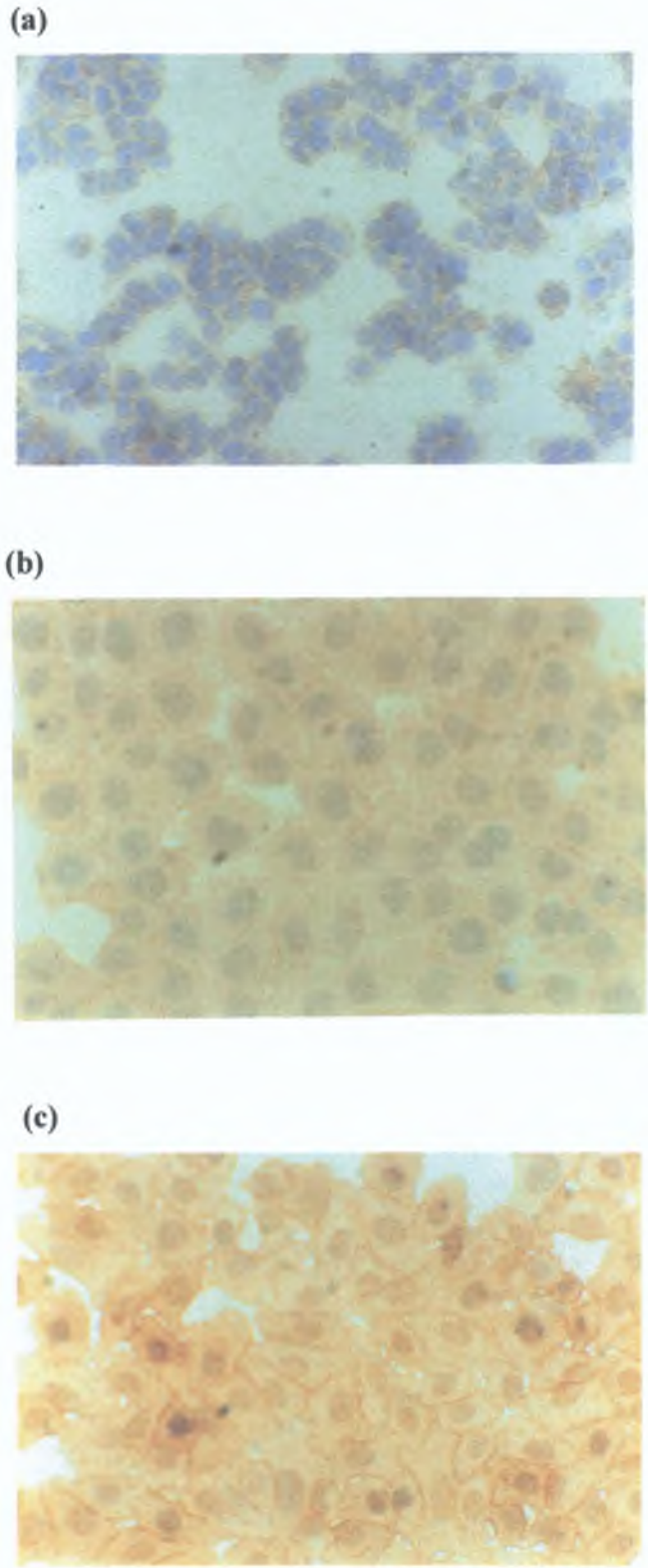
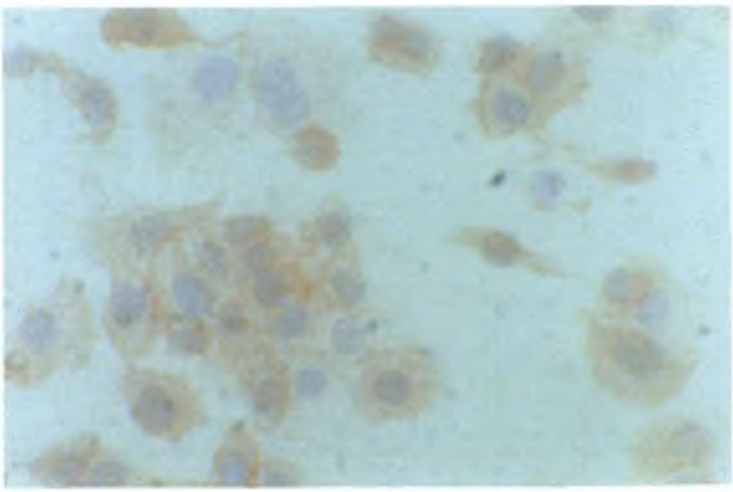


Fig. 3.10.7.a Immunocytochemical staining for β_1 integrin subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

(a)



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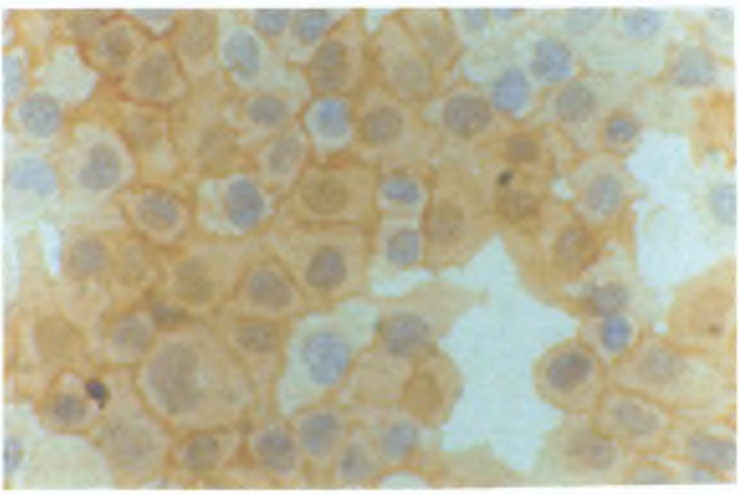
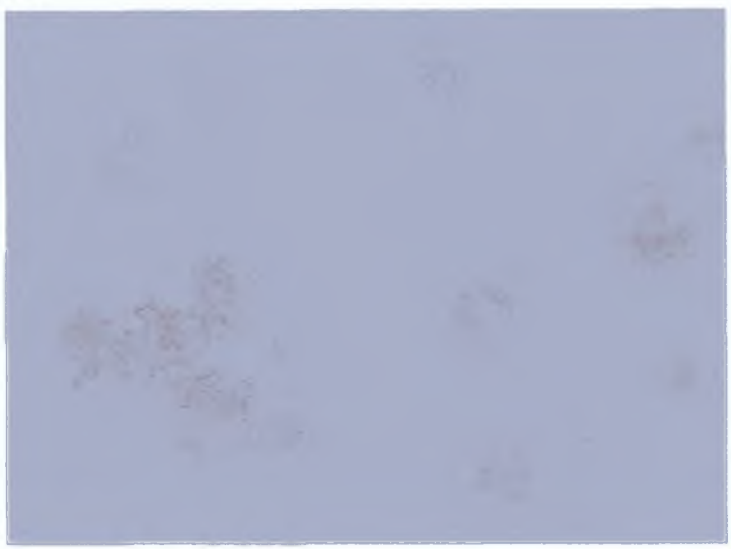


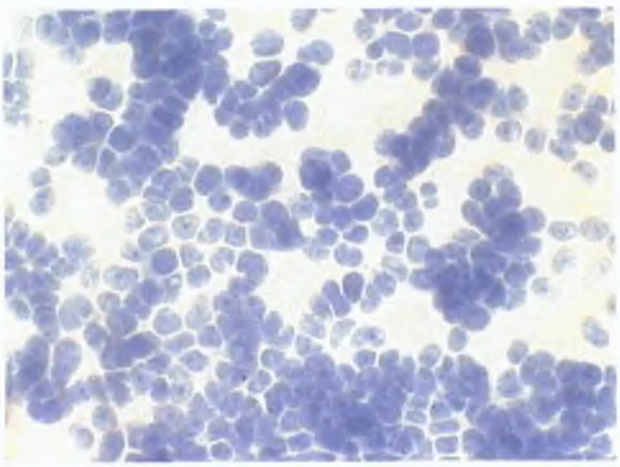
Fig. 3.10.7.b Immunocytochemical staining for β_1 integrin subunit in the DLKP and its MDR variants (40x) (a) DLKP (b) DLKP-A (c) DLKP long term melphalan-selection variant (d) DLKP melphalan-pulse selection variant (see next page)

(c)

(d)



(a)



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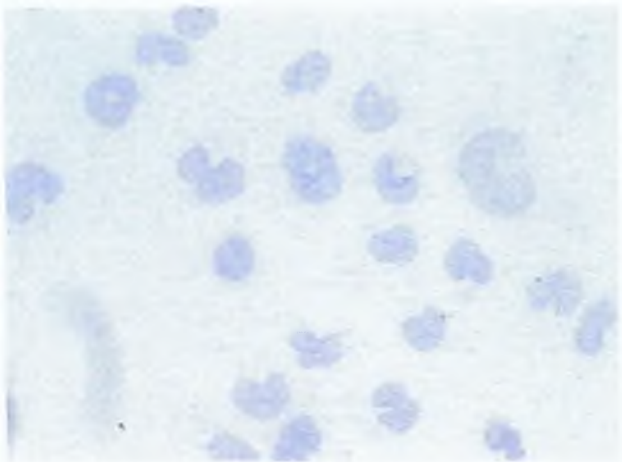


(c)

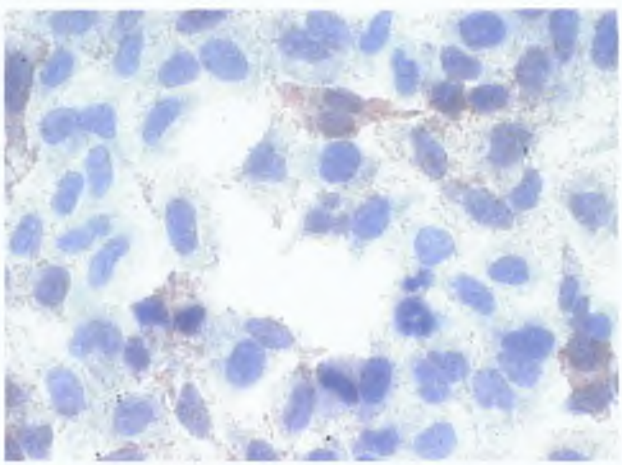


Fig. 3.10.8.a Immunocytochemical staining for integrin β_4 subunit in the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

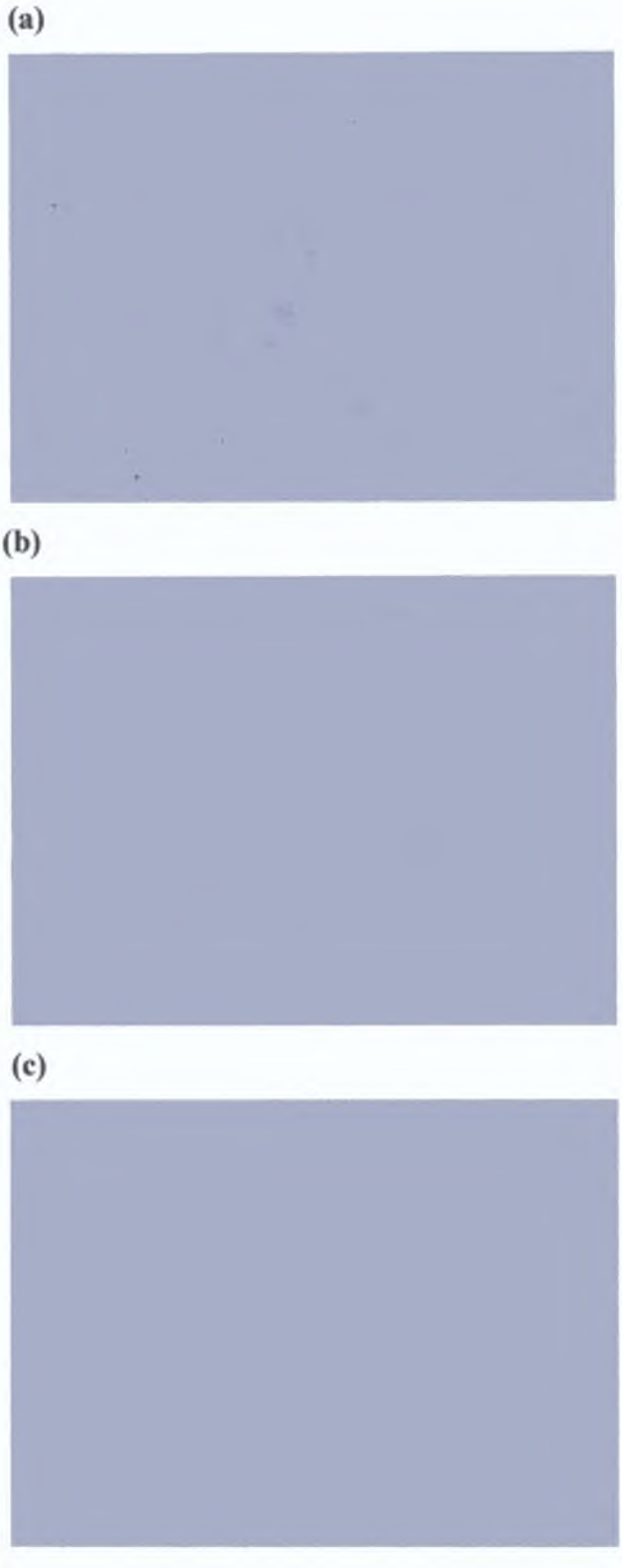


Fig. 3.10.8.b Immunocytochemical staining for β_4 integrin subunit in DLKP and its MDR variants (40x) (a) DLKP cell line (b) DLKP long term melphalan-selection variant (c) DLKP melphalan-pulse selection variant

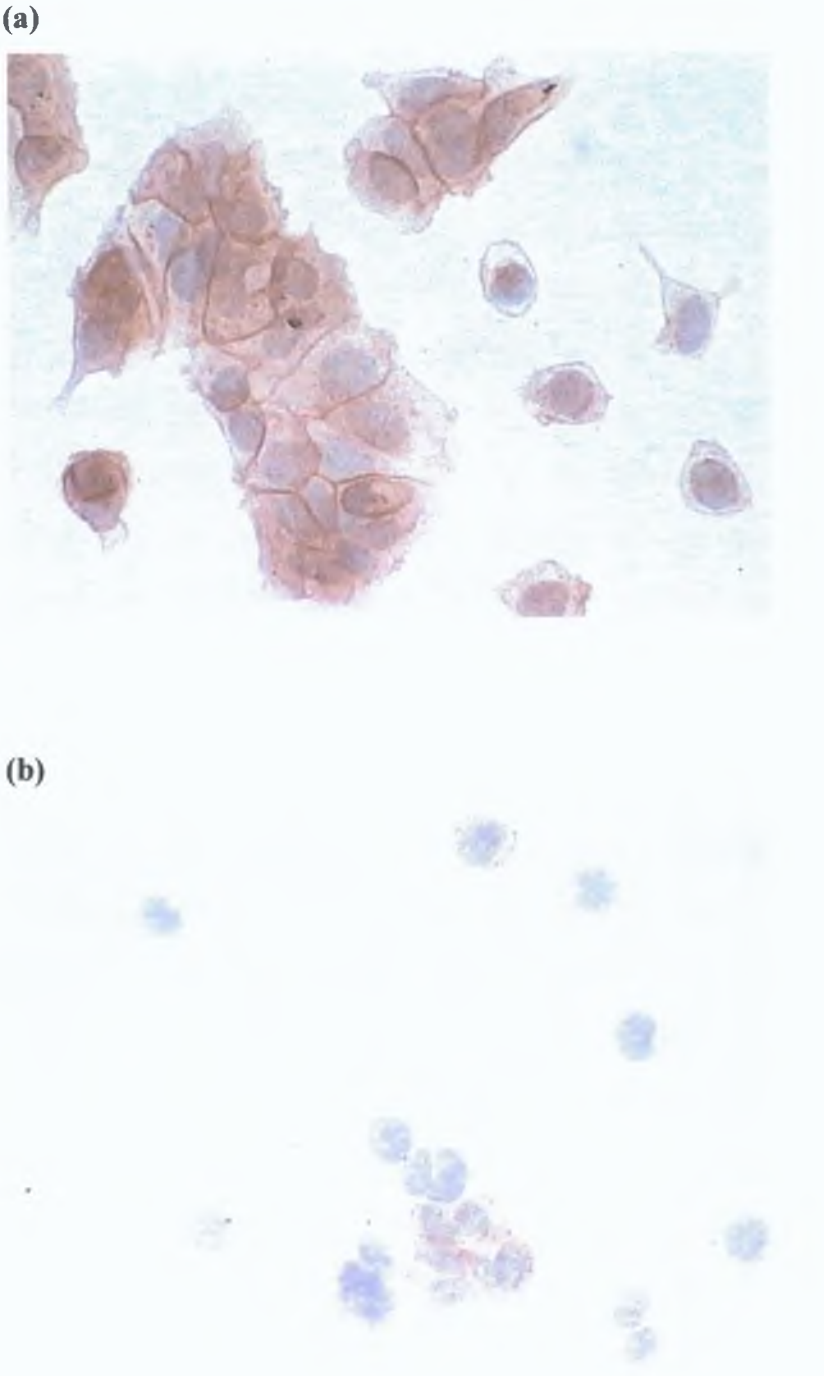
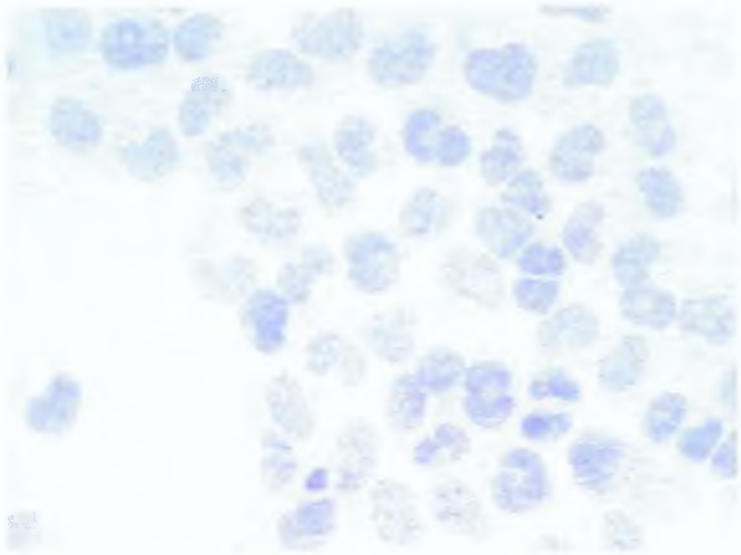


Fig. 3.10.9 Immunocytochemical staining for E-cadherin in the RPMI-2650 cell line and its MDR variants (40x) (a) MCF-7 cell line, served as positive control (b) RPMI-2650 (c) RPMI-2650 taxol-resistant variant (d) RPMI-2650 melphalan-resistant variant (see next page)

(c)



(d)

Integrin subunit	RPMI-2650	RPMI-2650 taxol-resis. variant	RPMI-2650 mel-resis. variant	DLKP	DLKP long term mel.-sele. variant	DLKP mel.-pulse sele. variant
α_1	-	±	±	ND	ND	ND
α_2	++	clonal	+++	clonal	+++	±
α_3	±	±	±	ND	ND	ND
α_4	++	-	-	-	-	-
α_5	±	±	+	ND	ND	ND
α_6	±	±	+	ND	ND	ND
β_1	++	+++	++++	++	+++	+
β_4	-	-	+	-	-	-
E-cadherin	+	-	-	ND	ND	ND

Table 3.10 Summary of the immunocytochemical staining for integrin subunits in the RPMI-2650, DLKP cell lines and their MDR variants

3.11 RT-PCR of integrin α_2 and β_1 subunits

From the results obtained from the immunocytochemical studies shown in section 3 10, interest was concentrated on the expression of α_2 and β_1 integrin subunits at the RNA level in the RPMI-2650 parental cell line and its MDR variants

The results of RT-PCR analysis in figure 3 11 showed that the RPMI-2650 melphalan-resistant variant had the highest expression of both α_2 and β_1 integrin subunits compared to the other two cell lines The lowest level of β_1 integrin subunit expression was observed in the RPMI-2650 while the RPMI-2650 taxol-resistant variant displayed the lowest level of α_2 integrin subunit expression The results obtained for RT-PCR analysis were consistent with the results from the immunocytochemical studies

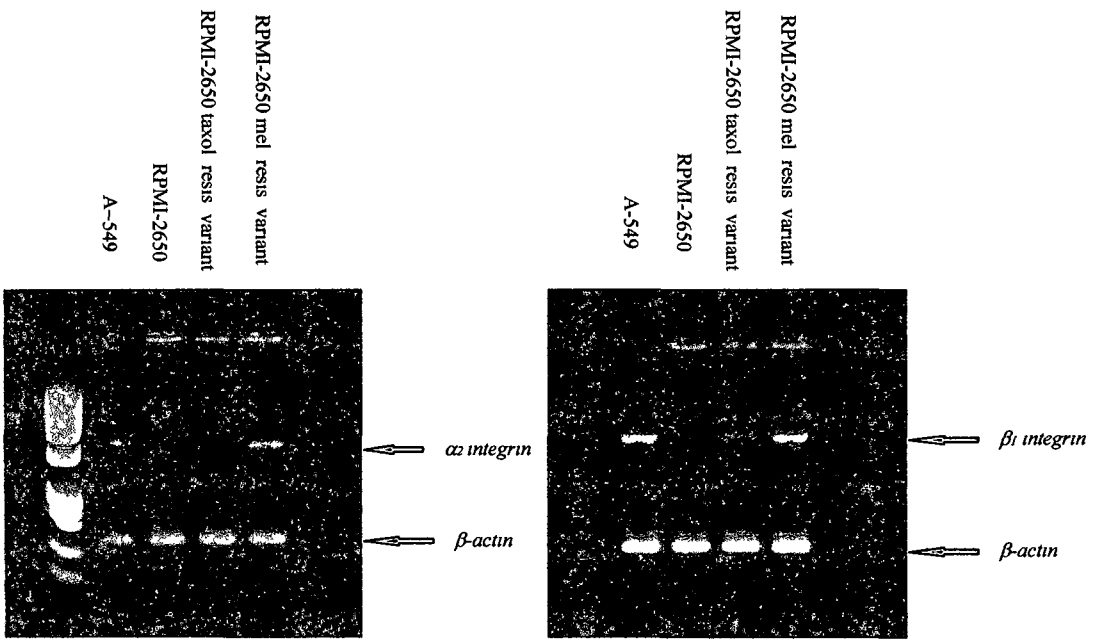


Fig. 3.11 RT-PCR analysis of α_2 and β_1 integrin subunits in the RPMI-2650 cell line, its taxol-resistant and melphalan-resistant variants A-549 served as positive control (The marker for β_1 integrin is the same as α_2 integrin) (The RT-PCR analysis was repeated three times RNA extraction was performed once)

3.12 Adhesion assays in the RPMI-2650 MDR variants

Cells utilise integrins to interact with extracellular matrix components such as collagen type IV, laminin and fibronectin To detect adhesiveness of the RPMI-2650 parental cell line and its MDR variants to collagen type IV, laminin, fibronectin and matrigel, adhesion assay was carried out as described in section 2 12

3.12.1 Collagen type IV

As shown in figure 3 12 2, the RPMI-2650 melphalan-resistant variant was most adhesive to collagen type IV which is the major component of basement membrane The RPMI-2650 cell line had less adhesiveness than melphalan-resistant variant, but was more adhesive to collagen type IV than the RPMI-2650 taxol-resistant variant

3.12.2 Laminin

As shown in figure 3 12 1, the RPMI-2650 parental cell line and its MDR variants had very weak adhesiveness to laminin which is another major component of basement membrane The RPMI-2650 melphalan-resistant variant showed slightly stronger adhesiveness to laminin compared to the other two cell lines

3.12.3 Fibronectin

Fibronectin is the major component of extracellular matrix and very little fibronectin is found in the basement membrane Figure 3 12 1 showed that the RPMI-2650 melphalan-resistant variant was most adhesive to fibronectin The RPMI-2650 taxol-resistant variant was less adhesive than melphalan-resistant variant, but was more adhesive than the parental cell line

3.12.4 Matrigel

Matrigel is the artificial reconstituted basement membrane It basically contains every component of the natural basement membrane The adhesion assays with matrigel (Figure 3 12 2) showed that the RPMI-2650 melphalan-resistant variant was most adhesive to matrigel The RPMI-2650 taxol-resistant variant was less adhesive to matrigel than the melphalan-resistant variant, but was much more adhesive than the RPMI-2650 parental cells

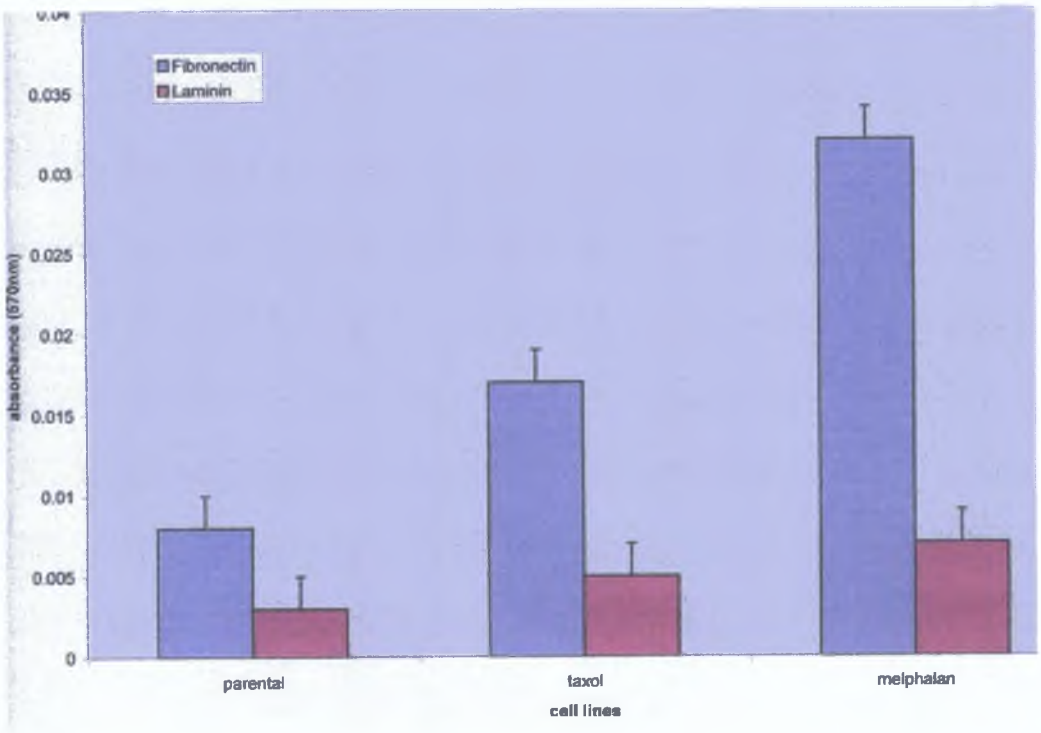


Fig. 3.12.1 Adhesion assays of the RPMI-2650 cell line, its taxol-resistant and melphalan-resistant variants with fibronectin and laminin

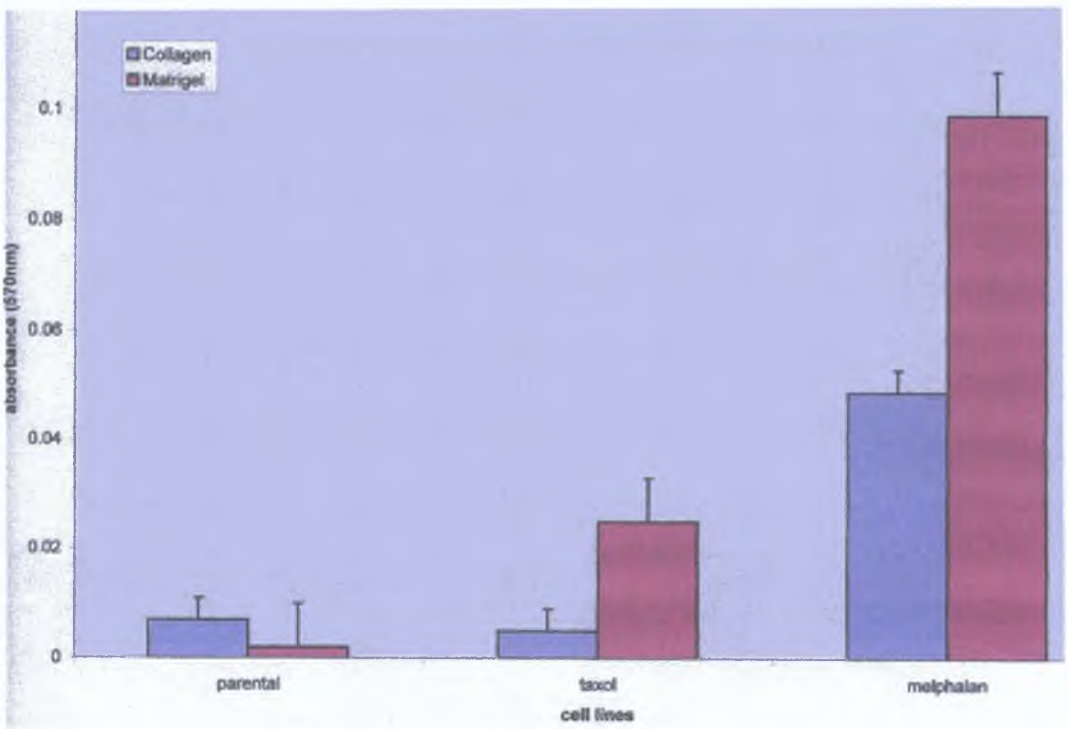


Fig. 3.12.2 Adhesion assays of the RPMI-2650 cell line, its taxol-resistant and melphalan-resistant variants with collagen type IV and matrigel

3.13 Invasion assays in the RPMI-2650 and DLKP MDR variants

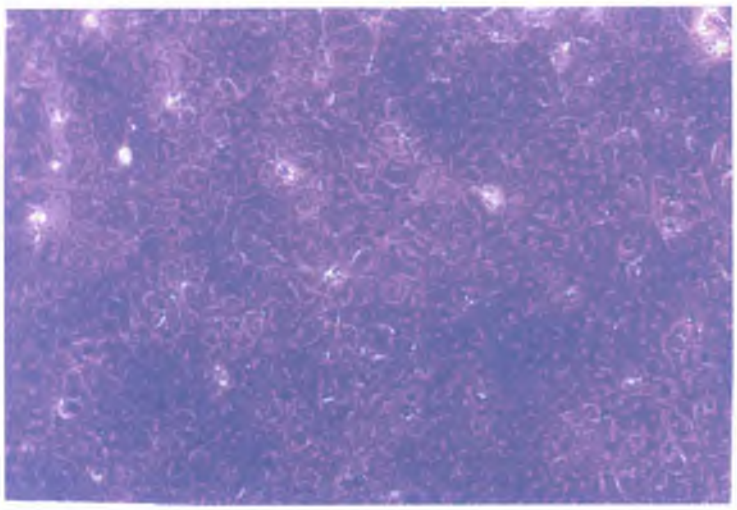
3.13.1 Invasion assays in the RPMI-2650 and its MDR variants

Adhesion of cells to extracellular matrix or basement membrane is the first step of cell invasion. Then cell will utilise different mechanisms to degrade extracellular matrix or basement membrane to move about. In order to investigate the invasive ability of the RPMI-2650 parental cell line and its MDR variants, invasion assays were carried out as described in section 2.13. Cell culture inserts which stand in medium-containing 24-well plates were coated with matrigel before cells were added. After a certain period of time, invasive cells can migrate through the miniholes on the bottom of the cell culture insert to the other side of the bottom. As instructed by Becton-Dickenson company where the cell culture inserts were bought, HT-1080, a highly invasive fibrosarcoma cell line and NIH-3T3, a poorly invasive cell line should be used as controls.

As shown in figure 3.13.2, the RPMI-2650 melphalan-resistant variant was significantly more invasive than the RPMI-2650 parental cell line and its taxol-resistant variant which were unable to invade through the bottom of the inserts.

Fig. 3.13.1 Invasion assays showing the cells invading through to the bottom side of the cell culture inserts (Top left: HT-1080, top right: NIH-3T3, lower left: RPMI-2650, lower middle: taxol-resistant variant, lower right: melphalan-resistant variant).

(a)



(b)

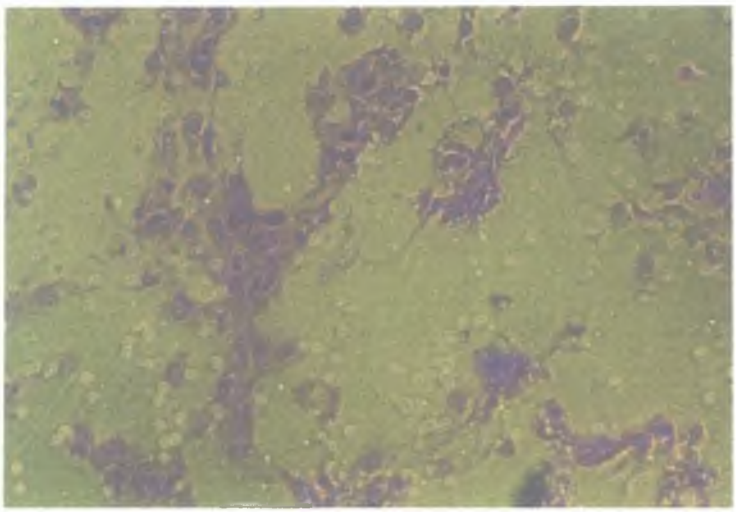


Fig. 3.13.2.a Invasion assays of (a) HT-1080 (b) NIH-3T3

(a)

(b)

(c)

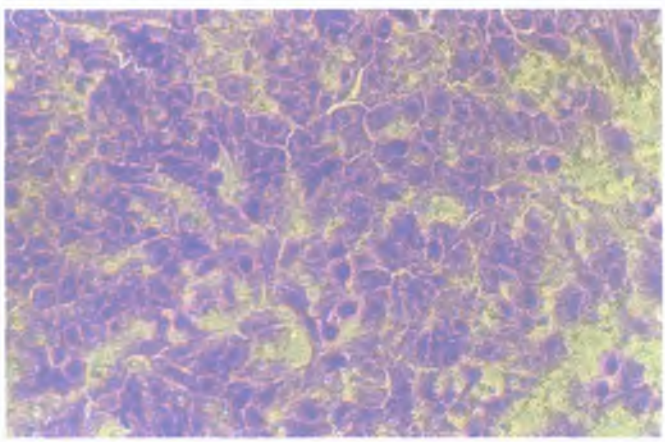


Fig. 3.13.2.b Invasion assays of the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

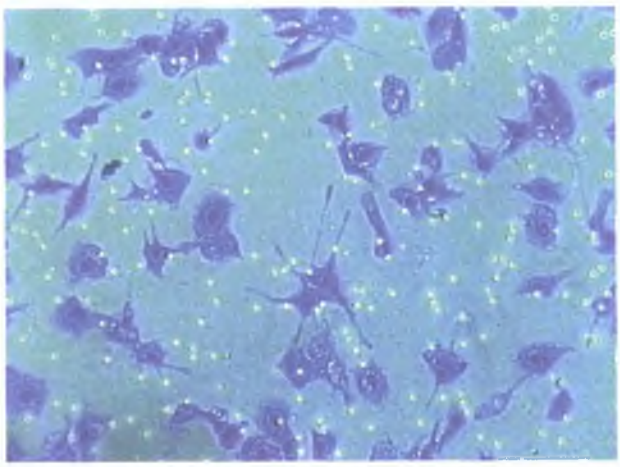
3.13.2 Invasion assays with sulindac and indomethacin

As shown in section 3 8 2, sulindac and indomethacin which are known to be MRP1 inhibitors can partially circumvent melphalan toxicity in RPMI-2650 and its melphalan-resistant variant To determine whether these two compounds can affect the invasiveness of this variant, invasion assays were carried out The results obtained demonstrate that the invasiveness of the RPMI-2650 melphalan-resistant variant was not affected by either sulindac or indomethacin (figure 3 13 2 c)

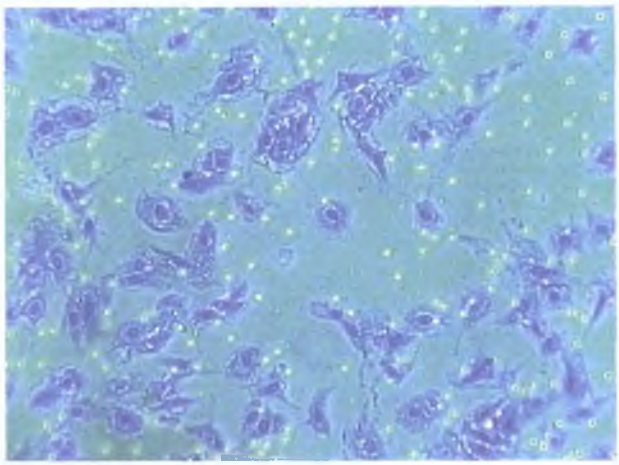
3.13.3 Invasion assays in DLKP and its MDR variants

As shown in figure 3 13 3, both the DLKP long term melphalan-selection and DLKP melphalan-pulse selection variants were more invasive than DLKP parental cell line, especially the long term selection variant

(a)



(b)



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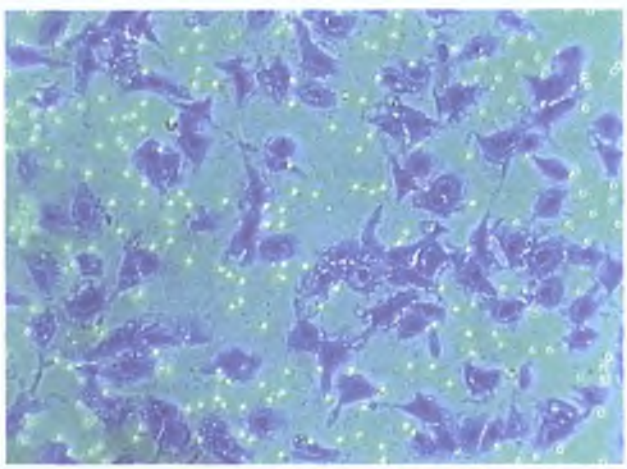
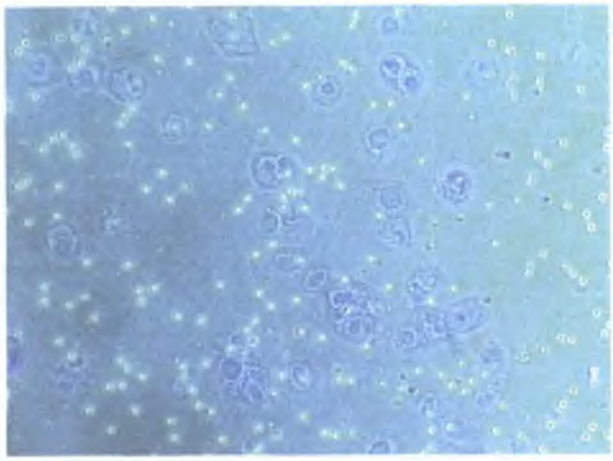


Fig. 3.13.2.c Invasion assays with sulindac and indomethacin in the RPMI-2650 melphalan-resistant variant (a) RPMI-2650 melphalan-resistant variant (b) with sulindac (c) with indomethacin

(a)



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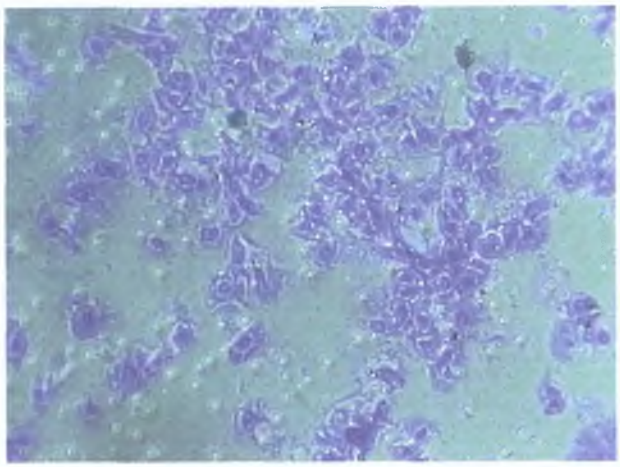

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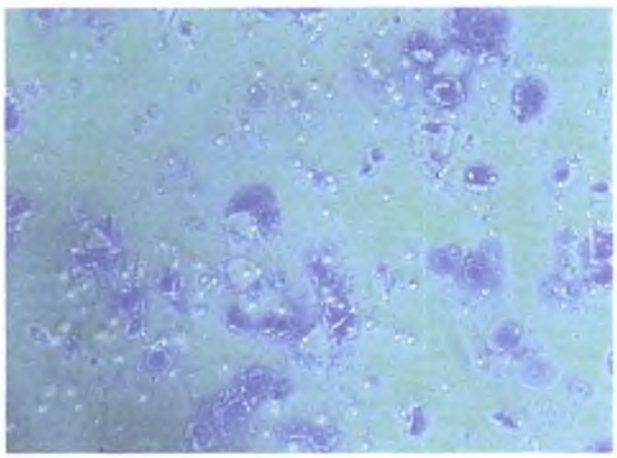


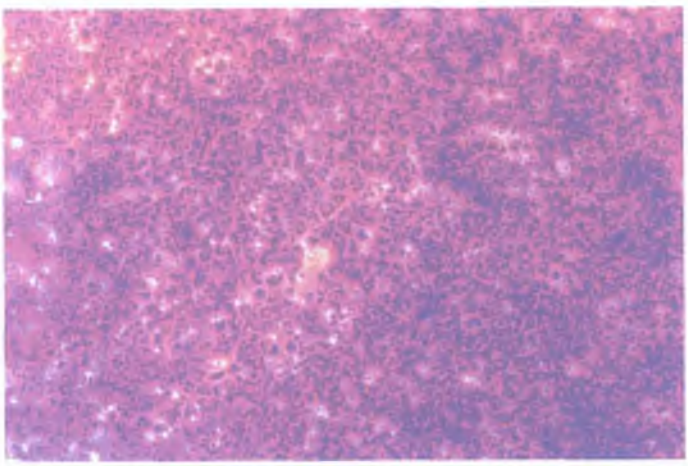
Fig. 3.13.3 Invasion assays in DLKP and its MDR variants (a) DLKP cell line (b) DLKP long term melphalan-selection variant (c) DLKP melphalan-pulse selection variant

3.14 Motility assays in the RPMI-2650 MDR variants

Motility assays were conducted to compare the locomotive ability of RPMI-2650 parental cell line and its MDR variants. The procedure used to demonstrate cell motility was similar to that used for the invasion assays except that the inserts were not coated with matrigel prior to the addition of the cells. The results obtained illustrated that the RPMI-2650 melphalan-resistant variant showed significantly higher motility than the RPMI-2650 taxol-resistant variant while the parental cells showed little or no motility (figure 3.14.1 and 3.14.2). HT-1080 and NIH-3T3 cell lines were used as positive and negative control respectively.

Fig. 3.14.1 Motility assays showing the cells migrating to the bottom side of the cell culture inserts (Top left: HT-1080, top right: NIH-3T3, lower left: RPMI-2650, lower middle: taxol-resistant variant, lower right: melphalan-resistant variant).

(a)



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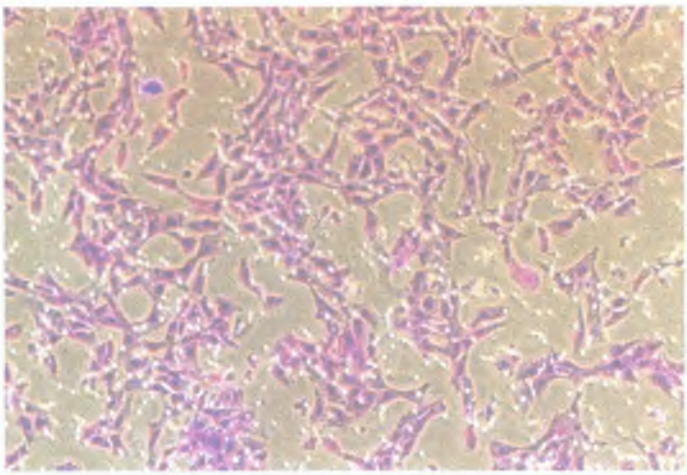
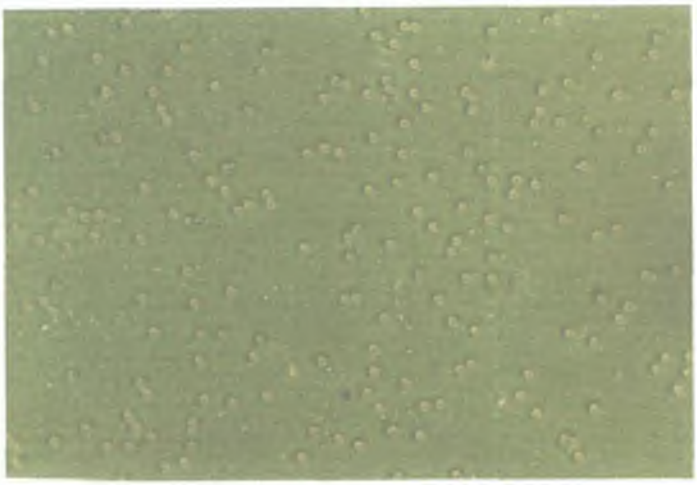


Fig. 3.14.2.a Motility assays of the (a) HT-1080 cell line (b) NIH-3T3 cell line

(a)



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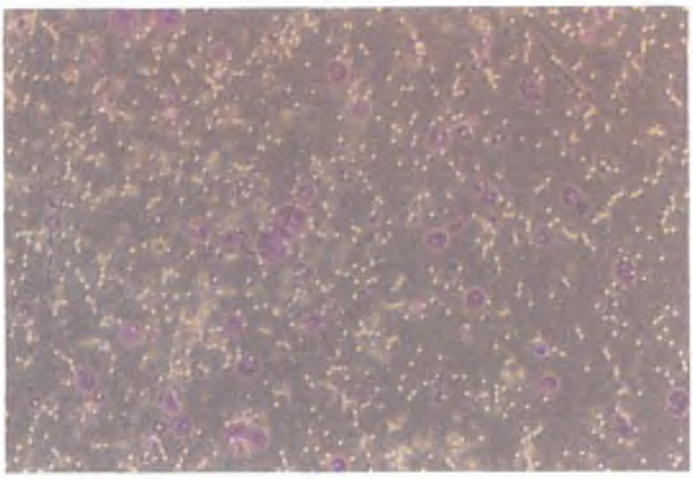

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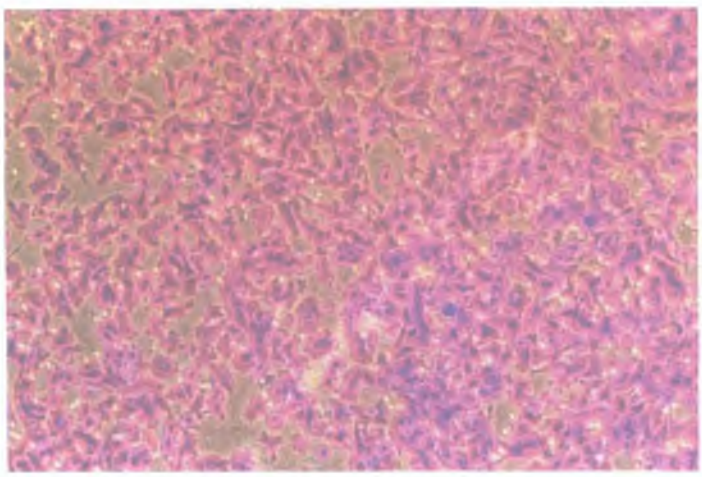


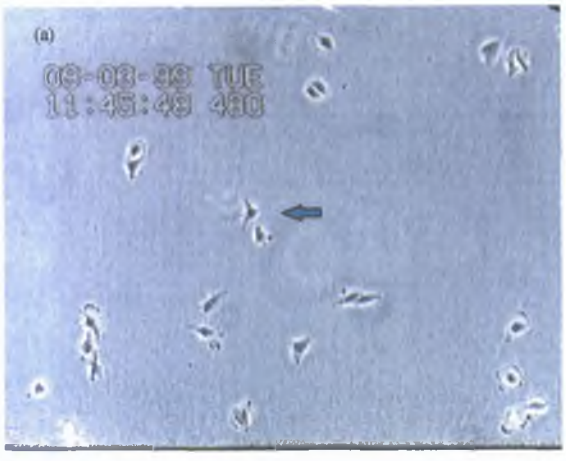
Fig. 3.14.2.b Motility assays of the RPMI-2650 cell line and its MDR variants (40x) (a) RPMI-2650 cell line (b) RPMI-2650 taxol-resistant variant (c) RPMI-2650 melphalan-resistant variant

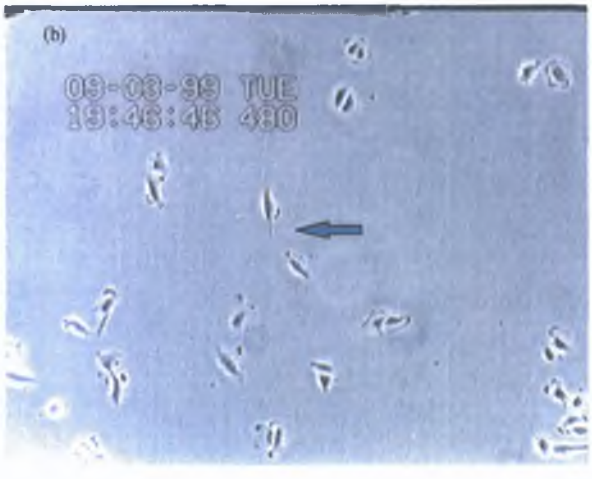
3.14.A Time lapse videomicroscopy

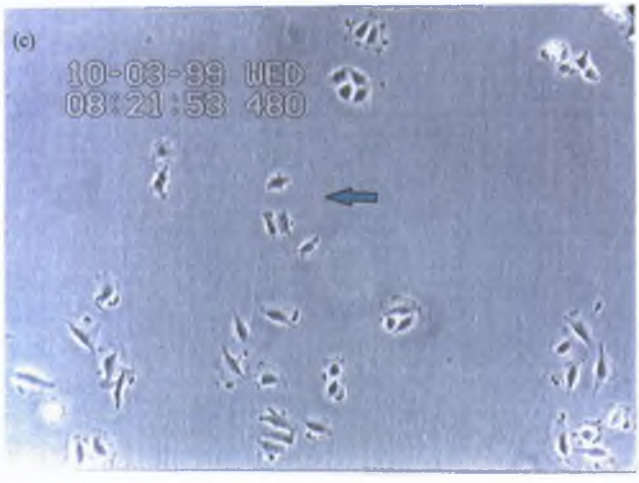
Time lapse videomicroscopy as outlined in section 2 11 was used to determine the speed of motility in the RPMI-2650 cell line and its MDR variants Cell motility was monitored by locating the initial position of the cell and the final position of the same cell and measuring the distance between the two positions Take the RPMI-2650 melphalan-resistant variant as an example, figure 3 14 A demonstrated the movement of the cells during certain time period Results indicate that the HT-1080 moved fastest, melphalan-resistant variant moved much faster than the taxol-resistant variant and the parental cell line RPMI-2650 cells showed lowest movement which was consistent with the results of the motility assays

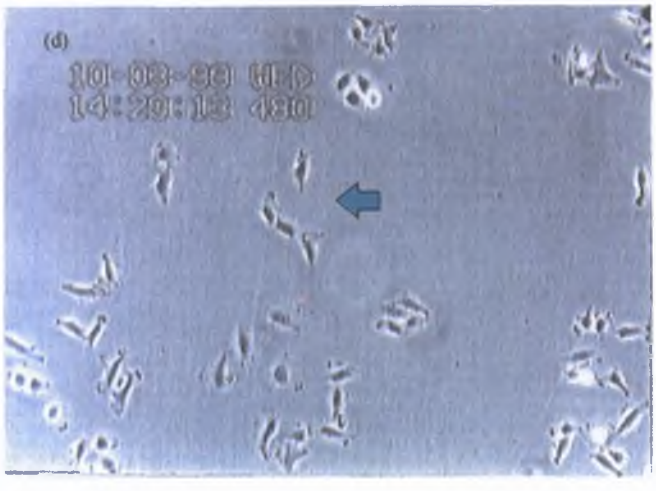
Cell line	Motility speed (μm/hour)
HT-1080	13 54 ± 11 8 (n=12)
RPMI-2650	0 55 ± 0 16 (n=13)
RPMI-2650 taxol-resistant variant	0 76 ± 0 31 (n=15)
RPMI-2650 melphalan-resistant variant	6 83 ± 2 63 (n=21)

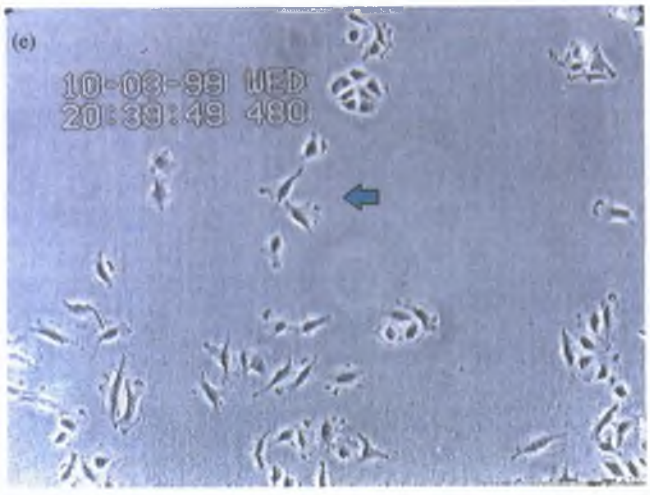
Table 3.14.A Motility speed of the RPMI-2650 cell line and its MDR variants, HT-1080 served as positive control (n value is the number of the cells monitored)


(a)


(b)


(c)
10-03-99 WED
08:21:53 480


(d)



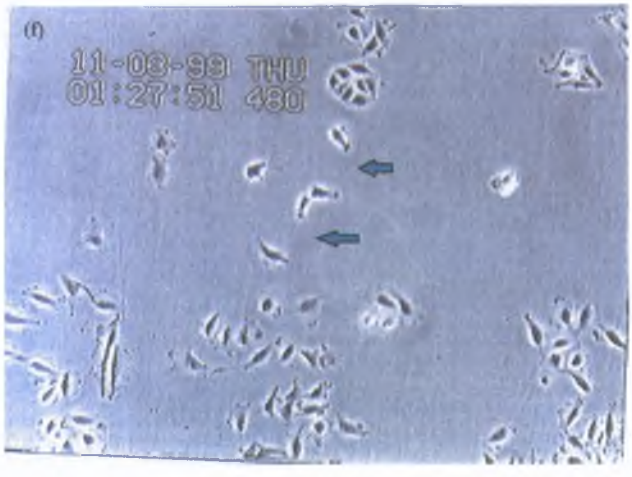


Fig. 3.14.A Pictures a-f demonstrated the movement of the RPMI-2650 melphalan-resistant variant cells during certain time period.

3.15 Studies of MMPs in the RPMI-2650, DLKP cell lines and their MDR variants

Matrix metalloproteinases (MMPs) which are secreted by cells, play an important role in degrading extracellular matrix. To investigate the mechanisms underlying the invasion phenotype of the RPMI-2650 parental cell line and its MDR variants, studies of MMPs were carried out as described in section 2.15.

3.15.1 Zymograph of MMPs in the RPMI-2650 cell line and its MDR variants

BHK (Baby hamster kidney) cell line which secrets MMP-2 and MMP-9 was used as the positive control in these studies. Figure 3.15.1 showed that the RPMI-2650 melphalan-resistant variant had a band for MMP-2 and a band for MMP-9 whereas the RPMI-2650 parental cells and taxol- resistant variant cells did not show any of these bands. However, the RPMI-2650 parental cell line showed three other bands which were not MMP-2 and MMP-9.

Fig. 3.15.1 Zymograph of proteinases in the RPMI-2650 cell line and its MDR variants, BHK serves as positive control lane 1- RPMI-2650; lane 2- RPMI-2650 taxol-resistant variant; lane 3- RPMI-2650 melphalan-resistant variant; lane 4 - BHK

3.15.2 Zymograph with proteinase inhibitors or enhancer in the RPMI-2650 cell line and its MDR variants

To confirm that the two bands of the RPMI-2650 melphalan-resistant variant were MMPs and also to detect the nature of the three bands visible for the RPMI-2650 parental cells, proteinase inhibitors were added to inactivate their corresponding substrates.

3.15.2.1 The effect of EDTA

EDTA is a chelating agent which can bind the zinc and calcium which are needed for the activation of MMPs. After the gel was incubated with EDTA, the two bands of the BHK cell line and the RPMI-2650 melphalan-resistant variant disappeared. Thus, all these bands proved to be MMPs. However, EDTA had no effect on the expression of the three bands of RPMI-2650 cell line (See figure 3.15.2.1).

Fig. 3.15.2.1 Zymograph of proteinases in the RPMI-2650 and its MDR variants with EDTA, BHK serves as negative control. lane 1- BHK ; lane 2- RPMI-2650 ; lane 3- RPMI-2650 taxol-resistant variant; lane 4 - RPMI-2650 melphalan-resistant variant

3.15.2.2 The effect of PMSF

PMSF is a serine protease inhibitor. After the gel was incubated with PMSF, only MMP-2 and MMP-9 bands of the melphalan-resistant variant and BHK were visible. The three bands of the RPMI-2650 became very weak. This suggests that the three bands of the RPMI-2650 cell line are serine proteinases.

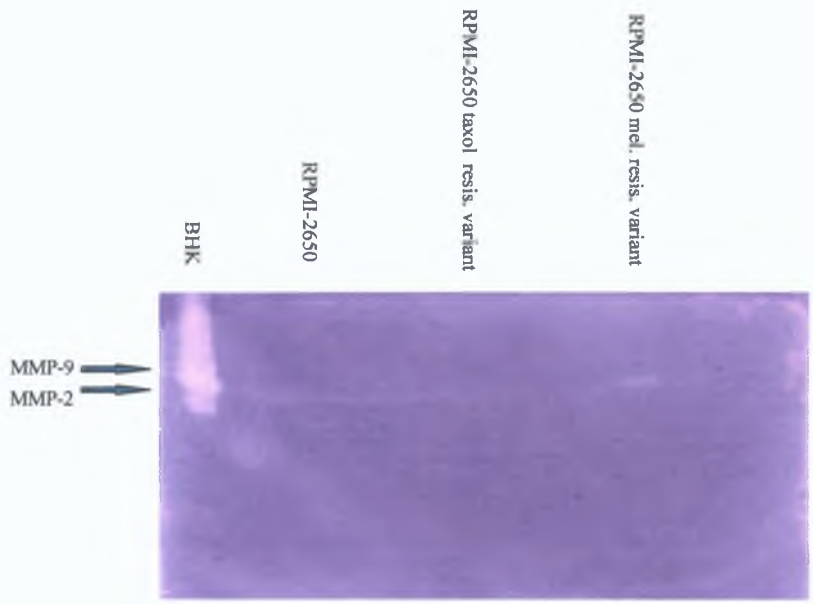


Fig. 3.15.2.2 Zymograph of proteinases with PMSF in the RPMI-2650 cell line and its MDR variants. BHK serves as positive control.

3.15.2.3 The effect of cysteine

If the three bands of the RPMI-2650 cell line were cysteine proteases, they would become stronger after the gel was incubated with cysteine. (Cysteine keeps cysteine protease in its reduced form, thus making it more active.) (Dalton and Brindley, 1997). The result shown in figure 3.15.2.3 suggests that the three bands of the RPMI-2650 on the zymograph are not cysteine proteases since no stronger bands appeared on the gel.

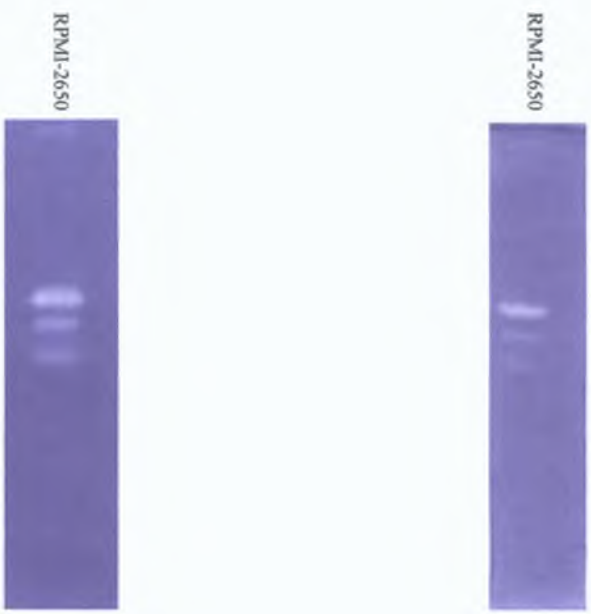


Fig. 3.15.2.3 Zymograph of proteinases (left: without cysteine in the RPMI-2650 cell line, right: with cysteine in the RPMI-2650 cell line).

3.15.3 Zymograghy of proteinases in the DLKP and its MDR variants

No band was visible in the DLKP cell line whereas DLKP long term melphalan-selection and melphalan-pulse selection variants expressed MMP-2 and MMP-9 at the similar level (figure 3.15.3).

Fig. 3.15.3 Zymograph of proteinases in the DLKP cell line and its MDR variants, BHK served as positive control. lane 1- BHK; lane 2- DLKP; lane 3- DLKP long term melphalan-selection variant; lane 4 - DLKP melphalan-pulse selection variant.

3.15.4 RT-PCR of MMP-2 and MMP-9

The expression of MMP-2 and MMP-9 was undetectable in the RPMI-2650 cell line and its MDR variants by RT-PCR as shown in figure 3.15.4.

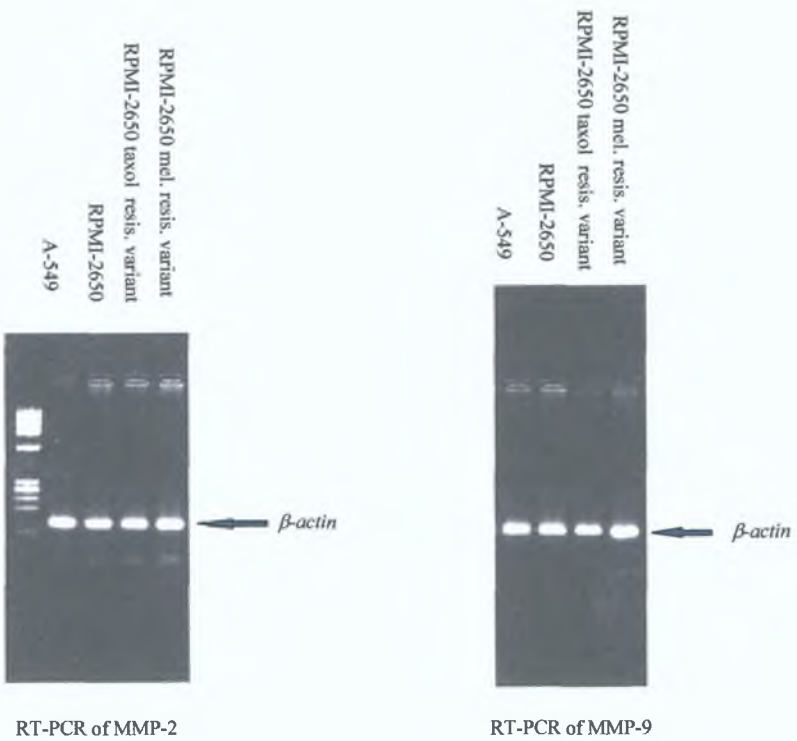


Fig. 3.15.4 RT-PCR analysis of MMP-2 and MMP-9 in the RPMI-2650 cell line, its taxol-resistant and melphalan-resistant variants. (The RT-PCR analysis was repeated twice. RNA extraction was performed once.)

3.15.5 RT-PCR of TIMP-1

The expression of TIMP-1 (MMP inhibitor) was undetectable in the RPMI-2650, its taxol-resistant and melphalan-resistant variants by RT-PCR (figure 3.15.5).

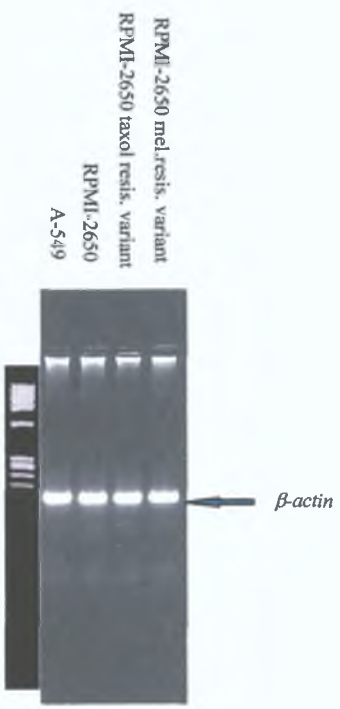


Fig. 3.15.5 RT-PCR analysis of TIMP-1 in the RPMI-2650, its taxol-resistant and melphalan-resistant variants. (The RT-PCR analysis was repeated twice. RNA extraction was performed once.)

3.16 Relationship between melphalan resistance and invasion phenotype in the HT-1080, NIH 3T3, A-549, DLKP, DLKP-C, COR-L23S and COR-L23R cell lines

Since RPMI-2650 melphalan-resistant variant was much more invasive than its parental cell line, studies were carried out to determine whether any relationship existed between melphalan resistance and cell invasion.

3.16.1 Toxicity assays with melphalan in the HT-1080, NIH-3T3, A-549, DLKP, DLKP-C, COR-L23S and COR-L23R cell lines

DLKP-C is a cisplatin-resistant variant of the parental DLKP cell line, a human lung carcinoma cell line. COR-L23R is an adriamycin-resistant variant of the parental COR-L23S cell line, a human large cell lung cancer cell line. As shown in table 3.16.1, A-549, DLKP-C, NIH-3T3 were quite resistant to melphalan while the rest of the cell lines did not display significant resistance to this drug.

Cell line	IC_{50} (nM)
A-549	48952 ± 9221
DLKP	2949 ± 139
DLKPC	10114 ± 2086
COR-L23S	6225 ± 983
COR-L23R	2064 ± 629
HT-1080	4751 ± 885
NIH-3T3	9996 ± 1804

Table 3.16.1 IC_{50} values of melphalan for the A-549, HT-1080, NIH-3T3, DLKP, DLKP-C, COR-L23S, COR-L23R cell lines

3.16.2 Invasion assays in the A-549, HT-1080, NIH-3T3, DLKP, DLKP-C, COR-L23S and COR-L23R cell lines

The results obtained by invasion assays showed that the HT-1080 and A-549 cell lines were the most invasive lines studied. The rest of the cell lines were not quite as invasive. Slight difference of invasiveness was observed between the COR-L23S and its resistant variant, COR-L23R. However, the DLKP-C cell line did appear to be more invasive than its parental cell line (figure 3.16.2).

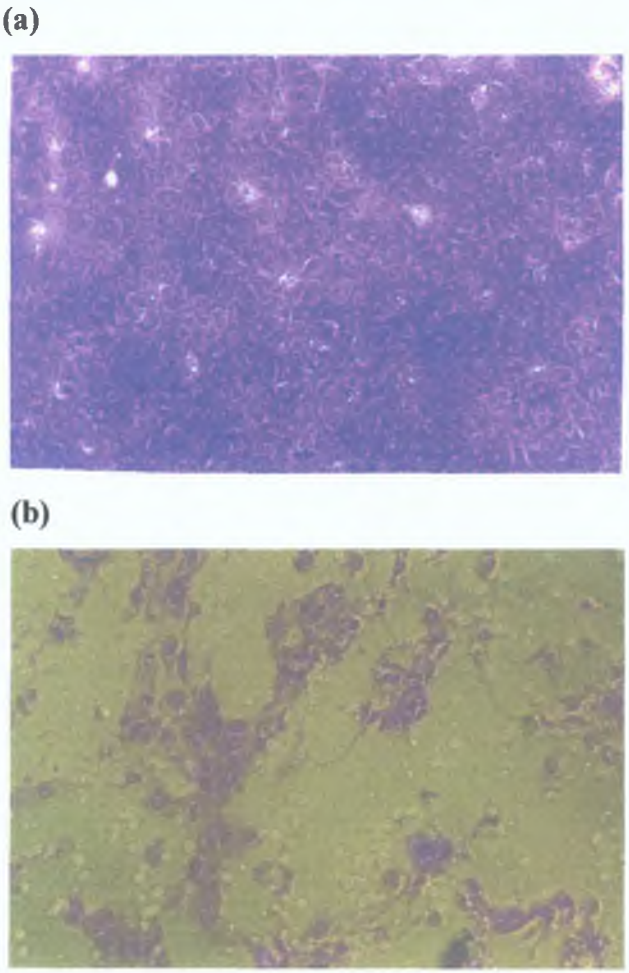
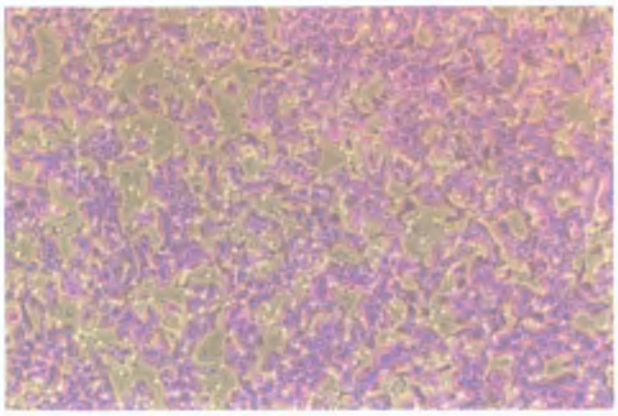
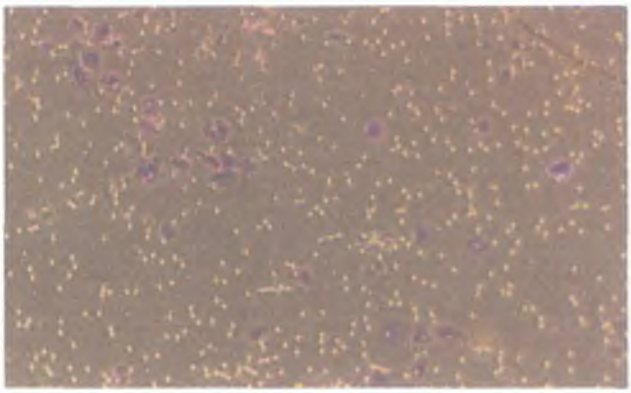


Fig. 3.16.2.a Invasion assays of (a) HT-1080 (b) NIH-3T3

(a)



(b)



(c)

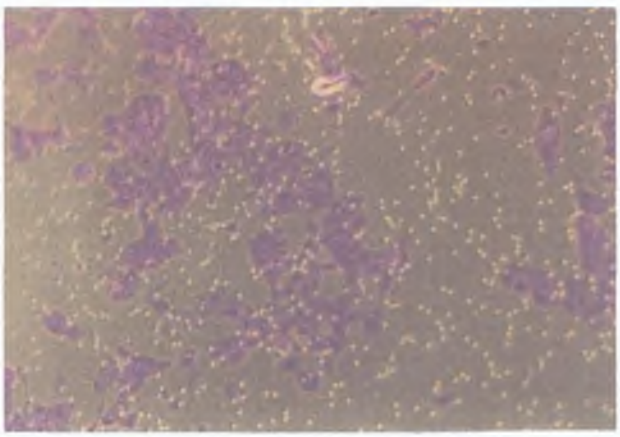
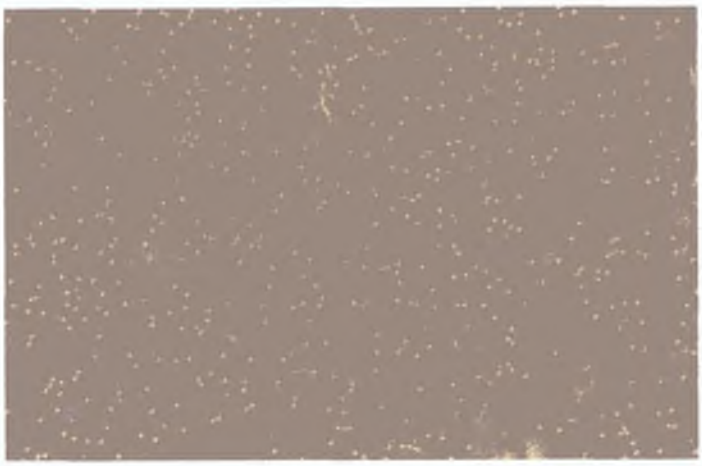


Fig. 3.16.2.b Invasion assays in the (a) A-549 (b) DLKP (c) DLKP-C

(a)



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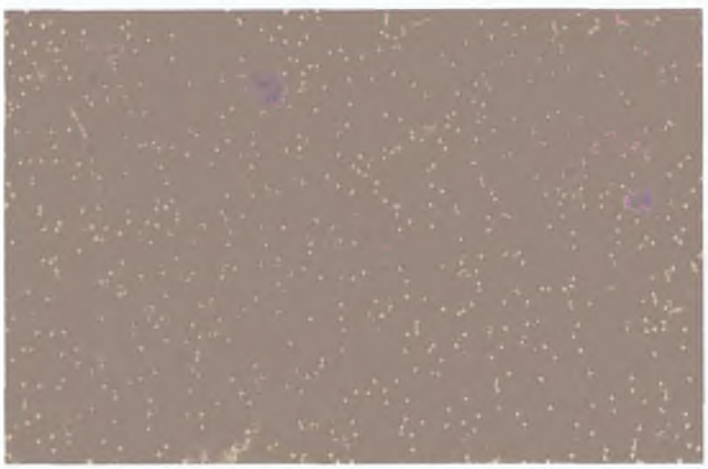


Fig. 3.16.2.c Invasion assays in the (a) COR-L23S (b) COR-L23R

3.16.3 Zymography of proteinases in the A-549, HT-1080, NIH-3T3, DLKP, DLKPC, COR-L23S and COR-L23R cell lines

To investigate the mechanism underlying the behaviour of invasion in these cell lines, zymography was carried out to detect proteinases secreted by these cell lines. As shown in figure 3.16.3.1 and 3.16.3.2, A-549 cell line only expressed MMP-2. HT-1080, NIH-3T3 and COR-L23S cell lines expressed both MMP-2 and MMP-9. No proteinase was detected in the COR-L23R, DLKP and DLKP-C cell lines.

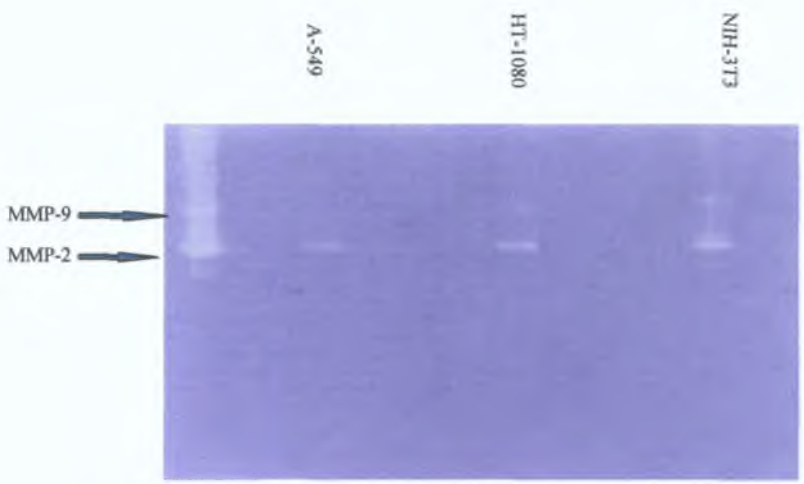


Fig. 3.16.3.1 Zymograph of proteinases in the A-549, HT-1080 and NIH-3T3 cell lines.

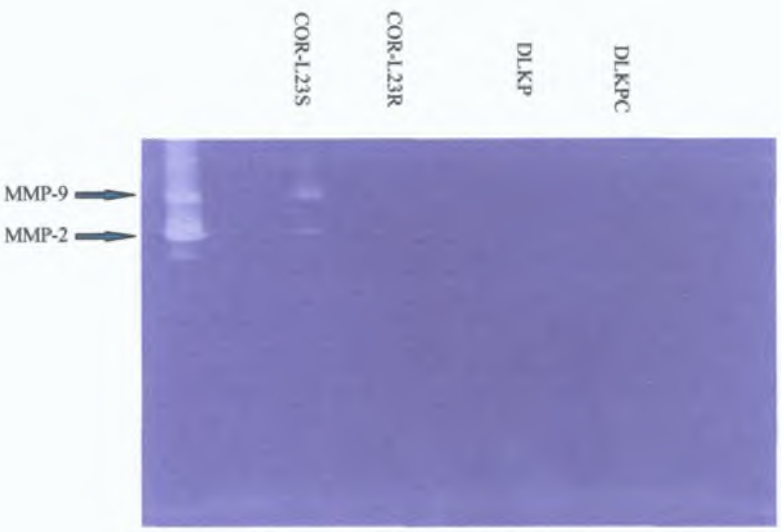


Fig. 3.16.3.2 Zymograph of proteinases in the COR-L23S, COR-L23R, DLKP and DLKP-C cell lines

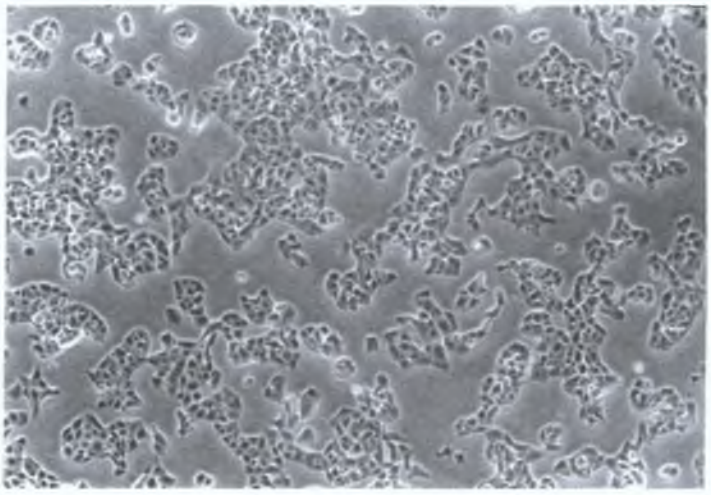
3.17 Studies in the subclones of the RPMI-2650 cell line

The apparent clonal expression of α_2 integrin subunit in the RPMI-2650 taxol-resistant variant led to the studies in subclones of the RPMI-2650 cell line which were established in this centre about ten years ago. Unfortunately, it was found later that all of these clones were contaminated by mycoplasma, so that the work was discontinued.

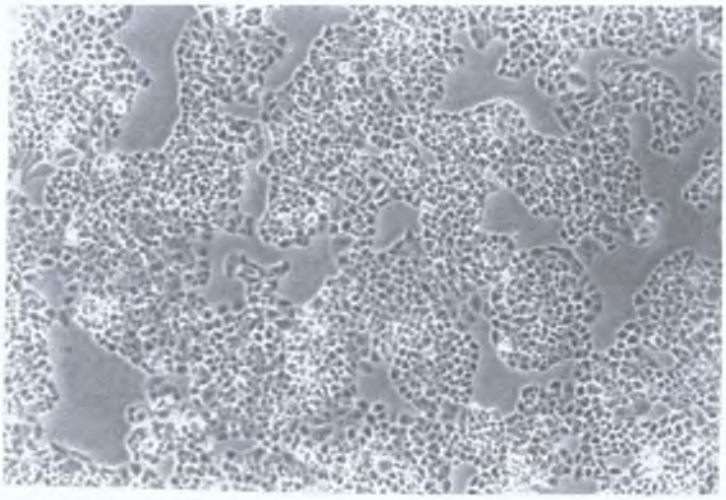
3.17.1 Morphological changes in the subclones of the RPMI-2650 cell line

As shown in figure 3.17.1, the morphology varied in the subclones of the RPMI-2650 cells, especially in clone 6.

(a)



(b)



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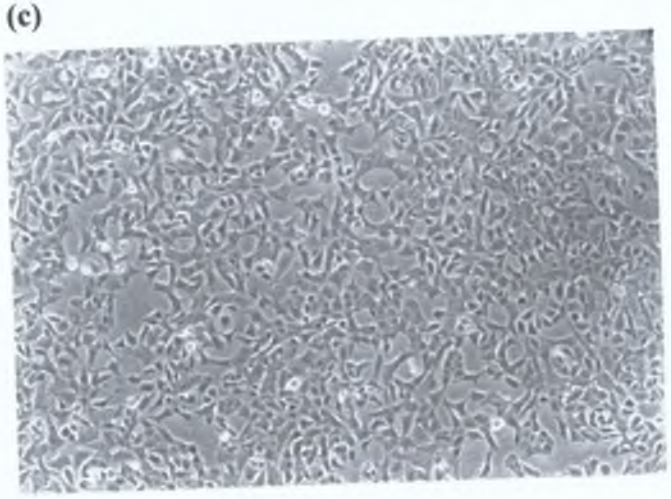
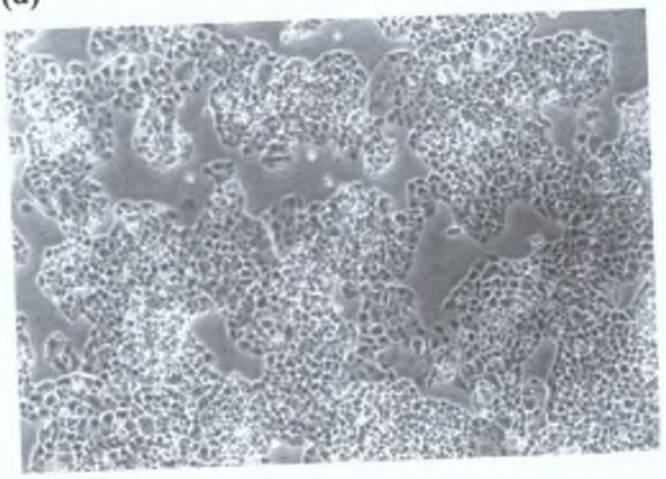
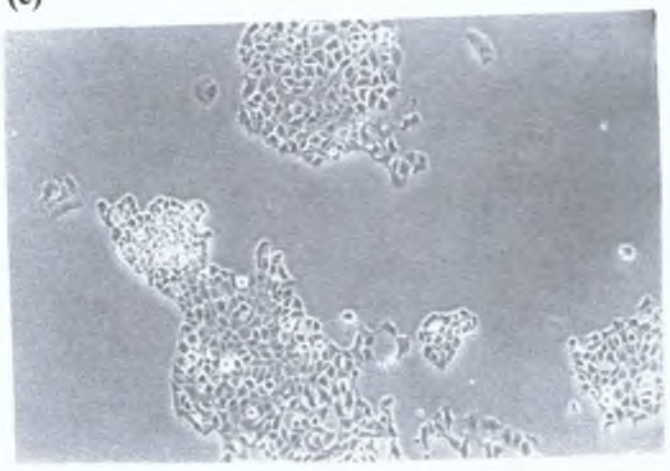


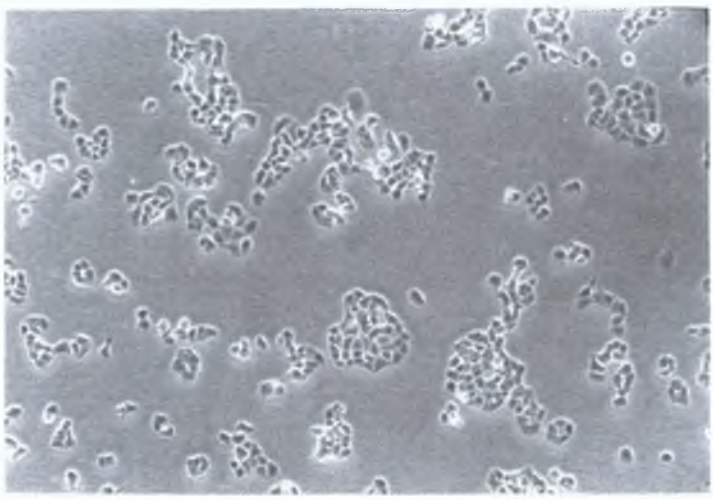
(d)



(e)



(f)



(g)

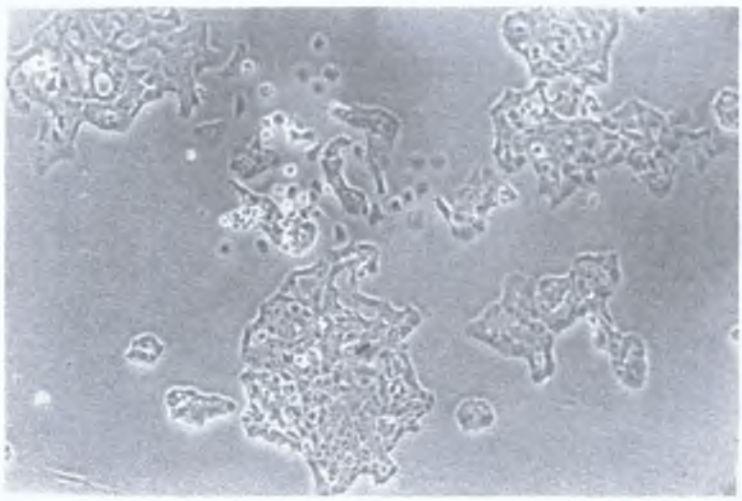


Fig. 3.17.1 Morphology of subclones of the RPMI-2650 cell line (10x) (a) clone 1 (b) clone 3 (c) clone 6 (d) clone 7 (e) clone 8 (f) clone 9 (g) clone 12

3.17.2 Immunocytochemistry studies of integrins and MDR-1

The immunocytochemistry studies showed that the expression of integrin subunits varied in different clones. The most significant variation was observed for α_5 and $\beta_1$integrin subunits which were significantly overexpressed in clone 6 and 9. Clone 6 was also found to overexpress MDR-1 (figure 3.17.2).

	Clone1	Clone 3	Clone 6	Clone 7	Clone 8	Clone 9
Integrin α_1	-	-	-	-	-	-
Integrin α_2	+	+	+	+	+	+
Integrin α_3	-	+	+	+	+	+
Integrin α_5	-	+	++++	++	+++	++++
Integrin α_6	-	+	+	+	-	-
Integrin β_1	-	+	++++	++	+++	++++
MDR-1	-	+	+++	+	+	+

Table 3.17.2 Immunocytochemistry for integrin subunits and MDR-1 in the subclones of the RPMI-2650 cell line

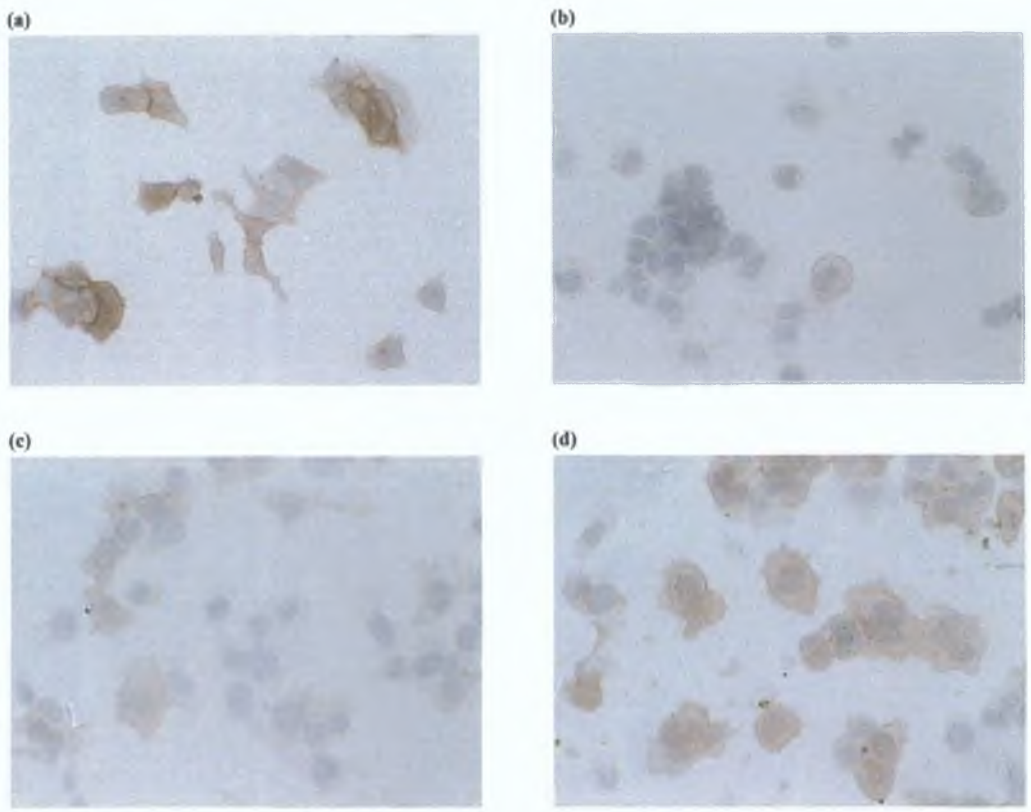


Fig. 3.17.2.a Immunocytochemistry for integrin α_5 subunit in the subclones of RPMI-2650 cell line (a) clone 6 (b) clone 7 (c) clone 8 (d) clone 9

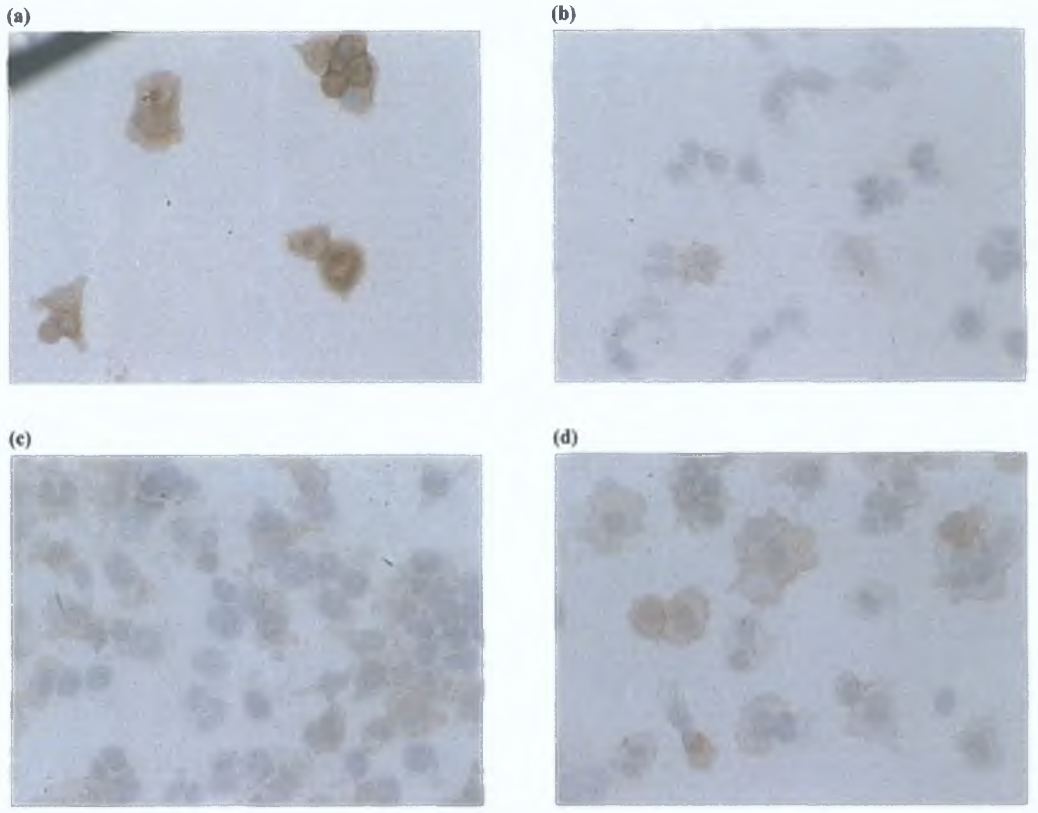
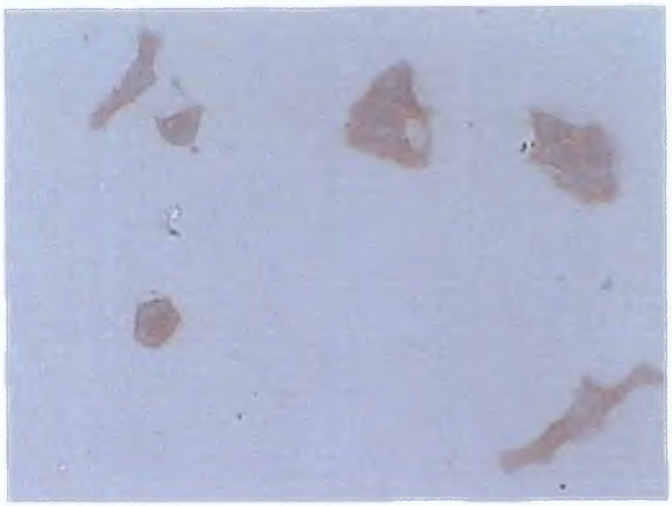


Fig. 3.17.2.b Immunocytochemistry for β_1 integrin subunit in the subclones of RPMI-2650 cell line (a) clone 6 (b) clone 7 (c) clone 8 (d) clone 9

(a)



(b)

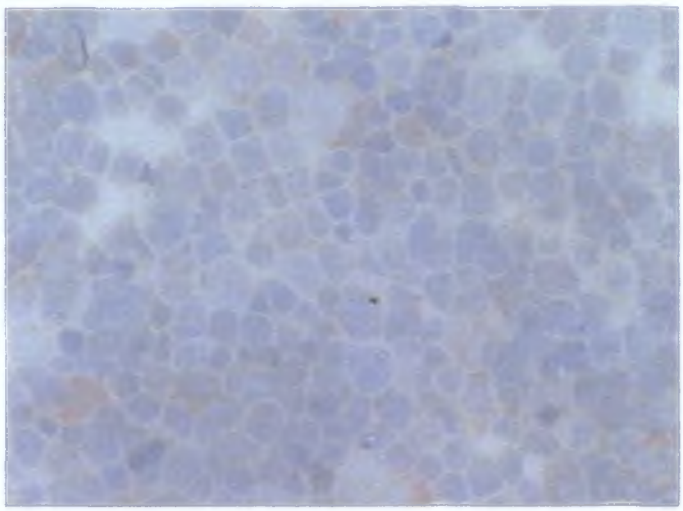


Fig. 3.17.2.c Immunocytochemical staining for MDR-1 in the clone 6 of RPMI-2650 cell line compared to RPMI-2650 parental cell line (40x) (a) clone 6 (b) RPMI-2650

3.17.3 Adhesion assays in subclones of the RPMI-2650 cell line with collagen type IV, laminin and fibronectin

Figure 3.17.3 illustrated that adhesiveness to collagen type IV, laminin and fibronectin varied significantly in the different clones of the RPMI-2650 cell line. Strong adhesion to collagen type IV and fibronectin was observed in clone 9.

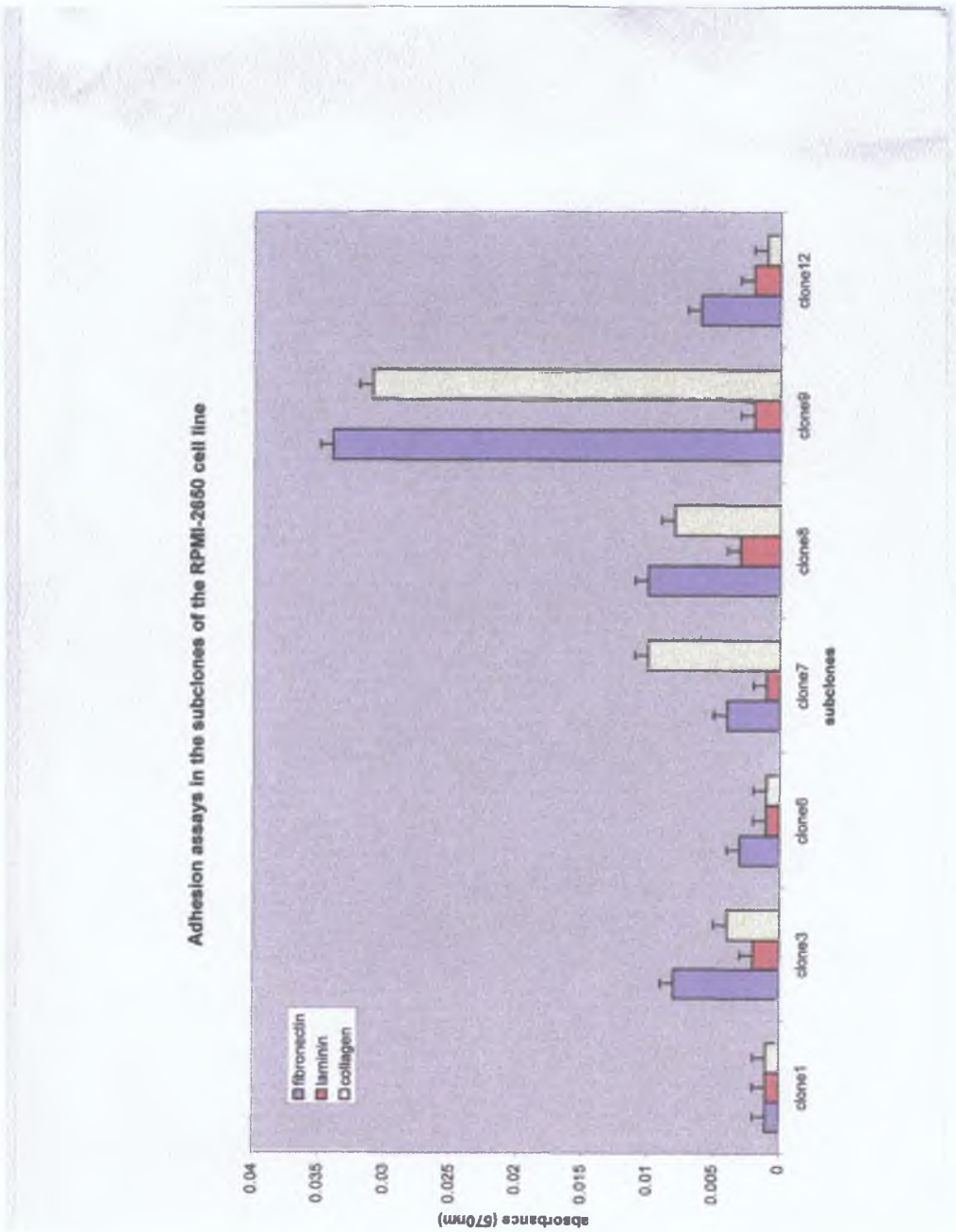


Fig. 3.17.3 Adhesion assays in subclones of the RPMI-2650 cell line with collagen type IV, laminin and fibronectin

4. Discussion

4.1 Toxicity of chemotherapeutic drugs in the SKLU-1, BT-20, RPMI-2650, A-549 and MX-1

As the initial part of this project, toxicity assays were carried out in the SKLU-1, BT-20, RPMI-2650, A-549 and MX-1 cell lines to determine the chemosensitivity of these cell lines to various chemotherapeutic agents (see section 3.1). The purpose of this study was to find a relatively sensitive cell line and also to determine if drug sensitivity or resistance patterns could be observed in certain kinds of cell lines, e.g. breast cancer cell lines such as BT-20 and MX-1 or lung cancer cell lines such as A-549 and SKLU-1. The panel also included a human nasal carcinoma cell line, RPMI-2650. None of these cell lines had been treated previously with chemotherapeutic drugs. The results of this study indicated that the A-549 cell line is much more resistant to chemotherapeutic drugs which are substrates of MRP family (such as adriamycin, vincristine, vinblastine, VP-16, melphalan and cisplatin) than the SKLU-1 cell line whereas the resistance to taxol and 5-FU which are not MRP substrates was similar in these two cell lines. These results agree with levels of MRP1 expression in A-549 and SKLU-1 cells reported by Berger *et al.* (1997). This group reported that among 15 NSCLC (non-small-cell lung cancer) cell lines investigated, A-549 expressed MRP1 at level comparable to drug-selected MDR cell lines while SKLU-1 cells expressed very low levels of MRP1.

To date, a number of studies have been carried out investigating the expression of oncogenes in the A-549 cell line. In one study, Kiefer *et al.* (1990) reported that v-src oncogene was highly expressed in the A-549. Lower levels of c-myc, v-erbB, c-raf1, c-Ha-ras, Ki-ras and N-ras were also expressed in this cell line. Whether the intrinsic resistance to the chemotherapeutic drugs in the A-549 cell line is related to the expression of these oncogenes needs to be clarified.

Of the two breast cell lines used in this study, more work has been carried out using the BT-20 cell line. This is an oestrogen-independent human breast adenocarcinoma cell line (Cappelletti *et al.*, 1991). Studies have shown that neither MRP1 nor Pgp are expressed in this cell line. The toxicity tests of this study showed that BT-20 cell line

was relatively sensitive to all the drugs tested with the exception of melphalan. No further studies were carried out to detect the mechanisms involved in its resistance to melphalan. To date, very little characterisation studies have been carried out in the human breast ductal carcinoma cell line, MX-1.

In contrast to the BT-20 cell line, the results obtained in this study showed that MX-1 cells are intrinsically resistant to many anticancer drugs. Overall, no generalisation can be made from these results in the breast and lung cancer cell lines regarding their sensitivities to chemotherapeutic drugs.

The results obtained demonstrated that a human nasal carcinoma cell line, RPMI-2650, was most sensitive to a wide range of drugs in all the cell lines investigated. Since the RPMI-2650 cell line was sensitive to a number of different chemotherapeutic drugs, work was carried out to establish resistant variants of this cell line.

4.2 Characterisation of RPMI-2650 and DLKP cell lines

4.2.1 Characterisation of RPMI-2650 cell line

RPMI-2650, the main cell line used throughout this work, was established by Moore and Sandberg in 1962 (Moore and Sandberg, 1963). This cell line was derived from a 52-year-old male. The pathological diagnosis of the tumour removed from his nasal septum was anaplastic squamous cell carcinoma. Subsequently, bilateral cervical lymph node metastases developed. A pleural effusion accumulated and it contained numerous tumour cells. Cell cultures were initiated from fresh pleural fluid. Moore and Sandberg found that there were a diploid number of chromosomes in the cell line and the karyotype was consistent with that of the normal human male. This finding was contrary to their experience with approximately 200 malignant cell specimens from effusions and biopsy specimens. In the rare instance a tumour had cells with 46 chromosomes, at least 1 chromosome was distinctly abnormal.

The results presented in this thesis indicate that Pgp and MRP1 are expressed in the RPMI-2650 cell line as demonstrated by Western blotting, immunocytochemistry and RT-PCR analysis. GST π, topoisomerase II α and metallothionein were also detected in this cell line by immunocytochemistry. However, the results obtained from toxicity assays with different groups of chemotherapeutic agents indicate that the RPMI-2650 cell line is very sensitive to anticancer drugs. This result suggests that the P-glycoprotein and/or MRP1 expressed in the cells may be non-functional. The most conclusive result to support this assumption is the result obtained from adriamycin distribution studies (see section 3.6.1). After a four-hour incubation period with adriamycin, strong adriamycin accumulation was noted in the nuclei of RPMI-2650 cells. This indicated that very little drug efflux was occurring in the cells which strongly suggested that P-glycoprotein and/or MRP1 were non-functional. Similarly, Marks *et al.* (1995) reported that treatment of K562 cells (human leukaemia cell line) and its epirubicin-selected MDR variant K562/E15B with sodium butyrate, a differentiating agent, resulted in an increase in P-glycoprotein expression in the K562/E15B cell line. However, no increase in drug resistance or decrease in rhodamine-123 accumulation was observed in the K562/E15B. They concluded from these studies that the P-glycoprotein was non-functional. They stressed the importance of examining not only the expression of P-glycoprotein, but also its functionality. The addition of verapamil or cyclosporin A did not lead to an obvious enhancement of adriamycin accumulation in the RPMI-2650 parental cell line whereas a significant enhancement was observed in the RPMI-2650 taxol- and melphalan-resistant variants (see section 3.6.2). This agrees with the report that the sensitivity of most intrinsically sensitive cells to cytotoxic drugs is not significantly affected by verapamil or cyclosporin A (Ford and Hait, 1993).

Previous studies have been carried out to investigate the expression of cytokeratins in the RPMI-2650 cells. Moll *et al.* (1983) have reported that cytokeratins 7, 8, 18, 19 are typical of simple epithelia while cytokeratins 1, 5, 6, 10, 11, 14-17 are typical of stratified epithelia. These authors also reported that the RPMI-2650 cell line contained cytokeratins 5, 7, 8, 17, 18, 19 which belong to complex pattern of cytokeratins. Sandoz Pharma (De Fraissinette *et al.*, 1995) carried out a study in the RPMI-2650 cell line and reported that the intermediate filament, vimentin, was strongly expressed

in RPMI-2650 whereas desmin which is mainly expressed in muscle cells was not. Normally, epithelial cells express cytokeratins but not vimentin. These results suggested that the RPMI-2650 cell line expressed inappropriate intermediate filament which may be an indicator of its malignancy. They also reported that the cells were not polarised (differentiation into apical and basal parts), had no cilia and were not connected by tight junctions which indicated that the RPMI-2650 cells express transformed or undifferentiated phenotypes.

The patient from whom this cell line was derived developed cervical lymph node metastases and the tumour finally affected the lung. However, experiments reported in this thesis demonstrated the non-invasiveness of RPMI-2650 *in vitro* (see section 3.13). All these results suggested that the final outcome of metastases depends on the interaction of the host and the tumour. RPMI-2650 cells may be activated by the environment of the host whereas they were non-invasive *in vitro*.

Like many other cancer cell lines, the RPMI-2650 line has a heterogeneous population. The concept of tumour progression was introduced by Foulds (1954) who stated that tumour progression is a process of independent, stepwise and permanent changes in one or more different properties of a malignant tumour. The process of progression is thought to result from genetic instability, leading to continuous emergence of various clones. Antigen expression, immunogenic specificity, karyotype, DNA content, degree of differentiation, morphology, growth characteristics, drug sensitivity, hormone-dependency and metastatic potential are some of the characteristics which can change as a tumour develops. The mechanisms of cellular interaction among subpopulations of tumours have not been elucidated. Its understanding can help to clarify tumour evolution, spontaneous tumour regression, involution of the primary tumour in metastatic cancer, development of resistance to therapy and remission and relapse. In this study, different clones derived from RPMI-2650 parental cells showed different morphology, Pgp and integrin expression and adhesiveness to the extracellular matrix. The overexpression of Pgp in clone 6 indicates that although cells can acquire resistance after exposure to chemotherapeutic drugs, small population of resistant cells are already present in the tumour before chemotherapy (see section 3.17.2). It may thus be concluded that repeated low-dose chemotherapy with tumour which is dominated by chemosensitive subpopulations

may lead to a better outcome of treatment whereas the application of an initial maximal tolerated dose may reduce the chemosensitive populations to an extent which these populations can no longer inhibit the growth of the resistant population. The dogma that maximal tolerated drug dose used in a regimen of cancer treatment gives the optimal outcome may not always be true (Aabo, 1996). In the same manner, metastasis favours the survival and growth of a few subpopulations of cells that pre-exist within the parental cells (Fidler and Kripke, 1977). Thus, metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells (Talmadge *et al.*, 1982; Fidler and Talmadge, 1986). Chen *et al.* (1994) reported the establishment and characterisation of 117 clones derived from the primary culture of cells obtained from biopsy tissue of an aggressive human squamous cell lung tumour. These clones exhibited different phenotypes with respect to the expression of integrins, attachment to ECM, tumourigenicity and experimental metastasis. The matastatic ability of different clones of RPMI-2650 with different properties of integrin expression and adhesiveness to ECM should be studied further.

4.2.2 Characterisation of DLKP cell line

DLKP, a poorly differentiated squamous lung cancer cell line, was established from a lymph node metastasis biopsy by bronchoscopy in this centre (Law *et al.*, 1992). Results obtained from toxicity assays indicated that the DLKP cells were very sensitive to adriamycin, vincristine, VP-16 and cisplatin (Clynes *et al.*, 1992). RT-PCR analysis showed that while the cells expressed low levels of mrp1, no mdr-1was detected (NicAmhlaoibh *et al.*, 1999). The results of RT-PCR and Western Blotting analysis presented in this thesis confirm these findings (see section 3.3 and 3.5). Adriamycin distribution studies demonstrated a high level of adriamycin accumulation in the nuclei of DLKP cells after two-hour-incubation with this drug (Cleary *et al.*, 1997), suggesting that adriamycin was not effectively pumped out from the cell by MRP1. An adriamycin-selected variant (DLKP-A) (Clynes *et al.*, 1992) and a carboplatin-selected variant (DLKP-C) (Irene Cleary, Ph.D. thesis, DCU, 1995) of DLKP have also been established. These variants were used as controls throughout this work.

Similar to the RPMI-2650 cell line, DLKP was derived from a secondary site of the original tumour. However, invasion assays demonstrated a low level of invasiveness in this cell line and no MMP-2 and MMP-9 was detected by zymography. This suggests that, similar to RPMI-2650, DLKP was metastatic *in vivo* but not *in vitro*. The explanation for this could be the interaction of organ microenvironment and cancer cells.

Grant (1993) reported that cytokeratin 18 was not detected in DLKP cell line by immunofluorescence, possibly due to its poor differentiation state. Again, immunofluorescence staining was not observed using anti-cytokeratin pan (a combination of all the cytokeratins) as the primary antibody in this cell line (Geraldine Grant, Ph.D. thesis, DCU, 1993).

Similar to the RPMI-2650 cell line, DLKP cells also display a heterogeneous population. McBride *et al.* (1998) reported that DLKP contains three morphologically distinct populations. These subclones displayed different growth characteristics, chromosome numbers and adhesiveness to ECM.

4.3 Taxol resistance

Taxol, a potent anticancer drug first extracted from the bark of the Pacific yew tree, has become increasingly important in the treatment of ovarian cancer, breast cancer and non-small cell lung cancer. Studies carried out with taxol-resistant tumour cells have clearly demonstrated that the cellular transport of taxol and its microtubule binding activity are important factors in the development of resistance to taxol. Recently, studies have also shown that a panel of oncogenes are involved in the taxol resistance (see later part of this section).

Findings in the RPMI-2650 taxol-resistant variant	Methods used
Overexpression of Pgp	Western blotting, RT-PCR, Immunocytochemistry
Down-regulation of MRP1	Western blotting, RT-PCR
Lack of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5	RT-PCR
Cross-resistant to adriamycin, vincristine, vinblastine and VP-16, but not significantly resistant to melphalan, cadmium chloride, cisplatin and 5-FU	Toxicity assay
Very weak adriamycin accumulation in the nuclei	Adriamycin distribution study
Cyclosporin A seems to be more effective than verapamil on circumventing adriamycin toxicity in this variant	Circumvention studies
Increase of c-myc expression, undetectable level of c-Ha-ras	RT-PCR
Undetectable level of LRP	Immunocytochemistry
Similar expression level of GST π, metallothionein and topoisomerase II α as the RPMI-2650 parental cells	Immunocytochemistry
Decreased expression of cytokeratin 18	Immunofluorescence

Table 4.3 Summary of findings in the RPMI-2650 taxol-resistant variant

Numerous studies indicate that taxol resistant cancer cell lines overexpress P-glycoprotein, but not MRP (Van Ark-Otte *et al.*, 1998; Huang *et al.*, 1997; Dumontet *et al.*, 1996; Bhalla *et al.*, 1994). In an attempt to identify the mechanisms of resistance in the RPMI-2650 taxol-resistant variant, alterations in the levels of a number of MDR markers were investigated in this thesis. Overexpression of P-glycoprotein was detected in the RPMI-2650 taxol-resistant variant by Western blotting, immunocytochemistry and RT-PCR compared to its parental cell line (section 3.3.1.1; 3.4.1.1; 3.5.1.1). Western blotting and RT-PCR demonstrated that the level of MRP1 expression was greatly decreased in the RPMI-2650 taxol-resistant variant compared to its parental cell line (section 3.3.2.1; 3.5.2.1). The expression of

mrp2-mrp5 was undetectable by RT-PCR in the taxol-resistant variant (section 3.5.3.1; 3.5.4; 3.5.5; 3.5.6). The exact mechanism of down-regulation of MRP1 in the taxol-resistant variant is unknown. Other investigators have also observed decreased levels of MRP1 in cell lines and tumours that overexpress Pgp or *vice verse*. For example, in OAW42-S cells, a drug-sensitive clone of the ovarian cancer cell line OAW42, MRP1was expressed at a higher level than in OAW42-A1 and OAW42-A cell lines which are two adriamycin-selected variants. However, Pgp was overexpressed in OAW42-A and OAW42-A1 cells, but not in OAW42-S cells (Moran *et al.*, 1997). Larkin *et al.* (1998) carried out immunohistochemical analysis on pre- and post-treatment samples from breast cancer patients. The results showed that in tumours where intense MRP1 positivity was observed, the level of Pgp expression was low. The expression of another resistance marker LRP was also investigated in the RPMI-2650 taxol-resistant variant by immunocytochemistry, however, no significant level of LRP was observed (see section 3.4.1.3). These results suggest that P-glycoprotein, but not LRP and MRP family members, plays a major role in the drug resistance phenotype in the RPMI-2650 taxol-resistant variant.

Fluorescence microscopy was carried out to determine if the resistance phenotype in the RPMI-2650 taxol-resistant variant was due to a reduction in cellular drug accumulation. The studies revealed that adriamycin predominantly localised in the nucleus of the RPMI-2650 parental cell line whereas very faint nuclear fluorescence was observed in the taxol-resistant cells (section 3.6). Faint cytoplasmic fluorescence was also observed in these cells, consistent with the localisation of drug within cytoplasmic vesicles. This result together with the results obtained from circumvention studies with verapamil and cyclosporin A (section 3.8) strongly suggest that P-glycoprotein is involved in drug resistance in the RPMI-2650 taxol-resistant variant. Numerous studies have been carried out to determine the effect of chemosensitisers on taxol resistance. In one study, Lehnert *et al.* (1993) tested the potential usefulness of currently available chemosensitisers for clinical reversal of Pgp-mediated taxol resistance. The 8226/Dox6 human myeloma cells, which overexpress Pgp and are approximately 40-fold resistant to taxol, were used to evaluate the effects of cyclosporin A, verapamil, quinidine and quinine on taxol resistance. Of these four modulators, cyclosporin A proved to be the most effective agent in reversing taxol resistance in the 8226/Dox6 cells, which agrees with the

result obtained from circumvention studies reported in this thesis. Cyclosporin A and its analogue SDZ PSC 833 were also found to be more effective than verapamil in modulating efflux of the Pgp substrate, rhodamine 123 in vinblastine-selected, Pgp-overexpressing Chinese hamster ovary CHO cell lines (Pétriz *et al.*, 1997).

The cross resistance profile (as shown in section 3.2.4.1) of the RPMI-2650 taxol-resistant variant illustrates that this variant is cross resistant to a number of chemotherapeutic drugs (*i.e.* it displays MDR). This variant also displays a cross resistance pattern consistent with the overexpression of P-glycoprotein. Taxol-resistant cell lines which overexpress P-glycoprotein usually exhibit cross resistance to adriamycin, vincristine, vinblastine, VP-16 and show little or no cross resistance to alkylating agents, platinum drugs and antimetabolites. In this study, the RPMI-2650 taxol-resistant variant was found to be resistant to taxol (226-fold) and cross resistant to adriamycin, vincristine, vinblastine and VP-16. However, there was no significant resistance to melphalan, cisplatin and 5-FU.

In a study on taxol-resistant populations of the human sarcoma cell line MES-SA (Dumontet *et al.*, 1996), all the resistant cell populations were found to be cross resistant to the tubulin depolymerising agents vincristine and vinblastine. Among these populations, MDR-1-positive clones demonstrated resistance to adriamycin and VP-16 which are substrates for the Pgp efflux pump whereas MDR-1-negative clones displayed sensitivities to adriamycin and VP-16. Taxol-resistant clones from a human ovarian carcinoma cell line 2008 were established. The 2008/17 clone displayed a P-glycoprotein-mediated drug resistance phenotype. Similar to the results obtained with the RPMI-2650 taxol-resistant variant, this clone was found to be highly cross-resistant to adriamycin, VP-16 and vincristine, but not to cisplatin (Parekh *et al.*, 1997).

A taxol-resistant subline (HL-60/ TAX 1000) of the myeloid leukaemia cell line HL-60 has also been shown to be cross-resistant to vincristine, adriamycin, but sensitive to the antimetabolite Ara-C (Bhalla *et al.*, 1994). In a study on the murine macrophage like cell line J774.2, a taxol-resistant subline J7/Tax-50 was developed (Roy *et al.*, 1985). J7/Tax-50 was shown to be 800-fold resistant to taxol and to

display cross-resistance to colchicine, vinblastine, adriamycin and actinomycin D, but remained sensitive to the alkylating agent bleomycin.

No difference in the expression of topoisomerase II α was observed by immunocytochemistry in the RPMI-2650 cell line and its taxol-resistant variant (see section 3.4.1.5). This would rule out the possible involvement of topoisomerase II in the resistance to adriamycin and VP-16 which exert cytotoxicity on topoisomerase II in the taxol-resistant variant. No significant difference in the expression of metallothionein was observed by immunocytochemistry in the RPMI-2650 parental cell line and its taxol-resistant variant which indicates that metallothionein does not play a major role in drug resistance in this taxol-resistant variant (section 3.4.1.6).

Microtubules are dynamic polymers and alterations in assembly and disassembly dynamics play a key role in the antimitotic effects of depolymerising agents, such as vincristine and stabilising agents, such as taxol. Thus, a number of mechanisms may be involved in the induction of resistance to these agents: (1) reduced binding of the drug (Boggs *et al.*, 1993). (2) reduced amounts of the target molecule tubulin (Minotti *et al.*, 1991) and (3) altered dynamic properties of microtubules (Rowinsky *et al.*, 1988).

In agreement with this, other studies have reported that taxol-resistant cancer cell lines that do not overexpress P-glycoprotein have altered α- or β-tubulin isotypes. Schibler *et al.* (1986) reported the isolation of 139 Chinese hamster ovary cell mutants resistant to taxol. Of these mutants, 59 exhibited an absolute requirement for taxol for normal growth and division, 13 had a partial requirement and 69 grew normally without the drug. Two dimentional gel analysis of whole cell proteins revealed "extra" spots representing altered tubulins in 13 of the mutants. Six of these had an altered α-tubulin and seven had an altered β-tubulin. Cabral *et al.* (1986) also reported that, when compared with wild-type cells, taxol-dependent mutants had normal arrays of cytoplasmic microtubules but formed much smaller mitotic spindles in the presence of taxol. When deprived of the drug, however, complete assembly of the mitotic spindle apparatus did not occur in these mutants. Thus, the defects leading to taxol

dependence in these mutants with defined alterations in α- and β-tubulin appear to result from the inability of the cells to form a functional mitotic spindle.

In section 3.4.2 of this thesis, immunofluorescence studies revealed that the RPMI-2650 taxol-resistant cells displayed slightly fainter α- and β-tubulin fluorescence compared to the RPMI-2650 parental cells. The results of studies carried out to date regarding tubulin content in taxol-resistant cell lines are conflicting. Jaffrezou *et al.* (1995) noted no difference in total tubulin content between the parental K562 cell line and its 9-fold taxol- resistant variant KPTA5. However, they found that the taxol-resistant cells presented a 2-fold increase in both 5β-tubulin mRNA expression and in the corresponding tubulin protein class IV isotype content, as revealed by RT-PCR and immunostaining respectively. Dumontet *et al.* (1996) reported that total tubulin content was significantly decreased in one of the taxol-resistant variants of MES-SA sarcoma cells. Reduced expression of the 5β and the β4 tubulin isotype transcripts in this variant was also observed compared to the parental cells.

Ohta *et al.* (1994) established a taxol-resistant variant (H69/TX1) of the human small-cell lung cancer cell line H69. No significant levels of Pgp were detected in this variant. However, altered α-tubulin isoform and increased acetylation of α-tubulin was observed in the H69/TXl cells compared to the parental cells. Kavallaris *et al.* (1997) selected the human lung cancer cell line A-549 with taxol resulting in cell variants that were 9- and 17-fold resistant. These cells displayed an altered ratio of classes I, II, III and IVa β-tubulin isotypes. Four taxol-resistant ovarian tumour-bearing ascites also displayed significant increases in class I, III and IVa β-tubulin isotypes as compared to untreated primary ovarian tumours.

Although the mechanism underlying the alteration in tubulin content in taxol resistance is not clear, reduced amounts of the target molecule, tubulin, may contribute to taxol resistance. In mammals, there are at least six isoforms of α-tubulin and a similar number of β tubulin isoforms, each encoded by a different gene (Sullivan, 1988). The role of the various tubulin isotypes remains controversial. Functional differences between tubulin isotypes have not been identified. The

alteration in the content of the isotypes of α and β-tubulin is not explored in this thesis.

Microtubules are composed of α-tubulin and β-tubulin which have 12 and 8 cysteine residues respectively (Cowan *et al.*, 1983). Oxidation of tubulin sulfhydryl groups has been shown to prevent the polymerisation of tubulin and the formation of microtubules (Ikeda *et al.*, 1978). Subsequent reduction of sulfhydryl moieties restores the ability of tubulin monomers to polymerise and form microtubules. Further evidence of a crucial role for sulfhydryls in microtubule assembly comes from studies using BSO, a potent inhibitor of glutathione synthesis, and CDNB, a compound which causes the rapid consumption of GSH. Exposure of the human lymphoid cell line 3T3 to both BSO and CDNB resulted in a complete loss of microtubules within the cells since the sulfhydryl groups of tubulins could not be reduced (Leung *et al.*, 1989). Preincubation of the 3T3 cells in taxol prevented the disaggregation of microtubules when GSH was subsequently lowered by CDNB (Chou *et al.*, 1984). Liebmann *et al.* (1993) reported that the depletion of glutathione by BSO antagonised the cytotoxicity of taxol and this effect was not due to altered uptake of drug. It has been suggested that the cysteine residues in α- and β-tubulin may undergo increased oxidation of their sulfhydryl groups when cellular glutathione levels are reduced, thereby altering their ability to polymerise. The effect of BSO on the RPMI-2650 taxol-resistant cells was not explored in this work. GST activity assay revealed that the RPMI-2650 taxol-resistant cells had only slightly higher GST activity than the parental cells, thus suggesting that GST does not play a major role in mediating resistance in the taxol-resistant variant.

Apart from Pgp overexpression and the development of altered α- or β-tubulin isotypes, reports have suggested that other mechanisms may be involved in taxol resistance. It was reported that taxol is metabolised by the cytochrome P-450 system and its metabolism may be altered by other drugs affected by this system (Rahman *et al.*, 1994). Consequently, this system may also be involved in the mechanisms of resistance to taxol. It has also been reported that a good correlation exists between the presence of a 135kDa phosphoglycoprotein and resistance to taxol (Samar *et al.*, 1985). In this report, a taxol-resistant subline was developed from the murine

macrophage-like cell line J774.2. Analysis of plasma membranes by SDS gel electrophoresis followed by silver staining revealed the presence of a prominent protein with an approximate molecular weight of 135,000 daltons in the resistant cell line which was absent from the parental cell line.

In section 3.4.2.3, the results of immunofluorescence demonstrated that the RPMI-2650 taxol-resistant variant displays a significant decrease in cytokeratin 18 levels compared to the RPMI-2650 parental cells. No significant difference in the levels of vimentin between these two cell lines was noted. Parekh *et al.* (1995) reported that the cisplatin-resistant human ovarian 2008/c13* cell line contained markedly lower levels of cytokeratin 18 when compared to the cisplatin-sensitive 2008 cell line. Transfection of full-length cytokeratin 18 cDNA into the cisplatin-resistant 2008/C13* cells resulted in a marked increase in sensitivity to cisplatin. Thus, a relationship exists between cytokeratin 18 and the anticancer drug. (Cytokeratin dependent drug resistance (C-MDR) has been introduced in section 4.4). So far, no study regarding the relationship between the cytokeratin 18 and taxol resistance has been reported. Although the mechanism underlying the decrease of cytokeratin 18 in the RPMI-2650 taxol-resistant variant is not clear, it is possible that cytokeratin 18 may be involved in mediating taxol resistance. Vimentin does not appear to play a role in mediating taxol resistance in the RPMI-2650 taxol-resistant variant since no alteration in vimentin levels was noted.

Many recent reports focus on the relationship between apoptosis, oncogene expression and taxol resistance. Rasouli *et al.* (1998) reported that Raf-1 protein kinase may have a profound influence on the level of taxol-induced apoptosis. In the 12 human cervical tumour cell lines studied, Raf-1 kinase activity was inversely correlated with the level of cytotoxicity induced by taxol. It was shown that Raf-1 kinase acts to suppress taxol-induced apoptosis. This data suggests that the clinical effectiveness of taxol could be improved by the use of Raf-1 kinase inhibitors. Other oncogenes have also been shown to be involved in taxol resistance. Human c-erbB-2 gene which encodes P185 protein was transfected into MDA-MB-435 human breast cancer cells. The P185-overexpressing MDA-MB-435 transfectants were more resistant to taxol than the parental cells. No increase in P-glycoprotein expression was observed in the P185-overexpressing MDA-MB-435 transfectants. This data

demonstrated that overexpression of c-erbB-2 could lead to intrinsic taxol resistance independent from P-glycoprotein (Yu *et al.*, 1996). It was found that taxol activates P34Cdc2 kinase in MDA-MB-435 breast cancer cells, leading to cell cycle arrest at the G2/M phase and subsequently, apoptosis. Overexpression of P185 ErbB2 in MDA-MB-435 cells resulted in the upregulation of P21Cip1, which associates with P34Cdc2, inhibits taxol-mediated P34Cdc2 activation, delays cell entrance to G2/M phase, and thereby inhibits taxol-induced apoptosis (Yu *et al.*, 1998). Huang *et al.* (1997) demonstrated by immunoblot analysis that the taxol-resistant variant HL-60/TAX1000, derived from the human leukaemia cell line HL-60, had higher Bcl-2 and Bcl-xl levels than HL-60. Transfection of bcl-2 or bcl-xl into the HL-60 cells significantly reduced taxol-induced apoptosis. C-myc also appears to be involved in resistance to many chemotherapeutic drugs (Niimi *et al.*, 1991; NicAmhlaoibh *et al.*, 1999). Preliminary results of RT-PCR analysis described in section 3.5.7 suggests that overexpression of c-myc might also be involved in taxol resistance in the RPMI-2650 taxol-selected variant.

4.4 Melphalan resistance

Melphalan is an alkylating agent which results in cross-linking between DNA strands or linkages between bases within the same strand of DNA (section 1.2.2). Melphalan has been used extensively in the treatment of a number of tumours. For multiple myeloma, no available treatment is curative, but about two thirds of patients achieve a stable response to combination chemotherapy (MacLennan *et al.*, 1994). Standard chemotherapy (melphalan and prednisone, MP) has been the main regimen of multiple myeloma for about 3 decades (Huang *et al.*, 1999). However, it is no longer considered the "gold standard". Chemotherapy including adriamycin and carmustine with the alkylating agents cyclophosphamide and melphalan achieves a higher stable response rate than conventional treatment with melphalan and prednisone without additional haematological toxicity (MacLennan *et al.*, 1994).

Chemotherapy is applied to almost all patients with advanced breast cancer. Response rates have been increased by the use of combination of taxoids and anthracyclines and/or alkylating agents. To improve access to chemotherapy, orally administered drugs are very useful for those patients when access to hospital is limited by financial considerations, long journeys or patient reluctance. In the past, only alkylating agents (cyclophosphamide, chlorambucil, melphalan) could be administered orally. Development of new oral drugs will simplify maintenance regimens on an outpatient basis (Marty *et al.*, 1999).

The three-drug combination of melphalan, etoposide (VP-16) and carboplatin followed by autologous stem-cell rescue has been recently evaluated by the French Society of Pediatric Oncology in treatment of pediatric Wilm's tumour patients (Pein *et al.*, 1998). About half of the patients tested remained disease-free for 3 years, indicating that this regimen is satisfactory.

Melphalan has also been extensively used in colorectal cancer hepatic metastases, melanoma, ovarian, testes and thyroid cancers, as well as in leukaemia, lymphoma and conditioning regimens for bone marrow transplantation (Samuels and Bitran, 1995).

There are many mechanisms involved in the drug resistance to melphalan. The most recent reports emphasised the enhancement of GS-X pump activity in melphalan resistance. The GS-X pump is an ATP-dependent export pump for organic anions such as cysteinyl leukotrienes, glutathione disulfide and glutathione S-conjugates. Studies by Müller *et al.* (1994) and Leier *et al.* (1994) have provided important evidence that the multidrug resistance-associated protein (MRP) gene encodes a GS-X pump. Jedlitschky *et al.* (1996) demonstrated MRP1-mediated ATP-dependent transport for the monochloro-mono[^{3}H]glutathionyl melphalan which proved again that glutathione conjugation is an important way to detoxify melphalan. Ishikawa *et al.* (1996) reported that in a cisplatin-resistant variant (HL-60/R-CP) of the human myelocytic leukaemia HL-60 cell line, MRP1 was overexpressed. Culturing the HL-60/R-CP cells in cisplatin-free medium resulted in a reduction in mrp1 mRNA level, but the level could be induced to rise within 30 hours by exposure to cisplatin and heavy metals such as arsenite, cadmium and zinc. γ-glutamylcysteine synthetase

expression in the cisplatin-resistant cells was also induced within 24 hours exposure to cisplatin, resulting in a significant increase in cellular GSH level since γ-glutamylcysteine synthetase catalyses the first step in GSH formation. HL-60/R-CP cells were found to be cross-resistant to melphalan, chlorambucil, arsenite and cadmium. These observations suggested that elevated expression of the MRP/GS-X pump and increased GSH biosynthesis may play an important role in mediating resistance to cisplatin, melphalan and heavy metals.

To date, newly identified MRP1 homologues, cMOAT (MRP2), MRP3, MRP4, MRP5, MRP6 have not been linked directly to melphalan resistance. However, it is quite possible that cMOAT and MRP3 could be responsible for melphalan resistance. cMOAT is also a GS-X pump whose substrate specificity is very similar to that of MRP1. It also contributes to the transport of some anticancer drugs and heavy metals. Mutant rats which did not express normal cMOAT showed a reduced biliary clearance of methotrexate (Masuda *et al.*, 1997), mercury, cadmium and arsenite (Kool *et al.*, 1999). Cells transfected with cMOAT cDNA were found to transport vinblastine (Evers *et al.*, 1998). Moreover, overexpression of the cMOAT gene has been found in several cisplatin-resistant cell lines (Taniguchi *et al.*, 1996; Kool *et al.*, 1997) and transfection of cMOAT-antisense constructs into liver cells was reported to confer an increased sensitivity to cytotoxic drugs (Koike *et al.*, 1997). All these observations strongly suggest that cMOAT may confer resistance to melphalan.

MRP3 has also been shown to be a GS-X pump. Of all the MRP family members, MRP3 displays the strongest homology to MRP1 (58% amino acid identity). MOR/P, the lung adenocarcinoma cell line and KB-3-1, an epidermoid carcinoma cell line, showed high expression of MRP3 together with high expression of cMOAT (Kool *et al.*, 1997). Several adriamycin-resistant sublines of a non-small cell lung cancer cell line, SW 1573/S1, and the cisplatin-resistant colon carcinoma cell line, HCT8/DDP, have also been found to overexpress MRP3 (Kool *et al.*, 1997). MRP3 is also able to confer resistance to methotrexate, VP-16 (etoposide), teniposide (Kool *et al.*, 1999) and vincristine (Young *et al.*, 1999). All these results suggest that MRP3 is likely to play a role in melphalan resistance although direct evidence needs to be established.

The RPMI-2650 melphalan-resistant variant was established by stepwise selection of the RPMI-2650 parental cells in increasing concentrations of melphalan. DLKP melphalan-resistant variants were obtained by either continual or pulse selection of DLKP to melphalan as described in section 2.5. The main results reported in this thesis are summarised below:

Findings in the RPMI-2650 melphalan-resistant variant	Methods used
Slight increase of Pgp expression	RT-PCR, Western Blotting and Immunocytochemistry
Overexpression of MRP1	RT-PCR and Western Blotting
Overexpression of cMOAT(MRP2) and MRP3	RT-PCR and Immunocytochemistry
Increase of c-myc expression, undetectable level of c-Ha-ras	RT-PCR
Cross-resistant to cadmium chloride, adriamycin, vinblastine, vincristine, VP-16, cisplatin, but not to taxol and 5-FU	Toxicity assay
Weak adriamycin accumulation in the nuclei, could be reversed by verapamil and cyclosporin A	Adraimycin distribution study
Verapamil seems to be more effective than cyclosporin A in circumventing adriamycin toxicity in this variant. Both sulindac and indomethacin can circumvent adriamycin toxicity in this variant.	Toxicity assay
Slightly higher activity of GST than the RPMI-2650	GST assay
Undetectable level of LRP expression	Immunocytochemistry
Similar levels of expression of GSTπ, metallothionein and topoisomerase II α as RPMI-2650	Immunocytochemistry

Table 4.4.1 Summary of findings in the RPMI-2650 melphalan-resistant variant.

Findings in DLKP long-term melphalan-selected variant	Methods used
Undetectable level of Pgp expression	RT-PCR and Western Blotting
Increase of MRP1 expression	RT-PCR and Western Blotting
Cross-resistant to cadmium chloride, cisplatin, adriamycin, vinblastine, but not to taxol	Toxicity assay
Undetectable level of cmoat (mrp2) expression	RT-PCR

Table 4.4.2 Summary of findings in the DLKP long term melphalan-selected variant

Findings in DLKP melphalan-pulse selected variant	Methods used
Undetectable level of Pgp expression	Western blotting
Increase of MRP1 expression	Western blotting
Cross-resistant to cadmium chloride, cisplatin, adriamycin, but not to taxol and vinblastine	Toxicity assay

Table 4.4.3 Summary of findings in the DLKP melphalan-pulse selected variant

In an attempt to identify the mechanisms of MDR in the RPMI-2650 and DLKP melphalan-resistant variants, alterations in a number of MDR markers were investigated. Western blotting analysis demonstrated MRP1 and cMOAT (MRP2) overexpression and a slight increase in Pgp expression in the RPMI-2650 melphalan-resistant variant compared to the parental cell line (see section 3.3.1.1, 3.3.2.1 and 3.3.3). RT-PCR analysis demonstrated the overexpression of mrp1, cmoat (mrp2), mrp3 and a slight increase of Pgp at mRNA levels in the RPMI-2650 melphalan-resistant variant compared to the parental cells (see section 3.5). Results of fluorescence microscopy studies showed that the intensity of adriamycin nuclear fluorescence was greater in the RPMI-2650 cells than in the melphalan-resistant variant (see section 3.6). This implicates decreased drug accumulation as a mechanism of resistance in the melphalan-resistant variant. Overexpression of MRP1, cMOAT (MRP2), MRP3 or possibly P-glycoprotein may lead to the decreased accumulation of adriamycin in the variant. Results obtained from fluorescence microscopy studies also demonstrated that adriamycin accumulation can be increased by verapamil and cyclosporin. This result together with the results of circumvention studies with verapamil and cyclosporin A support the hypothesis that MRP family members may play a major role in drug resistance in the RPMI-2650 melphalan-resistant variant. Previous studies have shown that although cyclosporin A was more effective than verapamil on circumventing adriamycin resistance in vincristine-selected, Pgp-overexpresing myelocytic leukaemia cell line HL60/vinc (McGarth *et al.*, 1988), verapamil was the more effective agent on reversing either adriamycin or etoposide (VP-16) resistance in adriamycin-selected, MRP1-overexpressing HL60/ADR (Germann *et al.*, 1997). Other studies performed in several different MRP1-overexpressing cell lines including small cell lung cancer cell line H69AR, fibrosarcoma cell line HT-1080/DR4 and large cell lung cancer cells COR-L23R have also suggested that verapamil was somewhat more effective than cyclosporin A as a chemosensitiser (Germann *et al.*, 1997; Barrand *et al.*, 1993; Cole *et al.*, 1989). The results of the circumvention studies reported in this thesis are consistent with the data mentioned above (see section 3.8). Similar to the results obtained for the RPMI-2650 melphalan-resistant variant, MRP1 was found to be overexpressed in the DLKP long term melphalan-selected and melphalan-pulse selected variants as detected by Western blotting and RT-PCR (section 3.3.2.2, 3.5.2.2). Significant levels of Pgp were not detected at either protein or mRNA levels in the DLKP cells and its

melphalan-resistant variants (section 3.3.1.2; 3.5.1.2). This suggests that MRP1 may be responsible for melphalan resistance in these variants.

The circumvention studies with sulindac and indomethacin also suggest that MRP family members may play a role in melphalan resistance in the RPMI-2650 melphalan-selected variant (section 3.8). Sulindac and indomethacin are nonsteroidal antiinflammatory drugs (NSAIDs) which are among the most frequently used medical drugs. They are used primarily as analgesics, anti-inflammatories and anti-pyretics. Their main known mode of action is through inhibition of the cyclo-oxygenase-mediated production of prostaglandins, but this is not thought to be sufficient to explain their wide variety of actions. There is currently widespread interest in their potential roles as chemotherapy modulators. It has been reported that indomethacin may reverse MRP-mediated efflux of adriamycin and vincristine via inhibition of the MRP pump and inhibition of glutathione-S-transferase (Draper *et al.*, 1997). The effect of combining a range of NSAIDs with a variety of chemotherapeutic drugs on cytotoxicity was examined in the human lung cancer cell lines DLKP, A-549, COR-L23R and in a human leukaemia line HL60/ADR. Sulindac and indomethacin significantly increased the cytotoxicity of the anthracyclines, as well as VP-16 and vincristine, but not cisplatin, cyclophosphamide, bleomycin and taxol (Duffy *et al.*, 1998). In the MRP1-overexpressing HL60/ADR and COR-L23R cell lines, a significant increase in cytotoxicity was observed in the presence of sulindac and indomethacin. It was found that sulindac and indomethacin were potent inhibitors of [^{3}H]-LTC_4 transport into inside-out plasma membrane vesicles prepared from MRP-expressing cells, of adriamycin efflux from preloaded cells and of glutathione-S-transferase activity which agreed with the results of Draper *et al.* mentioned above (Duffy *et al.*, 1998). Sulindac and indomethacin were believed to reverse MRP1-mediated efflux of drug by competitive inhibition of MRP1; whether these two compounds can inhibit cMOAT (MRP2)- and MRP3-mediated drug efflux is unknown. In section 3.8, the results showed that indomethacin is more cytotoxic than sulindac to the RPMI-2650, DLKP cell lines and their MDR variants. Treatment with sulindac or indomethacin resulted in increased cytotoxicity of melphalan in the RPMI-2650 melphalan-resistant variant, which again suggests the involvement of MRP1 in this variant.

Cross resistance studies revealed that the RPMI-2650 melphalan-resistant variant was 11-fold resistant to melphalan compared to its parental cell line and also cross resistant to cadmium chloride, adriamycin, vincristine, vinblastine and VP-16 (see section 3.2.4.2). Very low level of cross resistance to taxol, 5-FU and cisplatin was observed. Since Pgp (but not MRP) is related to taxol resistance, the slightly increased expression of Pgp in the RPMI-2650 melphalan-resistant variant might explain the low resistance to taxol in this variant. The RPMI-2650 melphalan-resistant variant showed extremely high resistance to cadmium chloride which is a substrate for MRP1, cMOAT and possibly MRP3. This variant also showed high resistance to adriamycin (which is substrate of MRP1, cMOAT, MRP3 and Pgp), to vinblastine (which is substrate of MRP1, cMOAT (MRP2) and Pgp) and to VP-16 (which is substrate of MRP1, MRP3 and Pgp). The cross resistance profile also strongly suggests the involvement of MRP1, cMOAT and MRP3 in multidrug resistance in the RPMI-2650 melphalan-resistant variant.

Compared to the RPMI-2650 melphalan-resistant variant, the DLKP melphalan long term and pulse selected variants exhibited low level resistance to melphalan which probably relates more to the clinical situation. Cross resistance patterns of these variants showed high resistance to cadmium chloride and cisplatin (see section 3.2.4.3 and 3.2.4.4). These variants were also resistant to adriamycin and VP-16. Resistance to vinblastine was observed in the DLKP long term melphalan-selected variant, but not in pulse selected variant, suggesting that very low level resistance to melphalan and/or very low level of MRP1 overexpression might not be able to induce vinblastine resistance. No significant resistance to taxol was observed in these two variants, suggesting once again that Pgp was not involved in melphalan resistance in these variants. All these results suggest the involvement of MRP1 and/or its homologue in melphalan resistance in the DLKP melphalan-selected variants.

Studies on the role played by LRP in melphalan resistance were based on the observation that LRP is widely expressed in untreated multiple myeloma and it is associated with a low probability of response and shorter survival in patients treated with conventional MP (melphalan combined with prednisone) regimen. It was found that LRP is an independent predictor for response and survival in patients treated with MP regimen (Raaijimakers *et al.*, 1998). Also, it was reported that the melphalan-

resistant variant 8226LR5 of the human myeloma cell line RPMI-8226 overexpressed LRP without any other MDR proteins (Raaijimakers *et al.*, 1998). A mitoxantrone-selected, highly LRP-positive cell population of RPMI-8226 also showed cross resistance to melphalan (Raaijimarkers *et al.*, 1998). It was hypothesised that vaults (LRP is the major protein of vaults) which localise in the nuclear membrane and nuclear pore complexes are involved in the nucleo-cytoplasmic exchange of melphalan which exerts its main cytotoxic effect in the cell nucleus. LRP was undetectable in both RPMI-2650 and its melphalan-resistant variant (see section 3.4.1.3), suggesting that LRP does not play a major role in drug resistance in this variant.

Some *in vitro* models of melphalan resistance have involved glutathione-mediated pathways, a finding observed in some rodent cell lines such as Chinese hamster L1210 leukaemia cell line (Ahmad *et al.*, 1987) and human cancer cell lines, including ovarian (Green *et al.*, 1984), myeloma (Gupta *et al.*, 1989; Bellamy *et al.*, 1991) and prostate cells (Bailey *et al.*, 1992).

Green *et al.* (1984) reported that they developed a human ovarian cancer cell line 1847^{ME} which was 4-fold resistant to melphalan compared to the parental cell line A1847. No detectable difference in melphalan uptake between the A1847 and 1847^{ME} cell lines was noted. However, glutathione levels were found to be 2-fold greater in the 1847^{ME} cells than in the A1847 cells. Similarly, murine L1210 cancer cell lines with acquired resistance to melphalan were shown to have elevated GSH levels (Suzukake *et al.*, 1983). Several other independent studies using tumour models have also found an association between increased glutathione levels and resistance to melphalan and other alkylating agents (Ball *et al.*, 1966; Calcutt *et al.*, 1983; Somfai-Relle *et al.*, 1984; Suzukake *et al.*, 1983). Thus, GSH has been shown to play a crucial role in the resistance to melphalan in some cell lines.

In order to reverse GSH mediated resistance, buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, has been used to deplete or lower GSH levels. Skapek *et al.* (1988) demonstrated that depletion of intracellular glutathione in the human medulloblastoma-derived cell line, TE-671, could

significantly increase the activity of melphalan. Somfai-Relle *et al.* (1984) also demonstrated that L1210 leukaemia cells resistant to melphalan could be rendered sensitive by BSO-mediated reduction in cellular glutathione. Similar observations were seen with the melphalan-resistant human ovarian cancer cell line, 1847^{ME}, which demonstrated an increased response to melphalan following depletion of glutathione (Green *et al.*, 1984). Ozols *et al.* (1991) also demonstrated that the survival of mice inoculated i.p. with NIH:OVCAR-3 ovarian cancer cells (derived from a patient clinically refractory to alkylating agents) and treated with melphalan could be extended by pretreatment with BSO.

There are also examples in which the development of resistance to melphalan or other alkylating agents is associated with increased expression or activity of the enzyme glutathione S-transferase. Batist *et al.* (1986) demonstrated an increase in the total activity of GST in human breast cancer cells from 3.6nmol/min/mg protein in the parental line to 161nmol/min/mg protein in the resistant line. Bolton *et al.* (1991) reported that they purified GST isozymes from mouse liver and analysed the metabolic products of the reaction of melphalan with glutathione in the presence of GST isozymes. They found that only isozymes of the α GST class catalysed the conjugation of melphalan with glutathione whereas μ or π isozyme appeared to have no effect. Thus, they suggested that increased levels of GST α isozyme may represent a specific mechanism whereby cells can acquire resistance to melphalan. Hall *et al.* (1994) also reported that a Chinese hamster ovary cell line, CHO-Chl, generated by exposure to chlorambucil and demonstrating a greater than 20-fold collateral resistance to melphalan showed increased expression of GST α. Their results suggested that overexpression of GST α may be directly responsible for the development of resistance to bifunctional alkylating agents. In order to further define the function of human GST α, Leyland-Jones *et al.* (1991) transfected cDNA of GST α into human MCF-7 breast cancer cells. However, the transfected cells failed to show any increased resistance to melphalan, adriamycin or cisplatin. Their results indicated that expression of human GST α by itself in MCF-7 human breast cancer cell line does not confer resistance to the chemotherapeutic drugs.

Elevated levels of GST π have also been shown in cultured cell lines resistant to melphalan (Robson *et al.*, 1987). Ban *et al.* (1996) reported that they transfected GST π antisense cDNA into the human colon cancer cell line M7609. They found that the sensitivity of the antisense transfectants to adriamycin, cisplatin, melphalan and etoposide was increased compared to those of the parental cells. They concluded that GST π was responsible for resistance to melphalan. However, Moscow *et al.* (1989) transfected a GST π experssion vector into MCF-7 cells and showed that the cells acquired resistance to etoposide, but there was no change in the cells' sensitivity to cisplatin and melphalan. Another group, Nakagawa *et al.* (1990) transfected a GST π expression vector into NIH-3T3 cells that had been transformed with H-ras and found that although resistance to adriamycin had increased, the cells did not acquire resistance to alkylating agents.

The results of GST activity assays in section 3.7 showed that the RPMI-2650 melphalan-resistant variant had a slightly higher GST activity than the parental cells. No significant difference in GST π expression was observed by immunocytochemistry between the RPMI-2650 cell line and its melphalan-resistant variant. GST α and μ were not detected in either of these cell lines. All the results obtained indicate that GST may play a minor role in drug resistance in the RPMI-2650 melphalan-resistant variant. Cellular glutathione content assays were not carried out for this study. In order to confirm the results, Western blotting of GST π and cellular glutathione content assay should be carried out. Grant (1993) reported that GST activity was decreased approximately 3-fold in the DLKP-C cell line (carboplatin-resistant variant) compared to the DLKP parental line. No significant difference was noted between the DLKP and DLKP-A (adriamycin-resistant variant) cells (Geraldine Grant, Ph.D. thesis, DCU, 1993).

Decreased influx and increased efflux which lead to decreased drug accumulation has also been associated with melphalan resistance. Evidence has shown that melphalan efflux occurs by a process different from the influx mechanism. Two leucine-preferring amino acid transporters have been identified as melphalan transporters and are designated system L and ASC. System L is a sodium-independent transporter and system ASC is a sodium dependent transporter (Begleiter *et al.*, 1979; Vistica, 1979).

Melphalan uptake studies in melphalan-resistant L1210 cells have demonstrated both a lower velocity of melphalan uptake and a lower affinity of the carrier for melphalan compared to the parental line. The results demonstrated an association between altered system L transport and melphalan resistance. Another melphalan-resistant cell line MelRMCF-7 was developed by Moscow *et al.* (1993) from the parental MCF-7 cell line. MelRMCF-7 was found to be 30-fold resistant to melphalan. Monitoring the cellular uptake of melphalan (50μM) over a 30 minutes period revealed a 4-fold decrease in the MelRMCF-7 cells compared with the parental cell line. Their results suggested that melphalan resistance was mediated by decreased capacity of system L amino acid transporter. Enhanced melphalan efflux has also been associated with melphalan resistance. Overexpression of MRP family members may be responsible for enhanced melphalan efflux.

Reduced DNA cross-linking is another mechanism of melphalan resistance. The relationship between DNA cross-linking and resistance to melphalan has been studied in the resistant variant, ZCR9, of the L1210 leukaemia cell line (Zwelling *et al.*, 1981). This cell line, selected with cisplatin, was found to be cross resistant to melphalan. In the resistant line, interstrand cross-linking by cisplatin or melphalan was reduced relative to the parental cell line. DNA-protein cross-linking by cisplatin was also reduced whereas DNA-protein cross-linking by melphalan was similar to that of the parental cells. These results suggest that DNA cross-linking correlates with tumour sensitivity to alkylating agent. Misonidazole (MISO), a 2-nitroimidazole compound in clinical trial as an oxygen-mimetic radiosensitiser of hypoxic tumour cells, has been shown to enhance the effectiveness of melphalan (Taylor *et al.*, 1983). The study showed that enhanced efficiency of DNA cross-link formation can account for the sensitisation to melphalan following treatment with MISO. In a melphalan-resistant melanoma cell line MM253-12M, a 50% decrease in melphalan-DNA binding was observed (Parsons *et al.*, 1981). The degree of cross-linking in the MM253-12M cells was also 50% less than that seen in the MM253 parental cells. Thus, the decrease in melphalan binding to DNA was almost sufficient to account for the decrease of cross-linking in the resistant cell line. In addition, increased DNA repair capacity of the cells may also contribute to melphalan resistance.

Metallothioneins (MTs) play a role in heavy metal detoxification, regulation of intracellular levels of Cu and Zn, free-radical scavenging, protection from ionising radiation and also in the control of growth and differentiation (see section 1.5.4). MTs have been shown to be induced by melphalan. The role of metallothionein was proven when it was demonstrated that transfection of murine cells with the metallothionein cDNA conferred resistance to cisplatin as well as to melphalan (Kelley *et al.*, 1988). Conflicting results, however, have been reported that murine C127 cells, after being transfected with MT, showed no resistance to cisplatin (Schilder *et al.*, 1990). Additionally, no significant difference in MT expression level was observed between untreated and cisplatin-treated ovarian cancer patients (Murphy *et al.*, 1991). In this thesis, the results obtained by immunocytochemistry (section 3.4.1.6) show no significant difference in the metallothionein expression between the RPMI-2650 parental cells and its melphalan-resistant variant. A possible explanation for these variable results is that the contribution of metallothionein to drug resistance may vary from tumour to tumour. Metallothioneins exist in all eukaryotic cells as well as in some prokaryotic cells. Consequently, all human cells will show positive immunostaining for metallothioneins. This would suggest that even minor difference in the level of MT expression between different cells may contribute to the resistance phenotype. Thus the results of immunocytochemistry may not be sensitive or precise enough to draw any conclusion. To determine the role of metallothionein in the RPMI-2650 melphalan-resistant variant, further studies need to be carried out including Western blotting and RT-PCR.

In the past 5 years, it has been found that cytokeratin expression may be related to resistance to a panel of chemotherapeutic drugs including melphalan. This finding was initiated by the observation that epithelial-derived untreated tumour cells are in some cases 10 to 2000 times more resistant to various chemotherapeutic agents than hematopoietic cell lines. Interest was then focused on cytokeratin because a major feature of epithelial cells is the expression of cytokeratins. Later it was reported that drug resistance in epithelial cells was due in part to cytokeratin 8 and 18. Bauman *et al.* (1994) compared the survival of mouse L fibroblasts lacking cytokeratins with that of L cells transfected with cytokeratin 8 and 18 (LK 8+18) in the presence of chemotherapeutic drugs. The expression of cytokeratin 8 and 18 conferred a MDR phenotype in cells exposed to mitoxantrone, adriamycin, methotrexate, melphalan and

vincristine. LK 8+18 cells were 6.5 times more resistant to melphalan than the parental cells. Drug resistance could not be attributed to altered growth characteristics, altered drug accumulation or less DNA damage in the transfected cells. Anderson *et al.* (1996) obtained similar results after transfecting cytokeratin 8 and 18 into murine NIH-3T3 cells. Since these studies, cytokeratin dependent drug resistance (C-MDR) has been established as a unique resistant mechanism in epithelial cells. C-MDR phenotype is defined as the ability of cytokeratin expression to alter cell survival by at least five-fold increase in the IC_{50}. To date, cytokeratin 8 and 18 has been related to drug resistance to adriamycin, mitoxantrone, bleomycin, mitomycin C and melphalan (Cress *et al.*, 1996).

Bichat *et al.* (1997) reported that similar levels of cytokeratin 8 and 18 were expressed in the human breast cancer cell line MCF7 and its adriamycin-resistant variant MCF7R. However, cytokeratin 19 was expressed in the MCF7S cell line and not in the MCF7R variant, while vimentin was highly expressed in MCF7R and weakly expressed in MCF7S. This suggested that other cytokeratins and intermediate filament might be involved in multidrug resistance.

From the data listed above, it seems that the drug-cytokeratin interaction initiates a cascade of events for an improved cellular response to potentially lethal damage. To support this assumption, Anderson *et al.* (1996) reported that the cytokeratin-positive cell lines were protected from apoptosis while the cell lines without cytokeratins were susceptible to apoptosis in response to mitoxantrone exposure. The exact mechanisms underlying C-MDR need to be elucidated.

Although cytokeratin 18 and vimentin were expressed in the RPMI-2650 melphalan-resistant variant, no significant difference in cytokeratin 18 and vimentin expression was observed in the parental cell line and its variant. These results suggest that cytokeratin and vimentin do not play a major role in drug resistance in the RPMI-2650 melphlan-resistant variant.

Various mutant oncogenes have been shown to affect the ability of tumour cells to express drug resistance properties. Expression of mutated oncogenes also results in inappropriate cellular proliferation. There are three main methods whereby normal

cellular genes (proto-oncogenes) are mutated to their oncogenic counterpart. The methods include: (1) deletion or point mutation in the coding sequence of gene resulting in a hyperactive protein produced in normal amounts. (2) gene amplification leading to the over-production of a normal protein (3) chromosomal rearrangement whereby a gene is under transcriptional regulation of an actively transcribed gene (Alberts *et al.*, 1994). Oncogenes can be grouped into two main categories - those that increase the rate of cell division and those that influence cell death. The latter group can be sub-divided into genes that suppress or induce apoptosis. The balance of these genes in the cell determines the cell's ability to survive the attack of chemotherapy. The products of oncogenes are parts of a cascade of proteins leading from the plasma membrane through the cytoplasm and into the nucleus (Bishop, 1991). Three biochemical mechanisms are known whereby the genes exert their transforming capacities. These mechanisms include (a) phosphorylation of proteins on serine, threonine or tyrosine residues, (b) transmission of signals by GTPases, or (c) transcription from DNA (Bishop, 1991). Molecular signals at the plasma membrane are transmitted through to the nucleus via kinases in the cytoplasm as part of a signal transduction cascade. The primary cascade involved in signal transduction initiates signalling through the family of small nucleotide binding proteins, namely small G-proteins, to effector proteins that eventually transmit the signal to the nucleus. The initiating proteins in this event are the ras proteins which are members of the small G-proteins. It has been reported that spontaneous apoptosis can be suppressed by the expression of c-Ha-ras (Wyllie *et al.*, 1987) which rapidly up-regulates the survival factors bcl-xL and bcl-2 (Kinoshita *et al.*, 1995). C-myc is a member of the nuclear oncoproteins and acts as a transcription factor (as a heterodimeric partner to Max) to promote cell proliferation and play a role in cell cycle (Pardee, 1989). The Myc-Max heterodimer can activate several promoters, including that of p53 (D'Amico and McKenna, 1994). myc usually stimulates proliferation, but if growth is inhibited by other factors, myc-induced apoptosis will occur. Therefore it has a dual role to play in the cell. Transfection of c-myc oncogenes into NIH-3T3 mouse fibroblasts resulted in increased resistance to such drugs as cisplatin, melphalan, cyclophosphamide and adriamycin (Niimi *et al.*, 1991). Transfection of c-Ha-ras into NIH-3T3 cells resulted in increased resistance to melphalan and cyclophosphamide (Niimi *et al.*, 1991). In addition, cells that have an impaired ability to undergo apoptosis would be expected to be resistant to chemotherapeutic agents. The overexpression of the bcl-2 oncogenes

has been shown to inhibit apoptosis in several cell types, and also to produce cells that are less sensitive to alkylating agents (Collin *et al.*, 1992). Preliminary results of RT-PCR analysis (described in section 3.5.7) revealed the overexpression of c-myc in the RPMI-2650 melphalan-resistant variant compared to its parental cell line. c-Ha-ras was not detected in either of these cell lines. It is possible that c-myc might be involved in drug resistance in the melphalan-resistant variant. Increased levels of c-myc have been demonstrated in the DLKP-C cell line compared to DLKP parental line. However, decreased levels were found in the adriamycin-selected DLKP-A cell line. Slight alterations in c-Ha-ras level were observed between the DLKP, DLKP-C and DLKP-A cell lines (Roisin NicAmhlaoibh, Ph.D. thesis, DCU, 1997).

4.5 Drug resistance and cancer invasion

Almost all types of cancer are characterised by their ability to progress toward increased malignancy. In terms of their evolution, three aspects of cancer behaviour are critical: proliferation, invasiveness and metastasis, and resistance to chemotherapy. For example, colon cancer can derive from *in situ* carcinoma which is proliferative and resistant to chemotherapy. It has to acquire the ability to invade tissue and metastasise to be a cancer. Another example, small cell lung cancer is sensitive to chemotherapy, but is proliferative, invasive and metastatic at diagnosis. The evolution of this tumour would be to acquire resistance to chemotherapy.

All these factors contribute to the slow pace of progress made in developing more effective cancer treatments. Unfortunately, studies of metastasis and drug resistance have generally proceeded along separate pathways of research. However, it is possible that drug resistance and cancer invasion/metastasis are linked to each other.

The evidence is growing that most malignant cancers arise as the end result of an accumulation of genetic mutations involving a number of oncogenes and recessive tumour suppressor genes. Many of these genes encode transcription factors which can act to regulate the expression of other genes. Some of these genes could affect the expression of drug resistance, and not just cell growth, differentiation, invasion and metastasis. Moreover, highly malignant cancers have a higher number of mutant

oncogenes and tumour suppressor genes than their benign counterparts. Hence, highly metastatic cancers have a greater chance of acquiring a drug resistant phenotype.

For example, transfection of the ras oncogene into rat embryo fibroblast cells resulted in the development of a malignant and invasive phenotype characterised by the production of MMP-9. Marked pulmonary metastases were also observed following injection of the transfected cells into the rat tail vein (Pozzatti *et al.*, 1986). Burt *et al.* (1988) demonstrated that transfection of v-H-ras into rat liver epithelial cells resulted in increased resistance to adriamycin and vinblastine. Transfection of c-H-ras into mouse fibroblasts NIH-3T3 resulted in increased resistance to cisplatin, melphalan and cyclophosphamide (Niimi *et al.*, 1991; Isonishi *et al.*, 1991). These results suggested that ras could affect the phenotypes of both invasion and drug resistance. In some cases, co-transfection of two oncogenes, such as ras and c-myc, into cells was required for the acquisition of metastatic capacity (Lee *et al.*, 1985). c-myc, v-raf and c-erbB2 have also been reported to play a role in resistance to cisplatin (Sklar, 1988), adriamycin, vinblastine (Burt *et al.*, 1988) and taxol (Yu *et al.*, 1998). Similarly, tumours overexpressing the bcl-2 oncogene were also shown to express increased resistance to various chemotherapeutic drugs (Reed, 1994; Fisher *et al.*, 1993) since many drugs have their effect by inducing apoptosis and bcl-2 acts as an inhibitor of apoptosis. With respect to tumour suppressor genes, studies have demonstrated that the absence of the wild-type p53 suppressor gene renders cells more resistant to various DNA damaging chemotherapeutic drugs. This may be due to the failure of the cells to undergo drug-induced apoptosis (Lowe *et al.*, 1993). The absence of p53 and Rb-1 has also been demonstrated to increase the ability of proliferation and invasion in tumour cells (Li *et al.*, 1996; Valente *et al.*, 1996).

A question that is often asked in relation to drug resistance is: why can oncogenes and tumour suppressor genes increase drug resistance in the cells? This question was partially answered by the finding that the promoter for the human mdr-1 gene was shown to be a target for the c-Ha-Ras-1 oncogene and the p53 tumour suppressor gene products. A mutant p53 specifically stimulated the mdr-1 promoter and wild-type p53 exerted specific repression. These results suggested that the mdr-1 gene could be activated during tumour progression and may be associated with mutations in ras and p53 (Chin *et al.*, 1992). Observations from clinical work have been consistent with

this statement. Benhattar (1996) carried out a study on seventeen tumours from patients treated with 5-FU for unresectable colorectal hepatic metastasis. The results obtained showed that colorectal tumours with mutated p53 had little or no response to chemotherapeutic treatment. This may be due to the significant correlation between mutant p53 and mdr-1 expression (de Kant *et al.*, 1996). Moreover, elevated mdr-1 gene expression was also found to accompany transfection of v-raf (Burt *et al.*, 1988). Kerbel *et al.* (1988 and 1990) reported the so called "growth dominant" phenomenon which refers to the ability of the metastatic tumour cells to gradually displace their non-metastatic cellular counterpart at the primary tumour site so that primary tumours may become progressively overgrown by the metastatic clone. This indicates that metastatic cells have survival advantages compared to non-metastatic cells. It is well known that the pattern of gene expression in the metastatic cells can be very different from their non-metastatic precursors. A large family of diverse genes can be switched on including genes encoding proteases, adhesion molecules and growth factors. These genes could also include those which affect drug resistance. If so, then metastatically competent tumour cells would be more drug resistant than their benign counterparts. Hence primary tumours could evolve to become increasingly drug resistant in the absence of drug exposure and selection.

Genetic instability is recognised as an important aspect of development of tumour heterogeneity and malignancy. It was demonstrated that metastatic variants were generated more rapidly in the highly metastatic B16F10 mouse melanoma cell line than in the less metastatic B16F1 cell line (Hill *et al.*, 1984). It was also observed that the highly metastatic B16F10 cell line generated variants resistant to methotrexate at higher rates than the B16F1 cell line. Thus it was concluded that the B16F10 cells possessed an increased ability to amplify DNA since resistance to methotrexate usually results from gene amplification. Hence, the higher rate of generation of drug-resistant variants corresponds to the higher rate of generation of metastatic variants (Cillo *et al.*, 1987). Similar results were also obtained in the KHT fibrosarcoma cell line. It was observed that the highly metastatic KHT 35L1 cell line was more resistant to methotrexate than the KHT parental cell line. The likely reason for this increased resistance is that the KHT 35L1 cell line generates variants resistant to methotrexate at higher rate than the KHT parental cells (Cillo *et al.*, 1989). The association between the rates of generation of drug-resistant and metastatic variants supports the

hypothesis that the mechanism of gene amplification may be involved in the generation of both phenotypes. However, Yamashima and Heppner (1985) found no correlation between metastatic ability and the rate of spontaneous mutation in three murine mammary tumour cell lines. They also showed that reproducing the selection procedure for B16F19 melanoma cell line did not result in increased resistance of this cell line to methotrexate (Jang and Hill, 1991). This suggested that these two phenotypes do not necessarily arise in parallel.

In the case of P-glycoprotein overexpression related drug resistance, Greene *et al.* (1997) reported that highly metastatic subclones of the prostate cancer cells, PC-3M, expressed higher levels of bFGF, IL-8, MMP-2, MMP-9 and mdr-1 mRNA than low metastatic subclones (Greene *et al.*, 1997). Similar results were obtained with the CT-26 murine colon cancer where subcutaneous tumours were found to be sensitive to adriamycin while metastases growing in the liver or lung were not (Wilmann *et al.*, 1992). A direct correlation was observed between the increased resistance to adriamycin of CT-26 and expression levels of mdr-1. Once removed from the lung, the tumour cells reverted to a sensitive phenotype similar to the parental cells. This indicated that increased resistance to adriamycin in the CT-26 cells in lung metastases was not due to selection of resistant subpopulations. This conclution is supported by results obtained from an experiment that involved implantation of CT-26 cells from lung metastases into the subcutis of syngeneic mice. Adriamycin-sensitive tumours were produced in the mice. In parallel studies, adriamycin-sensitive CT-26 cells from subcutaneous tumours developed the resistant phenotype when they were inoculated intraveneously and grew in the lung (Wilmann *et al.*, 1992). Moreover, in patients with colon carcinoma, high levels of Pgp expression were found on the invasive edge of the primary tumour and in lymph node, lung, and liver metastases (Weinstein *et al.*, 1991). Further investigations need to be carried out in order to identify organ-specific factors that can up-regulate expression of mdr-1 (or other resistant mechanisms).

The relationship between the metastatic potential of a tumour and its capability to escape treatments has not only been observed by the clinicians. The relationship between these two phenotypes has also been demonstrated by two types of experiments: (1) metastatic cells develop drug resistance more readily than non-metastatic cells as the series of experiments carried out by Cillo *et al.* mentioned

above. (2) Studies have demonstrated that at least in certain cases, tumour cells selected for resistance to chemotherapeutic drugs are more metastatic relative to non-resistant cells.

The adriamycin-resistant murine fibrosarcoma UV-2237M-ADMR cell line has been found to display more metastases than its parental UV-2237M cell line (Giavazzi *et al.*, 1983). Similarly, the MCF-7/AdrR cell line, an adriamycin-resistant variant of human bresat cancer cell line, MCF-7, was found to be highly metastatic compared to its parental cell line. The analysis of expression of lysosomal cysteine proteases showed that alterations of cysteine protease inhibitor activities contribute to increased levels of cathepsin activities in MCF-7/AdrR cells (Scaddan and Dufresne, 1993). Lücke-Huhle (1994) reported that the methotrexate-resistant variant of rat adenocarcinoma BSP73ASML was metastatic in contrast to the BSP73AS parental cell line. BSP73ASML cells also underwent amplification of the dihydrofolate reductase (DHFR) gene which results in the development of methotrexate resistance at an accelerated rate in contrast to its parental cell line. The capacity for gene amplification in metastatic BSP73ASML cells was correlated with a deletion in the p53 gene and enhanced expression of the c-myc oncogene. Haga *et al.* (1997) developed a cadmium-resistant HT-1080 cd-R cell variant and found that it was significantly more invasive than the parental HT-1080 cells. The HT-1080 cd-R cells showed increased expression of MMP-9. A more metastatic spread to the lungs was observed in mice inoculated i.v. with B16/col/R cells, a Pgp-overexpressing colchicine-resistant variant of B16 melanoma cells. B16/col/R cells displayed higher motility and a higher capacity to grow in the kidney and spleen than the B16 cells (Staroselsky *et al.*, 1996). Cisplatin resistance has also been correlated with enhanced metastasis. A variant of the fibrosarcoma cell line OR-32SK established from cisplatin-treated mice had a significantly higher level of metastasis to the lung compared to the parental cells, which may be related to the overproduction of MMP-9 (Choi *et al.*, 1999). Similar results were also obtained with a cisplatin-resistant cell line (Mitsumono *et al.*, 1998). In this thesis, the RPMI-2650 melphalan-resistant variant and DLKP melphalan-resistant variants were found to be more invasive than their parental cells, confirming that drug treatment does affect invasive behaviour of the cells.

In a study by Weinstein (1991), in all but 1 of the 95 cases of primary colon adenocarcinomas, invading carcinoma cells were present at the leading edge of the tumour. This subpopulation of invasive carcinoma cells expressed Pgp in 47 of the 95 specimens. Cases were grouped on the basis of the presence (Group 1, 47 cases) or absence (Group 2, 48 cases) of Pgp^+ invasive carcinoma cells. A significantly greater incidence of vessel invasion and lymph node metastases was noted in Group 1 cases. This finding indicated that Pgp^+ invasive colon cancer cells may have an increased potential for dissemination. Bradley and Ling (1994) reported that strong expression of Pgp was associated with a highly vascular stroma in rat liver carcinoma models.

Cytokeratins have also been found to be correlated with drug resistance in epithelial cells (see section 4.4). In some cancers, particularly malignant melanoma and breast carcinoma, there is a strong indication that overexpression of keratin or vimentin leads to augmentation of motility and invasiveness *in vitro* (Hendrix *et al.*, 1996). It has been reported in melanoma that the coexpression of vimentin and keratin 8 and 18 is associated with metastatic behaviour (Hendrix *et al.*, 1992). A375P, a human melanoma cell line of low invasive potential, expresses vimentin, but is keratin-negative. Co-transfection of A375P cells with cDNAs for both keratin 8 and 18 resulted in a 30-fold increase in experimental lung metastases when mice were injected i.v. with the transfectants (Hendrix *et al.*, 1992). In a study by Chu *et al.*, (1991), the highly metastatic melanoma cell line, C8161, which endogenously expresses both vimentin and keratins 8 and 18, was genetically manipulated to produce disrupted keratins. A three- to four-fold decrease in the invasive and migratory ability was observed in the genetically manipulated cells compared to the parental cells. The keratin-positive human breast cancer MCF-7 cell line (of poor invasive ability) was transfected with vimentin cDNA, with a resultant increase in the invasiveness. Similarly, MDA-MB-231, a highly invasive and metastatic cell line, positive for both vimentin and keratin 8 and 18, was treated with antisense oligonucleotides to vimentin and resulted in a 70% reduction in the migration of this cell line (Hendrix *et al.*, 1996).

Additional evidence linking the coexpression of vimentin and keratin expression with metastasis includes: (a) a non-metastatic rat pancreas adenocarcinoma expressed only vimentin whereas a metastatic variant contains a high amount of keratins in addition

to vimentin (Ben-Ze'ev *et al.*, 1986); (b) solid epithelial tumours contain keratin filaments exclusively. However, when these cells are present in ascitic or pleural fluid, they coexpress vimentin (Ramaekers *et al.*, 1983); (c) cells from murine sarcoma ascites were found to coexpress keratins and vimentin, while cells from the solid tumour of the same sarcoma expressed only vimentin (Gunther *et al.*, 1984) and (d) in rat ascites hepatoma, a spontaneously derived metastatic line contained both keratins and vimentin, but the poorly metastatic counterpart expressed only vimentin (Kinjo *et al.*, 1984). Therefore, it is apparent that coexpression of keratin and vimentin is related to invasion and metastasis.

There have been several reports which indicate that cell lines selected *in vitro* for drug resistance usually manifest a non-tumorigenic phenotype *in vivo* or are strongly suppressed in their ability to form metastases (Ganapathi *et al.*, 1987; Sircar *et al.*, 1987; Kawai *et al.*, 1990). It is possible, however, that a different picture may emerge when drug resistant variants are selected *in vivo.* In fact, there is evidence to suggest that tumour cells selected for drug resistance *in vivo* are more malignant. An interesting example of this comes from studies initiated in the laboratory of Teicher *et al.* (1990). These investigators established variants resistant to alkylating agent from a murine mammary carcinoma cell line called EMT-6 by serial passage in Balb/C mice. The sublines selected expressed high levels of resistance to cyclophosphamide, cisplatin and carboplatin. Of considerable interest was the finding that the drug resistant sublines failed to express their resistance properties *in vitro* in monolayer cell cultures (Teicher *et al.*, 1990). In subsequent studies, Teicher *et al.* (1993) found that the EMT-6 drug resistant variants were much more efficient at forming spontaneous lung metastases after subcutaneous injection of the cells than the drug-sensitive parental cell lines. These results suggested the new mechanism of drug resistance "only *in vivo*". In subsequent studies, it was found that if the EMT-6 drug-resistant variants were grown under more *in vivo*-like conditions in tissue culture, *i.e.* as three-dimensional spheroids, they expressed their resistance properties in a manner similar to that observed *in vivo* (Kobayashi *et al.*, 1993).

This form of acquired drug resistance is termed "multicellular drug resistance". It was shown that multicellular drug resistance can be induced in EMT-6 mammary tumour cells by a single transient exposure to agents such as cyclophosphamide or cisplatin

(Graham *et al.*, 1994). These experiments were undertaken to address why it usually takes a long time to isolate drug-resistant variants in cell culture under ideal selection conditions while drug resistance can develop in an accelerated fashion in the clinical setting (Cadman, 1989). Therefore studies were carried out to determine if drug resistance could be induced at a faster rate by exposure to drug in three-dimensional culture systems. The results indicated that this may indeed be the case (Graham *et al.*, 1994). EMT-6 tumour cells were selected with cyclophosphamide in monolayer versus three-dimentional culture conditions for 1 month. Resistance to cyclophosphamide was induced but could only be detected in the three-dimentional culture system (Graham *et al.*, 1994). Jardillier *et al.* (1998) also reported that spheroids made from the human breast cancer cell line MCF-7 were more resistant to adriamycin than the same cells grown as monolayers. Two unidentified serine proteases were detected in media conditioned by spheroids of both MCF-7 and MCF-7 MDR variants but not in media conditioned by monolayers. All these results suggest that the nature of the inter-relationship of drug resistance and metastasis should probably be re-evaluated in the context of the multicellular drug resistance assays. However, it has also been reported that MDR variants could be established by pulse selecting sensitive parental cell lines growing as monolayers in flasks after four pulses (four-hour exposure of the cells to drug a week is one pulse) (NicAmhlaoibh *et al.*, 1999).

4.6 Mechanisms of invasion in RPMI-2650, DLKP and their MDR variants

Numerous studies have shown that dynamic interactions among integrins, ECM, proteases, cytoskeletal proteins and signalling molecules foster the invasive behaviour of the tumour cells. The experiments reported in this thesis include analysis of several proteins which may have an impact on invasion and metastasis. Increased expression of $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, decreased expression of $\alpha_4\beta_1$, stronger adhesion to collagen type IV, laminin, fibronectin and matrigel, expression of MMP-2 and MMP-9 and overexpression of c-myc might all contribute to the high invasiveness of the RPMI-2650 melphalan-resistant variant. Conversely, the overexpression of α_4 integrin subunit, low attachment to collagen type IV, laminin, fibronectin, matrigel and non- detectable expression of MMPs might be related to the non-invasiveness of the RPMI-2650 parental cell line. The decreased expression of α_2 integrin which might result in decreased attachment to collagen type IV, lack of cytokeratin 18, non-detectable expression of proteinases may contribute to the non-invasiveness of the taxol-resistant variant. The overexpression of α_2 and β_1 integrin subunits and the expression of MMP-2 and MMP-9 may contribute to the invasiveness of DLKP long term melphalan-selected variant. Overexpression of MMP-2 and MMP-9 was also observed in the invasive DLKP melphalan-pulse selected variant. More work needs to be carried out to further investigate the mechanisms underlying the relatively high invasiveness in these two DLKP low level melphalan-resistant variants.

In addition, recent experiments by Dr. Joanne Keenan in our laboratory have shown that glucose uptake in the RPMI-2650 melphalan-resistant variant was greater than in the taxol-resistant variant, while the lowest uptake was seen in the parental cells. Expression of glucose transporter GLUT1 has been associated with aggressive behaviour in some cancers. Overexpression of GLUT 1 was observed in the RPMI-2650 melphalan-resistant variant and to a lesser extent in the taxol-resistant variant. The RPMI-2650 parental cell lines expressed little or no level of GLUT1. All these results are consistent with the motility behaviour of the RPMI-2650 cell line and its

MDR variants, *i.e.* more mobile cells use more glucose to supply their energy (personal communication with Dr. Joanne Keenan).

RPMI-2650 and DLKP are epithelial cell lines. The distinguishing features of epithelia are their polarised morphology, their attachment to an underlying basement membrane, the presence of specialised cell-cell contacts and their capacity for rapid self-renewal. Low levels of E-cadherin was found to be expressed in the RPMI-2650 cell line which suggests that E-cadherin may play a minor role in cell-cell interactions in the RPMI-2650 cells. St Croix and Kerbel *et al.* (1997) reported that E-cadherin was capable of upregulating P27KIP1, a cyclin-dependent kinase inhibitor which causes growth arrest in the G1 phase of the cell cycle and resistance to apoptosis induced by anticancer agents. However, no E-cadherin was detected in the taxol- and melphalan-resistant variants, indicating that E-cadherin does not play a major role in mediating drug resistance of these cell lines.

Immonucytochemistry studies showed that the expression of α_2, α_5, α_6, β_4 integrin subunits was increased in the RPMI-2650 melphalan-resistant variant whereas the expression of α_5 and α_6 was slightly increased in the taxol-resistant variant. These studies also showed that high levels of β_1 integrin subunit was expressed in the RPMI-2650 melphalan-resistant variant. The taxol-resistant variant was also found to express some β_1 integrin subunits while the lowest level was observed in the parental cells. The results obtained from RT-PCR analysis were consistent with the immunocytochemistry results. Expression of α_2 and β_1 integrin subunits was found to be increased in the DLKP long term melphalan-selected variant compared to DLKP parental cell line while no significant difference was noted in the pulse selected variant. Expression of the α_4 and β_4 integrin subunits was undetected in DLKP parental cell line and its melphalan-resistant variants.

The expression of $\alpha_1\beta_1$ integrin was also studied in the RPMI-2650 cell line and its MDR variants. $\alpha_1\beta_1$ integrin is a dual receptor for collagen type IV and laminin. The results illustrated that only trace amount of $\alpha_1\beta_1$ integrin was observed in the RPMI-2650 parental cell line and its taxol- and melphalan-resistant variants.

The expression of $\alpha_2\beta_1$ integrin varied greatly between the RPMI-2650 parental cell line and its MDR variants and also between DLKP and its MDR variants. $\alpha_2\beta_1$ integrin was uniformly stained in the RPMI-2650 parental cells whereas it appeared to be concentrated on some particular clones in the taxol-resistant variants. $\alpha_2\beta_1$ was overexpressed in the melphalan-resistant variants. The expression of $\alpha_2\beta_1$ appeared to be restricted to certain clones of the DLKP parental cells while uniformly intense staining for $\alpha_2\beta_1$ integrin was observed in the DLKP long term melphalan-selection variant. However, only weak staining was observed in the DLKP melphalan-pulse selected variant. Studies by Koukoulis *et al.* (1991) and Pignatelli *et al.* (1992) demonstrated that $\alpha_2\beta_1$ integrin expression was decreased in breast adenocarcinomas. Studies on other adenocarcinomas (colon, prostate, lung, pancreas and skin) have yielded similar findings regarding $\alpha_2\beta_1$ expression (Koretz *et al.*, 1991; Stallmach *et al.*, 1992; Hall *et al.*, 1991). These results suggested that $\alpha_2\beta_1$ integrin may function as a tumour suppressor for epithelial malignancies. In contrast, expression of $\alpha_2\beta_1$ integrin by malignant melanoma and rhabdomyosarcoma was markedly increased. These studies suggested that the function of the $\alpha_2\beta_1$-integrin may be cell type-dependent. It was found that erbB2 may contribute to the inability of SP1 protein to bind to the promoter of the α_2 integrin gene thereby reducing α_2-integrin gene expression. The modest expression of $\alpha_2\beta_1$ integrin in the RPMI-2650 and DLKP agrees with the report which states that $\alpha_2\beta_1$ expression is decreased in epithelial carcinomas. The mechanisms underlying the localisation of $\alpha_2\beta_1$ integrins in particular clones of the RPMI-2650 taxol-resistant variants and the DLKP cells are unknown. It appears that the RPMI-2650 melphalan-resistant variant utilises more $\alpha_2\beta_1$ to strongly adhere to collagen type IV compared to the parental cell line and the taxol-resistant variant. The DLKP long term melphalan-selected variant may also use more $\alpha_2\beta_1$ integrin in the same manner.

$\alpha_3\beta_1$ integrin is a versatile integrin which can bind to collagen type IV, laminin and fibronectin. $\alpha_3\beta_1$ was found to be expressed at similar level in the RPMI-2650 cell line and its MDR variants. Previous studies have shown that $\alpha_3\beta_1$ is a low-affinity receptor for type IV collagen (Kuhn and Eble, 1994). It was also demonstrated that binding to type IV collagen is initiated via the $\alpha_2\beta_1$ or $\alpha_1\beta_1$ integrin and then $\alpha_3\beta_1$

integrin is recruited to focal contacts following cell adhesion (DiPersio *et al.*, 1995). Most of the reports to date have indicated that reduced expression of $\alpha_3\beta_1$ integrin is likely to be involved in tumour metastasis although increases in $\alpha_3\beta_1$ expression has also been reported in cutaneous malignant melanoma (Natali *et al.*, 1993).

$\alpha_4\beta_1$ integrin which recognises fibronectin and VCAM-1 (vascular cell adhesion molecule-1) may influence metastatic process at various stages. The detachment of tumour cells from the primary tumour and the invasion of the surrounding tissue represent the onset of tumour metastasis. It was reported that at the primary tumour site, expression of α_4 integrin inhibits the ability of melanoma cells to break loose. This could be achieved either by strengthening of homotypic adhesion to adjacent tumour cells or by down regulation of MMPs that are required for tumour cell migration through the extracellular matrix (Holzmann *et al.*, 1998). Intense staining for $\alpha_4\beta_1$ integrin was observed by immunocytochemistry in the RPMI-2650 cell line whereas $\alpha_4\beta_1$ was not detected in the RPMI-2650 MDR variants. This may explain the non-invasiveness of the RPMI-2650 cells by matrigel invasion assay. Qian *et al.* (1994) also reported that matrigel invasion by $\alpha_4\beta_1$-positive cells of B16 melanoma cell lines was reduced. In contrast, VCAM-1 positive endothelial cells co-localise with tumour cells expressing $\alpha_4\beta_1$ integrin at later stages of metastasis, thus enhancing the metastatic capacity of the tumour cells. No $\alpha_4\beta_1$ integrin staining was observed in either the DLKP cell line or its melphalan-selected variants, indicating that this integrin may not be involved in either melphalan resistance or high invasiveness in these variants.

Damiano *et al.* (1999) reported that the drug-sensitive 8226 human myeloma cell clones which expressed both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, were relatively more resistant to the apoptotic effects of adriamycin and melphalan. These results are not entirely consistent with the results obtained for the melphalan-resistant variants of RPMI-2650 and DLKP. This indicates that either overexpression or underexpression of particular integrins in resistant cells may be cell-type specific.

$\alpha_5\beta_1$ integrin is the major receptor for fibronectin. Forced expression of the $\alpha_5\beta_1$ integrin in tumour cells has been shown to reduce tumorigenicity (Giancotti and Ruoslahti, 1990; Varner *et al.*, 1995). Conversely, decreased $\alpha_5\beta_1$ expression has been shown to increase the tumorigenicity of CHO cells (Schreiner *et al.*, 1991). $\alpha_5\beta_1$ is the integrin that controls the assembly of fibronectin matrix. Consistent with the above observation, $\alpha_5\beta_1$ integrin was modestly expressed in the RPMI-2650 parental cell line. No significant difference in the $\alpha_5\beta_1$ integrin expression was seen between the RPMI-2650 and its taxol-resistant variant. However, the melphalan-resistant variant was found to express higher levels of $\alpha_5\beta_1$. It seems that the melphalan-resistant variant utilises more $\alpha_5\beta_1$ integrins to enable the cells to attach strongly to fibronectin.

Nista *et al.* (1997) reported that the adriamycin-resistant MCF-7 variant expressed more $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins and was more adhesive to fibronectin than MCF-7 parental cell line.

Both $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins are receptors for laminin. It was reported that inhibiting $\alpha_6\beta_1$ function or its expression reduces the invasive and metastatic ability of several types of tumour cells (Lin *et al.*, 1993; Rabinovitz *et al.*, 1995; Shaw *et al.*, 1996). It was also found that high $\alpha_6\beta_4$ integrin expression was related to more advanced clinical stage of colon carcinoma and thyroid carcinoma (Falcioni *et al.*, 1994, Serini *et al.*, 1996). Expression of the $\alpha_6\beta_4$ integrin in RKO cells, a β_4-deficient rectal carcinoma cell line, significantly increased the ability of these cells to invade matrigel (Chao *et al.*, 1996). Expression of the $\alpha_6\beta_4$ integrin in MDA-MB-435 cells, a human breast carcinoma cell line that lacks β_4 integrin sbunit also resulted in a dramatic increase in invasion (Rabinovitz and Mercurio, 1996). The results obtained in the RPMI-2650 melphalan-resistant variant were very consistent with the above observations. Higher levels of α_6 integrin subunit were expressed in the melphalan-resistant variant than in the RPMI-2650 parental line and taxol-resistant variant. High levels of β_4 integrin subunit were also expressed in the melphalan-resistant variant whereas no β_4 was detected in the other two cell lines. The increase in α_6 integrin subunit expression could be due to the increase in the β_4 integrin subunit in the RPMI-2650 melphalan-resistant variant. Apart from that, the melphalan-resistant variant

utilises more $\alpha_6\beta_4$ or $\alpha_6\beta_1$ to attach to laminin. The expression of $\alpha_6\beta_4$ probably contributes to the high invasiveness of the RPMI-2650 melphlan-resistant variant.

Narita *et al.* (1998) investigated alterations in the expression of integrins in adriamycin-resistant MCF-7 (MCF-7/ADR) cells. Expression of α_6 integrin subunit was found to be increased whereas the expression of α_2 was decreased in the MCF-7/ADR cells. MCF-7/ADR cells also showed increased attachment to laminin, but not to collagen or fibronectin. The migration activity of MCF-7/ADR cells was higher than that of the parental cells. A significant inhibitory effect on the migration of MCF-7/ADR cells was observed by the addition of antibodies to α_6 and β_1 integrin subunits.

Duensing *et al.* (1996) studied alterations in β_1 integrin family expression and adhesiveness to ECM proteins in the human renal carcinoma cell line Caki-1. They compared the results with the vinblastine-resistant sublines Caki-1/V1 and Caki-1/V10 cultured in the presence of 1ng/ml and 10ng/ml vinblastine respectively. Their results indicated that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins were expressed in the Caki-1/V10 subline whereas untreated and Caki-1/V1 cells did not express these integrins. $\alpha_6\beta_1$ was found to be decreased in the vinblastine-resistant sublines. Attachment of both Caki-1/V1 and Caki-1/V10 cells to collage type IV was significantly increased compared to the parental cells. Both Caki-1/V1 and Caki-1/V10 cells also exhibited increased adhesion to fibronectin. No studies with regard to invasiveness were carried out.

Overall, it seems that overexpression or underexpression of integrin subunits varies in different drug resistant variants. Therefore, each resistant variant should be analysed on an individual base.

The results of invasion assay with sulindac or indomethacin showed that neither sulindac nor indomethacin could affect the invasive behaviour of RPMI-2650 melphalan-resistant variant, indicating that the inhibition of MRP1-mediated transport of endogenous substances such as leukotrienes might not correlate with the invasiveness of the cells.

4.7 Relationship between melphalan resistance and cell invasion in the HT-1080, NIH-3T3, A549, DLKP, DLKPC, COR-L23S and COR-L23R cell lines

Based on the observation that the RPMI-2650 melphalan-resistant variant was significantly more invasive than the taxol-resistant variant and its parental cell line, and also that the DLKP long term melphalan-selected variant was more invasive than the DLKP parental cell line, the following question was raised: is there any relationship between melphalan resistance and cell invasion? Since the melphalan-resistant variants of the RPMI-2650 and DLKP cell lines were found to overexpress MRP family members, another question that was raised was: is there any relationship between overexpression of MRP and cell invasion? Chemosensitivities to melphalan, expression of MRP and invasive behaviour (including secretion of MMPs) were thus studied in the HT-1080, NIH-3T3, A-549, DLKP, DLKPC, COR-L23S and COR-L23R cell lines.

HT-1080, a human fibrosarcoma cell line, is a highly invasive cell line which is widely used as positive control of invasion assay. The expression of MMP-2 and MMP-9 which was shown by gelatin zymograph probably plays an important role in the invasion behaviour of the HT-1080 cells. However, it was reported that HT-1080 is Pgp-negative and MRP1-negative (Vanhoefer *et al.*, 1997) which could explain the sensitivity of the HT-1080 to melphalan.

In the case of NIH-3T3, a murine fibroblast cell line, low invasiveness was observed, although both MMP-2 and MMP-9 were expressed. This suggests that other factors such as high activity of TIMPs maybe contribute to the weak invasiveness of this cell line. NIH-3T3 is approximately 2-fold more resistant to melphalan than the HT-1080. Breuninger *et al.* (1995) reported that no MRP1 was expressed in this cell line. The results obtained for the HT-1080 and NIH-3T3 cells indicate that neither melphalan resistance nor the expression of MRP is necessarily related to cell invasion. The same conclusion could be drawn in the COR-L23S and COR-L23R cell lines. COR-L23S, a human large cell lung cancer cell line, does not express Pgp and only expresses very low level of MRP1. COR-L23R, a MRP1-overexpressing adriamycin-resistant variant

of the COR-L23S, was found to be even more sensitive to melphalan than its parental cell line, COR-L23S. This indicates that although MRP1 overexpression is involved in melphalan resistance in some cell lines, it does not apply in all cases; it may also suggest that MRP1 may not play a major role in mediating melphalan resistance, and other MRP family members, such as MRP2 and MRP3, may play a role in melphalan resistance. COR-L23S was found to express MMP-2 and MMP-9 whereas nondetectable levels were observed in the COR-L23R cells. However, the COR-L23R cell line exhibited slightly more invasiveness than COR-L23S, indicating that proteinases other than MMP-2 and MMP-9 might be involved in its invasiveness.

In addition to melphalan-resistant variants of RPMI-2650 and DLKP, the human lung adenocarcinoma cell line, A-549, was found to overexpress MRPs 1-3. This cell line was also shown to be very resistant to melphalan and highly invasive. The cisplatin-resistant variant of DLKP, DLKP-C, was found to be cross resistant to melphalan and more invasive than the DLKP cells. Thus, it seems that melphalan-resistant cell lines (if this melphalan resistance is caused by the overexpression of MRP family members), with their resistance either intrinsic or acquired, are more invasive than melphalan-sensitive cell line. However, this does not mean that any highly invasive cell line, such as HT-1080, is resistant to melphalan.

Kiefer *et al.* (1990) reported that c-myc and c-Ha-ras were expressed in the A-549 cell line. C-Ha-ras and c-myc are believed to increase cell invasiveness (Ura, 1990). Giambernardi *et al.* (1998) also reported that overexpression of c-Ha-ras correlated with up-regulation of MMP-9. Expression of c-myc and c-Ha-ras was also reported to correlate with melphalan resistance (Niimi *et al.*, 1991). All these results suggest that c-H-ras or c-myc might link melphalan resistance to cell invasion.

4.8 Conclusions and future work

The establishment and characterisation of novel taxol- and melphalan-resistant variants of the RPMI-2650 cell line was the objective of the early work reported in this thesis. Investigation of the mechanisms underlying the high invasiveness of the RPMI-2650 and DLKP melphalan-resistant variants represented the objective of the later work of this project.

1. A 11X melphalan-resistant variant of the RPMI-2650 cell line was established. The cross resistance profile showed that it was resistant to cadmium chloride (54X), adriamycin (23X), vinblastine (24X), VP-16(26X), vincristine (6X) and cisplatin (2.6X), but displayed no significant resistance to taxol (1.7X) or 5-FU (1.1X).
2. Overexpression of MRP1 and cMOAT (MRP2) was detected by Western blotting, RT-PCR and immunocytochemistry in RPMI-2650 melphalan-resistant variant.
3. High levels of mrp3 mRNA were expressed in the RPMI-2650 melphalan-resistant variant as demonstrated by RT-PCR. Expression of mrp4 and mrp5 was undetectable in this cell variant.
4. Expression of Pgp (MDR-1) was slightly increased in the RPMI-2650 melphalan-resistant variant compared to the RPMI-2650 parental cell line.
5. A low level of adriamycin accumulated in the nuclei of the RPMI-2650 melphalan-resistant variant as detected by fluorescence microscopy. A significant increase in nuclear fluorescence was observed following treatment with verapamil or cyclosporin A in this variant.
6. Both verapamil and cyclosporin A can reverse adriamycin toxicity in the RPMI-2650 melphalan-resistant variant as determined by toxicity assays. Verapamil appears to be more effective than cyclosporin A in this cell line. Sulindac and indomethacin can also reverse melphalan toxicity in this variant.

On the basis of these results, it was concluded that overexpression of MRP1, and possibly cMOAT (MRP2) and MRP3, plays an important role in drug resistance in the RPMI-2650 melphalan-resistant variant.

7. Preliminary RT-PCR results demonstrated that c-myc expression was higher in the RPMI-2650 melphalan-resistant variant than in the parental cell line. C-Ha-ras was not detected in either of these cell lines.

It was concluded that c-myc may be involved in drug resistance in the RPMI-2650 melphalan-resistant variant.

Immunocytochemistry studies demonstrated that:

8. Expression of LRP was undetectable in the RPMI-2650 melphalan-resistant variant.
9. Topoisomerase II α was expressed at similar levels in the RPMI-2650 melphalan-resistant variant and the parental cell line.
10. Metallothionein was expressed at similar levels in the RPMI-2650 melphalan-resistant variant and the parental cell line.
11. GST activity in the RPMI-2650 melphalan-resistant variant was slightly higher than in the parental cell line as detected by GST activity assay.

On the basis of these results, it was concluded that LRP, topoisomerase II α, metallothionein and GST might not play an important role in drug resistance in the RPMI-2650 melphalan-resistant variant.

12. Cytokeratin 18, vimentin and tubulin in the RPMI-2650 melphalan-resistant variant were expressed at a similar level as in the RPMI-2650 parental cell line, indicating that these cytoskeletal proteins do not play a role in drug resistance in the RPMI-2650 melphalan-resistant variant.

13. The RPMI-2650 melphalan-resistant variant was shown to be highly invasive *in vitro* and the invasiveness was not affected by sulindac or indomethacin.
14. The RPMI-2650 melphalan-resistant variant exhibited higher adhesiveness to collagen type IV, laminin, fibronectin and matrigel.
15. Increased expression of $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ integrins, and decreased expression of $\alpha_4\beta_1$ integrin was observed in the RPMI-2650 melphalan-resistant variant.
16. The RPMI-2650 melphalan-resistant variant expressed MMP-2 and MMP-9 whereas the parental cells did not, as detected by zymography.

On the basis of these results, it was concluded that melphalan treatment can increase the invasiveness of RPMI-2650 cells by affecting its integrin and proteinase expression.

17. The RPMI-2650 melphalan-resistant variant showed high motility as detected by motility assay and videomicroscopy.

On the basis of this result, it was concluded that melphalan treatment can increase the motility of RPMI-2650 cells.

18. A 3.3X melphalan-resistant variant of the DLKP cell line was established by continual exposure to melphalan. It was cross resistant to cadmium chloride (9.8X), cisplatin (8.1X), adriamycin (1.8X) and vinblastine (1.9X), but not significantly resistant to taxol (1.3X).
19. A 2.1X melphalan-resistant variant of the DLKP cell line was established by pulse selection with melphalan. It was cross resistant to cadmium chloride (7.3X), cisplatin (3.1X), adriamycin (1.5X), but not significantly resistant to taxol (1.2X) and vinblastine (1.2X).
20. Overexpression of MRP1 was detected in the DLKP long-term melphalan-selection and melphalan-pulse selected variants by Western blotting and RT-PCR.
21. Expression of MDR-1 (Pgp) was not detected in these two variants by either Western blotting or RT-PCR.

On the basis of these results, it was concluded that MRP1, but not Pgp, plays an important role in melphalan resistance in the DLKP long term and pulse selected melphalan variants.

22. The DLKP long-term melphalan-selection variant was found to be more invasive than the melphalan-pulse selected variant which in turn was more invasive than the parental cell line.
23. Zymography showed that the DLKP long-term melphalan-selection variant and the DLKP melphalan pulse-selected variant expressed MMP-2 and MMP-9. These proteinases were not detected in the DLKP parental cell line.

24. Expression of β_1 and α_2 integrin subunits was increased in these two variants compared to the DLKP parental cell line. α_4 and β_4 integrin subunits were not detected in any of these cell lines.

On the basis of these results, it was concluded that melphalan treatment increased invasiveness of the DLKP cell line by affecting integrin and proteinase expression.

25. A 226X taxol-resistant variant of RPMI-2650 was established by continuous exposure to taxol. Studies showed that it was cross resistant to adriamycin (218X), vincristine (595X), vinblastine (94X) and VP-16 (20X), but not significantly resistant to melphalan (4.5X), 5-FU (3.6X), cadmium chloride (8.3X) and cisplatin (1.1X).
26. Overexpression of Pgp (MDR-1) was detected in the RPMI-2650 taxol-resistant variant by Western blotting, RT-PCR and immunocytochemistry.
27. Little or no MRP1 expression was observed in the RPMI-2650 taxol-resistant variant by Western blotting, RT-PCR and immunocytochemistry.
28. Significant levels of MRP2, MRP3, MRP4 and MRP5 were not detected in the RPMI-2650 taxol-resistant variant either by Western blotting or RT-PCR.
29. A low level of adriamycin accumulated in the nuclei of the RPMI-2650 taxol-resistant variants as detected by fluorescence microscopy. A significant increase in nuclear fluorescence was observed following treatment with cyclosporin A or verapamil in this variant.
30. Both cyclosporin A and verapamil could circumvent adriamycin toxicity in the RPMI-2650 taxol-resistant variant. Cyclosporin A was more effective than verapamil at circumventing resistance.

On the basis of these results, it was concluded that overexpression of Pgp plays an important role in drug resistance in the RPMI-2650 taxol-resistant variant.

31. c-myc was expressed at a higher level in the RPMI-2650 taxol-resistant variant than in the parental cell line as detected by RT-PCR. c-Ha-ras was not detected in either of these cell lines. It indicates that c-myc might be involved in drug resistance in the RPMI-2650 taxol-resistant variant.

Immunocytochemistry studies demonstrated that:

32. No expression of LRP was detected in the RPMI-2650 taxol-resistant variant.
33. Topoisomerase II α was expressed at a similar level in the RPMI-2650 taxol-resistant variant as in the parental cell line.
34. Metallothionein was expressed at a similar level in the RPMI-2650 taxol-resistant variant as in the parental cell line.
35. GST activity was slightly higher in the RPMI-2650 taxol-resistant variant than in the RPMI-2650 parental cell line as detected by GST activity assay.

On the basis of these results, it was concluded that LRP, topoisomerase II α, metallothionein and GST do not play a major role in drug resistance in the RPMI-2650 taxol-resistant variant.

36. As detected by immunofluorescence, tubulin content in the RPMI-2650 taxol-resistant variant was slightly less than the parental cell line. Expression of cytokeratin 18 was significantly decreased in the taxol-resistant variant. Vimentin was expressed at similar levels in the taxol-resistant variant and the parental cell line. These results indicate that cytoskeleton proteins may be involved in drug resistance in the RPMI-2650 taxol-resistant variant.

37. RPMI-2650 taxol-resistant variant was non-invasive as detected by invasion assays.
38. RPMI-2650 taxol-resistant variant exhibited low adhesiveness to collagen type IV, laminin, fibronectin and matrigel.
39. Decreased expression of α_2 integrin subunit was observed in the RPMI-2650 taxol-resistant variant compared to its parental line.
40. No proteinases were detected by gelatin zymography in the RPMI-2650 taxol-resistant variant.

On the basis of these results, it was concluded that taxol treatment did not affect the invasiveness of RPMI-2650 cells.

41. RPMI-2650 taxol-resistant variant exhibited modest motility as detected by motility assays and videomicroscopy.

On the basis of this result, it was concluded that taxol treatment can slightly increase the motility of the RPMI-2650 cell line.

Two main questions have been raised from these studies. Firstly, is MRP overexpression always associated with melphalan resistance? If not, what determines the particular mechanisms underlying melphalan resistance in a particular cell line? A similar question could be asked for cisplatin, another alkylating agent, often appears to share the same resistance mechanisms as melphalan, which several papers report that MRP overexpression was not observed in some cisplatin-resistant cell lines. Analysis of expression of MRP family members in other melphalan-resistant variants needs to be carried out. Since DLKP-C, a carboplatin-selected, cisplatin-resistant variant of DLKP has been established in this centre, it would be interesting to further investigate the mechanisms of cisplatin resistance in this variant. A second question needs to be answered is: how much does MRP1, cMOAT (MRP2) and MRP3 individually contribute to the melphalan resistance in the RPMI-2650 melphalan-resistant variant? Direct evidence may be obtained by knocking out each MRP member through genetic manipulation and rechecking the melphalan resistance in each MRP member-deficient cell line or by looking at properties of cell lines transfected with cDNAs of individual MRP family members.

In the DLKP long term melphalan-selected and melphalan-pulse selected variants, overexpression of MRP1 may contribute to their melphalan resistance. It has been shown that the melphalan-resistant variants of RPMI-2650 and DLKP cells all exhibit significant resistance to cadmium chloride, indicating that cadmium chloride may share some resistance mechanisms with melphalan.

Attempts to co-select RPMI-2650 cells with taxol and melphalan always failed, suggesting that cells cannot survive the effects of these two drugs. This might have clinical significance in chemotherapy. Further work needs to be carried out to confirm the lethal effects of the combination of these drugs, and perhaps animal trials should be carried out to study the drug reaction and pharmacokinetics.

Although interest in our laboratory have focused on the modulating effect of NSAIDs such as sulindac and indomethacin, the circumvention effect of these two drugs on melphalan cytotoxicity in the RPMI-2650 melphalan-resistant variant raises the following questions: are these two drugs the inhibitors of MRP1 only, or can they also inhibit other members of MRP family? Also, are these two drugs always effective in reversing MRP1-mediated drug resistance? If not, what limits their effects? Further work is required to establish the exact mechanisms by which NSAIDs exert their chemotherapy circumvention, e.g. are they substrates of MRP, or merely inhibitors?

It is concluded from the later work of this project that melphalan treatment can increase invasiveness of the RPMI-2650 and DLKP cells whereas taxol treatment cannot. This pointed out that chemotherapy can not only kill cancer cells, but can also result in induction or selection of cancer cells which are more malignant. The dogma that chemotherapy always plays an important role in cancer cure is not always true. From the work on the relationship between melphalan resistance and invasion phenotype in the HT-1080, NIH-3T3, A549, DLKP, DLKP-C, COR-L23S and COR-L23R cell lines in this study, there seems to be a trend that melphalan-resistant cell lines are generally more invasive than melphalan-sensitive cell lines. One question that needs to be answered is exactly what mechanisms link melphalan resistance to cell invasion, *i.e.* what events does melphalan treatment trigger to lead to increased cell invasion and metastasis? Another question is: How widely does melphalan treatment exert its effect on cell invasion? Is it on a few cell lines or tumours or on most cell lines or tumours? Intensive research, both scientifically and clinically, is required to answer these questions. Future work may start by finding out whether a common transcription factor exists to turn on two sets of genes, those responsible for melphalan resistance, e.g. MRP genes, and those responsible for increased cell invasion, e.g. MMP genes. Studies with the MRP gene promoter indicated that wild-type (wt) p53 markedly suppressed MRP promotor activity whereas mutant p53 had very little inhibitory effect. Wt p53 acts as a negative regulator of MRP gene transcription at least in part by diminishing the effect of a powerful transcription factor SP-1 (Wang and Beck, 1998). Binding sites for the transcription factors AP-1 and SP-1 were also found in MMP-9 gene which suggested that AP-1 and SP-1 were responsible for the induction of expression of MMP-9 (Sato and Seiki, 1993; Baylis *et al.* 1995). Sato *et al.* (1993) also reported that v-src activates the expression of MMP-

9 gene through the AP-1 site. All these results suggest that v-src, AP-1 or SP-1 may link MRP expression to cell invasion.

Moreover, it has been shown that the cisplatin-resistant variant, DLKP-C was more invasive than the DLKP parental cell line. Further studies are required to establish if a correlation exists between drug resistance and cell invasion in this cell line.

Finally, as discussed in section 4.5, an entirely different picture may emerge when drug resistant variants are selected *in vivo*. It would be of great interest to culture the MDR variants established in this study under more *in vivo*-like conditions such as three-dimensional spheroids to determine their drug resistance and cell invasion phenotypes.

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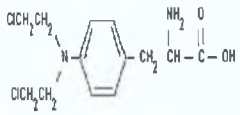
6. Appendices

6.1 Abbreviations

ADR	Adriamycin
ATCC	American Tissue Culture Collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BSO	Buthionine sulfoximide
cdc	Cell division cycle
CDK	Cyclin Dependent Kinase
cDNA	Complementry DNA
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTT	DiThiothretol
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamino tetra-acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
FITC	Fluorescein-isocyanate
5-FU	5-fluorouracil
GSH	Glutathione
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
HEPES	4-(2-hydroxethyl)-piperazine ethane sulphonic acid
IC_{50}	Inhibitory concentration 50%
Ig	Immunoglobulin
IGF	Insulin-like growth factor
KCl	Potassium chloride
kDa	Kilodalton
LRP	Lung resistance protein
MDR	Multidrug resistance
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium chloride

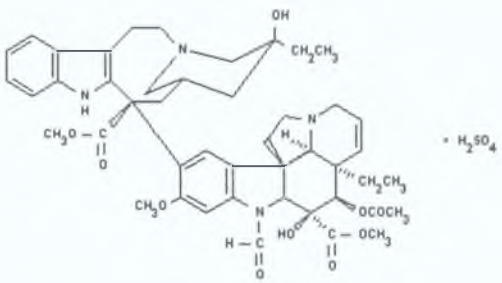
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
mRNA	Messenger RNA
MRP	Multidrug resistance associated protein
MT	Metallothionein
MW	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCTCC	National Cell & Tissue Culture Centre
NEAA	Non essential amino acids
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
Pgp	P-glycoprotein
PKC	Phosphokinase C
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
TBE	Tris boric acid, EDTA buffer
TBS	Tris buffered saline
TGF	Transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
TNF	Tumour necrosis factor
UV	Ultraviolet
VCR	Vincristine
VEGF	Vascular endothelial growth factor
VLB	Vinblastine
VP16	Etoposide
v/v	Volume to volume ratio
w/v	Weight to volume ratio

6.2 Chemical structures of chemotherapeutic drugs



M2011

Melphalan



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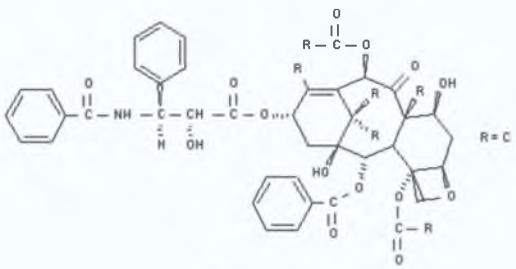
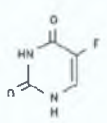
Vincristine



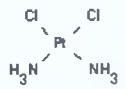
T7402

Taxol



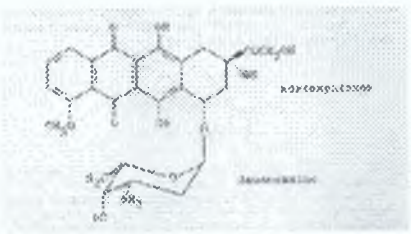
F0027

5-FU

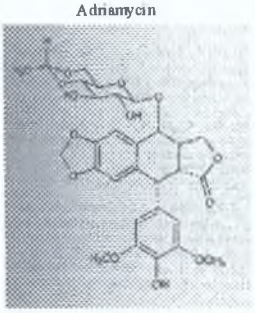


47,930-8

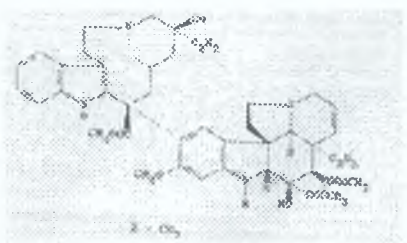
Cisplatin



Adriamycin

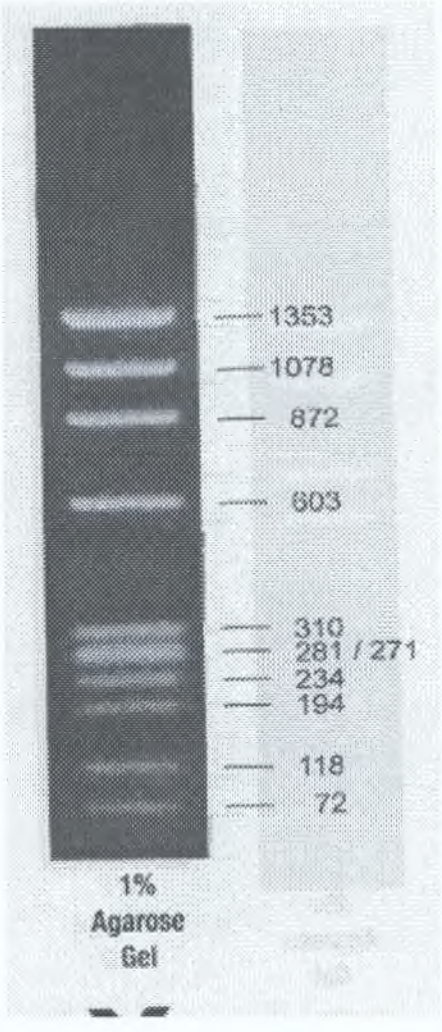


VP-16



Vinblastine

6.3 ϕ 174 DNA/Hae III marker used in RT-PCR



6.4 Molecular weight of the chemotherapeutic drugs

Adriamycin	543.54
Vincristine	824.9
Vinblastine	811
VP-16	588.57
Taxol	853.92
Melphalan	305.20
Cisplatin	300.05
Cadmium Chloride	183.32
5-FU	130.1