Anti-Cancer Drugs 2001, **12,** pp. 247–258

Preclinical report

Sequential gene expression of P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and lung resistance protein: functional activity of P-gp and MRP present in the doxorubicin-resistant human K562 cell lines

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Previous studies have reported that P-glycoprotein (P-gp), a transmembrane efflux pump involved in multidrug resistance (MDR), was overexpressed in the doxorubicin (Dox)-resistant human erythroleukemia cell line K562. Nevertheless, several results suggested that P-gp was not the only mechanism involved in these resistant cells. Sequential co-expression of other MDR-associated proteins was sometimes reported, as MDR-associated protein (MRP) and lung resistance protein (LRP), in different MDR cell lines. Thus, mRNA expression and stability of P-gp, MRP and LRP were analyzed, while their corresponding protein levels were quantified in correlation with functional assay, in the K562 cell line and two Dox-resistant variants (K562/R). Their P-gp content was in accordance with their degree of resistance, but not as much in the level of mRNA expression, suggesting a post-transcriptional regulation. On the other hand, MRP could play a minor role in MDR because of an unchanged expression in K562/R sublines. A surprising progressive disappearance of LRP in both resistant cells suggested that the original mechanism of drug redistribution may be operative, involving a negative role for LRP. [© 2001 Lippincott Williams & Wilkins.]

Key words: Doxorubicin, lung resistance protein, multidrug resistance-associated protein, P-glycoprotein.

This work was supported by the 'Ligue contre le Cancer du Limousin'.

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Introduction

The emergence of resistance to antineoplastic drugs represents a major obstacle to the successful treatment of cancer. Resistance often concerns a variety of unrelated chemical compounds and is then named multidrug resistance (MDR) or pleiotropic resistance. Several mechanisms may be involved in the development of MDR, including drug detoxification and alterations in drug targets.[1] Among these MDR mechanisms, the best characterized involves the overexpression of P-glycoprotein 170 (P-gp).[2] This transmembrane protein of 1280 amino acids is an ATP-dependent drug pump which expels agents such as vinca alkaloids, anthracyclines, epipodophylotoxins and taxol out of the cell. The overexpression of P-gp can be due to a positive transcriptional regulation as described by Labroille *et al.*[3] or to the amplification of the MDR gene.[4] In human cells, P-gp is encoded by a well-characterized DNA sequence, the *MDR1* gene, located on chromosome 7. Overexpression of P-gp occurs *in vitro* in numerous drug-selected cell lines, and *in vivo* in different types of solid tumors and leukemias.

Nevertheless, it has become more and more evident that alternative and/or additional mechanisms of MDR exist. Cole *et al.*[5] described a novel gene encoding a MDR-related protein (MRP) in an anthracycline-resistant but P-gp-negative lung cancer line. Like P-gp, MRP belongs to the ATP-binding cassette (ABC) family of membrane transport proteins and its mRNA encodes a protein of 1531 amino acids. P-gp and MRP share a

common molecular architecture, despite their low amino acid identity (less than 15%). Both proteins contain 12 large transmembrane domains and two intracellular ATP binding units.[6]

More recently, a novel resistance-associated gene encoding the lung resistance protein (LRP) was described by Scheper *et al.*[7] in a multidrug-resistant cell line that did not exhibit P-gp overexpression. LRP shares significant amino acids identity with the M_r 104 000 rat major vault protein.[8] Vaults are multisubunit ribonucleoproteins involved in nucleocytoplasmic transport, a finding which suggests that LRP may be involved in the intracellular transport of cytotoxic agents.[8] LRP overexpression is associated with resistance to doxorubicin (Dox), vincristine, carboplatin, cisplatin and melphalan,[9] but its role in MDR is unclear until now.

Studies on a multidrug-resistant leukemia cell line that overexpresses MRP have suggested that a substantial fraction of the protein may be present in the endoplasmic reticulum in addition to the plasma membrane.[10] Cell type-specific variations in MRP distribution between these two membrane compartments may explain why decreases in accumulation, as well as marked changes in intracellular drug distribution, have been observed in cell lines which overexpress MRP.[5] On the other hand, approximately 5% of vaults are nucleus-associated and localized to the nuclear pore complexes.[11] The majority of vaults, therefore, have a cytoplasmic localization.[12] Thus, LRP and/or vaults may regulate nucleocytoplasmic as well as vesicular transport of different substrates. Several studies, using clinical cancer specimens, as well as cancer cell lines displaying the MDR phenotype, have described a co-expression of two or three of these proteins, without any quantification of this phenomenon.[9,13] In addition, this phenomenon appears to be sometimes sequential.[14] However, it is not always clear whether overexpression of these proteins, generally studied in relationship to the mRNA level, is due or not to DNA amplification of the corresponding gene. Also, it is not clear either whether the difference in expression correlates with alterations in protein function. Moreover, no complete molecular analysis concerning an eventual amplification of these genes, their mRNA levels and stabilities was correlated with the quantification of the three proteins and functional assays in order to assess their respective role in the MDR phenotype.

The MDR phenotype in the K562 cell line, a well-known human myeloid leukemia cell line, has been related to the overexpression of P-gp, but this characterization has only been studied in one MDR K562 subline compared to the parental cells. Moreover, Bennis *et al.*[15] and Denis-Gay *et al.*[16] have observed that the reversal of the MDR phenotype in K562 resistant cells was not complete with verapamil, a P-gp inhibitor, suggesting the involvement of other mechanisms of resistance. Other transporter proteins could be localized in plasmic and intracellular membranes, as already mentioned in numerous studies.

The purpose of this study was to accurately quantitate the levels of expression of the three proteins (P-gp, MRP and LRP) and to correlate them with mRNA expression and stability, and with a possible amplification of the corresponding genes. Gene expression studies and functional tests were carried out to evaluate the specific contribution of P-gp and MRP in uptake and efflux of drugs.

Material and methods

Cell lines and culture conditions

Dox-sensitive K562[17] and their Dox-resistant counterparts[18] were grown in suspension in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (Seromed, Berlin, Germany) and 2 mM L-glutamine (Life Technologies, Cergy Pontoise, France). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The resistant cell lines were obtained by progressive adaptation of the parental sensitive cells (K562/S) to 0.2 μg/ml of Dox for K562/0.2R and then to 0.5 μg/ml of Dox for K562/0.5R cells (Robert *et al.*, unpublished). These resistant cell lines were maintained without drug for at least 1 month to avoid taking into account the direct effects of Dox on cells.

The human breast cancer cell line MCF-7 resistant to etoposide (VP-16) was also used as a positive control for MRP expression.[19] The cell line KB3.1, derived from a human squamous cell carcinoma,[20] was used as a positive control for expression of LRP (a gift from S Chevillard, CEA, France). Both cell lines were maintained as monolayer cultures in the same culture medium as K562 cell lines.

Chemosensitivity assay

Cytotoxicity of Dox in the presence or absence of cyclosporin A was measured by using the MTT assay. Briefly, 1000 K562/S cells or 2000 K562 resistant sublines were seeded in each well of 96-well plates. One day for sensitive cells, or 2 days later for the resistant ones, fresh medium containing Dox at concentrations ranging between 0.01 and 1 μg/ml for K562/S cells and between 0.1 and 100 μg/ml for resistant cells, with or without cyclosporin A (1 μM) was added. Incubation was performed for 2 h at 37°C.

The plates were then centrifuged, and cells were washed with buffered saline and allowed to grow further for 2 days in fresh medium. At this time, the surviving cells were estimated by MTT assay and the IC_{50} values (drug concentration responsible for 50% growth inhibition as compared with untreated cells) were calculated.

Functional assay of P-gp and MRP by flow cytometry

After one wash in PBS, cells were adjusted at 2×10^6 cells/ml in serum-free RPMI. Then, 250 μl of cell suspension (5×10^5 cells/sample) was distributed in eight test tubes for sensitive and both resistant cell lines, as described by Huet *et al.*[21] Cyclosporin A (1–4 μM) (Sigma-Aldrich, St Quentin Fallavier, France) and probenecid (10–40 μM), which are specific inhibitors of P-gp and MRP, respectively, were added to evaluate the effect of both proteins on rhodamine 123 uptake and efflux (Molecular Probes, Eugene, OR). Efflux of this dye was evaluated after 10 min and 1 h of incubation. For each sample, 10 000 viable cells were analyzed on a FACS Vantage flow cytometer (Becton Dickinson, Grenoble, France).

Monoclonal antibodies (mAbs)

For Western blot analysis, mAbs were purchased from Monosan (Uden, The Netherlands) in the MDR sampler pack. The murine mAb JSB-1 reacts with a conserved cytoplasmic epitope of the plasma membrane-associated P-gp.[22] The antibodies LRP-56 and MRPr1 react with internal epitopes of LRP and MRP proteins, respectively.[7,23] Rabbit anti-mouse or anti-rat (in the case of MRPr1) immunoglobulin (IgG1) peroxidase was purchased from Dako (Trappes, France). For flow cytometric analyses, we have used UIC2 (IgG2a) mAb (Immunotech, Marseille, France), which reacts specifically with an extracellular epitope of the human 170 kDa P-gp.[24] Flow cytometric analyses of MRP and LRP were carried out with MRPm6[23] and LRP-56 mAbs, which both react with internal epitope of MRP and LRP, respectively. A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse second antibody (Becton Dickinson, San Jose, CA) revealed the primary antibody specifically bound.

Western immunoblots and analysis of membrane proteins

Cells in exponential phase (48 h of culture) were washed twice with PBS and pelleted at 300 *g* for 10 min at 4°C. Then, after counting, 4×10^7 cells were washed with 40 mM Tris–HCl, pH 8, and pelleted as described before. Supernatants were removed and replaced by 250 μl of 5 mM Tris–HCl, pH 8, 6 mM $MgCl_2$ and 1 mM PMSF. After 10 min incubation on ice, cells were homogenized by sonication. Then we added 250 μl of 80 mM Tris, pH 8, 6 mM $MgCl_2$, 10 U DNase I (Life Technologies) and 1 mM PMSF. The mixture was left at room temperature for 30 min. The protein content of total cell lysates was determined by the Bradford procedure using bovine serum albumin as a standard.

Cell proteins were resolved by SDS–PAGE using the method of Laemmli[25] and electroblotted onto nitrocellulose (Amersham Pharmacia Biotech, Saclay, France). After blocking overnight at 4°C with 1% blocking reagent, the membranes were incubated for 1 h at room temperature with the indicated primary antibody. Detection was performed by chemiluminescence using a POD-conjugated secondary antibody and the 'BM chemiluminescence blotting substrate (POD)' kit (Roche, Meylan, France) according to the manufacturer's instructions. Chemiluminescence was detected for the indicated time with a Fuji X-ray film.

Quantitation of P-gp, MRP and LRP by flow cytometry

Immunofluorescent staining on Dox-sensitive and -resistant cells was first done to determine the concentration of UIC2, MRPm6 and LRP-56 necessary to saturate, respectively, all the P-gp, MRP and LRP sites. For MRP and LRP quantification with specific mAbs, which both recognize intracellular epitopes, preliminary fixation of cells in the logarithmic phase was performed in PBS/2% paraformaldehyde for 30 min at 4°C. Cells were then washed with PBS and permeabilized with 70% methanol for 30 min at 4°C. Then, for P-gp as for MRP and LRP expression analysis, cells were washed twice in PBS and 7×10^5 cells/sample were incubated for 1 h at 4°C with different concentrations of primary mAb ranging from 0.05 to 5 μg. Cells were then washed twice in PBS/1% BSA and the pellet was resuspended with 4 μg of a secondary antibody coupled to FITC diluted in PBS/1% BSA. After 30 min of incubation at 4°C, cells were washed twice with PBS/1% BSA and resuspended in 400 μl of PBS. In the case of P-gp, 10 μg of propidium iodide was added to exclude dead cells from fluorescence measurement just before analysis. An isotypic control was realized to determine non-specific binding. In parallel, to quantitate accurately the expression levels of P-gp, MRP and LRP at the surface of the different K562 cells, the 'Qifikit' (Dako) was used. It contains series of beads coated with different, but well-defined quantities of

mouse mAbs molecules. These calibration beads were labeled with the same FITC-conjugated anti-mouse secondary antibody as cells were. Consequently, the emitted fluorescence was correlated with the number of primary antibody molecules bound on the beads and on the cells at the saturating concentration of the primary mAb. The fluorescence of cells and beads was analyzed on a FACS Vantage flow cytometer (Becton Dickinson). For each sample, 10^4 cells were analysed after elimination of dead cells, debris and aggregates, with a flow rate of 1 000 cells/s^{-1}. For each immunostaining, the mean fluorescence of the corresponding negative control was subtracted from the mean fluorescence of the assay.

DNA and RNA manipulations

Genomic DNA of resistant and sensitive cells was extracted by the 'High Pure PCR Template Isolation' kit (Roche). It was digested with restriction enzymes purchased from Gibco/BRL (Life Technologies). Total cellular RNA was extracted from the sensitive and resistant cells by the 'High Pure RNA Isolation' kit (Roche). All aqueous solutions used in these manipulations were treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved. RNA concentration was estimated spectrophotometrically and 10 μg was electrophoresed on formamide–formaldehyde denaturing 1% agarose gel. Amplification of cDNA used as probes was performed with 5 U Taq DNA polymerase. All DNA manipulations, if not described, were carried out by standard procedures.

Southern hybridization

Chromosomal DNA was digested with restriction endonuclease *Eco*RI and analyzed by 0.8% agarose gel electrophoresis. The DNA was transferred from the gel onto Hybond-N+ filters (Amersham Pharmacia Biotech), essentially according to the method of Southern.[26] The filters were pre-hybridized at least 1 h at 65°C and then hybridized overnight at 65°C with an appropriate probe. Hybridization conditions are detailed in Results. After washing, the filters were autoradiographed with X-ray films. Amplified DNA products used as probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using the 'Random Primers DNA labeling system' kit (Life Technologies).

Northern hybridization

Total RNA was transferred from the denaturing gel onto Hybond-N+ filters by capillarity. Filters were hybridized overnight at 42°C in the presence of 50% formamide and ^{32}P-labeled DNA probes of the *MDR1*, *MRP*, *LRP* and human *β-actin* cDNAs. This last gene, which is expressed constitutively in most human cells, was used as an internal control for quantifying the amounts of RNA. As for Southern blots, autoradiograms were scanned by laser densitometry using Imagequant Software (Molecular Dynamics, Sunnyvale, CA) and were normalized in comparison of *β-actin* signal.

Stability of MDR1 and MRP mRNA

Sensitive and resistant cells were incubated in the presence of 4 μM actinomycin D (Sigma-Aldrich) at time 0 and total cellular RNA was isolated by the 'High Pure RNA Isolation' kit (Roche) every 3–4 h for 15 h. Total RNA (10 μg) was electrophoresed as described for Northern blot analysis, and the blot obtained was hybridized with *MDR1*, *MRP* and *β-actin* probes. Autoradiograms were then scanned by laser densitometry using ImageQuant Software (Molecular Dynamics).

RT-PCR amplification

An aliquot of 3 μg of RNA solution was heated at 70°C for 10 min with 1 μl of oligo-dT (500 μg/ml), ice cooled and subjected to first-strand DNA synthesis using 200 U of Superscript II, RNase H^- (Life Technologies). Each specific probe was amplified from first-strand cDNA in a 100 μl total volume reaction through 35 cycles of PCR, each cycle consisting in 30 s at 94°C, 30 s at the appropriate annealing temperature and 50 s at 72°C. A negative control without cDNA was also performed to check possible contamination. The primer sequences used for *MDR1*, *MRP* and *LRP* gene amplification are described in Table 1.

Table 1. Sequences of the three different couples of primers and length of the obtained product used for semi-quantitative RT-PCR and as probes for Northern blot and Southern blot hybridization

Probe	Sequence of primers	Length of the probe (bp)
MDR1	5′gATCTTgAAggggACCgCAATggA-3′ 5′gATgCATAgATCAgCAggAAAgCAgC-3′	922
MRP	5′-ggACCTggACTTCgTTCTCA-3′ 5′CgTCCAgACTTCCTTCATCCg-3′	291
LRP	5′-CCCCCATACCACTATATCCATgTg-3′ 5′-CTCgAAAAgCCACTCATCTCCTg-3′	407

The amplified probes were gel-purified with the 'gel extraction spin' kit (Genomed, Montreuil, France).

Semi-quantitative RT-PCR was performed as described above to estimate the transcription rate of the *LRP* gene, but the first-strand cDNA was sequentially diluted, ranging from no dilution to a 25-fold dilution. The product obtained was separated on non-denaturing 1.5% agarose gel.

DNA sequencing and analysis

DNA sequencing was performed by the dideoxy chain termination method[27] using an automated sequencer (ABI Prism 310; Perkin Elmer, Courtabœuf, France). Each probe was sequenced twice at least on both strands. Sequence comparisons were performed by searching the GenBank database at the Internet Web site.

Statistics

An unpaired Student's *t*-test of the Statview software was used for all experiments.

Results

Drug sensitivity of K562/S cells and K562 MDR sublines

K562/0.2R and K562/0.5R, Dox-resistant human erythroleukemia sublines, were established from K562/S cells.[17] Cytotoxicity, determined by the MTT assay (Table 2), exhibited an average 310-fold resistance to Dox for K562/0.2R cells and an average 510-fold resistance for K562/0.5R cells. The Dox IC_{50} of sensitive cells was 0.03 ± 0.005 μg/ml, and 10.1 ± 3.7 and 16.7 ± 5 μg/ml for K562/0.2R and k562/0.5R, respectively (each measure was done in duplicate 3 times). Dox IC_{50} values were evaluated in the presence of cyclosporin A (1 μM). As indicated in Table 2, cyclosporin A reduced by 3-fold the concentration of Dox required for 50% growth inhibition in both resistant sublines. Interestingly, cyclosporin A significantly increased the cytotoxicity of Dox by about 2-fold in the sensitive cell line.

Analysis of P-gp and MRP functional activity

The Dox-resistant cells, which express P-gp, accumulate less fluorescent rhodamine 123 than K562/S cells ($p < 0.05$) (Figure 1A). To test whether P-gp was the

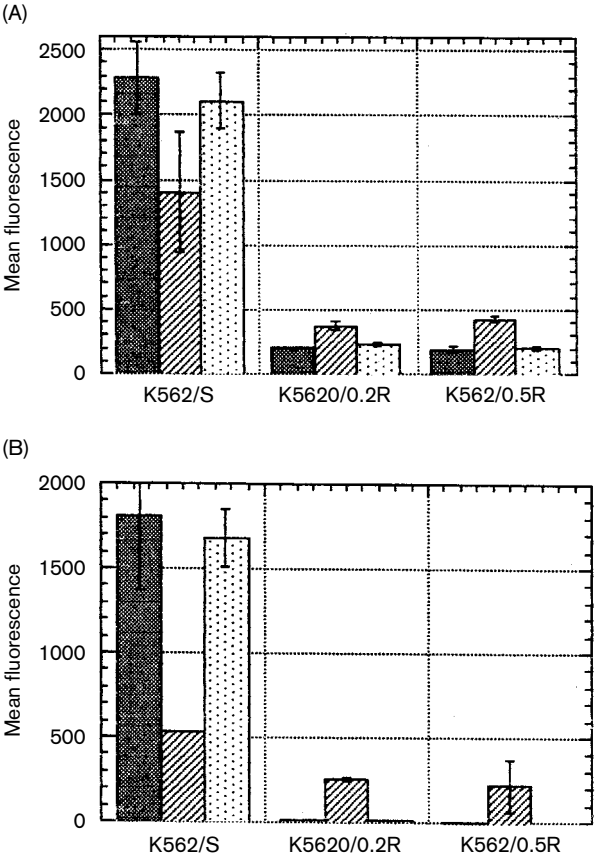

Figure 1. Analysis of P-gp and MRP activity by flow cytometry. (A) Analysis of rhodamine 123 accumulation in the absence of inhibitor (▬), with cyclosporin A (4 μM) (▨), with probenecid (40 μM) (▭). (B) Analysis of rhodamine 123 efflux in the absence of inhibitor (▬), with cyclosporin A (▨), with probenecid (▭).

Table 2. IC_{50} assessed in duplicate 3 times by the MTT assay and relative resistance in K562/S cells and both MDR variants: K562/0.2R and K562/0.5R sublines (mean ratio of IC_{50} of K562/MDR variant to K562/S cells)

Drug	IC_{50}			Relative resistance	
	K562/S	K562/0.2R	K562/0.5R	K562/02R	K562/0.5R
Dox	0.03 ± 0.005	10.1 ± 3.7	16.7 ± 5	311	512
Dox and cyclosporin A (1 μM)	0.07 ± 0.003	6.4 ± 3	13.3 ± 4	85	176

only mechanism involved in reduced dye accumulation, incubations were performed in the presence of cyclosporin A at non-toxic concentrations, which have been shown to have no effect on MRP-mediated drug efflux. In the case of K562/0.2R and K562/0.5R subline cells, a significant increase ($P<0.05$) of rhodamine 123 accumulation was obtained, but incorporation remained much lower than in sensitive cells (Figure 1A). Thus, cyclosporin A treatment failed to completely restore drug accumulation in the resistant cells. Interestingly, the addition of this P-gp-specific inhibitor to K562/S cells caused an important decrease (40%, $P<0.05$) in fluorescent dye accumulation, although P-gp could be detected neither by flow cytometry nor by Western blot analyses. This decrease was not dependent on tested cyclosporin A concentration in the range of 1–4 μM. Probenecid treatment was also tested to characterize the role of MRP in drug uptake. No significant difference was observed between probenecid-treated and -untreated cells. The study of rhodamine 123 efflux showed that a great proportion of the dye stayed in sensitive cells, whereas fluorescence corresponding to this dye was almost undetectable in both resistant cell lines after 1 h dye efflux (Figure 1B). In addition, we were able to show that MDR cells expelled the dye very quickly (less than 10 min; data not shown). The inhibition of P-gp by cyclosporin A allowed a better retention of the dye which remained, however, at a very lower level than in K562/S cells. By contrast, cyclosporin A treatment induced an important efflux of rhodamine 123 out of sensitive cells ($p<0.05$), in a dose-independant manner. The addition of probenecid, i.e. the inhibition of MRP, had no significant effect on rhodamine 123 efflux by the three cell lines. The mean level of fluorescence in K562/S cells stayed unchanged as compared to untreated cells ($p<0.05$), as in MDR cells.

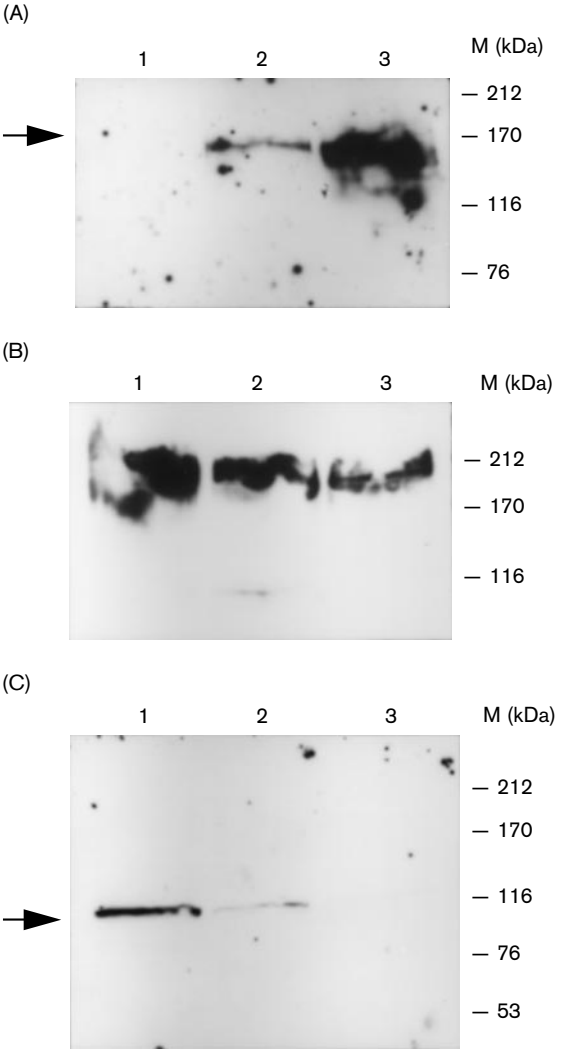

Figure 2. Western blot analysis of P-gp, MRP and LRP in whole cell lysates (100 μg) of K562 cell lines. Lane 1, K562/S cells; lane 2, k562/0.2R cells; Lane 3, K562/0.5R cells; lane M, molecular standardization (kDa). (A) Detection of P-gp with mAb JSB-1. (B) Detection of MRP with mAb MRPr1. (C) Detection of LRP with mAb LRP-56.

Analysis of P-gp, MRP and LRP expression

As a first step in the investigation of the expression of P-gp, MRP and LRP in K562 resistant cells, different Western blot analyses were carried out. Immunoblotting on K562/S cells and both MDR sublines with P-gp-specific antibody showed bands to the expected M_r 170 000 only in K562/0.2R and K562/0.5R cells (Figure 2A, lanes 2 and 3). Moreover, the intensity of the signal increased with the resistance degree of cells. These results were corroborated by the quantification of the expression of P-gp by flow cytometry, after staining with UIC2. The amount of this protein in sensitive cells was too weak to be detected (Table 3). The calculation of P-gp antigen density (as indicated in Materials and methods) showed that 4.5×10^5 P-gp sites/cell were present at the K562/0.2R cell surface and 1.02×10^6 sites/cell on K562/0.5R cells (each measure was done at least in duplicate).

To investigate whether the increased P-gp expression in the resistant cell lines was accompanied by increased MRP and/or LRP protein levels, total cell lysates were tested on Western blot with the mAbs MRPr1 and LRP-56, generated against intracellular epitopes of MRP and LRP, respectively. The analysis of expression of MRP and LRP was done comparatively in control cells known to express these proteins (data

Table 3. Quantification of P-gp, MRP and LRP sites/cell in each cell line by flow cytometry, by interpolation on the calibration curve obtained with Qifikit calibration beads (Dako)

	K562/S	K562/0.2R	K562/0.5R
P-gp sites	ND	$450 \pm 3.1 \times 10^3$	$1020 \pm 4.4 \times 10^3$
MRP sites	$20.2 \pm 1.05 \times 10^3$	$20.4 \pm 4.4 \times 10^3$	$16 \pm 3.11 \times 10^3$
LRP sites	$2.7 \pm 0.117 \times 10^3$	ND	ND

Cells were stained with UIC2, MRPm6 and LRP-56 antibodies, respectively, and revealed by FITC–secondary antibody. ND: non detectable. Each quantification was done at least in duplicate.

not shown). Protein analysis of the cell lines with MRPr1 showed the presence of an expected M_r 190 000–200 000 protein in all three cell lines (Figure 2B). Whereas a signal corresponding to a M_r 110 000 protein was revealed with LRP-56 antibody in sensitive and, to a much lesser extent, in K562/0.2R cells (Figure 2C, lanes 1 and 2, respectively), and it was undetectable in K562/0.5R (Figure 2C, lane 3). In the case of MRP, the results obtained by Western blot analysis were corroborated by cytometric quantification (Figure 3). The estimated number of MRP sites was not significantly different between the three cell lines ($p < 0.05$), i.e. about 2×10^4 sites/cell (Table 3). In parallel, the number of LRP sites achieved statistical significance between sensitive and MDR sublines ($p < 0.05$). LRP sites were estimated to be 2.7×10^3 in sensitive cells and became undetectable in both MDR sublines (Table 3).

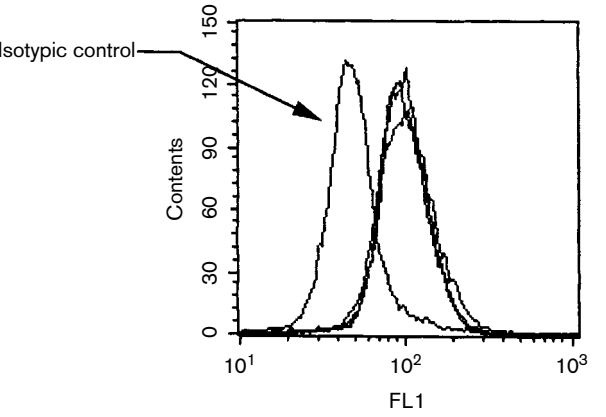

Figure 3. MRP quantification by measurement of green fluorescence by flow cytometry. For cell samples (on the right), the three histograms obtained were about on the same channel, resulting in non-disjunct distributions. The mean fluorescence values corresponding to the MRP sites were estimated after subtracting of mean fluorescence of the isotypic control (histogram on the left).

Evaluation of P-gp, MRP and LRP mRNAs

A differential expression of proteins may be attributed to a variation in transcription of the corresponding genes. Total RNAs from K562 parental and MDR variant cells were analyzed for expression of *MDR1*, *LRP* and *MRP*. Using Northern blotting, K562 cells expressed a 4.5 kb transcript that hybridized to the *MDR1* probe (Figure 4). When compared with parental cells, K562/0.2R and K562/0.5R cells showed approximately 8- and 9-fold increased levels of *MDR1* transcripts, respectively. The parental and variant K562 cell lines were also examined for MRP expression by Northern blotting (Figure 4). K562/0.5R cells showed a significant 1.5-fold increase ($p < 0.05$) in the level of *MRP* expression as compared with sensitive and K562/0.2R cells, which possess a similar MRP expression. By contrast, 2.7 kb *LRP* transcripts, which were clearly apparent in the control line KB3.1 (data not shown), were undetectable in sensitive and resistant K562 cells. This is why semi-quantitative RT-PCR, a more sensitive method, was used to determine the relative *LRP* mRNA levels. These transcripts were detected in the three sublines, but their levels were more important in parental cells, for

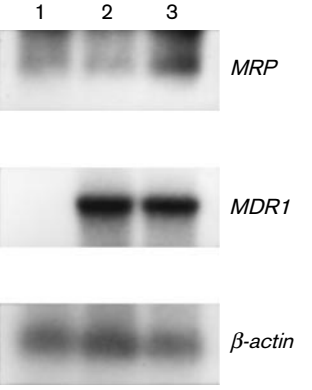

Figure 4. Expression of *MDR1*, *MRP* and *LRP* mRNAs. Total RNA (10 μg/lane) from parental (lane 1) and variant cells (lane 2, K562/0.2R; lane 3, K562/0.5R) was resolved by electrophoresis, transferred to nylon membranes, and then hybridized with different ^{32}P-labeled human probes. Blots were reprobed with human *β-actin* to assess RNA loading.

which they were detected up to the 25-fold dilution. For resistant cells, only few *LRP* transcripts could be detected, up to the 10-fold dilution (data not shown).

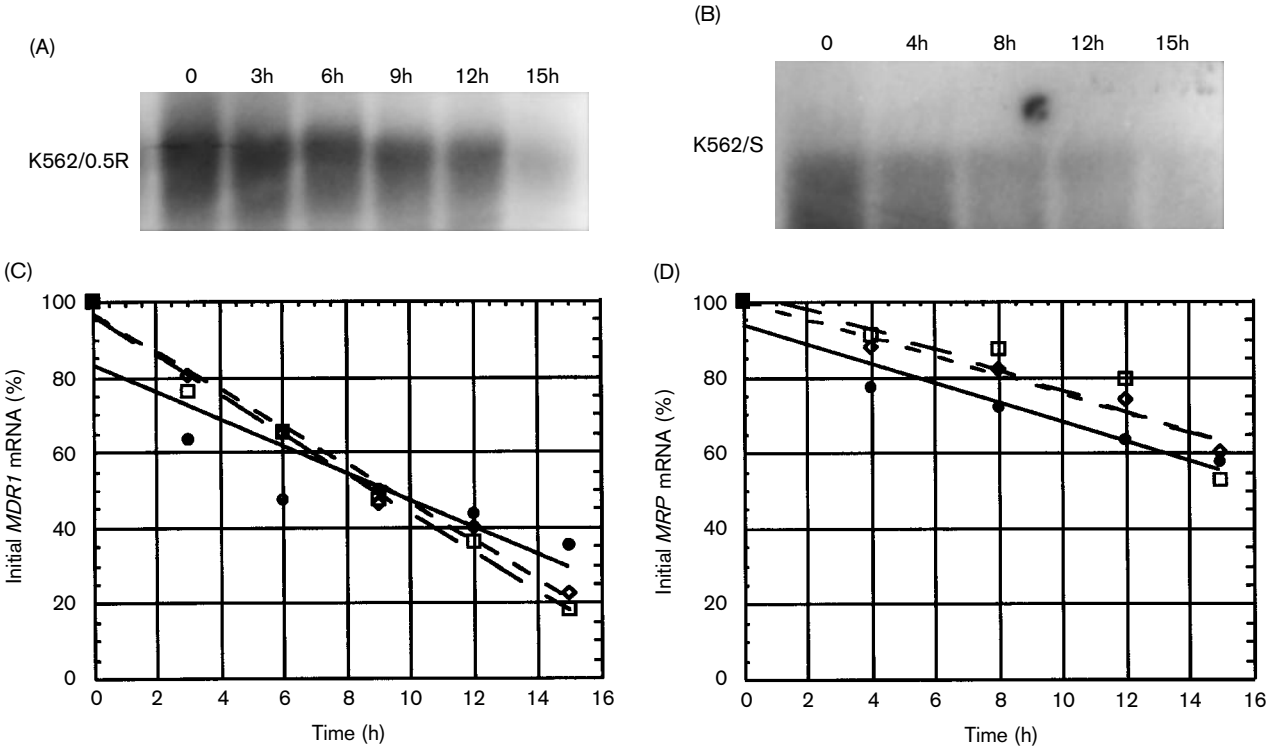

Figure 5. Determination of *MDR1* and *MRP* mRNA half-life. (A) Northern blot for *MDR1* transcript levels as a function of time in K562/0.5R cells. (B) Blot obtained with K562/S mRNA for the *MRP* gene. (C) Quantitation of Northern blots for experiments in (A) (% of *MDR1* mRNA remaining at each time point) in K562/S cells (◇), K562/0.2R cells (●) and K562/0.5R cells (□). (D) Quantitation of Northern blots for experiment in (B): K562/S cells (◇) and of blots obtained with K562/0.2R (●) and K562/0.5R (□) variants (data not shown).

Stability of MDR1 and MRP mRNA

The increase in *MDR1* transcripts with the degree of resistance could be related to an altered turnover of the corresponding mRNA. So we evaluated the stability of the *MDR1* transcript by measuring the rate of disappearance of this mRNA in control and actinomycin D-treated cells (4 μM) for up to 15 h. The same determination was done for the *MRP* transcripts in all three cell lines. Representative Northern blots (Figure 5) showed the decrease of *MDR1* and *MRP* mRNAs as a function of time in sensitive and MDR cell lines. The quantification of two independent Northern blots showed that the half-life of the *MDR1* mRNA was 535 ± 65 min in K562/S cells, 785 ± 227 min in K562/0.2R cells and 567 ± 33 min in K562/0.5R cells. There was no statistical difference between the half-lives of *MDR1* mRNA in sensitive and resistant cells. For *MRP* mRNA, half-life was estimated to be 912 ± 108 min in K562/S cells, 840 ± 336 min in K562/0.2R cells and 1010 ± 220 min in K562/0.5R cells. As for *MDR1* mRNA, the difference of MRP mRNA half-life between sensitive and MDR cells was not statistically significant. Thus the increase in P-gp expression with the degree of resistance of the cells cannot be attributed to a modification in mRNA stability.

Analysis of MDR1, MRP and LRP gene amplification

To assess the possibility that different gene copy numbers may account for the observed mRNA levels, *MDR1*, *MRP* and *LRP* gene amplifications in the three sublines were sought using the Southern blot method after digestion with *Eco*RI. As for Northern blot analysis, DNA levels were evaluated comparatively to human *β-actin*. The *MDR1* probe hybridized with two bands of approximately 8100 and 3000 bp in the three cell lines, as indicated in Figure 6(A). No difference was observed in the intensity of the signal between K562 cell lines; the signal, as compared with *β-actin*, indicated that the *MDR1* gene was not amplified in these variants. Likewise, the two signals of approximately 12 000 and 5100 bp, obtained with the *MRP* probe (Figure 6B), displayed a similar intensity

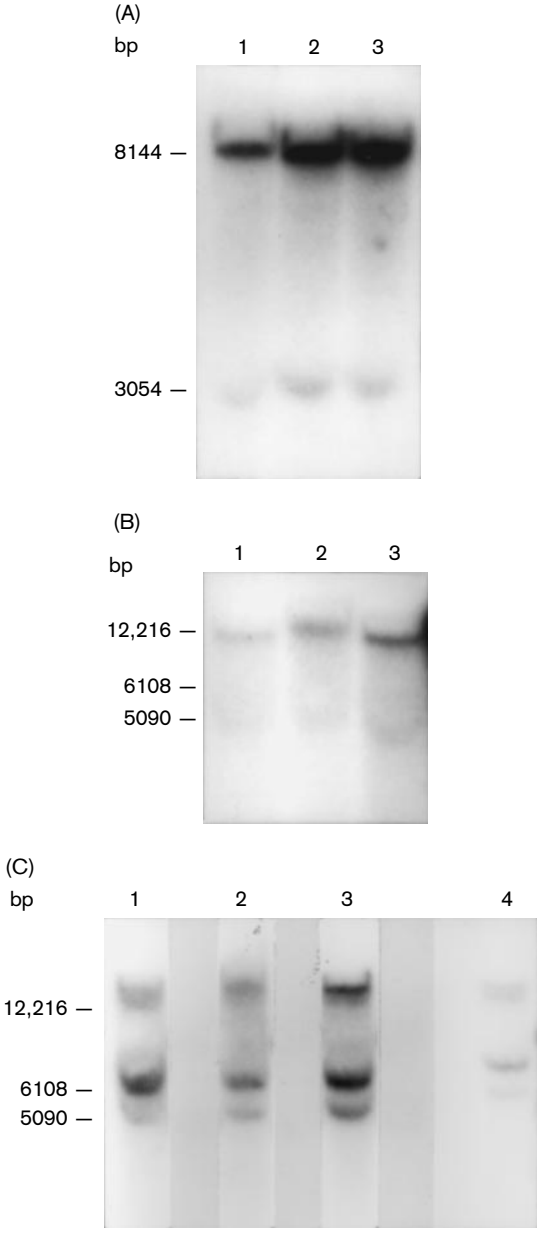

Figure 6. Analyses of *MDR1*, *MRP* and *LRP* genes amplification in K562 sensitive and resistant cells from 10 μg of genomic DNA. Lane 1, genomic K562/S DNA; lane 2, genomic K562/0.2R DNA; lane 3, K562/0.5R genomic DNA. (A) Analysis of the *MDR1* gene, (B) analysis of the *MRP* gene and (C) analysis of the *LRP* gene, compared with KB3.1-positive control cells (lane 4).

between parental and both MDR sublines. Finally, in K562/0.2R and K562/0.5R cells, *LRP* DNA levels (three signals of approximately 13 000, 6200 and 5100 bp) relative to *β-actin*, were similar to those observed in the sensitive cell line (Figure 6C). Thus, these results show unambiguously that the MDR phenotype in K562 cells cannot be ascribed to an amplification of any of these genes.

Discussion

Dox-sensitive K652 cells (a human myeloid leukemia cell line) and their Dox-resistant counterparts have been the target of much research over the past decade, concerning their cross-resistance to agents typically affected by MDR.[15,28] Among these, previous data[15] demonstrated that, in addition to the classical model of Dox extrusion by P-gp, intracellular redistribution of drug inside cytoplasmic vesicles takes place in the K562/0.2R variant, preventing the drug reaching its nuclear target, unlike sensitive cells. The authors have shown that verapamil was able to restore Dox incorporation in the human leukemia K562 resistant cell line to the level reached in sensitive cells, but not to completely recover drug sensitivity. In the same way, Denis-Gay *et al.*[16] observed an uncomplete restoration of rhodamine 123 incorporation, in K562 resistant cells, after inhibition of P-gp by verapamil. In the present study, the use of another well-known P-gp inhibitor, cyclosporin A, confirmed the published data. The inhibition of P-gp by the antagonist was unable to completely restore either rhodamine 123 accumulation or its efflux in the two K562 resistant cell lines at the level observed in K562/S cells. Moreover, in these cells, cyclosporin A provoked both a significant decrease of rhodamine 123 incorporation and an increase of its efflux. These values remained higher or lower, respectively, than those observed for K562 resistant cells. The fast changes observed in rhodamine 123 fluorescence (a mitochondrial potential sensitive dye) of K562/S cells might be attributed to cyclosporin A cytotoxicity and produced an alteration of mitochondrial membrane potential through its well-known effect with mitochondrial permeability transition (MPT). This hypothesis can be excluded because concentrations of cyclosporin A used (1–4 μM), routinely employed by other authors, are not toxic and inhibited MPT, causing a mitochondrial hyperpolarisation.[29] Moreover, in the presence of 1 μM cyclosporin A, Dox IC_{50} values (measured with MTT, a metabolic activity assay) of K562/S cells increased about 2-fold. Consequently, the changes of the rhodamine 123 fluorescence and of Dox IC_{50} for K562 sensitive cells in the presence of cyclosporin A may rather be due to a spontaneous drug efflux. Cyclosporin A and Dox or rhodamine 123 were indeed added to sensitive cells at the same time, without delay. On the contrary, Donenko *et al.*[30] have already observed that a preincubation with verapamil for 5

days of sensitive Sa180 cells did indeed raise their resistance level to Dox by approximately 2-fold. In the same way, increases of *MDR1* mRNA were obtained within 8 h of addition of P-gp antagonists with other cell lines,[31] suggesting that a feedback mechanism is involved in the regulation of P-gp expression. In our case, we have shown that P-gp was undetectable on the plasmic membrane of sensitive K562 cells. Thus, this protein could not be involved in this fast and spontaneous phenomenon. The cells did not have a sufficient delay to synthesize the P-glycoprotein which was undetectable in these cells. We can suppose that another mechanism still present in K562/S cells is involved in this spontaneous efflux and/or reduced cytotoxicity of the drugs.

In order to explain these results we have investigated the molecular changes of the major proteins considered as responsible for the MDR phenotype (P-gp, MRP and LRP). *MDR1* overexpression appears to be an important determinant in the acquisition of the drug-resistance phenotype in numerous tumor cells. In the present study, we have confirmed the preliminary results on P-gp expression in K562 cells after staining with MRK16 antibody.[16] The protein was undetectable in the sensitive line, modestly increased in the K562/0.2R variant and highly overexpressed in the K562/0.5R variant. At the mRNA level, the *MDR1* transcripts were barely detectable in the sensitive line and 8- to 9-fold similarly overexpressed in the two resistant variants. There is, therefore, no direct proportionality between mRNA overexpression and the quantity of membrane P-gp. Several hypotheses can be proposed to explain this data, such as modification in the translation efficiency of the *MDR1* mRNA and/or to a difference in the stability of the P-gp, and/or to a preferential localization of P-gp in intracellular compartments in the K562/0.5R cell line. It is remarkable that no *MDR1* gene amplification occurs in these resistant cell lines; most resistant *in vitro* cell lines studied up to now have indeed shown an amplification of this gene,[32] while it has never been observed in human tumors overexpressing the *MDR1* gene.

Therefore, other mechanisms different from P-gp overexpression must be responsible for the spontaneous drug efflux (or resistance) of parental sensitive K562 cells in the presence of cyclosporin A and/or involved in drug resistance of K562 variant cell lines. Cell lines selected *in vitro* for drug resistance have often been reported to display simultaneously several drug resistance mechanisms.[13,20] In our study, we have examined *LRP* and *MRP* gene amplification and corresponding mRNA and protein expression.

With regard to MRP, this protein was expressed in K562 sensitive cells as already observed by Zhou *et al*.[14] These authors observed a marked overexpression of the *MRP* gene at the mRNA level during the first steps of selection of a resistant line with the drug homoharringtonine. During further selections of more resistant cells, this overexpression was lost to the profit of *MDR1* overexpression and *MRP* expression returned to the basal level of sensitive cells.[14] In our case, we only observed a slight over-transcription (1.5-fold) of *MRP* in the highest resistant cell line (K562/0.5R) and only at the mRNA level, relative to the *β-actin* messenger, not due to a difference in mRNA stability. This slight overexpression was not translated at the protein level, since we failed to detect any difference in MRP concentration between the three cell lines. These results and the fact that treatment with probenecid did not alter the low rhodamine 123 uptake exhibited by our MDR cells suggest that MRP plays, if any, a minor role in the MDR phenotype of these cell lines. In addition, these data were supported by other results[33] where the delay of rhodamine 123 efflux by MRP was 4 h at least. In our case, this dye efflux was almost complete after 1 h, indicating that MRP was not involved in this phenomenon. However, we cannot exclude an internal protein redistribution between plasma and cytoplasmic membranes.

Concerning LRP, it is remarkable that this protein, which has been shown to be associated with MDR, progressively disappeared from K562 cells conversely with the increased resistance to Dox. LRP is part of ribonucleoprotein complexes involved in nucleocytoplasmic transport and trafficking, and we can wonder why its presence becomes unnecessary or even harmful in our Dox-resistant cells. LRP has been shown to be increased in several MDR cell lines selected with Dox[9] and to play a key role in these cells in intracellular drug redistribution.[8] The redistribution of Dox in the K562/0.2R cell line already mentioned[15] could be mediated by the disappearance of LRP rather than by its presence, which might constitute a new mechanism of resistance. Indeed, the role of P-gp and other transport proteins in drug intracellular redistribution has never been studied with much attention; it is known, however, that P-gp is present in cytoplasmic membrane systems where it could play a role in drug redistribution.[3] On the other hand, the fact that LRP was only detected in sensitive cells could explain the partial spontaneous resistance (and/or efflux capacity) to the drugs in the presence of cyclosporin A into the K562 sensitive cell line. LRP could be involved in this phenomenon, but its disappearance in resistant sublines would prevent this feedback mechanism.

Finally, comprehensive studies on the role of transport proteins in drug redistribution are needed

to obtain a more complete view on spontaneous or induced drug resistance and their complementary role undergoing these processes.

Conclusion

In conclusion, the current study revealed that the doxorubicin resistance mechanism of K562 cells was in partly due to overexpression of P-gp. On the other hand, MRP played, if any, a minor role in the MDR phenotype of these cells, and LRP disappearance could be involved in the redistribution of doxorubicin. Further studies are needed to obtain more insights about the mechanisms of intracellular drug sequestration.

Acknowledgments

We are grateful to Dr P Pélissier and Dr D Delourme for fruitful discussion and their critical reading of the manuscript, and Dr C Jayat-Vignoles for her excellent assistance with the flow cytometric analyses.

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(Received 4 December 2000; accepted 27 December 2000)