MRP IS FREQUENTLY EXPRESSED IN HUMAN LUNG-CANCER CELL LINES, IN NON-SMALL-CELL LUNG CANCER AND IN NORMAL LUNG

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The multidrug resistance-associated protein (MRP), a new membrane transporter related to non-Pgp multidrug resistance, is overexpressed in some drug-selected cancer-cell lines. The role of MRP in unselected cell lines and in human cancer is unknown. MRP gene expression, determined by RNase protection assay and chemosensitivity to doxorubicin, etoposide and cisplatin, determined by MTT assay, were assessed in 18 non-drug-selected lung-cancer cell lines (10 small-cell lung cancer, 6 non-small-cell lung cancer, and 1 carcinoid). MRP gene expression was also investigated in normal lung tissue and primary non-small-cell lung cancer. All cell lines except one and all normal lung tissues and primary non-small-cell lung cancers expressed detectable levels of MRP. Expression was significantly lower in cell lines than in normal and neoplastic lung. MRP protein expression was also assessed by immunohistochemistry using the monoclonal antibody MRPr1; comparable levels of expression were observed between mRNA and protein in cell lines; however, in tumor samples intense staining was observed in tumor cells as well as in infiltrating normal cells in tumors, making the results less comparable to those obtained by RNase expression. MRP expression did not directly correlate with function in a calcein accumulation assay in 2 unselected cell lines. No gene amplification was observed by Southern-blot analysis, in the unselected cell lines or in tumor samples. In general, in cell lines, MRP gene expression was correlated with lower chemosensitivity to doxorubicin and etoposide, but not to cisplatin. However, MRP expression did not directly correlate with MRP function as assessed by a calcein accumulation assay in one of 2 unselected cell lines examined. Our results suggest that MRP may be implicated in drug resistance in unselected lung-cancer cell lines and its role in normal lung and primary lung cancer warrants further investigation in patients undergoing chemotherapy.

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Cell lines selected *in vitro* for resistance to one of a group of natural product anti-cancer agents (e.g., anthracyclines, vinca alkaloids, epipodophyllotoxins, colchicine, taxol) often display cross-resistance to the entire group of drugs. This type of drug resistance is called multidrug resistance (MDR) and is frequently associated with the overexpression of Pgp, encoded by the *MDR1* gene. Pgp is a transmembrane protein that actively pumps cytotoxic drugs out of the cytoplasm of cells, thereby reducing the interaction with their intracellular targets. MDR is associated with decreased intracellular drug accumulation.

Several cell lines selected *in vitro* for resistance to one of the above-mentioned drugs do not overexpress the *MDR1* gene, despite having a defect in drug accumulation (Nakagawa *et al.*, 1992). In some of these cell lines a lower expression and/or reduced activity of topoisomerase II (Schneider *et al.*, 1994; Giaccone, 1994) can be seen, but frequently multiple mechanisms of drug resistance are found (Schneider *et al.*, 1994). Recently, a new transmembrane drug-transporter gene has been identified, which is overexpressed in some lung-cancer cell lines resistant to doxorubicin (Cole *et al.*, 1992; Barrand *et al.*, 1994; Zaman *et al.*, 1993), and other cell lines also selected with other MDR drugs (Schneider *et al.*, 1994; Slovak *et al.*, 1993; Slapak *et al.*, 1994a; Krishnamachary and Center, 1993; Doyle *et al.*, 1995). The product of this new gene, called *MRP*,

after MDR-associated protein, belongs to the ATP-binding cassette (ABC) superfamily of membrane proteins, to which Pgp also belongs. Over 50 ABC transporters are known, and each one is relatively specific for a given substrate. MRP is a 1531-amino-acid *M_r* 190,000 N-glycosylated integral membrane phosphoprotein that is encoded by a 6.5-kb mRNA. Despite the similarities, MRP shares less than 15% amino-acid identity with Pgp (Cole *et al.*, 1992). Transfection of the *MRP* gene induced overexpression of MRP and determined an increase in resistance to doxorubicin, vincristine and VP16, but not to cisplatin (Grant *et al.*, 1994), or taxol and mAMSA (Zaman *et al.*, 1994). Moreover, expression of cDNA library transfer of doxorubicin-resistant HL60R cells into NIH/3T3 cells established *MRP* as the dominant acting resistance gene in determining doxorubicin resistance in transfected cells (Kruh *et al.*, 1994). Although reduced drug accumulation is usually described in cell lines with *MRP* overexpression, altered intracellular distribution has also been observed, which may be due to vesicular drug entrapment (Cole *et al.*, 1992, 1994; Krishnamachary *et al.*, 1993; Slapak *et al.*, 1994b).

In the doxorubicin-resistant SCLC cell line H69/AR, in which MRP was identified (Cole *et al.*, 1992), and in other cell lines overexpressing MRP (Slovak *et al.*, 1993; Slapak *et al.*, 1994a; Binaschi *et al.*, 1995; Doyle *et al.*, 1995), amplification of the gene was observed, a mechanism infrequently present in MDR, which is more often due to transcriptional activation.

Unselected lung-cancer cell lines and lung tumors express undetectable or very low levels of *MDR1* gene and the drug resistance of this type of cancer is probably due to other mechanisms (Lai *et al.*, 1989). Although a reduced expression of topoisomerase II and mutations (Giaccone *et al.*, 1992; Binaschi *et al.*, 1992) have both been described, they cannot explain all instances of multidrug resistance seen in lung-cancer cell lines. As overexpression of the *MRP* gene was seen in drug-resistant human lung-cancer cell lines, it may represent a possible mechanism of drug resistance in this tumor.

Lung cancer is the most lethal malignancy in men and women in the US and its treatment has only very little impact on survival. Two major clinical forms of lung cancer are described: SCLC and NSCLC, the former being very sensitive to chemotherapy and the second rather refractory. Although chemotherapy can achieve over 90% response rates in SCLC, relapses occur in the vast majority of patients and are chemotherapy-refractory. SCLC and NSCLC exhibit 2 distinct types of drug resistance, acquired and intrinsic, respectively.

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Abbreviations: SCLC, small-cell lung cancer; NSCLC, non-small-cell lung cancer; VP16, etoposide; VM26, teniposide; Pgp, P-glycoprotein; MDR, multidrug resistance.

We investigated the correlation between MRP m-RNA expression and chemosensitivity of unselected human lung-cancer cell lines. In addition, we assessed the expression of this gene in normal lung tissue and NSCLC of patients undergoing operation, and investigated the protein expression in selected cell lines and samples by immunohistochemistry.

MATERIAL AND METHODS

Cell lines and cytotoxicity assay

A total of 19 human lung-cancer cell lines were used in this study, comprising 1 carcinoid, 12 SCLC and 6 NSCLC cell lines (Table I). All the cell lines were developed at the NCI-Navy Medical Branch, except the GLC4 cell lines, which were kindly provided by Dr. E. de Vries (Groningen, The Netherlands). All cell lines were grown in the medium which best supported their growth, and were cultured in a 5% CO₂ and 95% air incubator at 37°C. Cell lines were negative for Mycoplasma contamination.

With the exception of the SCLC cell line GLC4/ADR, none of the cell lines had been exposed *in vitro* to drug selection. GLC4/ADR was obtained from GLC4 and made resistant to doxorubicin by stepwise increasing concentrations of doxorubicin up to a drug concentration of 1.18 µM (de Jong *et al.*, 1990). This cell line is respectively 225, 72 and 66 times more resistant to doxorubicin, VM26 and VP16, than the parental cell line (de Jong *et al.*, 1990), and non-cross-resistant to cisplatin. GLC4/ADR has been shown to be a Pgp-negative MDR cell line, with overexpression of the *MRP* gene, associated with gene amplification (Zaman *et al.*, 1993).

Chemosensitivity testing was performed using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, as previously described (Giaccone *et al.*, 1992). Drugs were added 24 hr after cell plating, for a 4-day continuous drug exposure assay. The duration of the test encompassed at least 2 doubling times of all cell lines tested.

Cisplatin (Bristol Myers, Brussels, Belgium) and doxorubicin (Farmitalia Carlo Erba, Nivelles, Belgium) were solubilized in 0.9% NaCl. VP16 (Eli Lilly, Amsterdam) was solubilized in DMSO. All solutions were stored at -20°C and diluted in culture medium before addition to the cultures. All other chemicals were of standard analytical quality.

Extraction of nucleic acids

Like the preparation for the MTT assay, medium of cell lines was refreshed 24–48 hr before harvesting for nucleic acid extraction; cell lines growing as floating aggregates were also gently disaggregated at the same time, in order to obtain exponential growth. Total RNA and DNA were simultaneously extracted from exponentially growing cells or frozen tissue sections by cell lysis with guanidine isothiocyanate and centrifugation in a cesium chloride gradient. Normal lung tissues and tumor samples were collected during surgery for NSCLC, snap-frozen immediately after resection in liquid nitrogen and preserved at -80°C until use. Then, 70–100 cryosections 20 µm thick were cut for nucleic acid extractions, and the first and last sections were used for histologic assessment (H. and E. staining).

RNase protection assay

MRP cDNA, obtained as previously described (Zaman *et al.*, 1993), was kindly provided by Dr. G. Zaman (Netherlands Cancer Institute, Amsterdam). [α -³²P]labeled RNA transcripts complementary to MRP mRNA sequences were transcribed from EcoRI-linearized DNA from pMRP-5' inserted into the EcoRI site of pGEM3-Zf(-) (Promega, Leiden, The Netherlands), using SP6 RNA polymerase. RNase protection was carried out as described (Burger *et al.*, 1994b). Protected probe was visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. Experiments were repeated at least twice. In all experiments a probe for γ -actin was included as control for RNA loading. The amount of MRP mRNA relative to the amount of γ -actin mRNA was

TABLE I - CHARACTERISTICS, CYTOTOXICITY, AND MRP GENE EXPRESSION OF HUMAN LUNG-CANCER CELL LINES. MTT CYTOTOXICITY EXPERIMENTS WERE PERFORMED AT LEAST 3 TIMES BY 4-DAY CONTINUOUS DRUG EXPOSURE; STANDARD DEVIATIONS WERE <20%. THE MRP EXPRESSION WAS INVESTIGATED BY RNase PROTECTION ASSAY. THE RELATIVE MRP EXPRESSION WAS ESTIMATED IN RELATION TO THE γ -ACTIN EXPRESSION. EXPERIMENTS WERE PERFORMED AT LEAST TWICE; STANDARD DEVIATIONS WERE <30%

Cell line	Type	Growth pattern	Medium	IC ₅₀ (µM)			Relative MRP gene expression
				ADR	VP16	DDP	
NCI-H187	SCLC-c	Floater	R10	0.026	0.600	1.000	0.01
NCI-H345	SCLC-c	Floater	HITES	0.022	2.1	2.5	0.00
NCI-N417	SCLC-v	Floater	R10	0.013	3.700	2.000	0.17
NCI-H446	SCLC-v	Float/at.	HITES	nd	nd	nd	0.21
NCI-H1436	SCLC-c	Floater	5H	0.062	0.320	0.110	0.13
NCI-H1450	SCLC-c	Floater	5H	0.064	0.365	0.480	0.27
NCI-H1618	SCLC-c	Floater	5H	nd	nd	nd	0.13
NCI-H1622	SCLC-c	Floater	5H	0.471	4.710	5.570	0.50
NCI-H1963	SCLC-c	Floater	2.5H	nd	nd	nd	0.05
NCI-H1994	SCLC-c	Floater	5H	nd	nd	nd	0.03
NCI-H226	Squamous	Attached	R10	0.033	0.730	1.030	0.24
NCI-H322	BAC ¹	Attached	R10	0.467	22.600	6.340	0.31
NCI-H358	BAC ¹	Attached	R10	0.044	1.040	3.340	0.03
NCI-H676	Adenoca.	Floater	A4	0.776	31.200	4.360	0.04
NCI-H1355	Adenoca.	Floater	A4	0.144	1.770	5.760	0.05
NCI-H460	Large-cell ²	Attached	R10	0.055	0.130	0.377	1.00
NCI-H727	Carcinoid	Attached	R10	0.848	74.300	5.220	0.21
GLC4	SCLC-v	Fl./attach.	R10	0.008	0.288 ³	0.55	0.03
GLC4/ADR	SCLC	Floater	R10	1.800	19.165 ³	0.20	1.5

¹BAC, bronchioloalveolar carcinoma. ²Large-cell carcinoma with neuroendocrine properties. ³de Jong *et al.*, 1990. SCLC-c, classic small-cell lung cancer; SCLC-v, variant small-cell lung cancer; R10, RPMI1640 + 10% FCS; A4, ACL-4 serum-free medium; HITES, serum-free medium; 2.5H, HITES + 2.5% FCS; 5H, HITES + 5% FCS. nd = not determined. ADR = doxorubicin, VP16 = etoposide, DDP = cisplatin.

calculated by densitometric scanning of autoradiograms. Because substantial differences in RNA loading were sometimes observed, several exposure times of films were employed, in order to remain in the linear range of detection of the densitometer for each sample tested.

Southern-blot analysis

Genomic DNA (10 µg) was digested with 2 restriction enzymes, EcoRI and PstI (Boehringer Mannheim, Almere, The Netherlands) and then run on a 0.8% agarose gel. Normal human spleen DNA was used as a control. The DNA was then transferred to a synthetic nylon membrane (GeneScreen Plus, NEN, Boston, MA) and hybridized to a ³²P-labeled probe. The final wash was at 55°C for 15 min in 2 × SSC (buffer 20×: NaCl, 3M; Na₃citrate · 2H₂O, 0.3 M) – 1% SDS.

The same RNA probe as used for RNase protection experiments was also employed for Southern hybridization. Additionally, a longer fragment (4235–5596 nucleotides), kindly provided by Dr. G. Zaman, was also employed, with similar results. This cDNA probe was ³²P-labeled with a random primer kit (specific activity, 2–5 × 10⁸ cpm/µg DNA) (random primers DNA labelling system).

Immunohistochemistry

Cryostat sections (4 µm thick) of tissue samples and cytopins of human lung cancer cell lines were used for immunohistochemistry. Slides were air-dried overnight and fixed in acetone for 10 min at room temperature, pre-incubated with 2% normal rabbit serum (Dako, Glostrup, Denmark), then incubated for 1 hr with the MAb MRPr1 (5 µg/ml) diluted in 1% BSA/PBS at room temperature (Flens *et al.*, 1994). This antibody does not cross-react with the human Pgp (Flens *et al.*, 1994). Slides were next incubated for 1 hr with a peroxidase-conjugated rabbit-anti-rat immunoglobulin (1:25 Dako) + 2% human pool serum. For development of the bound peroxidase, slides were incubated for 3–5 min at room temperature in freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) and 0.07% (v/v) hydrogen peroxide in PBS and rinsed with tap water. Slides were counterstained with hematoxylin, rinsed in water, dehydrated and mounted. Negative controls for tumor tissues included staining with an irrelevant antibody (mouse myeloma immunoglobulin IgG₁) diluted at a final concentration of 10 µg/ml (not shown).

Daunorubicin and calcein accumulation assays

Cells (0.3–0.7 × 10⁶) were incubated for 60 min at 37°C in 0.5 µM daunorubicin (Sigma, St. Louis, MO) at pH 7.4 with or without the Pgp modulator bepridil 16 µM. Calcein accumulation experiments were performed by loading cells for 10 min with 0.5 µM calcein-AM (Molecular Probes, Eugene, OR) with or without the MRP modulator probenecid (1 mM), and in some experiments calcein efflux was measured after 60 min of efflux. After the cells had been washed, the fluorescence of calcein was measured with a 530-nm band-pass filter, and that of daunorubicin with a 575-nm filter (Becton Dickinson, Mountain View, CA; FACScan flow cytometer, excitation at 488 nm). Details of the methods employed are given elsewhere (Feller *et al.*, 1995a, b).

RESULTS

Cytotoxicity experiments

The cell-killing effects of 2 topoisomerase-II inhibitors, doxorubicin and VP16, and the alkylating agent cisplatin, were tested on most human lung-cancer cell lines during 4 days of drug exposure. Large differences were found in drug sensitivities among the unselected human lung-cancer cell lines (Table I). The range of IC₅₀ values was particularly large for VP16 and doxorubicin, and less for cisplatin.

The SCLC cell lines were in general more sensitive than the NSCLC cell lines, with the exception of NCI-H460, an NSCLC

cell line with neuroendocrine properties; NSCLC lines with neuroendocrine properties are known to be more sensitive than those which lack these features (Gazdar *et al.*, 1992). The differences in cytotoxicity between SCLC and NSCLC cell lines were, however, statistically significant only for cisplatin, and after the exclusion of NCI-H460 ($p < 0.05$; Kruskal-Wallis test), suggesting that histology had only a limited influence on chemosensitivity in the set of cell lines that we analyzed. In addition, the carcinoid cell line NCI-H727 was rather resistant to the drugs tested, its sensitivity pattern resembling that of the NSCLC cell lines, as already reported (Gazdar *et al.*, 1992).

The patterns of drug sensitivities in 14 unselected cell lines were remarkably correlated, regardless of the mechanism of action of the 3 drugs tested. The R correlation coefficients of linear regression analysis were 0.74 for the logarithms of IC₅₀ values of doxorubicin-VP16 ($p = 0.004$), 0.557 for doxorubicin-cisplatin ($p = 0.038$), and 0.80 for VP16-cisplatin ($p = 0.0007$), respectively. Similar results have been previously reported in unselected human lung-cancer cell lines (Giaccone *et al.*, 1992; Gazdar *et al.*, 1992).

MRP expression in human lung-cancer cell lines, and correlation with cytotoxicity

The expression of MRP mRNA was analyzed by a sensitive RNase protection assay (Fig. 1). Correction of the RNA loading was performed by quantification of expression of the γ -actin gene, and the relative MRP expression was indicated by the MRP/ γ -actin ratio (Tables I, II). The MRP transcript was detectable in all the cell lines except one (NCI-H345) at the expected protected size of 244 bp, with a wide range of intensity of gene expression.

Among the cell lines tested, there did not appear to be any difference in MRP expression between SCLC and NSCLC cell lines. In the group of SCLC cell lines, NCI-H1622, the most resistant to all 3 drugs tested, had also the highest relative MRP expression, while the SCLC cell line NCI-H345, with intermediate chemosensitivity, was the only human lung-cancer cell line with undetectable MRP expression. Interestingly, the very sensitive NSCLC cell line NCI-H460 had the highest expression of all the unselected cell lines. In these experiments, GLC4/ADR, which displays MRP overexpression, as already reported (Zaman *et al.*, 1993), was used as a control. Cytotoxicity results for each cell line were analyzed in relation to their MRP expression (Table I). In general, higher IC₅₀ values of doxorubicin and VP16, but not cisplatin, were often associated with higher relative MRP gene expression levels. However, this association was borderline statistically significant only for doxorubicin ($n = 15$, $R = 0.479$, $p = 0.07$; Pearson correlation test) (Fig. 2), and was significant only with the exclusion of NCI-H460 ($n = 14$, $R = 0.592$, $p = 0.026$).

MRP mRNA expression in human normal lung and NSCLC

MRP expression was assessed on total RNA extracted from 5 normal lung samples and 6 NSCLC samples; 1 tumor sample and 1 normal lung sample were derived from the same patient. Interestingly, there was a striking difference in MRP expression between unselected human lung-cancer cell lines, normal lung and NSCLC resected from patients ($p = 0.0112$; Kruskal-Wallis test) (Tables I, II). There was no significant difference in γ -actin expression between cell lines and tissue samples (Fig. 3).

There was no statistically significant difference in levels of expression MRP transcript between tumors and normal lung ($p = 0.27$), although the number of samples was small. Cell lines had significantly lower MRP gene expression than normal lung ($p = 0.0379$) and NSCLC ($p = 0.0117$). In the only instance in which normal lung and tumor were available from the same patient, a slightly higher expression was present in the normal lung than in the tumor (Table II).

FIGURE 1 – Representative experiments of MRP mRNA expression of human lung cancer cell lines by RNase protection assay. 10 μ g of total RNA was hybridized using a probe complementary to sequences at the 5' end of the MRP mRNA, and a probe for γ -actin, used to control for RNA loading. See "Material and Methods" for details. Experiments were performed at least twice; standard deviations were <30%. In the right panel of the figure the drug sensitive GLC4 and its non-Pgp MDR subline GLC4/ADR are shown. Both probes before treatment with RNase are also shown.

TABLE II – MRP GENE EXPRESSION IN NORMAL LUNG AND NSCLC. MRP mRNA EXPRESSION WAS ASSESSED BY RNase PROTECTION ASSAY, AND CORRECTED BY THE EXPRESSION OF THE γ -ACTIN GENE. EXPERIMENTS WERE PERFORMED AT LEAST TWICE; STANDARD DEVIATIONS WERE <30%

Tissue sample	Histology	Sex/age	Relative MRP gene expression
IT-4	Normal lung	M/62	0.17
IT-20 ¹	Normal lung	M/65	0.41
IT-23	Normal lung	M/69	0.27
IT-26	Normal lung	M/55	0.52
IT-44	Normal lung	M/49	0.38
IT-7	Adenocarcinoma	M/59	0.28
IT-9	Squamous-cell ca.	M/59	1.78
IT-11	Squamous-cell ca.	M/67	1.13
IT-19	Squamous-cell ca.	M/57	1.11
IT-21 ¹	Squamous-cell ca.	M/65	0.12
IT-47	Squamous-cell ca.	M/72	0.43

¹Derived from the same patient.

MRP immunohistochemistry

An anti-MRP MAb (Flens *et al.*, 1994) was used to stain a selected number of cell lines and patient tissue samples which had substantially different MRP expression levels by RNase protection assay. Staining results, taking into consideration the number and intensity of stained cells from at least 2 independent experiments, were classified as negative (–), weakly positive (+/–), positive (+), or strongly positive (++) . The

staining was mainly limited to the cell membrane, although some cytoplasmic staining was also observed. The results of staining of cell lines were comparable to those obtained by RNase protection (Table III).

In the histological sections of tumors, tumor cells as well as non-neoplastic cells were stained, although with different intensities (Fig. 4). The results obtained by RNase protection and immunohistochemistry were less similar than those of the cell lines. Because of the positive staining observed in non-neoplastic cells, and particularly the strong staining of macrophages, as described previously (Thomas *et al.*, 1994), the use of bulk techniques may not be warranted in order to quantify MRP expression in tissues derived from solid tumors.

Southern-blot analysis

Because overexpression of the *MRP* gene is associated with gene amplification in *in vitro*-selected cell lines resistant to doxorubicin (Cole *et al.*, 1992; Zaman *et al.*, 1993), Southern-blot analysis was performed in 10 unselected cell lines, 6 normal lung and 7 NSCLC samples. Experiments included DNA from GLC4/ADR, known to overexpress the *MRP* gene and to display gene amplification (Zaman *et al.*, 1993). None of the unselected human lung-cancer cell lines showed amplification of the *MRP* gene (not shown). Furthermore, no amplification was found in NSCLC and in normal lung tissue, and no rearrangement of the gene was observed when 2 different restriction enzymes were used (EcoRI and PstI) (not shown).

FIGURE 2 – Correlation between the relative MRP mRNA expression and of the logarithms of the IC₅₀ values of doxorubicin (panel *a*), VP16 (panel *b*), and cisplatin (DDP; panel *c*). Each point represents one cell line. MTT experiments were repeated at least three times; standard deviations were <20%. The R correlation coefficients for each graph were 0.479 ($p = 0.07$), 0.119 ($p = 0.67$), and -0.429 ($p = 0.11$), respectively. When the cell line NCI-H460 was omitted, the correlation coefficients varied as follows: 0.592 ($p = 0.026$), 0.374 ($p = 0.188$), -0.354 ($p = 0.214$), respectively.

FIGURE 3 – MRP mRNA expression of NSCLC specimens (T) and normal lung (N) by RNase protection assay. 10 μ g of total RNA was hybridized using a probe complementary to sequences at the 5' end of the MRP mRNA, and a probe for γ -actin, used to control for RNA loading. See "Material and Methods" for details. Histology of tumors is displayed (SQ = squamous cell carcinoma, AD = adenocarcinoma). Both probes before treatment with RNase are also shown. Experiments were performed at least twice; standard deviations were <30%.

Accumulation assays

In order to assess the activity of Pgp and MRP in human lung-cancer cell lines, we have determined the accumulation of daunorubicin with or without Pgp modulator (16 μ M bepridil) and of the MRP substrate calcein with or without the MRP

TABLE III – COMPARISON OF IMMUNOHISTOCHEMISTRY AND RNase PROTECTION IN HUMAN LUNG CANCER CELL LINES AND TUMORS. RNase PROTECTION EXPERIMENTS WERE PERFORMED AT LEAST TWICE; STANDARD DEVIATIONS WERE <30%. STAINING RESULTS WERE CLASSIFIED AS NEGATIVE (–), WEAKLY POSITIVE ($\pm$), POSITIVE (+), OR STRONGLY POSITIVE (++)

	Staining results	Relative MRP mRNA expression
Cell lines		
GLC4/ADR	++	1.5
GLC4	$\pm$	0.03
NCI-H187	–	0.01
NCI-H209	$\pm^1$	nd
NCI-H322	+	0.31
NCI-N417	$\pm^1$	0.17
NCI-H460	+	1
Tumors		
IT9	++ ²	1.78
IT19	+ ³	1.11
IT21	$\pm^4$	0.12
IT47	++ ⁵	0.43

¹Cytoplasmic staining also observed. ²Strong staining of tumor cells, normal tissue and stroma (see Figure 4b). ³Positive tumor with weakly positive lung epithelial cells and strongly positive macrophages (see Figure 4d). ⁴Weakly positive tumor cells, positive macrophages. ⁵No normal tissue observed. nd = not done.

modulator probenecid (1 mM) (Feller *et al.*, 1995a, b). Table IV shows that no significant activity of either Pgp or MRP was detected in the unselected lung-cancer cell lines (see Table III for mRNA and protein expression). In the positive control cell line GLC4/ADR, a high MRP activity was seen.

DISCUSSION

Drug resistance to multiple drugs is a major cause of failure of medical treatment of several malignancies, including lung cancer. The mechanisms of drug resistance in lung cancer are still rather obscure. Even though Holzmayer *et al.* (1992) found a significant difference in response to chemotherapy and MDR1 expression in a very limited number of SCLC patients, MDR1 expression cannot explain the resistance pattern of most lung cancers (Lai *et al.*, 1989).

Several investigators reported that reduced levels of topoisomerase II might be responsible for decreased sensitivity to topoisomerase-II inhibitors in human lung-cancer cell lines (Giaccone, 1994). There are, however, strong indications that multifactorial mechanisms may coexist in determining drug resistance in lung cancer cells.

FIGURE 4 – Immunohistochemistry of the cell lines GLC4/ADR (panel a) and GLC4 (panel c), and sections of two squamous cell lung carcinomas (IT9 and IT19, see also Table III) using a monoclonal antibody raised against MRP. The staining of GLC4/ADR is strongly membranous with a spot next to the nucleus, while the parental cell line has only weakly cytoplasmic staining. Both tumor sections have positive tumor cells (t), and in both also stromal cells (s) and alveolar macrophages (a) stain strongly with the antibody used. Scale bars: 25 μ m.

TABLE IV – DAUNORUBICIN ACCUMULATION WITH OR WITHOUT BEPRIDIL, AND CALCEIN ACCUMULATION WITH OR WITHOUT PROBENECID IN HUMAN LUNG-CANCER CELL LINES

Cell line	Daunorubicin accumulation ratio ¹	Calcein accumulation ratio ¹	Calcein efflux ratio ²
NCI-H187	0.88	0.96	0.98
NCI-H322	1.05	nd	nd
NCI-H460	0.97	1.05	1.09
NCI-N417	1.01	nd	nd
GLC4	nd	0.98	1.07
GLC4/ADR	nd	2.27	2.1

¹Ratios are accumulation of daunorubicin or calcein with modulator, divided by accumulation without modulator. ²Data are ratios of calcein retained after 60 min efflux with probenecid divided by retention without probenecid. –nd, not determined. –All data are means from 2–3 experiments (SD $\leq$ 15%).

Cole *et al.* (1992) have identified an increased expression of the new putative transmembrane drug transporter MRP in a doxorubicin-selected H69 human SCLC cell line. In this cell line, amplification of the *MRP* gene was observed, which was probably the cause of the overexpression. Zaman *et al.* (1993) reported overexpression associated with amplification of the *MRP* gene in GLC4/ADR, another doxorubicin-resistant SCLC cell line, while levels of *MRP* expression observed in doxorubicin-resistant cell lines derived from SW1573 NSCLC cells were similar to those of parental cells, indicating that overexpression of *MRP* cannot account for all forms of non-Pgp MDR. The pattern of cross-resistance of cells overexpressing *MRP* was slightly different from that of cells overexpressing Pgp. In *MRP* transfectants, cross-resistance to vinblastine and colchicine was low (Cole *et al.*, 1994), while it was low (Cole *et al.*,

1994) or null (Zaman *et al.*, 1994) to taxol and null to mAMSA, mitoxantrone and 9-alkyl-anthracyclines (Zaman *et al.*, 1994). Other doxorubicin-resistant cell lines which overexpress MRP have also displayed a relative lack of cross-resistance to taxol (Binaschi *et al.*, 1995) and vinblastine (Nakagawa *et al.*, 1992). Transfection experiments strongly suggested that MRP is a plasma membrane drug-efflux pump (Zaman *et al.*, 1994), as transfected cells have reduced sensitivity to MDR drugs and lower drug accumulation.

Since results obtained in drug-resistant selected lung-cancer cell lines may not directly reflect the human situation, we assessed the expression of the *MRP* gene in a panel of unselected human lung-cancer cell lines, lung tumors and normal lung tissues. In our study, MRP appeared to be widely expressed in all SCLC cell lines but one, and in all normal and neoplastic tissues. A wide expression of MRP has been already reported in a number of normal tissues (Zaman *et al.*, 1993), in leukemias and in normal blood cells (Burger *et al.*, 1994a; Abbaszadegan *et al.*, 1994), with high expression levels in chronic lymphocytic and prolymphocytic leukemia (Burger *et al.*, 1994b). No correlation was found between levels of MRP expression and treatment status (Burger *et al.*, 1994a). Interestingly, in a panel of 7 human glioma cell lines, a correlation was found between MRP expression, VP16 accumulation and cytotoxicity (Abe *et al.*, 1994). Unlike leukemic cell lines, these cells do not overexpress *MDR1*. No amplification was found in either of the studies (Burger *et al.*, 1994a; Abe *et al.*, 1994). Remarkably, in human neuroblastoma cell lines and primary tumors, MRP overexpression paralleled overexpression/amplification of *N-myc*, a marker of aggressiveness in this disease; treatment with retinoic acid could diminish *N-myc* and *MRP* expression, but not *MDR1* expression (Bordow *et al.*, 1994).

In our study we identified a significantly lower MRP expression in cell lines than in tissues. This could not be explained by a difference in actin expression. There was apparently no difference in MRP expression between SCLC and NSCLC cells, although the number of cell lines analyzed is relatively small.

Interestingly, in general higher *MRP* gene expression was correlated to lower cytotoxicity of doxorubicin. However, this correlation was only of borderline significance, with a relatively poor correlation coefficient ($R = 0.524$, $p = 0.045$). NCI-H460 cell line, an NSCLC with neuroendocrine properties, is a highly sensitive and rapidly proliferating cell line that expresses low levels of topoisomerase II and appreciable amounts of *MDR1* (Lai *et al.*, 1989; Giaccone *et al.*, 1992). The higher chemosensitivity of NSCLC with neuroendocrine properties has been reported in cell lines (Gazdar *et al.*, 1992) and in patients (Graziano *et al.*, 1989). Other factors are likely to have much more influence on the high chemosensitivity of this cell line than the expression of the presently known drug-resistance genes and histology.

The absence of activity of MRP, as detected by calcein accumulation or efflux assay (Feller *et al.*, 1995a, b) is unexpected, given the clearly enhanced MRP expression in this cell line. This issue requires more research, with other more sensitive probes for MRP function (Broxterman *et al.*, 1995).

Histology might be important for expression of MRP; squamous-cell carcinomas of the lung show higher *MRP* gene expression than the other histological types by Northern blotting (Ota *et al.*, 1995). In this study there was an indication that high MRP RNA expression may be a poor prognostic factor for survival in patients who received post-operative chemotherapy including MDR drugs.

Because of the small number of cases examined in our study, no correlation between MRP expression and histological type of NSCLC can be made.

No amplification was seen in our study in the unselected cell lines and in tissues, indicating that probably *MRP* gene amplification is an infrequent event, and transcriptional activation is a more frequent cause of *MRP* overexpression. Human myeloid leukemia cell lines exposed to progressively higher doxorubicin concentrations overexpressed first *MRP* and later *Pgp*, indicating that different levels of drug resistance may be induced by overexpression of these 2 genes (Slapak *et al.*, 1994b). Similar findings were observed in SCLC H69/VP cells (Brock *et al.*, 1995). In GLC4 cells progressively exposed to increasing doxorubicin concentrations, *MRP* overexpression and reduced topoisomerase II were early events (Versantvoort *et al.*, 1995).

We also performed immunohistochemistry with an MAb (Flens *et al.*, 1994). Interestingly, most of the cell lines showed some membranous staining, but cytoplasmic staining was also seen on some occasions. The correlation between RNase protection and immunohistochemistry intensity of staining was good for cell lines. However, in tumor samples staining was also observed in normal cells surrounding tumor cells and in alveolar macrophages, as already described by the *in situ* hybridization technique (Thomas *et al.*, 1994). Although in

general the tumors displaying high RNA expression also had intense immunocytochemical staining, the presence of positive normal cells in the samples might influence the results obtained with RNase protection and similar bulk techniques, by either diluting the *MRP* expression level or increasing it if the sample includes an abundance of macrophages (Thomas *et al.*, 1994).

In conclusion, *MRP* might play a role in resistance to MDR drugs such as doxorubicin in unselected lung-cancer cell lines. Given the wide expression in lung-tumor material and the absence of amplification of the *MRP* gene, evaluation of expression levels may be of interest in patients with lung cancer undergoing chemotherapy. The *MRP* gene product has been shown to be a primary-active ATP-dependent export pump for conjugates of lipophilic compounds with glutathione and several other anionic residues (Leier *et al.*, 1994), suggesting that *MRP* overexpression can cause multidrug resistance by promoting the export of drug modification products from cells.

Prospective studies of *MRP* expression in patients with lung cancer undergoing chemotherapy containing MDR drugs appears to be warranted.

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