Altered MRP is associated with multidrug resistance and reduced drug accumulation in human SW-1573 cells

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Summary We have analysed the contribution of several parameters, e.g. drug accumulation, MDRI P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and topoisomerase (topo) II, to drug resistance in a large set of drug-resistant variants of the human non-small-cell lung cancer cell line SW-1573 derived by selection with low concentrations of doxorubicin or vincristine. Selection with either drug nearly always resulted in MDR clones. The resistance of these clones could be explained by reduced drug accumulation and was associated with a decrease rather than an increase in the low MDRI mRNA level. To test whether a decrease in MDRI mRNA indirectly affected resistance in these cells, we introduced a MDRI-specific hammerhead ribozyme into wild-type SW-1573 cells. Although this led to a substantial reduction in MDRI mRNA, it did not result in resistance. In all resistant clones we found an altered form of the multidrug resistance-associated protein (MRP), migrating slightly slower during SDS-polyacrylamide gel electrophoresis than MRP in parental cells. This altered MRP was also present in non-P-gp MDR somatic cell hybrids of the SW-1573 cells, demonstrating a clear linkage with the MDR phenotype. Treatment of crude cellular membrane fractions with N-glycanase, endoglycosidase H or neuraminidase showed that the altered migration of MRP on SDS-PAGE is due to a post-translational modification. There was no detectable difference in sialic acid content. In most but not all doxorubicin-selected clones, this MDR phenotype was accompanied by a reduction in topo IIα mRNA level. No reduction was found in the clones selected with vincristine. We conclude from these results that selection of the SW-1573 cell line for low levels of doxorubicin or vincristine resistance, predominantly results in MDR with reduced drug accumulation associated with the presence of an altered MRP protein. This mechanism can be accompanied by other resistance mechanisms, such as reduced topo IIα mRNA in case of doxorubicin selection.

Keywords: multidrug resistance; post-transcriptional modification; multidrug resistance-associated protein; reduced drug accumulation

Selection of the human non-small-cell lung cancer cell line SW-1573 for resistance to doxorubicin can result in two types of multidrug resistance (MDR): high-level resistance due to P-glycoprotein encoded by the MDRI gene (Baas et al., 1990), a well-defined form of MDR (reviewed by Endicott and Ling, 1988; Roninson, 1991; Schinkel and Borst, 1991; Gottesman and Pastan, 1993; Moscow et al., 1993); and low-level resistance associated with a complex phenotype that includes a decrease rather than an increase in MDRI expression, a decrease in topoisomerase (topo) IIα mRNA and a decreased drug uptake (Eijdems et al., 1992). This form of non-P-gp MDR was first identified in three independent SW-1573 mutants (Keizer et al., 1989; Baas et al., 1990; Kuiper et al., 1990) and it results in resistance to drugs affecting the function of topoisomerase II (topo II) such as daunorubicin, VP16-213 and m-AMSA [4'-9-acridinylamino)methanesulphon-m-anisidin], to the membrane-active compound gramicidin D and to drugs affecting the polymerisation of microtubules such as the vinca alkaloids and colchicine (Keizer et al., 1989; Baas et al., 1990; Kuiper et al., 1990; Eijdems et al., 1992). In somatic cell fusions we have shown that the MDR phenotype, the reduced drug accumulation and the reduction in MDRI P-gp mRNA are transferred together to drug-sensitive SW-1573 cells, but that the alteration in topo IIα is not genetically linked to the non-P-gp MDR phenotype (Eijdems et al., 1992).

Several other non-P-gp MDR cell lines have been selected (reviewed in Cole, 1992) and are being used to identify the mechanism(s) underlying this form of resistance. Cole et al. (1992), identified the multidrug resistance-associated protein (MRP) gene, which was amplified and overexpressed in one of these non-P-gp MDR cell lines, H69AR. Subsequently, overexpression of the MRP gene was reported in other non-P-gp MDR cell lines (Krishnamachary and Center, 1993; Slovak et al., 1993; Zaman et al., 1993; Barrand et al., 1994; Schneider et al., 1994), suggesting a role for MRP, a member of the ATP-binding cassette transporter superfamily, in these cell lines as well. Recent transfection studies have proved that MRP can confer resistance to a broad range of natural product drugs (Grant et al., 1994; Zaman et al., 1994) by extruding the drugs from the cells (Zaman et al., 1994).

Since the multiplicity of alterations in the SW-1573 non-P-gp MDR cell lines described thus far remains puzzling, we have isolated a series of new resistant variants with drug concentrations just above the IC50 of the parental cell line to study the following questions:

1. What is the predominant form of drug resistance obtained by low-level drug selection?
2. Are known transporters such as MDRI P-glycoprotein or MRP involved in the low-level MDR present in the resistant variants?
3. Is down-regulation of MDRI mRNA an obligatory feature of low-level MDR in these variants?
4. Is the observed resistance frequently associated with a decrease in topo IIα mRNA level?
5. Does vincristine and doxorubicin selection result in the same resistance phenotype?

Material and methods

Chemicals

Doxorubicin (doxorubicin hydrochloride), vincristine sulphate, ouabain and digitonin (50% pure) were purchased from Sigma (St Louis, MO, USA), daunorubicin hydrochloride was obtained from Specia (Paris, France), [G-
1Hvinristine sulphate (sp. act. 4.8 Ci mmol⁻¹) from Amer sham (UK) and [G-1H]daunorubicin (sp. act. 1.6 Ci mmol⁻¹) from DuPont de Nemours (Germany).

Cell culture

The resistant cell lines described in this study were derived from the human non-small-cell lung cancer cell line SW-1573, originally isolated and characterised by Dr A Leibovitz (Scott and White Clinic, Temple, TX, USA). Cell line S1ou is a subclone of the drug-sensitive parental cell line S1 (a SW-1573 subtype which is morphologically homogeneous) transfected with the α subunit of the murine sodium/potassium exchanger (Eijdems et al., 1992). This clone has similar growth characteristics and drug sensitivity as the original SW-1573 S1 cell line. The sensitivity for MDR drugs was measured in the presence of ouabain, to ascertain that the MDR clones were derived from S1ou. Resistant cell lines were isolated as single-cell clones from cell line S1ou in a single step at 25 nM and at 40 nM doxorubicin or at 20 nM and at 25 nM vincristine. For nomenclature we took the drug concentration (nm) used for selection followed by an unique clone number that is preceded by a ‘V’ when the clones were selected with vincristine. When the growth rate of the selected clones was similar to that of the parental cells without drug they were analysed. To generate stable transfec tants containing the MDR1-specific hammerhead ribozyme, drug-sensitive S1 cells were transfected with the 196 MDR1 ribozyme construct with linked neomycin resistance marker, designed by Kobayashi et al. (1993), following a standard calcium phosphate precipitation technique (Graham and Van der Eb, 1973). Control transfections were performed with pGEM5Zf(-) DNA. After 3 weeks of selection with G-418 (300 μg·ml⁻¹), individual clones were picked and propagated separately under G-418 selection. All cells were grown as monolayers in Ham’s F-10 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units ml⁻¹) and streptomycin (50 μg ml⁻¹). Cells were maintained in humidified air/5% carbon dioxide at 37°C. All cells were free of Mycoplasma as tested by the use of the Gen-Probe rapid Mycoplasma detection system (Gen-Probe, San Diego, CA, USA).

Assay of drug resistance

Clonogenic survival assays were carried out as follows: cells were plated at 80 cells per well in 24-well dishes (tissue culture cluster 3424; Costar, Cambridge, MA, USA) in the continuous presence of an increasing concentration of drugs. Cells were grown for 8 days, fixed and stained with 0.2% crystal violet (merck 820603) in 3.7% glutaraldehyde. The percentage of cells that were able to produce a colony of >50 cells was used as a measure of cell survival. The resistance was calculated as the ratio of IC₅₀ of the resistant cell line to the IC₅₀ of the parental cell line.

Cellular drug accumulation

Steady-state accumulation of drugs was measured according to Skovsgaard (1990), modified by Broxterman et al. (1988). Adherent cells in the logarithmic phase of growth were try spun, washed and resuspended at densities of 0.2–0.5 x 10⁶ cells ml⁻¹ (daunorubicin) or 0.5–1.5 x 10⁶ cells ml⁻¹ (vincris
tine) in Dulbecco’s modified essential medium (DMEM) without bicarbonate containing 20 mM N-2-hydroxyethylpiperazine-N’-2ethanesulphonic acid (Hepes), 10% fetal calf serum (pH 7.35 ± 0.05) and divided into 0.5 ml portions. [G-1H]Vinristine (diluted with unlabeled vincristine to a vincristine concentration of 0.5 μM) or [G-1H]Daunorubicin (diluted with unlabeled daunorubicin to a daunorubicin concentration of 0.5 μM) was added and cells were incubated at 37°C for 60 min. In the same experiment the daunorubicin accumulation was compared with maximal daunorubicin binding to the cells upon permeabilisation of the plasma membrane with digitonin (Versantvoort et al., 1992). Digitonin (20 μM) was added 5 min before the end of the incubation time with daunorubicin. Drug uptake was stopped by addition of ice-cold phosphate-buffered saline (PBS), supplemented with 10% growth medium, and after two cell washes the cells were transferred to liquid scintillation fluid Opti-Phase III (LKB, Bromma, Sweden) and radioactivity was measured. Values were corrected for amount of cell-associated radioactivity at time zero at 0°C.

RNAse protection assay

Cytoplasmic RNA was isolated by a Nonidet P-40 lysis procedure (Sambrook et al., 1989). The RNAse protection assay was carried out according to Zinn et al. (1983) modified by Baas et al. (1990). Ten micrograms of total cytoplasmic RNA from each SW-1573 derivative was used or 10 μg of Escherichia coli tRNA as a negative control (Boehringer Mannheim, Germany). The protected probe was visualised by electrophoresis through a denaturing 6% acrylamide gel containing 8 μM urea, followed by autoradiography. The following probes were used: MDR1, a 301 nucleotide MDR1 cDNA fragment (nucleotide positions 3500–3801; Chen et al., 1986); topo IIa, a 174 nucleotide topo IIa cDNA fragment (nt positions 1343–1517; Tsu-Pfiffigelder et al., 1988); MRP, a 244 nucleotide MRP cDNA fragment (nucleotide positions 239–483; Zaman et al., 1993). In all experiments a probe for γ-actin (Enoch et al., 1986) was included as an internal control. To determine the intensity of the signals for MDR1, topo IIa, MRP and γ-actin mRNA bands, the autoradiographs were scanned on a Bioimage analysis system (Millipore, USA).

Protein immunoblot analysis

Total cell lysates were made by lysis of cells in 10 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris–HCl pH 7.4 and 0.5% (w/v) sodium dodecyl sulphate (SDS) supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF), leupeptide (2 μg ml⁻¹), pepstatin (1 μg ml⁻¹) and aprotinin (2 μg ml⁻¹). DNA was sheared by sonication. Crude cellular membrane fractions were prepared as described previously (Gerlach et al., 1987) and protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). The protein samples were dissolved in sample buffer [65 mM Tris–HCl pH 6.8, 2.5% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol], separated on a 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and transferred onto nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) by electroblotting. The blots were probed with the monoclonal anti-MRP antibody, MRPl, raised against a bacterial fusion protein containing amino acids 192–360 of MPR (Flens et al., 1994). Antibody was visualised with peroxidase-conjugated rabbit anti-rat immunoglobulins (Dako, Copenhagen, Denmark) followed by enhanced chemiluminescence detection (Amer sham, UK).

Analysis of protein-linked oligosaccharides

For the removal of N-linked oligosaccharides, crude cellular membrane fractions were incubated with 2.67 mM 1 μl⁻¹ N-glycanase (a mixture of endoglycosidase F and peptide-N-glycosidase F; Boehringer Mannheim, Mannheim, Germany)
overnight at 30°C as described previously (Schinkel et al., 1993). High-mannose oligosaccharides were removed by incubation of the crude cellular membrane fractions with 2 units ml⁻¹ endoglycosidase H (Endo H; Boehringer Mannheim, Mannheim, Germany) in sodium citrate pH 5.5 (end concentration 50 nM) and 0.2% (w/v) SDS. Incubations were performed overnight at 37°C. For the removal of steric acids, crude cellular membrane fractions were incubated with 10 units ml⁻¹ neuraminidase (type 8) overnight at 4°C.

Results

MDR is frequently selected with low levels of doxorubicin

SW-1573 clones, resistant to low levels of doxorubicin, were isolated in a single-step procedure using 25 nM, 30 nM and 40 nM doxorubicin (IC₅₀ of the parental cells is 15 nM). After 3 months of selection, 34 clones were isolated and cultured in the presence of doxorubicin. All clones were analysed for their level of mRNA for MDR1 P-gp and topo IIα (see Figure 1) and a representative subset of ten clones was chosen and analysed in detail. As expected, all ten clones showed significant resistance to doxorubicin (Table I). With the exception of clone 5R30, all clones also showed some degree of vincristine resistance (Table I). We conclude that most clones have a MDR phenotype of the non-P-gp variety, as MDR1 mRNA is reduced rather than elevated in most of them (see below).

Drug accumulation is reduced in the doxorubicin-selected SW-1573 clones

To investigate whether drug resistance correlated with reduced drug accumulation in these cell lines, daunorubicin and vincristine accumulation was determined (Table II). All but two of the doxorubicin-selected clones (25.26 and 40.8) showed a reduced steady-state accumulation of daunorubicin compared with the drug-sensitive parental cell line (Table II). In most clones this accumulation defect for daunorubicin was due to a drug gradient over the plasma membrane because the drug uptake increased after permeabilisation of the cell membrane by digitonin (Table III). Despite the lack of an apparent daunorubicin accumulation defect, clone 40.8 showed a significant increase of drug accumulation in the presence of digitonin. No significant differences were found

| Table I Drug resistance in the SW-1573 clones selected at a low doxorubicin or vincristine concentration |
|-----------------------------------------------|-----------------------------------------------|
| **Cell line** | **Resistance factor (RF)** | **Doxorubicin RF** |
|----------------|-----------------------------|---------------------|
| **Doxorubicin-selected** | | |
| 25.10 | 2.3 ± 0.7 (4) | 1.7 ± 0.4 (4) |
| 25.26 | 6.5 ± 1.3 (4) | 3.2 ± 1.1 (5) |
| 5R30 | 4.1 ± 0.1 (2) | 1.1 ± 0.1 (2) |
| 30.3A | 2.4 ± 0.7 (3) | 3.0 ± 2.0 (5) |
| 30.10 | 3.1 ± 0.9 (9) | 3.4 ± 1.4 (9) |
| 30.14 | 4.3 ± 1.6 (6) | 3.0 ± 1.2 (5) |
| 40.3 | 4.8 ± 1.5 (3) | 2.8 ± 0.9 (4) |
| 40.8 | 4.2 ± 1.7 (4) | 2.2 ± 0.8 (3) |
| 40.10 | 3.6 ± 1.5 (3) | 2.8 ± 1.0 (4) |
| 1R50b | 6.1 ± 0.9 (5) | 3.1 ± 0.8 (5) |
| 30.3M | 3.7 ± 1.2 (3) | 4.6 ± 1.0 (3) |
| **Vincristine-selected** | | |
| 20V1 | 3.1 ± 1.0 (3) | 10.6 ± 1.0 (3) |
| 20V2 | 3.0 ± 0.8 (3) | 9.9 ± 2.1 (3) |
| 20V5 | 2.9 ± 0.2 (3) | 10.1 ± 2.5 (3) |
| 25V3 | 2.9 ± 0.8 (3) | 10.3 ± 1.3 (3) |
| 25V4 | 2.9 ± 0.7 (3) | 12.2 ± 1.8 (3) |
| 25V5 | 2.9 ± 0.9 (3) | 12.9 ± 1.1 (3) |

*The resistance factor = IC₅₀ cell line/IC₅₀ parental cell line (IC₅₀ parental cell line S1 = IC₅₀ parental cell line S10u). *The data are the mean resistance factor ± s.d. or the range in case of two experiments. The number of experiments (clonogenic survival assay) is in parentheses. Each experiment was performed in duplicate. *The resistance factor differs significantly from the resistance factor of the parental cell lines with at least 95% confidence. *Results for the original non-P-gp MDR cell line 1R50b are also shown.

**Figure 1 MDR1 P-glycoprotein and topoisomerase IIA mRNA levels measured by RNase protection assays. (a) MDR1 mRNA levels. (b) Topoisomerase IIA mRNA levels.** Clones are grouped by doxorubicin concentration used for selection. The identifying numbers of the clones are given on the x-axis. The mRNA levels are expressed relative to the mRNA level of the drug-sensitive parental cell line S10u. The mRNA level of the parental cell line is 100% by definition. For the clones that were analysed in at least three independently isolated RNA preparations assayed in independent experiments, the mean mRNA (%) ± s.d. (error bar) is given. See also Table II for the ten clones analysed in detail.
Table II  Topoisomerase IIα and MDR1 mRNA levels of representative doxorubicin-selected SW-1573 cell lines and clone S1rib as determined by RNase protection

| Cell line | Topo II mRNA level (%)a | MDR1 mRNA level (%)a |
|-----------|--------------------------|-----------------------|
| 25.10     | 70 ± 14                  | 36 ± 13               |
| 25.26     | 58 ± 18b                 | 17 ± 3b               |
| 5R30      | 46 ± 5b                  | 6 ± 1b                |
| 30.3A     | 13 ± 2b                  | 23 ± 11b              |
| 30.10     | 56 ± 38                  | 18 ± 14b              |
| 30.12     | 108 ± 29                 | 34 ± 20b              |
| 30.14     | 49 ± 13b                 | 7 ± 1b                |
| 40.3      | 29 ± 11b                 | 0.8 ± 0.8b            |
| 40.8      | 123 ± 4                  | 6 ± 2b                |
| 40.10     | 45 ± 10b                 | 15 ± 11b              |
| 1R50b     | 13 ± 3b                  | 15 ± 6b               |
| S1rib     | ND                       | 13 ± 7b               |

aThe mRNA level of the parental cell line is 100% by definition. The data were obtained from three independently isolated RNA preparations assayed in three independent experiments and are presented as the mean mRNA (%) ± s.d. The mRNA level is significantly reduced compared with the level of the parental cell line with at least 95% confidence.

between the pH of the resistant and the parental cell lines (data not shown). This excludes a contribution of a pH increase to the decreased accumulation of the weak base daunorubicin in the resistant cell lines. In addition, no differences in DNA content were found between any of the resistant clones and the parental cells (data not shown), excluding the possibility that the decrease in daunorubicin accumulation was due to a decreased amount of target DNA in the resistant cells.

Most resistant variants also accumulated less vincristine than the parental cells (Table III). This vincristine accumulation is corrected for differences in cell volume between resistant and parental cells as an increase in cell size might result in an increase in vincristine uptake and prevent the detection of an accumulation defect. A significant decrease in vincristine accumulation was detected for the clones isolated at the higher doxorubicin concentrations and a tendency towards decreased vincristine accumulation was found for the clones isolated at lower doxorubicin concentrations (25 nM/30 nM). Two clones, 5R30 and 25.26, showed no vincristine accumulation defect. Clone 5R30 was also not resistant to vincristine. Clone 25.26, however, is unusual in that it has a clear MDR phenotype despite its lack of a detectable accumulation defect for both daunorubicin or vincristine. There are two possibilities for the altered behaviour of clone 25.26. The first explanation is that this clone is multidrug resistant owing to a single mutation, and resistance in this clone is also due to a restricted access of drug to target. The alternative explanation would be that clone 25.26 carries two independent mechanisms for drug resistance, one conferring resistance to doxorubicin, like 5R30, and a second mutation conferring resistance to vincristine. This mutation, however, would have occurred in the absence of the selecting drug. In view of the very low frequency of spontaneously arising vincristine-resistant clones, we consider the scenario with two independent mutations highly unlikely. We assume that resistance in 25.26 is due to the restriction of drug to target, which somehow does not show up in our drug accumulation assays. Therefore, we have not included this clone in the statistical analysis regarding drug accumulation described below.

The results in Table II show that the resistance of the new clones is associated with the decreased drug accumulation in a qualitative sense. To analyse whether the level of drug accumulation also correlated with the degree of drug resistance in a quantitative sense, correlation coefficients were calculated for the parental cell line S1ou and its resistant derivatives. The unusual clone 25.26 was excluded from this analysis. A correlation coefficient of −0.76 was obtained for vincristine accumulation and resistance, indicating that resistance is due to reduced drug accumulation (P < 0.01). For the relation between reduced daunorubicin accumulation and resistance to the related anthracycline doxorubicin a low, non-significant, correlation coefficient of −0.44 was found (P > 0.05). This suggests that additional factors, such as alterations of topo IIα, affect doxorubicin resistance in these clones.

MDR1 P-gp mRNA levels are not increased in the SW-1573 clones with low-level MDR

MDR1 P-gp mRNA levels were measured in all 34 doxorubicin-selected clones by a RNase protection assay (see Figure 1 for all clones and Table II for the ten clones analysed in detail). None of the resistant clones had elevated MDR1 P-gp mRNA levels. On the contrary, nearly all clones showed some degree of MDR1 mRNA reduction, whereas a decrease of MDR1 mRNA levels of more than 50% was detectable in seven out of the ten clones isolated at 25 nM doxorubicin, in 14 out of the 16 clones selected at 30 nM and in all eight clones selected at 40 nM.

The reduction of MDR1 P-gp mRNA levels correlates with reduced vincristine accumulation

All ten doxorubicin-selected clones analysed, showed a significant reduction of MDR1 P-gp mRNA (Table II). To test whether a quantitative relation with drug resistance was present, correlation coefficients were calculated. The reduction of MDR1 P-gp mRNA level and vincristine resistance showed a low, non-significant, correlation coefficient of −0.47 (P > 0.05). The reduction of MDR1 P-gp mRNA level and decreased vincristine accumulation showed a non-significant correlation coefficient of 0.44 (P > 0.05). However, omission of the data for clones 25.26 and 5R30, which do not show a vincristine accumulation defect, raises this last value to 0.91 (P < 0.01).

Reduction of topoisomerase IIα gene expression is an additional event in the development of low-level doxorubicin resistance

To determine the frequency of topo IIα mRNA reduction, all 34 doxorubicin-selected clones were analysed in RNase protection assays. The topo IIα mRNA levels of the resistant clones varied from parental levels to less than 20% of parental levels (see Figure 1b and Table II for the ten clones analysed in detail). The decrease was at least 2-fold in 8 out of 16 clones isolated at 30 nM doxorubicin and in three out of seven clones isolated at 40 nM doxorubicin.

Statistical analysis of the data for the ten clones that were analysed in detail (Table II) showed a significant reduction of topo IIα levels in six clones (25.26, 5R30, 30.3, 30.14, 40.3, 40.10). This suggests that low doxorubicin concentrations can select for cells with lowered topo IIα mRNA levels.

FACS analysis showed no significant differences in cell cycle distribution between the clones and the parental cell lines that could account for the observed topo IIα mRNA reduction and the variation between the clones (data not shown). The alterations in topo IIα mRNA level were not due to changes in γ-actin levels, since similar results were obtained with a probe for β-glucuronidase as internal standard in the RNase protection experiments (data not shown). The only exception was clone 40.8, which had an increased cell size and increased ratio of γ-actin to β-glucuronidase mRNA in the absence of a decreased topo IIα mRNA level.

MDR clones selected at low levels of vincristine do not exhibit the decrease in topoisomerase IIα mRNA

To test whether a similar type of non-P-gp MDR could be obtained with vincristine as with doxorubicin, we selected an additional set of clones in a single step with 20 or 25 nM
vincristine (IC_{50} of the parental cell line is 2.5 nm). These were the lowest concentrations that still fully eliminated parental background clones. After 6 months of selection, six clones were isolated and cultured in the presence of vincristine. The analysis of these clones is summarised in Figure 2 and Tables I and IV. The vincristine-selected clones resemble their doxorubicin-selected counterparts in several characteristics, i.e. resistance to both doxorubicin and vincristine (Table I), decreased drug accumulation (Table III) and a reduced MDR1 P-gp mRNA level (Figure 2). However, they differ from the doxorubicin-selected clones in two properties: they lack the reduced topo IIα mRNA level (Figure 2) and they have a much higher vincristine than doxorubicin resistance (Table I). Whereas the average ratio doxorubicin/vincristine resistance was 1.6 (range 0.8–4.0) for the ten doxorubicin-selected clones, it was only 0.3 (range 0.2–0.3) for the six vincristine-selected ones (Table I).

The reduction of MDR1 P-gp mRNA does not contribute to drug resistance

We showed that the reduction of MDR1 P-gp mRNA was co-transferred with the MDR phenotype in somatic cell fusion experiments (Eijdems et al., 1992) and was present in all MDR SW-1573 cells in this study (Figures 1 and 2, Table II). The MDR1 P-gp extrudes drugs from cells and one would therefore not expect a decrease in MDR1 expression to increase resistance. To exclude exotic indirect effects, we artificially reduced the MDR1 in the parental drug-sensitive cells by transfection of a hammerhead ribozyme specific for MDR1 (Kobayashi et al., 1993, 1994). Transfected cells were selected for G-418 resistance, as a neomycin phosphotransferase gene was present in the expression vector. One of ten G-418-resistant subclones, clone Sl rib, showed a reduced MDR1 mRNA level similar to that of the SW-1573 non-P-gp MDR cells (Table II), but no detectable decrease or increase of sensitivity to doxorubicin, vincristine or VP16-213 was found for this clone in clonogenic survival assays (data not shown). These results indicate that, despite the observed down-regulation of MDR1 mRNA in the MDR cell lines, reduced MDR1 mRNA levels do not contribute to drug resistance in the SW-1573 cells.

Table III Daunorubicin and vincristine accumulation in SW-1573 clones selected at low doxorubicin or vincristine concentration

| Cell line | Daunorubicin accumulation | Vincristine accumulation |
|-----------|--------------------------|------------------------|
|           | Control pmol 10^4 cells | Digitonin pmol 10^4 cells | % | % |
| Doxorubicin-selected | | | | |
| Slou | 215 ± 11 | 100 | 207 ± 30 | 21.1 ± 6.7 | 100 |
| 1R50b | 146 ± 27 | 68 | 198 ± 41 | 11.1 ± 3.9 | 52 |
| 25.10 | 180 ± 31 | 84 | 239 ± 19 | 15.3 ± 4.8 | 70 |
| 25.26 | 196 ± 22 | 91 | 196 ± 40 | 26.5 ± 5.9 | 125 |
| 5R30 | 156 ± 25 | 72 | 215 ± 42 | 19.8 ± 0.5 | 94 |
| 30.3A | 130 ± 23 | 60 | 221 ± 51 | 11.6 ± 3.7 | 55 |
| 30.10 | 156 ± 15 | 72 | 258 ± 16 | 12.3 ± 6.4 | 58 |
| 30.12 | 165 ± 19 | 77 | 208 ± 16 | 13.8 ± 4.1 | 65 |
| 30.14 | 182 ± 26 | 85 | 208 ± 50 | 9.1 ± 2.6 | 43 |
| 40.3 | 142 ± 25 | 66 | 195 ± 34 | 4.13 ± 2.0 | 20 |
| 40.8 | 202 ± 29 | 94 | 250 ± 32 | 11.4 ± 3.7 | 54 |
| 40.10 | 172 ± 29 | 80 | 230 ± 21 | 10.3 ± 3.3 | 49 |

Vincristine-selected

| Cell line | Daunorubicin accumulation | Vincristine accumulation |
|-----------|--------------------------|------------------------|
|           | Control pmol 10^4 cells | Digitonin pmol 10^4 cells | % | % |
| Slou | 331 ± 12 | 100 | 290 ± 43 | 27.9 ± 8.6 | 100 |
| 20V2 | 175 ± 29 | 53 | 284 ± 36 | 7.4 ± 1.7 | 30 |
| 25V4 | 154 ± 13 | 47 | 296 ± 21 | 7.2 ± 1.7 | 28 |

*The data are from 3–8 independent experiments each performed in quadruplicate. The mean accumulation ± s.d. is given. *The vincristine accumulation is corrected for differences in cell volume compared with the parental cell line as measured by a Coulter counter calibrated with microspheres. The standard deviation is obtained from the standard deviations of both the vincristine accumulation and the cell volume. *The accumulation differs significantly from that in the Slou cells or the accumulation after addition of digitonin differs significantly from that without addition of digitonin (P < 0.05) according to the unpaired Student's t-test. *As the drug accumulation data for the doxorubicin- and for the vincristine-selected cell lines were determined in independent experiments, the drug accumulation of the drug-sensitive parental cell line for both data sets is given.

Figure 2 RNase protection assays to quantify MDR1 P-gp, MRP and topoisomerase IIα mRNA levels in the SW-1573 clones selected with vincristine. The vincristine concentration used for selection (nm) is used for nomenclature, followed by an identifying clone number. For comparison, RNA of the original non-P-gp MDR cell line 1R50b was also assayed. Ten micrograms of total cytoplasmic RNA from each SW-1573 derivative was used. The γ-actin signal was comparable for the independent experiments. On a longer exposure a very faint band of MDR1 mRNA signal was detectable in the vincristine-selected clones and was similar to the signal in the 1R50b cell line.

MRP mRNA levels in the resistant SW-1573 clones

All new MDR clones showed a slight increase in MRP mRNA level (Figures 2 and 3, Table IV). For most individual clones this small increase was not statistically significant, as also observed in initial experiments with the resistant cell
line 1R50b (Zaman et al., 1993). We observed large variations in independently isolated RNA preparations, illustrated by the standard deviations in Table IV, e.g. 1R50b 129% ± 79. Only two individual clones, 30.12 and 30.3M, showed a significant increase in MRP mRNA (with at least 95% confidence; Table IV and Figure 3). In 30.3M, obtained by continued doxorubicin selection of 30.3A, this increase was due to transcriptional activation of the MRP gene (Eijdems et al., 1995a), which was accompanied by an increase in both doxorubicin and vincristine resistance compared with 30.3A (Table I). This suggests that MRP can be involved in low-level drug resistance in the SW-1573 cell lines. Despite large variability in MRP mRNA levels in the individual clones, all clones taken as a group had a significant increase of MRP mRNA level relative to the parental cells (t-test, P<0.01).

**An altered form of MRP in the MDR SW-1573 cells**

To test whether the slight increase in MRP mRNA found in the resistant variants was translated into an increased level of MRP protein, we analysed the level of MRP in these variants.

**Table IV MRP mRNA levels of low-resistant MDR SW-1573 cell lines selected with doxorubicin or vincristine**

| Cell line | MRP mRNA level (%)* |
|-----------|---------------------|
| Doxorubicin-selected | |
| 25.10 | 161 ± 102 |
| 30.12 | 218 ± 82 |
| 40.3 | 171 ± 57 |
| 1R50b | 129 ± 79 |
| 30.3M | 292 ± 64 |
| Vincristine-selected | |
| 20V2 | 198 ± 86 |
| 25V4 | 189 ± 73 |

*The MRP mRNA of the parental cell line is 100% by definition. The data were obtained from three independently isolated RNA preparations assayed in three independent experiments and are presented as the mean mRNA (%) ± s.d. The mRNA level differs significantly from that of the parental cell line with at least 95% confidence.

![Figure 3](image) **Figure 3** RNase protection assay to quantify MRP mRNA levels in the SW-1573 clones selected with doxorubicin. The doxorubicin concentration used for selection (nm) is used for the nomenclature, followed by an identifying clone number. For comparison, RNA of the original non-P-gp MDR cell line 1R50b was also assayed. Ten micrograms of total cytoplasmic RNA from each SW-1573 derivative was used. The positions of the protected fragments of MRP and γ-actin mRNA as well as the size (nucleotides) of the molecular weight markers are indicated (M).

![Figure 4](image) **Figure 4** Western blot analysis of MRP in SW-1573 cells. (a and b) MRP in total cell lysates of drug-sensitive cell line Slou and low-resistance SW-1573 cell lines. The cell lines were selected with doxorubicin (25.10, 30.3A, 30.3M, 30.12, 40.3 and 1R50b) or vincristine (20V2 and 25V4), or were derived by somatic cell fusion between cell line 1R50b and drug-sensitive, parental cell line Slou (F6.1 and F6.3). (c) Crude cellular membrane fractions of Slou, 1R50b, 30.3M and 20V2, incubated in the absence (−) or presence (+) of N-glycanase. The protein samples (30 μg of protein per lane for a and b, and 20 μg for c) were size fractionated in a 7.5% polyacrylamide gel containing 0.5% SDS, transferred to a nitrocellulose membrane and MRP was detected by incubation with monoclonal antibody MRPr1. The size (kDa) and position of molecular weight markers are indicated.
apparent molecular mass of 190–200 kDa was present, whereas parental MRP runs at 180 kDa (Figure 4a and b). The relative amounts of the 180 kDa and the 190–200 kDa forms of MRP varied somewhat in different clones. The increase in resistance level in cell line 30.3M compared with 30.3A (Table I) was accompanied by a nearly complete replacement of the 180 kDa protein by the 190–200 kDa protein (Figure 4a). The protein with altered mobility was also detectable in the non-P-gp MDR cell hybrids F6.1 and F6.3 (Figure 4b), which were derived by somatic cell fusion of the non-P-gp MDR cell line 1R50b with drug-sensitive cell line S1ou (Eijdems et al., 1992). This shows that the presence of the 190–200 kDa MRP protein is linked to the non-P-gp MDR phenotype in the SW-1573-derived cell lines.

To analyse the nature of the altered behaviour of MRP on SDS-PAGE, crude cellular membrane fractions of the multidrug-resistant cell lines 1R50b, 30.3M (doxorubicin selected) and 20V2 (vincristine selected) were analysed (Figure 4c). In the membrane fractions, a single MRP band with a molecular weight in the range of 190–200 kDa was detectable in the resistant cells, in contrast to the 180 kDa protein detectable in the parental, drug-sensitive cell line S1ou. Similar to the results obtained with total cell lysates, no major increase in the amount of MRP and protein was detectable in the resistant cell lines (1R50b, 20V2). Only cell line 30.3M, which has a 3-fold elevated MRP mRNA, also showed an increase of MRP epitopes in the membrane fractions.

Treatment of the crude cellular membrane fractions with N-glycanase, which removes N-linked oligosaccharides, resulted in the appearance of a single band in the range of 150 kDa in both the parental and in the resistant cell lines (Figure 4c). This indicates that the altered mobility of MRP in the resistent cell lines is due to differences in post-translational modification and not to major differences in the primary structure in baculovirus.

Treatment of purified membrane fractions with endoglycosidase H (Endo H), which removes high-mannose oligosaccharides, did not affect the mobility of MRP either in the resistant cell lines or in the drug-sensitive cell line S1ou (data not shown). Removal of sialic acid with neuraminidase reduced the size of MRP protein in both the drug-resistant and the drug-sensitive cells to the same extent and did not alter the difference in mobility (data not shown). These results demonstrate that the 180 kDa and the 190–200 kDa MRP proteins both contain complex oligosaccharides and must have passed the medial Golgi compartment and reached the trans-Golgi network.

Discussion

We have analysed a large set of drug-resistant variants of the human lung cancer line SW-1573, selected with low concentrations of either doxorubicin or vincristine. We found that the predominant type of resistance is a form of MDR characterised by reduced drug accumulation and decreased MDR1 P-gp mRNA levels. Since the degree of resistance correlates with the extent to which drug accumulation is diminished, it seems likely that the lowered intracellular drug concentrations are mainly responsible for resistance in these variants. A diminished drug accumulation has also been observed in other non-P-gp MDR cell lines (reviewed by Cole, 1992), the H69/AR cell line being the only exception (Cole et al., 1991).

Overexpression of the MRP gene can confer multidrug resistance associated with reduced drug accumulation in human HeLa cells (Grant et al., 1994) and SW-1573 cells (Zaman et al., 1994). In several non-P-gp MDR cell lines a role for overexpression of MRP was suggested as well (Krishnamachary and Center, 1993; Slovak et al., 1993; Zaman et al., 1993; Barrand et al., 1994; Schneider et al., 1994). The MRP mRNA and MRP protein levels in most clones isolated in this study were increased at most 2-fold. At first sight, it seems unlikely that this could account for the complete MDR phenotype in these SW-1573 cells. However, our finding that an altered form of MRP is present in all our non-P-gp MDR clones and that this altered form segregates with the non-P-gp MDR trait in somatic cell fusion experiments strongly indicates that MRP is involved in the non-P-gp phenotype of these clones. To test this more directly, we are constructing a ribozyme directed against MRP mRNA.

The precise nature of the alteration of MRP resulting in altered migration in SDS-PAGE gels is not yet known. Our results show that this altered mobility is due to a change in the MRP-linked complex oligosaccharides. Post-translational modification is the most plausible explanation. This modification could either be directly responsible for MDR, e.g. result in a more active MRP drug pump, or it could be a consequence of other alterations affecting MRP, such as alterations occurring at the Golgi apparatus. Minor changes in the primary structure of MRP cannot be excluded yet.

A remarkable feature of the non-P-gp MDR SW-1573 clones is the paradoxical decrease of MDR1 mRNA levels. This was also reported for the H69-AR cell line (Cole et al., 1991), the non-P-gp MDR cell line from which the MRP gene was isolated (Cole et al., 1992). Although it is hard to believe that a decrease in MDR1 P-glycoprotein could result in an increased drug extrusion, the theoretical possibility existed that P-glycoprotein was extruding an endogenous compound required to induce the non-P-gp MDR mechanism in SW-1573 cells. Our ribozyme experiment excludes this far-fetched possibility. Hence, the down-regulation of MDR1 does not cause MDR in our resistant clones, but is a side-effect of the mutation causing the non-P-gp phenotype. In view of the alteration of MRP in the SW-1573 cells, the decrease in MDR1 mRNA could be an indirect effect of the post-translational modification of a regulator of MDR1 expression. The absence of increased MDR1 expression in the non-P-gp MDR cell line is not due to the inability of the SW-1573 cells to activate the MDR1 gene since we have shown that SW-1573 cells selected for higher levels of resistance invariably increase their expression of MDR1 (Baas et al., 1990).

The non-P-gp MDR phenotype with altered MRP, reduced drug uptake and reduced MDR1 mRNA was obtained both with doxorubicin and vincristine selection. However, the resistance spectra of the two types of clones differed (Table I). This suggests that cells may activate multiple resistance mechanisms in response to these low levels of selection. In the cases of doxorubicin selection, the supplementary resistance mechanism probably consists of alterations in topo IIa. Most of the MDR variants selected for doxorubicin resistance contained decreased topo IIa mRNA levels, and the clones with normal mRNA level may nevertheless contain decreased topo II enzyme activity (Eijdems et al., 1995b). In the case of vincristine selection, we have no clue as to the mechanism that contributes to the relatively high vincristine resistance.

Our experiments show that low-level resistance associated with altered MRP arises with high frequency in SW-1573 cells. Precise estimates of this frequency are not available, as the sensitive cells continue to divide slowly during mutant selection at low drug concentration. It is clearly much higher, however, than the activation of MDR1, which occurs at a frequency of 1-2 x 10^{-6} per cell in SW-1573 when higher drug concentrations are used for selection (Eijdems et al., 1992, and unpublished results). We infer from our results that resistance associated with altered MRP only protects against relatively low levels of resistance. This follows from the association of this type of resistance with other forms of low-level resistance, topo II alterations or unknown, and from the fact that selection for higher levels of resistance results either in an activation of MDR1 or in a transcriptional activation of MRP. The only clear example of MRP activation is clone 30.3M, which has a 3-fold increase in MRP mRNA. We have recently shown by RNA run-on experiments that this is due to increased transcription of MRP (Eijdems et al., 1995b).

In conclusion, we have shown that in the SW-1573 lung cancer cell line low levels of two different types of drug select...
for altered MRP accompanied by reduced drug accumulation and concomitant multidrug resistance. Although this MDR phenotype correlated well with reduced MDR1 mRNA, we excluded a contribution of this reduction to drug resistance. Our results also show that, even at the low drug concentrations used, multiple resistance mechanisms may coexist in resistant clones.

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