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Determining the different mechanisms of resistance in two human tumor cell lines of different origin: identification of P-glycoprotein and multidrug resistance associated protein

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Abstract

We have studied doxorubicin and vincristine cytotoxicity and doxorubicin accumulation in two human tumor cell lines (the erythroleukemia line K562 and the breast cancer line MCF7) and their variants selected for resistance to doxorubicin, as well as restoration of doxorubicin accumulation and sensitivity by verapamil and quinine. We have shown in the resistant variants that verapamil was able to completely restore doxorubicin accumulation and partially reverse doxorubicin resistance; quinine completely restored doxorubicin sensitivity in resistant cells, despite of a weak effect on doxorubicin accumulation.

The analysis of expression and activity of P-glycoprotein (P-gp) revealed that both lines presented a multidrug resistance phenotype, with a similar expression and activity of P-gp, but with different factors of resistance. This discrepancy could be due to the concomitant presence of other mechanisms of resistance, in addition to P-gp overexpression. We used Western blotting and RT-PCR and detected respectively the presence of multidrug resistance associated protein (MRP1) and of the mRNA of the *MRP*1 gene in K562 resistant cells, but only *MRP*1 mRNA in MCF7 resistant cells. We concluded that the lack of correlation between the degree of resistance and the expression of P-gp may result, at least in part, from the coexistence of two mechanisms of resistance in the same cell line.

Key words : multidrug resistance; doxorubicin; growth inhibition, P-glycoprotein; multidrug-resistance associated protein; verapamil; flow cytometry.

Introduction

Multidrug resistance is a common mechanism by which cancer cells escape the cytotoxic effects of chemotherapy. It is now generally admitted that the most common mechanism is due to over-expression of a high molecular weight membrane glycoprotein, termed P-glycoprotein (P-gp), which is responsible for the active extrusion of anticancer drugs as well as of many xenobiotics out of tumor cells. The involvement of P-gp (product of the MDR1 gene) in multidrug resistance has been extensively characterized, and gene transfection studies have demonstrated that expression of this protein confers a classical multidrug resistance phenoltype on many wild-type drug-sensitive cells (Gottesman and Pastan, 1993). At the beginning of the 90ies, it was generally believed that P-gp was the exclusive cause of multidrug resistance. However, several cell lines were isolated that displayed a multidrug resistance phenotype without P-gp expression. One such non-P-gp multidrug resistant cell line was H69 AR (derived from small cell lung cancer) wich displays resistance to many drugs and expresses another membrane protein, the multidrug resistance-associated protein MRP1 (Cole *et al*., 1992). This multidrug resistance –

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associated protein is also a membrane transporter of the ATP-binding cassette (ABC) family with 15% sequence identity with P-gp. Immunoblotting and immuno-precipitation analyses with MRP1-specific antibodies indicated that MRP1 was a 190 kDa membrane protein, and cell fractionation studies indicated that the protein was predominantly localized in the plasma membrane in resistant cells, but also in intracellular membrane compartments (Hipfner *et al.,* 1994 ; Barrand et al., 1995 ; Hipfner *et al*., 1999).

As several other tumor drug-resistant cell lines, K562/ADR and MCF7/ADR, selected with doxorubicin from the corresponding wild-type cells, exhibit a cross resistance pattern and overexpress P-gp, as previously shown (Bennis *et al.*, 1997). As commonly shown in MDR cells, anthracycline resistance in these cell lines is partially reversed by the calcium channel blocker verapamil; this occurs through restoration of drug accumulation in the cells. However, another widely used MDR modulator, quinine, is able to restore doxorubicin sensitivity and nuclear accumulation in resistant cells, but has practically no effect on whole cell drug accumulation and, in addition, does not inhibit azidopine binding to P-gp as verapamil and other MDR modulators do (Bennis *et al.,* 1995). We have shown that verapamil and quinine had differential effects on doxorubicin redistribution in K562/ADR cells, quinine providing an apparent release from cytoplasmic sequestration compartments to the nucleus, while verapamil directly interfered with P-gp at the plasma membrane level (Bennis *et al.,* 1995). Furthermore, during the characterization of these cell lines, we observed a lack of correlation between the level or the activity of P-gp and the degree of resistance of the cells to this drug. In order to explain and understand this discrepancy in MDR cell lines, we looked for the presence of MRP1 by Western blotting and for the expression of the MRP1 gene at the mRNA level by RT-PCR, in comparison to P-gp and MDR1 expression. Our results show that, in addition to the classical, P-gp-mediated mechanism of resistance, K562/ADR cells exhibit the atypical, MRP1-mediated, mechanism of resistance, which may explain the intracellular sequestration of doxorubicin in these cells. This is, however, not the case for MCF7/ADR cells in which another mechanism of resistance must be associated to the classical MDR one.

Materials and methods

Cell culture: MCF7 and MCF7/ADR cell lines (Batist et al., 1986) were cultivated as monolayers in Petri dishes, whereas K562 and K562/ADR cell lines (Tsuruo et al., 1986) were cultivated in suspension. All cell lines were grown with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotic mixture at 37°C in a humidified atmosphere containing 5% CO2. The cultures were replicated each week and the medium was changed every 2 or 3 days. The doxorubicin-resistant lines K562/ADR and MCF7/ADR were routinely maintained with 0.34 and 10 µM doxorubicin respectively.

cytotoxicity evaluation: A colorimetric assay using the tetrazolium salt MTT ((3,4,5 dimethylthiazol2-yl) 2,5 diphenyl tetrazolium bromide), was used to assess the cytotoxicity of doxorubicin in the various cell lines (Carmichael et al., 1987). For K562 cells, 1000 cells were seeded in each well of 96-well plates, incubation was performed 2 days later for 2 hours with doxorubicin at concentrations ranging between 0.001 and 1 µM for sensitive cells and 0.01 and 100 µM for resistant cells. For MCF7, 1500 sensitive or 2000 resistant cells were seeded similarly in 96-well plates and incubation with doxorubicin was performed 2 days later for sensitive cells and 3 days later for resistant cells, always for 2 hours, at concentrations ranging between 0.001 and 10 µM for sensitive cells and 0.01 and 100 µM for resistant cells. At the end of the incubation, the wells were rinsed (after centrifugation in the case of K562 cells) and the cells grown in fresh medium for 2 days (3 days for MCF7 resistant cells). At this

time, 300 µl of medium containing 0.5 mg/ml MTT were added in each well and the plates incubated at 37°C for 4 h. Medium was then removed and 200µl of DMSO were added to dissolve the formazan crystals. Absorbance was immediately determined on a two-wavelength Microplate Auto Reader (Biotek Instruments, Winooski, VT) at test and reference wavelengths of 570 and 630 nm, respectively. Blank controls without cells were subtracted from sample absorbance values. Triplicate determinations were always performed. Cytotoxicity was expressed as IC50, the concentration responsible for 50% growth inhibition as compared to cells incubated without drug. For evaluation of vincristine cytotoxicity, the same procedure was followed, except that incubations with this drug were performed for 24 h at appropriate concentrations.

Reversal of resistance to doxorubicin was studied with two modulators, verapamil and quinine, at concentrations ranging between 0.1 and 50 µM. The modulators were maintained for 2 hours in contact with the cells, during incubation with doxorubicin. Reversal of doxorubicin resistance was evaluated as the ratio of the IC50 obtained in the presence of doxorubicin alone to the IC50 obtained in the presence of doxorubicin and modulator.

Doxorubicin accumulation was evaluated after 2 hours of incubation of the appropriate numbers of cells with 17.2 µM doxorubicin, in the presence or absence of the modulators at concentrations ranging between 0.1 and 50 µM. The intracellular concentrations of doxorubicin were evaluated by spectrofluorometry after precipitation of the proteins with trichloroacetic acid, as already described (Huet *et al.,* 1992). In other experiments, the cells were exposed to doxorubicin at the concentrations required to produce 50% growth inhibition as determined in the cytotoxicity assay.

P-glycoprotein and MRP immunodetection by Western blotting: 4×107 cells were washed twice with phosphate saline buffer (PBS), harvested and pelleted at 900 g for 5 min after counting. Cell pellets were washed with 40 mM Tris and pelleted at 300 g for 5 min at 4°C. The supernatant was replaced by 250 µL of 5 mM Tris (pH 8.6) containing 6 mM MgCl2, 1.5 µl aprotinin (32.2 TUI/ml) and incubated for 10 min on ice. The lysis was completed by sonication, and then were added 250 µl of 80 mM Tris (pH 8) containing 6 mM MgCl2, 1.5 µl aprotinin and 10 UI DNase. The mixture was incubated for 30 min at room temperature before protein measure-ment. Protein measurement was done with Biorad reagent (Richmond, CA) using bovine serum albumin (BSA) as a standard. Cell proteins, 40 to 80 µg per lane, were resolved by SDS-PAGE using the method of Laemmli (1970). After electrophoresis, the proteins were transferred to a nitrocellulose membrane with an electro-blotting buffer system (Milliblot SDE, Millipore, Bedford, MA) by the method indicated by the manufacturer. The blots were incubated for 1 hour at 37°C in blocking buffer (0.9% NaCl, 10 mM Tris HCl pH 7.5, 0.02% sodium azide, 5% dry milk, 3% IgG-free BSA, 0.2% Tween 20), then incubated overnight at 4°C in blocking buffer, either with the anti-P-gp C219 antibody (Kartner et al., 1985, purchased from Centocor, Malvern PA), or with the anti-MRP1 MRPr1 antibody (Flens et al., 1994, purchased from Novus Biologicals, Littleton, CO). The blots were washed with Tris saline buffer, and incubated with alkaline phosphatase- conjugated rabbit anti-mouse IgG in blocking buffer at room temperature for 90 min, then washed one more time in Tris saline buffer, and finally developed with a substrate of alkaline phosphatase, BCIP (5 bromo 4 chloro, 3 indolyl-phosphate) at 0.5 mg/ml. The signals were analyzed with a video camera (Kodak DC120 Zoom Digital Camera, Rochester, NY, USA) coupled with a micro-computer, using the Kodak Digital Science 1D Image Analysis Software. Signal intensities were recorded and expressed in arbitrary units.

P-glycoprotein immunodetection by flow cytometry: Single cell suspensions originating from cell cultures in logarithmic phase of growth were washed with PBS and pelleted at 300 g for 10 min. For monoclonal

antibodies reacting with cytoplasmic epitopes, cells were permeabilized by addition of 5 ml of acetone/methanol solution (75/25). After 10 min of incubation at room temperature, cells were washed in PBS first, then in PBS containing 1% BSA (PBS-BSA). 100 µl of cell suspension at 4×106 cells/ml in PBS-BSA were incubated at 4°C for 1 hour with monoclonal antibodies reacting with external epitopes (MRK16 (Hamada and Tsuruo, 1992) and UIC2 (Mechetner and Roninson, 1992), both purchased from Immunotech, Marseilles, France). The second-layer staining was carried out with fluorescein isothiocyanate (FTIC)-labeled goat anti mouse IgG (Becton Dickinson, Franklin Lakes, NJ) (1/25 dilution in PBS-BSA 10% NGS) for 30 min. Finally, the cells were washed twice with PBS-BSA and pelleted at 300 g for 10 min before resuspension in PBS. Fresh cells were washed in PBS-BSA and incubated at 4°C for 1 hour with UIC2 (20 µg/ml), MRK16 (20 µg/ml), or IgG2a isotypic control (20 µg/ml) in PBS-BSA. After this step, cells were resuspended in 400 µl PBS plus 40 µl propidium iodure (PI) (0.5 mg/ml) to exclude dead cells.

Evaluation of P-gp activity by flow cytometry: To study P-gp function, we measured the intracellular accumulation of some P-gp substrates (doxorubicin, rhodamine 123, fluo3 A-M) in MCF/ADR and K562/ADR cells in the presence or absence of verapamil. The cells, still in exponential phase of growth, were washed with PBS, pelleted at 300 g for 10 min and adjusted at 2×106 cells/ml in serum-free culture medium. The modulator was used at the concentration of 10 µM, incubated for 90 min simultaneously with the fluorochrome used at 0.1 µM for rhodamine 123 and at 10 µM for fluo3 A-M and doxorubicin.

Analysis by flow cytometry: Multiparametric analysis were carried out on cell suspensions using a FacScan (Becton Dickinson) equipped with a 15 mW, air-cooled argon ion laser and tuned to 488 nm excitation wavelength. Forward scatter and side scatter were collected on linear scale for 10,000 events, and used to exclude dead cells and cell aggregates from analysis. Logarithmically amplified FITC and PI fluorescence were collected through sand pass filters (530 nm ± 15 nm for FITC and 585 ± 21 nm for PI and doxorubicin). Calculation of logarithmically amplified fluore-scence means was performed in arithmetic mode using the Lyses computer program (Becton Dickinson).

MRP1 and MDR1 gene expression evaluation by reverse transcription polymerase chain reaction (RT-PCR): Total cellular RNAs from exponen-tially growing cells were extracted by 6 M guanidine isothiocyanate and centrifuged at 150,000 g for 17 h on a 5.7 M cesium chloride layer. RNAs were washed twice in 70% ethanol and resuspended in TES buffer (Tris 10 mM, pH 7.4, EDTA 5 mM, SDS 1%). They were quantified by spectrometric absorbance measure-ment at 260 nm and 280 nm, precipitated in ethanol / 3 M sodium acetate (2/1, v/v) and stored at -20°C until use. Complementary DNAs (cDNAs) were synthesized with 1 µg of total cellular RNA and 2.5 µM random hexameres in 20 µl of a solution containing 1 × PCR buffer, 5 mM MgCl2, 1 mM of each deoxynucleoside trisphosphate, 1 U RNase inhibitor, 2.5 U reverse transcriptase. Reverse transcription was carried out at 42°C for 1 h. The samples were then left at 99°C for 10 min, followed by a 4°C quick chill.

A 157 bp MDR1 specific fragment was amplified using two oligonucleotides with the following sequences, according to Noonan et al. (1990) : sense : 5' CCC ATC ATT GCA ATA GCA GG 3' ; antisense : 5' GTT CAA ACT TCT GCT CCT GA 3'. PCR was performed by adding 5 µl of the RT products to 45 µl of amplification reaction buffer (1× PCR buffer, 250 ng of each primer, 2.5 U Ampli Taq DNA polymerase, 1.35 mM MgCl2, 0.2 mM of each deoxynucleoside trisphosphate). Samples were first incubated at 94°C for 5 min and submitted to 20 cycles, using a Perkin-Elmer 480 thermo-cycler. Each cycle included the following steps: 94°C 1 min; 58°C 15 sec; 72°C 15 sec. Then, a final extension of 5 min at 72°C was performed, and samples were cooled down to 4°C

before loading. For detection of the MRP1 gene, a 460 pb MRP1-specific fragment was amplified using two oligonucleotides with the following sequences, sense : 5' GGT GCC CGT CAA TGC TGT TAC CA 3' ; antisense 5' CGA TTG TCT TTG CTC TTC ATG TG 3', and the PCR was realized in the same conditions as described above for the MDR1 gene. As a control, a 80 pb fragment of □-actin cDNA was amplified in a separate tube in the same conditions as above using the following primers chosen after the gene sequence : sense : 5' GAG AAG ATG ACC CAG ATC ATG T 3' ; antisense : 5' CAG AGG CGT ACA GGG ATA GCA C 3'.

The RT-PCR products were qualitatively characterized by loading 10 µl of the reaction volume on a 8% polyacrylamide gel stained with ethidium bromide. The signals were analyzed with a video camera coupled with a micro-computer. Signal intensities were recorded and expressed in arbitrary units.

Results

The two human cell lines were characterized by the usual features of the MDR phenotype (Table 1 and Figure 1): cross resistance between an anthracycline, doxorubicin, and a vinca- alkaloid, vincristine; decrease of drug accumula-tion when the cells were exposed to a given concentration of doxorubicin (17.2 µM); overexpression of P-glycoprotein (P-gp) as shown by Western Blotting using C219 antibody. We also noticed that resistant lines accumulated more doxorubicin than sensitive cells when they were exposed to their respective IC50, showing that the resistant line displayed intracellular tolerance to the drug, a phenomenon we have already noted in (Huet et al., 1992; Schott et al., 1993).

For the study of the reversal of resistance and of the restoration of drug accumulation in the resistant lines, we used verapamil and quinine at concentrations ranging between 0.1 and 50 µM. Verapamil only partially reversed drug resistance in K562/ADR and MCF7/ADR cells, even at the highest concentrations used, whereas a complete reversal of resistance to doxorubicin was noted with quinine at 50 µM in K562/ADR cells, but not in MCF7/ADR cells which exhibited some residual resistance (Table 2).

It is worth to mention that in both cell lines, the same concentrations of a given modulator provided the same degree of reversal of doxorubicin resistance. Doxorubicin accumulation was completely restored by 10 µM of verapamil in K562/ADR but not in MCF7/ADR cells, for which doxorubicin accumulation increased significantly without reaching the level obtained in the sensitive line. In both resistant cell lines, quinine was unable to increase significantly doxorubicin accumulation despite its major effect on drug cytotoxicity (Figure 2).

A semi-quantification of P-gp expression was obtained in both resistant cell lines by Western blotting using C219 antibody on serial dilutions of the whole cell extracts. P-gp was approximately 200-fold and 400-fold overexpre-ssed in K562/ADR and MCF7/ADR cells respectively, in comparison to the wild-type corresponding cells (Figure 1).

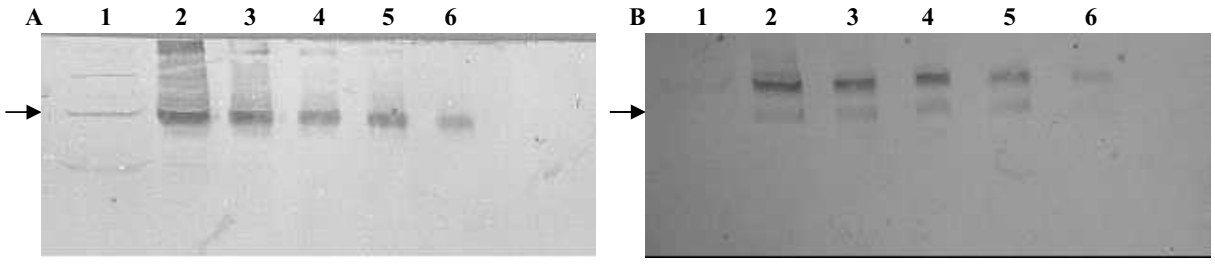

Figure 1. Western blots of P-glycoprotein in whole cell lysates of doxorubicin-sensitive and doxorubicin-resistar cells using the C219 antibody. **A**: MCF7 cells. Lane 1 corresponds to 800 µg proteins from sensitive cells, lane 2-6 correspond to 200, 20, 8, 4 and 2 µg proteins from resistant cells, respectively. **B**: K562 cells. Lane corresponds to 800 µg proteins from sensitive cells, lanes 2-6 correspond to 200, 80, 40, 16 and 8 µg protein from resistant cells, respectively. The arrow indicates the migration of a 170-kDa standard.

Table 1. Doxorubicin and vincristine cytotoxicity and doxorubicin accumulation in MCF7 and K562 sensitive and resistant cell lines. Results are means ± SD of values obtained in at least 3 independent experiments, each performed in triplicate.

	K562	K562/ADR	MCF7	MCF7/ADR
Doxorubicin IC50 (μM)	0.17 ± 0.02	2.58 ± 0.60	0.089 ± 0.004	109 ± 12
Resistance factor		15		1225
Vincristine IC50 (nM)	5.2 ± 0.4	970 ± 20	4.9 ± 0.3	3970 ± 310
Resistance factor		187		810
Doxorubicin accumulation (2-h exposure to 17.2 μM) (nmol/106cells)	1.20 ± 0.20	0.30 ± 0.03	4.0 ± 0.4	0.11 ± 0.03
Doxorubicin accumulation (2-h exposure to IC_{50}) (nmol/10^6cells)	0.020 ± 0.004	0.082 ± 0.007	0.065 ± 0.004	0.63 ± 0.06

Table 2. Reversal of doxorubicin cytotoxicity in MCF7 and K562 resistant cell lines by verapamil and quinine.

		K562 cells		MCF7 cells	
		IC_{50} (μM)	Reversal factor	IC_{50} (μM)	Reversal factor
Sensitive cells		0.17 ± 0.02		0.089 ± 0.004	
Resistant cells without revertant		2.58 ± 0.60		109 ± 12	
Verapamil	0.1 μM	2.06 ± 0.34	1.25	112 ± 7	0.97
	1 μM	1.96 ± 0.06	1.31	62 ± 5	1.76
	10 μM	0.69 ± 0.10	3.73	21 ± 1	5.19
	50 μM	0.50 ± 0.01	5.16	19 ± 2	5.73
Quinine	0.1 μM	2.01 ± 0.50	1.28	100 ± 11	1.09
	1 μM	1.89 ± 0.20	1.36	83 ± 3	1.31
	10 μM	0.40 ± 0.07	6.45	21 ± 1	5.19
	50 μM	0.19 ± 0.06	13.6	9 ± 1	12.1

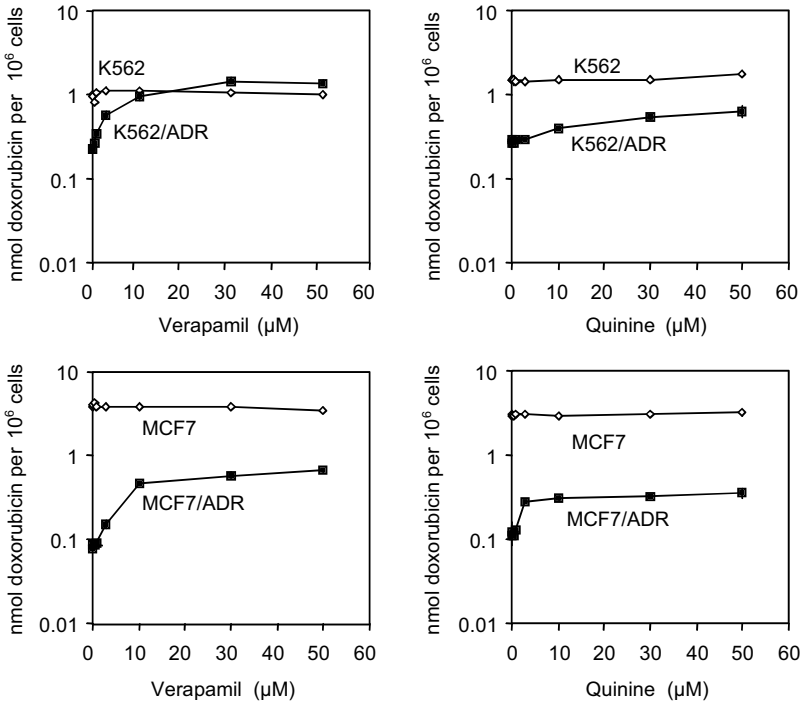

Figure 2. Comparison of doxorubicin accumulation in two human cell lines in the presence of increasing concentrations of verapamil and quinine. Doxorubicin (17.2 μM) and modulators were simultaneous incubated for 2 h, doxorubicin was extracted from the cells and evaluated by spectrofluo-rometry. Results are means ± SD of 3 independent experiments, each experiment being performed in triplicate.

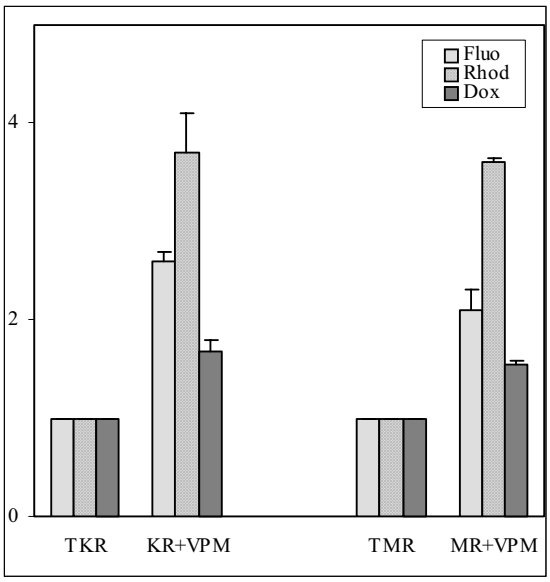

Figure 3. Expression of P-gp obtained with different antibodies used in flow cytometry. Results are means of three independent experiments.

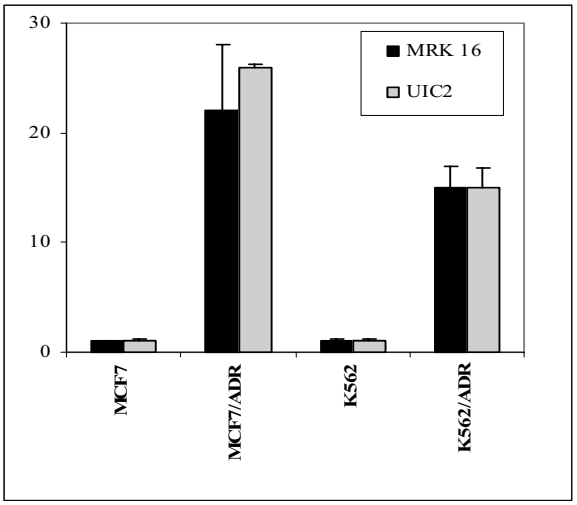

Figure 4. Activity of P-gp obtained with three substrats used in flow cytometry. Results are means of three independent experiments. TKR = control K562/ADR, KR+VPM = K562/ADR+VPM, TMR= control MCF7/ADR, MR+VPM = MCF7/ADR +VPM.

When studied by flow cytometry using immunodetection with MRK16 and UIC2 antibodies, which recognize extracellular epitopes of P-gp, the two resistant cell lines also presented a similar level of expression (Figure 3). The quantification data contrast with the important difference between the resistance factors exhibited by the two cell lines (15-fold for K562/ADR and 1225-fold for MCF7/ADR, table 1).

P-gp functionality was also studied by flow cytometry used a known P-glycoprotein substrate for determining the activity. Figure 4 shows the retention quantity of Rhodamine 123 (Rh 123), doxorubicin, and fluo 3 A-M staining fluorescence into the cells in presence or absence of reverter (verapamil 10 μM). In presence or absence of modulator, we noted on the parents lines the same fluorescence dyes ; however in MCF7/ADR and K562/ADR we observed a high fluorescence dyes with Rh 123 and Fluo 3 A-M (3 to 4 fold over retention) in presence of verapamil. With doxorubicin the fluorescence dyes is weak because this molecule is quenching between pair bases of DNA.

Discussion

We show in this paper that P-glycoprotein overexpression cannot explain the complete multidrug resistance phenotype exhibited by human tumor cells in culture after selection with doxorubicin. This results from the comparison between the important differences noted in

doxorubicin cytotoxicity between the K562/ADR and the MCF7/ADR cell lines, whereas P-gp expression and functional activity appeared similar in the two cell lines. This conclusion is in agreement with what we had previously shown on rat cell lines also selected with doxorubicin (Huet *et al*., 1992).

In an attempt to evaluate the origin of this discrepancy, which can be caused by the contribution of other drug transporters, we quantified in both cell lines the expression of MRP1, both at the mRNA and the protein levels. It appeared that both cell lines expressed the *MRP*1 gene, but that only the K562/ADR cell line contained detectable amounts of the protein by Western blotting.Whereas MRP1 cannot be considered as participating to the high level of resistance of MCF7/ADR cells, it can be concluded that MRP1 may be responsible for the intracellular redistribution of doxorubicin in K562/ADR cells.

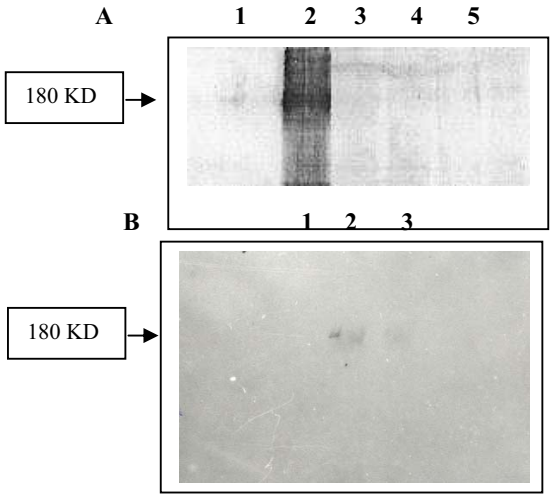

Figure 5. Western blots of MRP1 in whole cell lysates of doxorubicin-sensitive and doxorubicin-resistant cells using the MRPr1 antibody.
A: MCF7 cells. Lane 1 corresponds to 800 µg proteins from sensitive cells, 800 µg, lane 2 corresponds to a positive control, lanes 3, 4, 5 correspond respectively to 800, 400 and 200 µg proteins from resistant cells.
B : K562 cells. Lane 1 corresponds to 800 µg protein from sensitive cells, lanes 2 and 3 to 800 and 400 µg proteins from resistant cells, respectively.
The arrow indicates the migration of a 180-kDa standard.

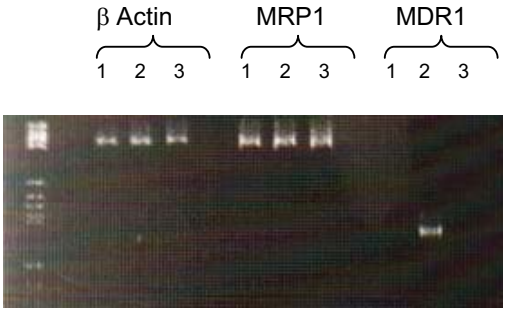

Figure 6. Expression of *MDR*1, *MRP*1 and β-actin genes by RT-PCR performed on RNAs extracted from MCF7 and K562 cells. Lanes 1, MCF7 cells; lanes 2, MCF7/ADR cells; lanes 3, K562/ADR cells.

In MCF7 cells, other mechanisms of multidrug resistance should be involved in addition to P-pg – mediated MDR, such as through alterations in the redox status of the cells (Kramer et al., 1988) or in topoisomerase II availability (Beck *et al*., 1987).

Another goal of this paper was to compare the effects of verapamil and quinine as multidrug resistance modulators in K562 and MCF7 cell lines selected with doxorubicin. We show that quinine remains able in both resistant cell lines to provide a complete reversal of doxorubicin resistance without significantly increasing drug accumulation. We had already suggested, for K562/ADR cells, that this might be due to a cellular redistribution of the drug in sequestration compartments (Bennis *et al*., 1995) and this has been confirmed by another group (Grandjean *et al*., 2001). Similar observations were made for MCF7/ADR cells, but, whereas MRP1 can be involved in doxorubicin sequestration in K562/ADR cells, this cannot be the case for MCF7/ADR cells, which do not show MRP1 expression at the protein level. The presence of a functional protein like P-gp in intracellular compartments has been mentioned for a long time (Abbaszadegan *et* al., 1996; Sognier *et al*., 1994); we hypothesize that it could be responsible for such drug redistribution in our resistant cells.

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