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Selection with melphalan or paclitaxel (Taxol) yields variants with different patterns of multidrug resistance, integrin expression and *in vitro* invasiveness

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Abstract

A melphalan-resistant variant (Roswell Park Memorial Institute (RPMI)-2650Ml) and a paclitaxel-resistant variant (RPMI-2650Tx) of the drug-sensitive human nasal carcinoma cell line, RPMI-2650, were established. The multidrug resistance (MDR) phenotype in the RPMI-2650Tx appeared to be P-glycoprotein (PgP)-mediated. Overexpression of multidrug resistant protein (MRP) family members was observed in the RPMI-2650Ml cells, which were also much more invasive *in vitro* than the parental cell line or the paclitaxel-resistant variant. Increased expression of α_2, α_5, α_6, β_1 and β_4 integrin subunits, decreased expression of α_4 integrin subunit, stronger adhesion to collagen type IV, laminin, fibronectin and matrigel, increased expression of MMP-2 and MMP-9 and significant motility compared with the parental cells were observed, along with a high invasiveness in the RPMI-2650Ml cells. Decreased expression of the α_2 integrin subunit, decreased attachment to collagen type IV, absence of cytokeratin 18 expression, no detectable expression of gelatin-degrading proteases and poor motility may be associated with the non-invasiveness of the RPMI-2650Tx variant. These results suggest that melphalan exposure can result in not only a MDR phenotype, but could also make cancer cells more invasive, whereas paclitaxel exposure resulted in MDR without increasing the *in vitro* invasiveness in the RPMI-2650 cells.

Keywords: Melphalan; Paclitaxel (Taxol); Multidrug resistance (MDR); Invasion; Multidrug resistance protein (MRP); Integrin; Matrix metalloproteinase (MMP)

1. Introduction

Melphalan and paclitaxel are chemotherapeutic drugs with different mechanisms of cytotoxic action. Melphalan, also called L-phenylalanine mustard, is an alkylating agent. The alkylating agents are highly reactive compounds that can form covalent bonds with a number of nucleophilic groups in proteins and nucleic acids. These reactions cause cross-linking between DNA strands or linkages between bases within the same strand of DNA, resulting in DNA synthesis inhibition. Melphalan is used in the treatment of multiple myeloma, melanoma, acute leukaemia, malignant lymphoma, ovarian, breast and colon cancer and Ewing's sarcoma.

Paclitaxel, a natural product originally isolated from the bark of *taxus brevifolia*, has significant antitumour activity in a number of human cancers, including advanced ovarian, breast and non-small cell lung carcinomas. Paclitaxel enhances the assembly of stable microtubules from tubulin dimers and inhibits their depolymerisation. Incubation of cells with paclitaxel leads to the formation of abnormal bundles of microtubules and results in the arrest of cells in the G_2/M phase of the cell cycle.

Despite advances in the use of chemotherapeutic drugs, treatment is frequently hindered by the development of resistance of tumour cells to the anticancer agents, often associated with the emergence of multidrug resistance (MDR). The mechanisms of MDR [1] include: the exclusion of drug from the cell by overexpression of either P-glycoprotein (Pgp) or various members of multidrug resistance protein (MRP) family;

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alteration in levels or properties of drug targets such as topoisomerase II; increased detoxification due to enhanced activity of glutathione S-transferase (GST); 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.

Invasion and metastasis are also factors responsible for many deaths from cancer. Invasion of the basement membrane is one of the critical steps in metastasis. Dynamic interactions among cell adhesion molecules (such as integrins), extracellular matrix (ECM) proteins (such as collagen type IV, laminin and fibronectin), proteases (such as matrix metalloproteinases (MMPs)), cytoskeletal proteins and signalling molecules all contribute to the invasive behaviour of the tumour cells.

There is evidence in the literature that invasion and metastasis may sometimes be related to the MDR phenotype. A relationship between these two phenotypes has been demonstrated by two types of experiment: firstly, in some cases, invasive/metastatic cells develop drug resistance more readily than non-invasive/metastatic cells [2,3]; secondly, some tumour cells selected for resistance to chemotherapeutic drugs are more invasive/metastatic relative to non-resistant parental cells [4–10]. Mechanisms underlying this relationship, or how frequently such a relationship occurs, remain unclear.

To further investigate the mechanisms of resistance to melphalan and paclitaxel and the effect of these drugs on cell invasion, a melphalan-resistant variant (Roswell Park Memorial Institute (RPMI)-2650Ml) and a paclitaxel-resistant variant (RPMI-2650Tx) of a human nasal carcinoma cell line, RPMI-2650, were established.

2. Materials and methods

2.1. Chemicals

Paclitaxel and VP-16 were obtained from Bristol-Myers Squibb (Dublin, Ireland), doxorubicin and 5-fluorouracil (5-FU) from Farmitalia Carlo Erba (Milton Keynes, UK) and vincristine, vinblastine and cisplatin from Lederle (Dublin, Ireland). All media and serum used in the maintenance of the cell lines were obtained from GIBCO BRL (Paisley, UK). Cyclosporin A was purchased from Sandoz (Basle, Switzerland). Reagents used in the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis were purchased from Promega (Southampton, UK). All other chemicals were obtained from Sigma (Dublin, Ireland).

2.2. Cell lines

The RPMI-2650 cell line (originally derived from the pleural effusion of a patient with an extensive malignant tumour of the nasal septum) [11] as well as the A549, HT-1080 and NIH-3T3 cell lines were obtained from the ATCC (American Type Culture Collection). The human ovarian 2008 cell line transfected with cDNA for *MRP3* was obtained from the laboratory of P. Borst, Amsterdam, The Netherlands. The RPMI-2650 melphalan- or paclitaxel-resistant variants were derived by continuous exposure of the parental cells to increasing concentration of melphalan or paclitaxel over a period of 9 or 6 months, respectively, starting from 1.25 μg/ml melphalan to a final concentration of 6.5 μg/ml and from 4 ng/ml paclitaxel to 200 ng/ml. The positive control cell line for Pgp detection was DLKP-A, an doxorubicin-selected variant of the DLKP lung carcinoma cell line [12]. All the cell lines used in this study with the exception of DLKP-A were cultured in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum (FCS) (10% new born calf serum for NIH-3T3), L-glutamine (2 mM), 1% sodium pyruvate and 1% non-essential amino acid. The DLKP-A cell line was cultured in DMEM/Ham's F12 with 5% FCS and 2 mM L-glutamine. COR-L23R, an MRP-overexpressing drug-resistant variant of a human lung carcinoma cell line, was a gift from P. Twentyman, Cambridge, UK. Antibiotics were not used in the growth media. All cell lines were free from mycoplasma as tested with the indirect Hoechst DNA staining method.

2.3. Antibodies

Pgp was detected by immunocytochemistry and western blotting and topoisomerase II α was detected by immunocytochemistry with MDR-1 (BRI, Ireland) and topoisomerase II α (BRI, Ireland) monoclonal mouse antibodies, respectively, which were developed in our laboratory [13,14]. The MRP1 and lung resistance related protein (LRP) monoclonal antibodies MRPr1 and LRP-56 were purchased from TCS, UK. The canalicular multi-specific organic anion transporter (cMOAT) (MRP2) and MRP3 monoclonal mouse anti-human antibodies were a gift from R. Scheper, Amsterdam, The Netherlands. The GST α (NCL-GST alpha) and GST μ (NCL-GST muM2) polyclonal antibodies were purchased from NOVO Lab, UK. GST π polyclonal antibody (A3600) was purchased from DAKO Ltd, UK. Expression of integrins in the cells was investigated by immunocytochemistry using a range of mouse monoclonal anti-human α_1, α_2, α_3, α_4, α_5, α_6, β_1 and β_4 integrin subunits antibodies (Serotec, UK).

2.4. Cytotoxicity assays

Toxicity to melphalan or paclitaxel and a range of other drugs in the RPMI-2650 cell line and RPMI-2650Ml or RPMI-2650Tx was determined by the acid phosphatase method which measures viable cell number

after 5–7 days of continuous exposure to drugs as previously described [15]. The circumvention assays in which cytotoxicity of the drug is compared in the presence or absence of another drug which is capable of blocking one or more of the efflux pumps were performed as outlined by Heenan and colleagues [15]. Toxicity assays were carried out at least three times for each cell line for each drug and each assay contained eight replica wells for each drug concentration.

2.5. Western blotting

Western blotting was performed by the method of Moran and colleagues [16].

2.6. Immunocytochemistry

Immunocytochemistry was performed using the ABC method as outlined by Moran and colleagues [16].

2.7. Immunofluorescence

Detection of α-tubulin, β-tubulin, cytokeratin 18 and vimentin was performed by indirect immunofluorescence studies as outlined by Moran and colleagues [16].

2.8. Intracellular doxorubicin distribution

Studies on the subcellular distribution of the drug were performed by the method of Cleary and colleagues [17].

2.9. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from pelleted cells was extracted with TRI-reagent (Sigma) according to manufacturer's instructions. The mRNAs investigated included those coding for MDR-1 (Pgp), MRP1, cMOAT (MRP2), MRP3, MRP4, MRP5, α_2 and β_1 integrin subunits. In all PCR reactions, the number of PCR cycles used was 30 cycles. The sequence primers for *mdr-1*, *mrp1*, *mrp2*, *mrp3*, *mrp4*, *mrp5* and α_2 and β_1 integrin subunits have been published previously [18–21]. *β-actin* was used as the endogenous control for all reactions. The products formed were analysed by agarose gel electrophoresis and densitometry (Imaging Densitometer, Bio-Rad, UK, model GS-670). The size of the resulting PCR products was determined by comparison with ϕ 174 DNA/*Hae III* marker (Promega).

2.10. Adhesion assays

For the adhesion assays, collagen type IV, fibronectin and laminin (Sigma) were diluted to 25 μg/ml with PBS while matrigel (Sigma), a reconstituted basement membrane, was diluted to 1 mg/ml. 250 μl aliquots were placed into each well of a 24-well plate (Costar, USA) and the plates were incubated at 4°C overnight. The following day, the supernatants were removed and the wells rinsed twice with sterile phosphate buffered saline (PBS). 0.5 ml sterile 0.1% bicinchoninic acid (BSA)/PBS (w/v) was added to each well and the plates were incubated at 37°C for 20 min to reduce non-specific binding. After 20 min, the plates were rinsed twice with PBS and the cells which were harvested from the flask were suspended in serum-free DMEM medium and plated at a concentration of 2.5×10^4 cells per well in triplicate and incubated at 37°C for 60 min. After 60 min, the medium was removed and the plate rinsed with PBS. The cells were then stained with 0.5 ml 0.25% crystal violet dye/well for 10 min. After 10 min, the plate was rinsed and allowed to dry. The dye was eluted with 200 μl 33% glacial acetic acid/well and 100 μl aliquot/well was transferred to a 96-well plate and the absorbance was read on enzyme linked immunosorbent assay (ELISA) reader at 570 nm.

2.11. Invasion assays and motility assays

Invasion assays were performed by a modification of the method described by Albini and colleagues [22]. Matrigel (Sigma) was diluted to 1 mg/ml in serum-free DMEM medium. 100 μl of 1 mg/ml matrigel was placed into each insert (Falcon) (8.0 μm pore size) which stood in wells of a 24-well plate (Costar). The inserts and the plate were incubated overnight at 4°C. The following day, cells were harvested and suspended in DMEM containing 5% FCS at a concentration of 1×10^6 cells/ml. The inserts were washed with serum-free DMEM, then 100 μl of the cell suspension was added to each insert and 250 μl of DMEM containing 5% FCS was added to the well underneath the insert. Cells were incubated at 37°C for 48 h. After this time period, the inner side of the insert was wiped with a wet swab to remove the cells while the outer side of the insert was gently rinsed with PBS and stained with 0.25% crystal violet for 10 min, rinsed again and then allowed to dry. The inserts were then viewed under the microscope.

The procedure for carrying out motility assays was identical to the procedure used for invasion assays with the exception that the inserts were not coated with matrigel.

2.12. Time-lapse videomicroscopy

Time-lapse videomicroscopy was carried out using a Nikon inverted microscope (Micron Optical, Ireland) equipped with phase-contrast optics, linked to a Mitsubishi CCD-100 colour video camera. Images were recorded on a video tape. Recording speed was set at 3.22 s/field, which at normal playback speed resulted in an acceleration factor of 160. Cells were seeded at

1×10^5 cells /ml in a 25 cm^2 flask 24 h before the recording was started. Cell motility was monitored by locating the initial and final positions of the cells during a specified time period.

2.13. Gelatin zymography

Zymography was used to assess the level of proteolytic activity of different proteases [23]. Gelatin is a substrate for MMPs, serine and cysteine proteases. The protein concentration of the serum-free medium in which cells were cultured was determined by the BCA protein assay (Pierce, IL) and 20 μg of supernatant protein was applied to non-reduced sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% gel containing 0.1% gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 at room temperature with gentle shaking for 30 min and incubated in substrate buffer (50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$) at 37°C for 18–24 h. Gels were then stained with 2.5 mg/ml Coomassie Blue for 2 h and destained in a mixture of acetic acid:isopropanol: distilled water (1:3:6) until clear bands were visible. Gelatinase activity was determined as distinct, clear bands.

3. Results

3.1. Cross-resistance profiles

The sensitivity of the RPMI-2650Tx or the RPMI-2650Ml to a range of cancer chemotherapeutic agents was determined (Tables 1 and 2). The highest levels of resistance in the RPMI-2650Tx cells were observed with vincristine, doxorubicin and its selecting agent, paclitaxel (Table 1). The low, but significant, levels of resistance to melphalan, 5-FU and cadmium chloride indicate the existence of additional resistance mechanisms. The results (Table 2) showed that the RPMI-2650Ml cells were 11 times more resistant to melphalan than the parental cells. This variant showed highest resistance to cadmium chloride and also exhibited resistance to doxorubicin, vinblastine, VP-16 and to a lesser extent to vincristine and cisplatin compared with its parental cell line. No significant resistance was observed to paclitaxel or to 5-FU. The RPMI-2650Ml was more resistant to vinblastine than vincristine while in the RPMI-2650Tx the situation was reversed.

3.2. MDR-1/Pgp detection

Western blotting (Fig. 1), immunocytochemistry (data not shown) and RT-PCR (Fig. 2) were used to detect the levels of MDR-1/Pgp in the RPMI-2650 cell line and its paclitaxel- or melphalan-resistant variants. All these methods produced consistent results indicating that MDR-1/Pgp was significantly overexpressed at the mRNA and protein levels in the RPMI-2650Tx, while only slightly elevated expression of MDR-1/Pgp was observed in the RPMI-2650Ml compared with the parental cells.

3.3. MRP detection

Results obtained from western blotting (Fig. 1), immunocytochemistry and RT-PCR (Fig. 2) indicated that MRP1 expression was decreased at both protein and mRNA levels in the RPMI-2650Tx compared with the parental cells. cMOAT (MRP2) and MRP3 were not detected in the RPMI-2650Tx. Increased expression of both MRP1 and cMOAT (MRP2) at the protein and mRNA levels was observed in the RPMI-2650Ml compared with the parental cells (Figs. 1 and 2). Expression of MRP3 in this variant was increased at the mRNA

Table 1
Comparison of chemosensitivity in the RPMI-2650 cell line and the RPMI-2650Tx variant[a]

Drug	IC_{50} (nM)		
	RPMI-2650	RPMI-2650Tx	Fold-resistance of the RPMI-2650Tx
Doxorubicin	18.5±3.5	4030±350	218
Vincristine	2.4±0.4	1427±377	595
Vinblastine	1.8±0.1	168±18	93
VP-16	23±3	464±53	20
Paclitaxel	2.3±0.9	519±18	226
Melphalan	582±10	2621±164	4.5
5-Fluorouracil (5-FU)	1624±164	5898±98	3.6
Cisplatin	1494±39	1600±267	1.1
Cadmium chloride	709±68	5891±465	8.3

[a] The IC_{50} values are the mean of a minimum of three repeat experiments.

Table 2
Comparison of chemosensitivity in the RPMI-2650 cell line and the RPMI-2650Ml variant[a]

Drug	IC_{50} (nM)		
	RPMI-2650	RPMI-2650Ml	Fold-resistance of the RPMI-2650Ml
Doxorubicin	18.5±3.5	425±70	23
Vincristine	2.4±0.4	15±2.4	6.3
Vinblastine	1.8±0.1	42±3.4	23
VP-16	23±3	587±60	26
Paclitaxel	2.3±0.9	3.9±1.1	1.7
Melphalan	582±10	6135±172	11
5-Fluorouracil (5-FU)	1624±164	1784±16	1.1
Cisplatin	1494±39	3874±125	2.6
Cadmium chloride	709±68	22038±218	31

[a] The IC_{50} values are the mean of a minimum of three repeat experiments.

level, but not at protein level (Figs. 1 and 2). MRP4 and MRP5 were not detected by RT-PCR in any of these cell lines.

3.4. Immunocytochemistry of LRP, GSTα, GSTπ, GSTμ and metallothionein

Immunocytochemistry showed no significant difference in metallothionein and GST π expression between the RPMI-2650 parental cells and its paclitaxel- and melphalan-resistant variants. LRP, GST α and GST μ were undetectable in any of these cell lines (data not shown).

3.5. Detection of α-tubulin, β-tubulin, cytokeratin 18 and vimentin

Possible altered expression of α- and β-tubulin, cytokeratin 18 and vimentin in the drug resistant cells was investigated by immunofluorescence. Results illustrated slightly more intense fluorescence for α-tubulin and β-tubulin in the RPMI-2650 parental cells than in the paclitaxel- or melphalan-resistant cells. The RPMI-2650 and the RPMI-2650Ml cells showed faint cytoplasmic fluorescence for cytokeratin 18 while little or no fluorescence staining was observed in the RPMI-2650Tx cells. Intense fluorescence for vimentin was observed in all of these cell lines (data not shown).

3.6. Subcellular location of doxorubicin

In order to investigate possible altered intracellular drug distribution in the resistant variants, fluorescence microscopy of doxorubicin-treated cells was performed (Fig. 3). Intense doxorubicin fluorescence was observed predominantly in the nuclei of the RPMI-2650 cells, whereas very faint doxorubicin nuclear fluorescence and speckles of cytoplasmic fluorescence were observed in the RPMI-2650Tx cells. Faint nuclear fluorescence was observed in the RPMI-2650Ml cells to a lesser extent than in the RPMI-2650Tx cells. The addition of verapamil and cyclosporin A resulted in a significant increase in the intensity of nuclear fluorescence in the RPMI-2650Tx and the RPMI-2650Ml with little visible increase in the parental cells.

3.7. Circumvention studies with verapamil and cyclosporin A

The Pgp-overexpressing cell line DLKP-A and MRP1-overexpressing cell line COR-L23R were used as

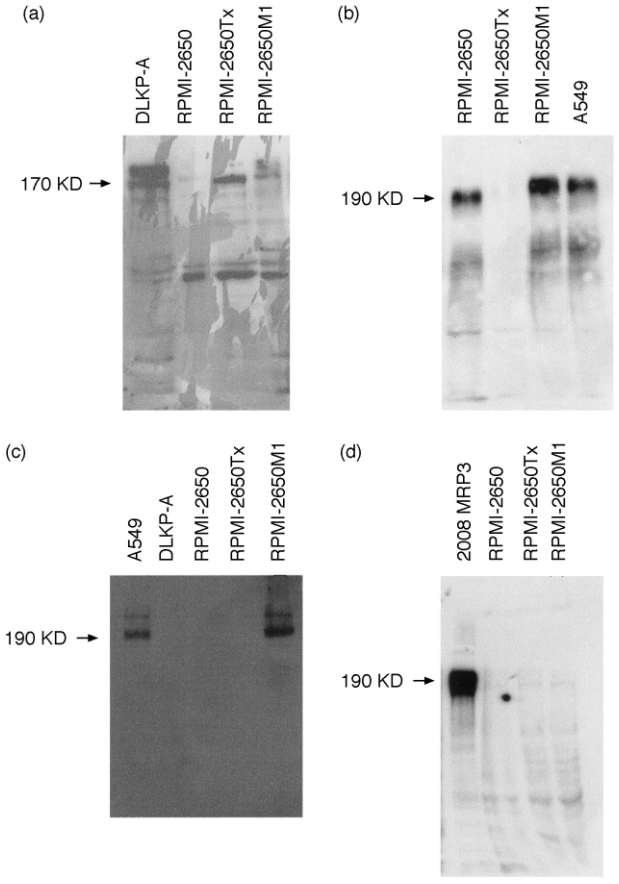

Fig. 1. Western blot of (a) Pgp, (b) MRP1, (c) cMOAT (MRP2) and (d) MRP3 in the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants.

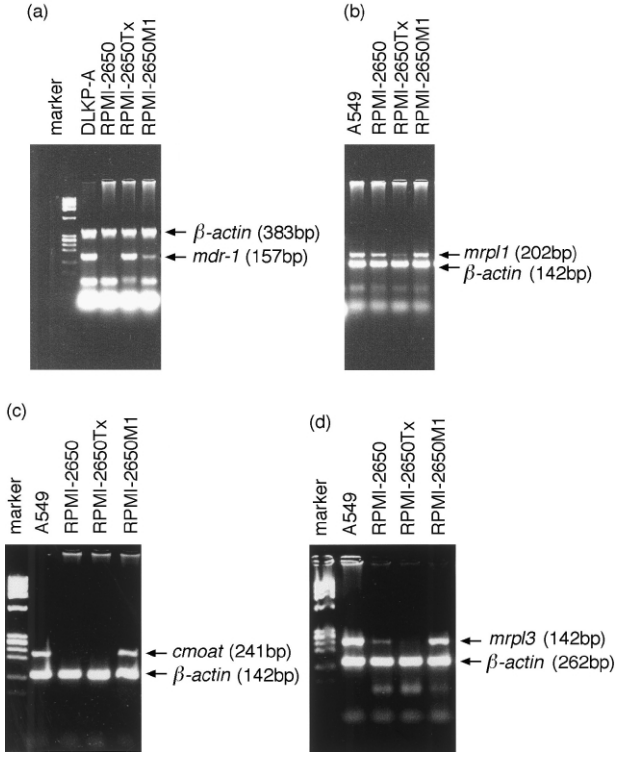

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of (a) *mdr-1*, (b) *mrp1*, (c) *cmoat* (*mrp2*) and (d) *mrp3* in the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants.

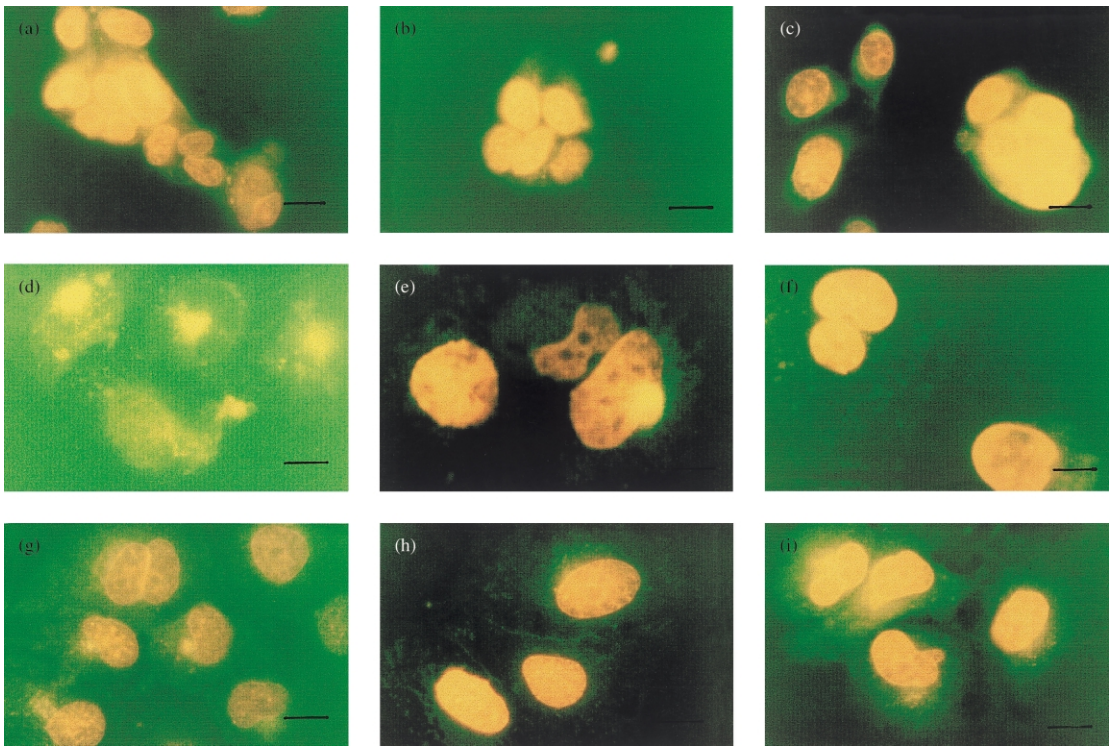

Fig. 3. Doxorubicin localisation in (a) the RPMI-2650 cell line (scale bar: 5 μm) and (b) after incubation with verapamil (scale bar: 5 μm) or (c) with cyclosporin A (scale bar: 5 μm), in (d) the RPMI-2650Tx (scale bar: 5 μm) and (e) after incubation with verapamil (scale bar: 5 μm) or (f) with cyclosporin A (scale bar: 5μm), in (g) the RPMI-2650Ml (scale bar: 5 μm) and (h) after incubation with verapamil (scale bar: 5 μm) or (i) with cyclosporin A (scale bar: 5 μm).

positive controls. The results of the circumvention experiments (Table 3) showed that neither verapamil nor cyclosporin A had a significant modulating effect on the sensitivity to doxorubicin in the RPMI-2650 cells. However, verapamil and cyclosporin A significantly increased doxorubicin sensitivity in the RPMI-2650MI and the RPMI-2650Tx variants respectively.

3.8. Detection of integrins

Immunocytochemistry was carried out to detect the presence of α_1, α_2, α_3, α_4, α_5, α_6, β_1 and β_4 integrin subunits in the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants. Results are summarised in Table 4. Uniform α_2 staining was observed in the RPMI-2650 cell line. The α_2 staining seemed to concentrate in certain subclones of the RPMI-2650Tx (Fig. 4). An increase in β_1 integrin expression was observed in the RPMI-2650Tx (Fig. 4). Compared with the parental cells, increased expression of α_2, α_5, α_6, β_1 and β_4 integrin subunits, especially the β_1 subunit, was observed in the RPMI-2650Ml cell line. Strong staining for α_4 integrin subunit was observed in the parental cell line, but not in its melphalan- and paxlitaxel-resistant variants. Results of the RT-PCR analysis on α_2 and β_1 integrin subunits (Fig. 5) are consistent with the results of immunocytochemistry.

3.9. Adhesion assays

Results obtained from the adhesion assays (Fig. 6) indicate that the RPMI-2650Tx was more adhesive to fibronectin, laminin and matrigel, but less adhesive to collagen type IV than the parental cell line. RPMI-2650Ml was more adhesive to collagen type IV, fibronectin, laminin and matrigel than the RPMI-2650 parental cell line and the RPMI-2650Tx cell line.

Table 3
Circumvention studies with verapamil and cyclosporin A in the RPMI-2650 cell line and its taxol- and melphalan-resistant variants[a]

Cell line	IC_{50} (nM)		
	ADR	ADR + verapamil (1 μg/ml)	ADR + cyclosporin A (1 μg/ml)
COR-L23R	2097±364	938±146	1435±129
DLKP-A	5750±275	441±157	1582±167
RPMI-2650	110±37	59±18	77±19
RPMI-2650Tx	5750±224	2171±195	497±118
RPMI-2650Ml	690±138	48±15	304±125

ADR, doxorubicin.
[a] The data were from three repeat experiments.

Table 4
Immunocytochemical detection of various integrin subunits in the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants[a]

Cell line	α_1	α_2	α_3	α_4	α_5	α_6	β_1	β_4
RPMI-2650	–	+	Trace	++	Trace	Trace	++	–
RPMI-2650Tx	–	Clonal	Trace	–	Trace	Trace	+++	–
RPMI-2650Ml	–	++	Trace	–	+	+	++++	+

[a] ++++ = very strong staining; +++ = strong staining; ++ = medium degree staining; + = weak staining; – = no staining.

3.10. Invasion assays

The RPMI-2650Ml was significantly more invasive than the RPMI-2650 parental cells and the RPMI-2650Tx which were unable to invade through the matrigel-coated bases of the inserts onto the surface of the outside walls (Fig. 7). Results of *in vitro* invasiveness for the positive control cell line HT-1080 and negative control cell line NIH-3T3 confirmed that conditions for the invasion assay were correct.

3.11. Motility assays/time-lapse videomicroscopy

Motility assays were conducted to compare the locomotive ability between the RPMI-2650 cell line and its drug resistant variants. Results showed that the RPMI-2650 cells were not motile, whereas the RPMI-2650Tx showed very poor motility and the RPMI-2650Ml exhibited significant motility (Fig. 7). Time-lapse videomicroscopy supported these results. The RPMI-2650Tx moved approximately 1.5 times faster than its parental cells, whereas the RPMI-2650Ml moved approximately 12 times faster than its parental cells (data not shown).

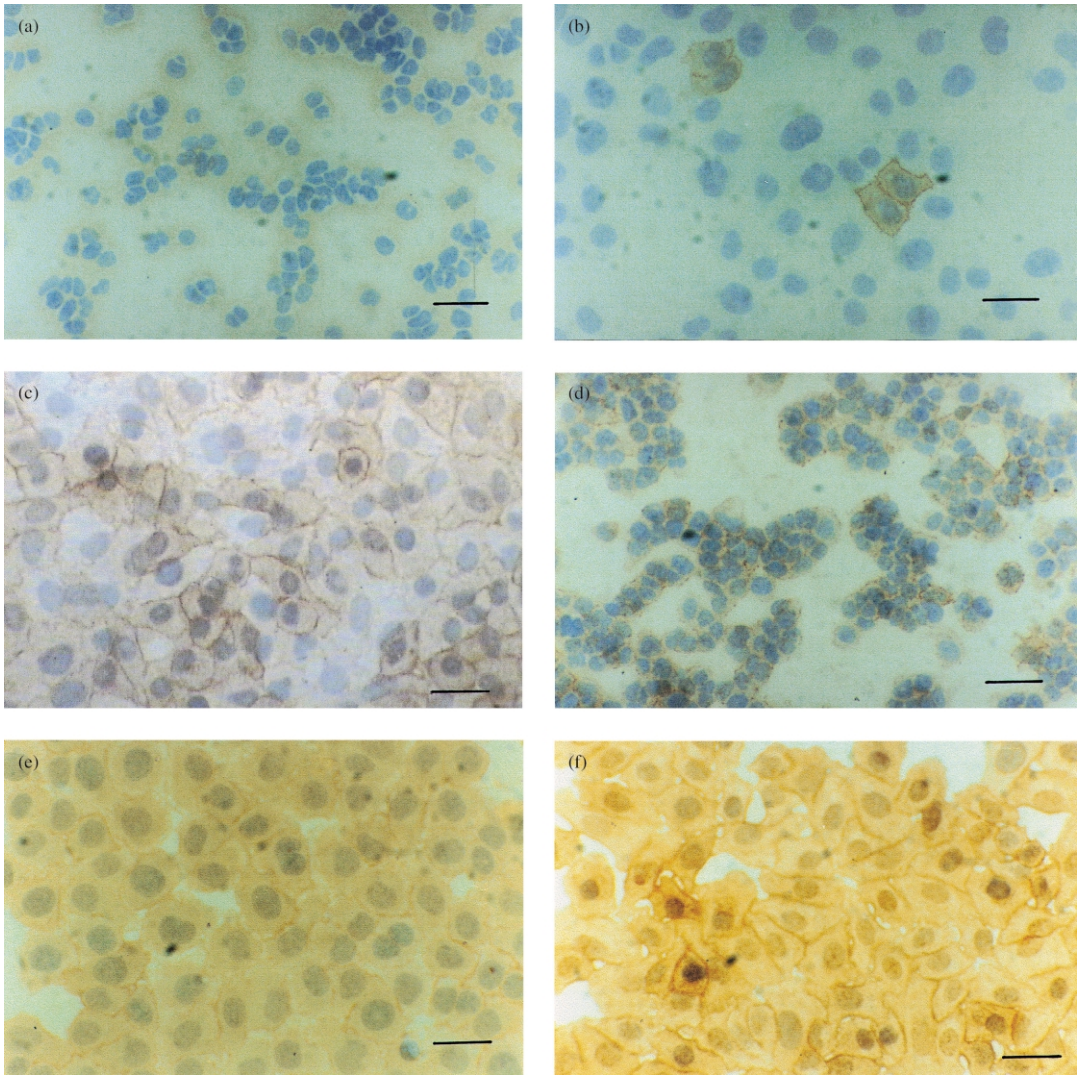

Fig. 4. Immunocytochemical staining for α_2 integrin subunit in (a) the RPMI-2650 cell line (scale bar: 25 μm), (b) the RPMI-2650Tx (scale bar: 25 μm) and (c) the RPMI-2650Ml (scale bar: 25 μm); for β_1 integrin subunit in (d) the RPMI-2650 cell line (scale bar: 25 μm), (e) the RPMI-2650Tx (scale bar: 25 μm) and (f) the RPMI-2650Ml (scale bar: 25 μm).

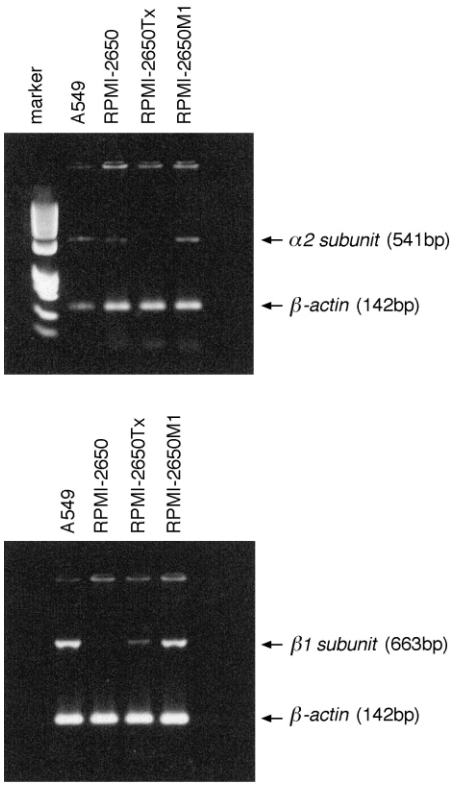

Fig. 5. RT-PCR analysis of α_2 (left panel) and β_1 (right panel) integrin subunits in the RPMI-2650 and its paclitaxel- and melphalan-resistant variants.

3.12. Assays of gelatin-degrading proteases by zymography

Zymography (using BHK-21 cells as a positive control for MMP-2 and MMP-9) indicates that the RPMI-2650Ml secretes MMP-2 and MMP-9, whereas the parental cells and the RPMI-2650Tx do not (Fig. 8). After the gel was incubated with ethylene diamine tetra acetic acid (EDTA), a chelating agent which can bind the zinc and calcium which are needed for the activation of MMPs, the bands of the BHK cell line and the RPMI-2650Ml disappeared, suggesting that these bands were MMPs. The bands in the parental RPMI-2650 cells did not, however, disappear. The three bands of the parental cells became very weak after the gel was incubated with a serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF), suggesting these bands were serine proteases (data not shown).

4. Discussion

In an attempt to identify the mechanisms of MDR in the RPMI-2650Ml and the RPMI-2650Tx variants, alterations in a number of MDR markers were investigated. Overexpression of MRP1, cMOAT (MRP2) (and possibly to some extent Pgp) demonstrated by western blotting and RT-PCR may lead to the decreased accu-

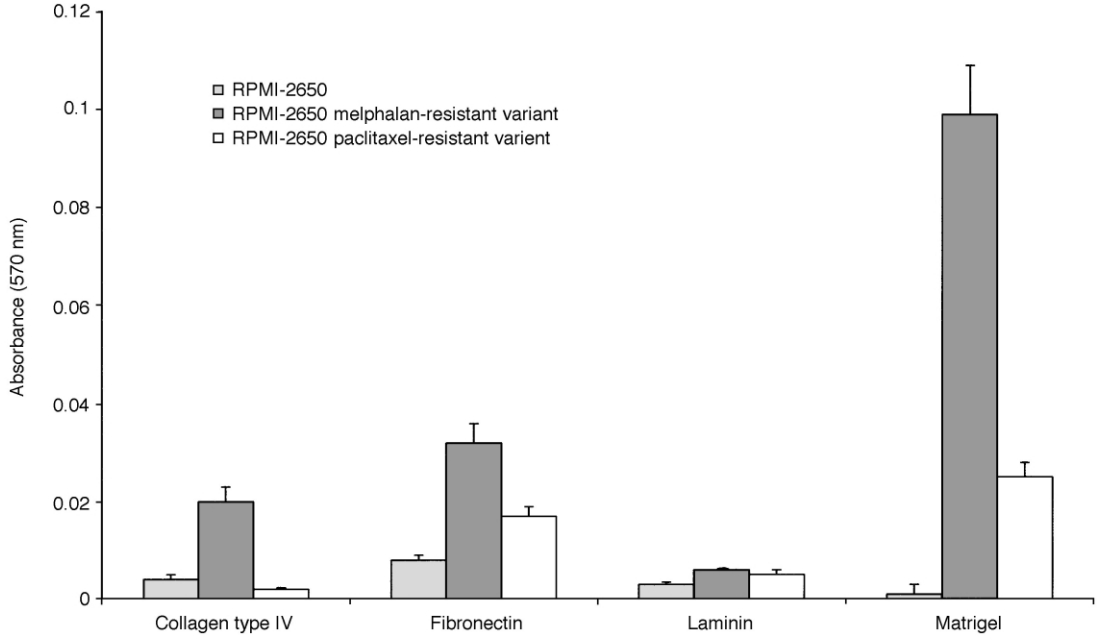

Fig. 6. Attachment of the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants to extra cellular matrix (ECM) proteins: collagen type IV, fibronectin, laminin and matrigel. Results are expressed as absorbance at 570 nm of stained attached cells. The data is shown as means ± standard deviations (S.D.) for a minimum of three experiments.

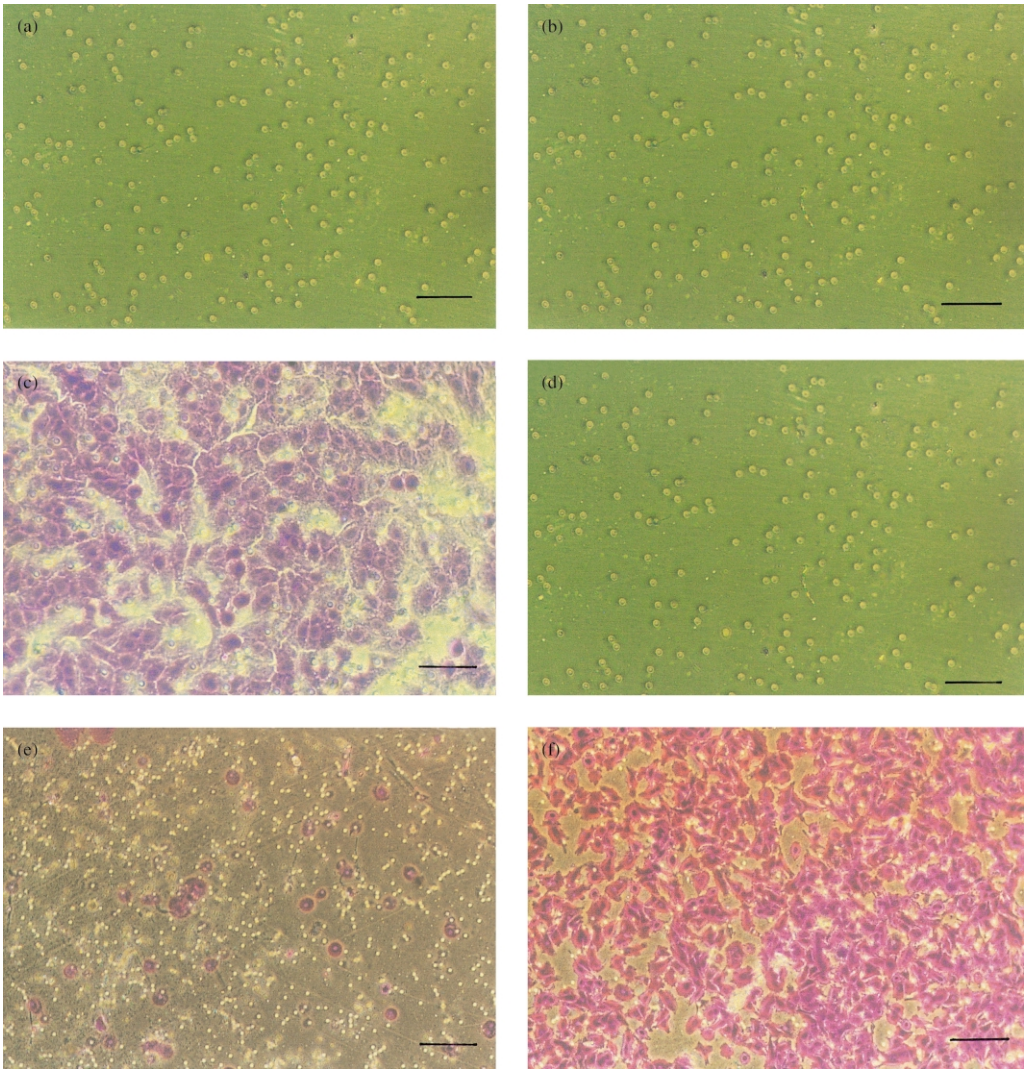

Fig. 7. Invasion assays of (a) the RPMI-2650 cell line (scale bar: 80 μm), (b) the RPMI-2650Tx (scale bar: 80 μm) and (c) the RPMI-2650Ml (scale bar: 80 μm). Motility assays of (d) the RPMI-2650 cell line (scale bar: 80 μm), (e) the RPMI-2650Tx (scale bar: 80 μm) and (f) the RPMI-2650Ml (scale bar: 80 μm).

mulation of drug in the RPMI-2650Ml. Fluorescence microscopy and circumvention studies demonstrated that doxorubicin accumulation or doxorubicin sensitivity can be increased by verapamil and cyclosporin A in the RPMI-2650Ml, supporting a role for efflux pumps in the drug resistance in this variant. Particularly, verapamil seems to be more effective than cyclosporin A in circumventing doxorubicin resistance in the RPMI-2650Ml cells. Previous studies have also shown that verapamil is somewhat more effective than cyclosporin A as a chemosensitiser in circumventing drug resistance in MRP1-overexpressing cells [24]. The RPMI-2650Ml was 11-fold more resistant to melphalan compared with its parental cell line. This variant showed very high resistance to cadmium chloride which is a substrate for MRP1 [25] and cMOAT [26], and high resistance to

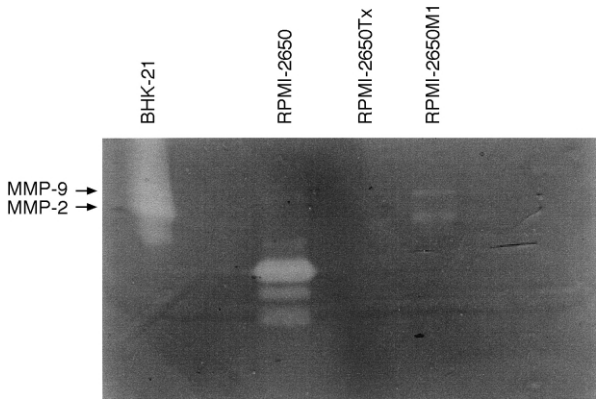

Fig. 8. Zymograph of gelatin-degrading proteases in the RPMI-2650 cell line and its paclitaxel- and melphalan-resistant variants. The BHK-21 cell line served as the positive control.

doxorubicin (a substrate of MRP1, cMOAT (MRP2), MRP3 and Pgp), vinblastine (a substrate of cMOAT (MRP2) and Pgp) and VP-16 (a substrate of MRP1, MRP3 [26] and Pgp). The cross-resistance profile is consistent with the involvement of MRP family members and Pgp (although its expression level is quite low) in multidrug resistance in the RPMI-2650Ml.

Jedlitschky and colleagues [27] demonstrated MRP1-mediated transport for the monochloro-mono[^{3}H] glutathionyl melphalan. Ishikawa and colleagues [25] reported that in a cisplatin-resistant variant (HL-60/R-CP) of the human myelocytic leukaemia HL-60 cell line, MRP1 was overexpressed. HL-60/R-CP cells were found to be cross-resistant to melphalan, chlorambucil, arsenite and cadmium. To date, cMOAT (MRP2), MRP3, MRP4, MRP5 and MRP6 have not been reported to link directly to melphalan resistance. This paper is the first report that melphalan exposure leads to the overexpression of cMOAT (MRP2) (at the mRNA level and protein level) and MRP3 (at the mRNA level).

The RPMI-2650Tx cell line was found to be resistant to paclitaxel (226-fold) and cross-resistant to doxorubicin, vincristine, vinblastine and VP-16. However, there was no significant resistance to melphalan, cadmium chloride, cisplatin and 5-FU, indicating the involvement of Pgp in its multidrug resistance phenotype. In agreement with this, overexpression of Pgp, but not MRP, LRP, topoisomerase II or GST, was detected both at protein and mRNA levels in RPMI-2650Tx. All these results, together with the results obtained from circumvention studies with verapamil and cyclosporin A suggest that Pgp plays a major role in the RPMI-2650Tx variant.

The results of immunofluorescence also demonstrated that RPMI-2650Tx displayed a decrease in cytokeratin 18 expression level compared with the RPMI-2650 parental cells and RPMI-2650Ml. Parekh and colleagues [28] reported that the cisplatin-resistant human ovarian 2008/C13* cell line contained markedly lower levels of cytokeratin 18 when compared with the sensitive parental cells. Transfection of full-length *cytokeratin 18* cDNA into this cell line increased sensitivity to cisplatin, thus suggesting a relationship between cytokeratin 18 expression and drug resistance. Any role for cytokeratin 18 in mediating paclitaxel resistance in the RPMI-2650Tx remains unclear.

Although the RPMI-2650 parental cells expressed MRP1 as detected by western blotting and RT-PCR, the results obtained from toxicity assays showed that they were very sensitive to various chemotherapeutic drugs. For doxorubicin, vincristine, vinblastine, paclitaxel, melphalan and 5-FU, they are more sensitive than the SKLU-1, BT-20, A-549 and MX-1 cell lines. These results suggest that MRP1 expressed in the cells may be non- or poorly-functional. The most conclusive result to support this assumption comes from the doxorubicin distribution studies. After a 4-h incubation with doxorubicin, strong accumulation of the drug was noted in the nuclei of the RPMI-2650 cells (Fig. 3). This indicated that MRP1 was poorly- or non-functional.

Tumour cells selected for resistance to chemotherapeutic drugs, e.g. doxorubicin [4,5] and methotrexate [6] have, in some instances, been reported to be more invasive or metastatic relative to non-resistant cells. Haga and colleagues [7] established 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 lung was observed in mice inoculated intravenously (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 [8]. 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 with the parental cells, which may be related to the overproduction of MMP-9 [9]. Similar results were also obtained with another cisplatin-resistant cell line [10]. Thus it seems that in some cases chemotherapy actually makes tumour cells more malignant.

Liotta [29] 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 ECM, such as integrins. The anchored tumour cells next secrete hydrolytic enzymes (or induce host cells to secrete enzymes) which can locally degrade the matrix. The third step is tumour cell locomotion into the region of the matrix modified by proteolysis. In this study, the results indicated that melphalan selection significantly alters the invasiveness of the cells *in vitro* whereas paclitaxel selection does not. Compared with the RPMI-2650 parental cells, increased expression of α_2, α_5, α_6, β_1 and β_4 integrin subunits which result in stronger adhesion to collagen type IV, laminin, fibronectin and matrigel, decreased expression of α_4 integrin subunit, and expression of MMP-2 and MMP-9 may all contribute to the high invasiveness of the RPMI-2650Ml variant. In the RPMI-2650Tx, the decreased expression of α_2 integrin subunit which results in decreased attachment to collagen type IV, lack of cytokeratin 18 and non-detection of gelatin-degrading proteases may be associated with its non-invasiveness.

$\alpha_2\beta_1$ integrin, which recognises collagen and laminin, is suggested to function as a tumour suppressor for epithelial malignancies [30]. However, Chen and colleagues [31] reported that low expression level of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins in a clone derived from a human squamous cell lung tumour and its failure to bind collagen may be

related to the lower metastatic potential of this clone. It is possible that the low expression level of $\alpha_2\beta_1$ integrin in the RPMI-2650Tx cells, resulting in its low adhesion to collagen type IV which is the major component of basement membrane, is related to the non-invasiveness of this variant, while the relatively higher expression level of $\alpha_2\beta_1$ integrin in the RPMI-2650Ml may be related to its invasiveness.

$\alpha_4\beta_1$ integrin which recognises fibronectin and vascular cell adhesion molecule-1 (VCAM-1) may influence the metastatic process at various stages [32]. It was reported that at the primary tumour site, expression of α_4 integrin subunit inhibits the ability of tumour cells to break loose [32]. This could be achieved either by strengthening of homotypic adhesion to adjacent tumour cells or by downregulation of MMPs. This may partially explain the non-invasiveness of the RPMI-2650 cells and the invasiveness of the RPMI-2650Ml. Antibody inhibition assays could be used to determine if specific integrins are directly involved in increasing invasiveness. Research by several other groups on MDR cell lines indicates that expression of integrin subunits varies in different drug resistant variants, suggesting expression of the particular integrins may be cell-type specific [33–35]. It is also worth noticing that certain integrins could induce either expression or activity of certain MMPs [36,37].

It is concluded from these studies that selection for melphalan resistance can increase invasiveness and motility of the RPMI-2650 cells, whereas paclitaxel exposure results in multidrug resistance without increasing *in vitro* invasiveness and motility. Research is in progress to establish if other anticancer drugs have similar effect in DLKP and in other cell lines. It must be emphasised that invasion and metastasis are complex *in vivo* phenomena, and that the *in vitro* invasion assay used is only a partial indicator of *in vivo* invasiveness. It is, however, generally accepted that the *in vitro* models have value in moving towards an understanding of invasion and metastasis [38]. This paper is the first report that melphalan exposure can increase cell invasiveness, while paclitaxel does not. It remains to be established exactly what mechanisms link melphalan resistance to cell invasion. Our data demonstrate multiple changes in the molecular phenotype of the melphalan-resistant cells, which also show increased invasiveness, but it remains unclear whether any or all of these changes are directly related to the invasive phenotype in the cell line. It is possible that a common transcription factor exists to turn on several functional sets of genes, including those responsible for melphalan resistance, e.g. MRP genes, and those responsible for increased cell invasion, e.g. MMP genes. Studies with MRP gene promoters indicated that transcription factors SP-1 and AP-1 modulate the transcriptional activity of MRP genes [39,40]. Binding sites for SP-1 and AP-1 were also found in *MMP-2* and *MMP-9* genes [41,42], suggesting that SP-1 or AP-1 may link MRP expression to cell invasion.

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References

1. Clynes M. *Multiple Drug Resistance in Cancer*. Dordrecht, The Netherlands, Kluwer Academic Publishers, 1998.
2. Cillo C, Dick JE, Ling V, Hill RP. Generation of drug-resistant variants in metastatic B16 mouse melanoma cell lines. *Cancer Res* 1987, **47**, 2604–2608.
3. Cillo C, Ling V, Hill RP. Drug resistance in KHT fibrosarcoma cell lines with different metastatic ability. *Int J Cancer* 1989, **43**, 107–111.
4. Giavazzi R, Miller L, Hart IR. Metastatic behavior of an adriamycin-resistant murine tumor. *Cancer Res* 1983, **43**, 5081–5086.
5. Scaddan PB, Dufresne MJ. Characterization of cysteine proteases and their endogenous inhibitors in MCF-7 and adriamycin-resistant MCF-7 human breast cancer cells. *Invasion Metastasis* 1993, **13**, 301–313.
6. Lücke Huhle C. Permissivity for methotrexate-induced DHFR gene amplification correlates with the metastatic potential of rat adenocarcinoma cells. *Carcinogenesis* 1994, **15**, 695–700.
7. Haga A, Nagase H, Kito H, Sato T. Invasive properties of cadmium-resistant human fibrosarcoma HT-1080 cells. *Cancer Biochem Biophys* 1997, **15**, 275–284.
8. Staroselsky AN, Mahlin T, Savion N, *et al*. Metastatic potential and multidrug resistance correlation in the B16 melanoma system. *J Exp Ther Oncol* 1996, **1**, 251–259.
9. Choi S, Okada F, Konoshi M, *et al*. Single treatment with cisplatin or UFT, but not their combination treatment enhances the metastatic capacity of mouse fibrosarcoma cells. *Anticancer Drugs* 1999, **10**, 235–243.
10. Mitsumoto M, Kamura T, Kobayashi H, Sonoda T, Kaku T, Nakano H. Emergence of higher levels of invasive and metastatic properties in the drug resistant cancer cell lines after the repeated administration of cisplatin in tumor-bearing mice. *J Cancer Res Clin Oncol* 1998, **124**, 607–614.
11. Moore GE, Sandberg AA. Studies of a human tumor cell line with a diploid karyotype. *Cancer* 1964, **17**, 171–175.
12. Clynes M, Redmond A, Moran E, Gilvarry U. Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A. *Cytotechnology* 1992, **10**, 75–89.
13. Moran E, Larkin A, Doherty G, Kelehan P, Kennedy S, Clynes M. A new MDR-1 encoded P-170 specific monoclonal antibody: (6/1C) on paraffin wax embedded tissue without pretreatment of sections. *J Clin Pathol* 1997, **50**, 465–471.
14. Moran E, Larkin AM, Woodman A, Doherty G, Clynes M. Antibodies generated to synthetic peptides specifically recognise their target antigens (P-170 and topoisomerase II α) by immunohistochemical techniques and western blotting. *Cytotechnology* 1996, **19**, 263.
15. Heenan M, O'Driscoll L, Cleary I, Connolly L, Clynes M. Isolation from a human MDR lung cell line of multiple clonal subpopulations which exhibit significantly different drug resistance. *Int J Cancer* 1997, **71**, 907–915.

16. Moran E, Cleary I, Larkin AM, *et al*. Co-expression of MDR-associated markers, including P-170, MRP and LRP and cytoskeletal proteins, in three resistant variants of the human ovarian carcinoma cell line, OAW42. *Eur J Cancer* 1997, **33**, 652–660.
17. Cleary I, Doherty G, Moran E, Clynes M. The multidrug-resistant human lung tumour cell line, DLKP-A10, expresses novel drug accumulation and sequestration systems. *Biochem Pharmacol* 1997, **53**, 1493–1502.
18. O'Driscoll L, Daly C, Saleh M, Clynes M. The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug-resistant cells. *Cytotechnology* 1993, **12**, 289–314.
19. Kool M, De Haas M, Scheper GL, *et al*. Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997, **57**, 3537–3547.
20. Milam SB, Magnuson VL, Steffensen B, Chen D, Klebe RJ. IL-1 beta and prostaglandins regulate integrin mRNA expression. *J Cell Physiol* 1991, **149**, 173–183.
21. Rozzo C, Chiesa V, Carida G, Pagnan G, Ponzoni M. Induction of apoptosis in human neuroblastoma cells by abrogation of integrin-mediated cell adhesion. *Int J Cancer* 1997, **70**, 688–698.
22. Albini A, Iwamoto Y, Kleinman HK, *et al*. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987, **47**, 3239–3245.
23. Johansson S, Smedsrod B. Identification of a plasma gelatinase in preparations of fibronectin. *J Biol Chem* 1986, **261**, 4363–4366.
24. Barrand MA, Rhodes T, Center MS, Twentyman PR. Chemosensitisation and drug accumulation effects of cyclosporin A, PSC 833 and verapamil in human MDR large cell lung cancer cells expressing a 190K membrane protein distinct from P-glycoprotein. *Eur J Cancer* 1993, **29A**, 409–415.
25. Ishikawa T, Bao J-J, Yamane Y, *et al*. Coordinated induction of MRP/GS-X pump and γ-glutamylcysteine synthetase by heavy metals in human leukemia cells. *J Biol Chem* 1996, **271**, 14981–14988.
26. Kool M, Van der Linden M, De Haas M, *et al*. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* 1999, **99**, 6914–6919.
27. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* 1996, **56**, 988–994.
28. Parekh H, Wiesen K, Simpkins H. Acquisition of taxol resistance via P-glycoprotein and non-P-glycoprotein-mediated mechanisms in human ovarian carcinoma cells. *Biochem Pharmacol* 1997, **53**, 461–470.
29. Liotta LA, Kleinerman J, Cataanzara P, Rynbrandt D. Degradation of basement membrane by murine tumor cells. *J Natl Cancer Inst* 1977, **58**, 1427–1439.
30. Zutter MM, Santoro SA. The ups and downs of alpha2beta1-integrin expression: contributions to epithelial cell differentiation and the malignant phenotype. *Curr Top Microbiol Immunol* 1998, **231**, 167–185.
31. Chen F-A, Alosco T, Croy BA, Narumi K, Percy DH, Bankert RB. Clones of tumour cells derived from a single primary human lung tumour reveal different patterns of β_1 integrin expression. *Cell Adhes Comm* 1994, **2**, 345–357.
32. Qian F, Vaux DL, Weissman I. Expression of the integrin $\alpha_4\beta_1$ on melanoma cells can inhibit the invasive stage of metastasis formation. *Cell* 1994, **77**, 335–347.
33. Nista A, Leonetti C, Bernardini G, Mattioni M, Santoni A. Functional role of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. *Int J Cancer* 1997, **72**, 133–141.
34. Narita T, Kimuka N, Sato M, Matsuura N, Kannagi R. Altered expression of integrins in adriamycin-resistant human breast cancer cells. *Anticancer Res* 1998, **18**, 257–262.
35. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999, **93**, 1658–1667.
36. Troussard AA, Costello P, Yoganathan TN, Kumagai S, Roskelley CD, Dedhar S. The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor- dependent upregulation of matrix metalloproteinase 9 (MMP-9). *Oncogene* 2000, **19**, 5444–5452.
37. Morini M, Mottolese M, Ferrari N, *et al*. The alpha3beta1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. *Int J Cancer* 2000, **87**, 336–342.
38. Murphy G, Gavrilovic J. Proteolysis and cell migration: creating a path? *Curr Opin Cell Biol* 1999, **11**, 614–621.
39. Zhu Q, Center MS. Evidence that SP1 modulates transcriptional activity of the multidrug resistance-associated protein gene. *DNA Cell Biol* 1996, **15**, 105–111.
40. Tanaka T, Uchiumi T, Hinoshita E, *et al*. The human multidrug resistance protein 2 gene: functional characterization of the 5′-flanking region and expression in hepatic cells. *Hepatology* 1999, **30**, 1507–1512.
41. Himelstein BP, Lee EJ, Sato H, Seiki M, Muschel RJ. Transcriptional activation of the matrix metalloproteinase-9 gene in an H-ras and v-myc transformed rat embryo cell line. *Oncogene* 1997, **14**, 1995–1998.
42. Ozanne BW, McGarry L, Spence HJ, *et al*. Transcriptional regulation of cell invasion. AP-1 regulation of a multigenic invasion programme. *Eur J Cancer* 2000, **36**, 1640–1648.