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# Mitochondrial localization and activity of P-glycoprotein in doxorubicin-resistant K562 cells

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MDR, multidrug resistance
PE, phycoerythrin
P-gp, P-glycoprotein 170

ABSTRACT

It is now well-established that P-glycoprotein 170 (P-gp), an efflux pump involved in multidrug resistance (MDR) is overexpressed at the plasma membrane of doxorubicin-resistant K562 leukemia cells. Nevertheless, several results suggested: (i) that P-gp-mediated drug efflux was not the only mechanism involved in resistance; (ii) that intracellular compartments could accumulate the drug, preventing it from reaching its nuclear targets; (iii) that agents able to reverse multidrug resistance may lead to intracellular drug redistribution. We have studied the localization of P-gp in mitochondria as well as its functional properties in this compartment. Using several monoclonal antibodies (MoAbs) directed against different P-gp epitopes, a protein was detected in the cytoplasm of two doxorubicin-resistant K562 sublines and, by confocal laser scanning microscopy, this protein was shown to co-localize in the Golgi apparatus and in mitochondria, in equivalent proportions. Purified mitochondria were isolated from K562 cell variants; the presence of a protein of about 170 kDa and reacting with several anti-P-gp antibodies was assessed in MDR cells by Western blotting and flow cytometry. Functional assays have shown that mitochondrial P-gp was involved in doxorubicin accumulation inside the organelle but not in its efflux, suggesting an orientation of P-gp in the mitochondrial membrane inverse to that observed in the plasma membrane. A potential role for mitochondrial P-gp in MDR cells would be to protect the nucleus from doxorubicin. This is the first demonstration of the presence and functional activity of P-gp in mitochondria of MDR cells.



## 1. Introduction

Resistance to antineoplastic drugs represents a major obstacle to the success of cancer chemotherapy. Frequently, drug resistance concerns simultaneously a variety of chemically unrelated compounds, giving rise to multidrug resistance (MDR) phenotypes. Different mechanisms of MDR have been identified, involving especially common drug detoxification pathways or alterations in common drug targets [1]. The first and the best characterized MDR mechanism is due to the over expression of a transmembrane active pump called P-glycoprotein 170 (P-gp), a member of the ATP-binding cassette (ABC) protein super family [2]. This protein of 1280 amino acids is able to expel various agents, such as anthracyclines, out of

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the cell. The overexpression of P-gp can be due to the amplification of the *MDR1* gene or to a positive transcriptional regulation. Overexpression of P-gp occurs in vitro in numerous drug-selected cell lines as well as in vivo in different types of solid tumors and leukemias.

In the past decade, the existence of alternative and/or additional mechanisms of multidrug resistance became obvious. Several ABC proteins, such as the MDR-related protein (MRP1) or the breast cancer resistance protein (BCRP) have been described [3]. Non-ABC proteins may also be involved, such as the lung resistance protein (LRP), which may play a role in the nuclear–cytoplasmic transport of cytotoxic agents [4]. In previous studies, we have shown that the MDR phenotype of doxorubicin-selected variants of the human myeloid leukemia cell line K562 was related to the overexpression of P-gp [5], occurring concomitantly with the disappearance of LRP protein [6]. In addition, it has been observed that the reversal of MDR in K562-resistant cells was not complete with P-gp inhibitors, such as verapamil or cyclosporine A [5], but the expression of oiher transporters was not likely to be involved in the accumulation and efflux of doxorubicin [6]. Thus, it could be hypothesized that oiher mechanisms of resistance were operative in these cell lines, especially through intracellular redistribution of drugs, as suggested by other studies (see for review [7]).

Although primarily located in the plasma membrane, P-gp has been detected also in the nucleus [8], in the Golgi apparatus [9] as well as in unidentified organelles [10–12]. Bennis et al. [5] observed a preferential accumulation of doxorubicin in subcellular components distinct from nucleus in doxorubicin-resistant K562 cells. The reversal of the MDR phenotype by quinine in these cells was not accompanied by the restoration of its cellular accumulation, but by the drug redistribution from these sequestration compartments to the nucleus. Quinine is a P-gp inhibitor, but it is also known to antagonize the mitochondrial permeability transition pore (PTP or megachannel). This multiprotein complex is involved in the transport of small molecules between the cytoplasm and the mitochondrial matrix and in the execution phase of apoptosis [13]. In addition, a study suggested that daunorubicin was sequestered in mitochondria in a MDR K562 cell variant [14]. These authors observed that the green fluorescence of daunorubicin was located in the perinuclear region and at the cell periphery. On the other hand, a similar distribution of the fluorescence was observed with rhodamine 123, a mitochondrial fluorescent probe. Thus, this organelle appears to be a good candidate for doxorubicin sequestration in K562 MDR cells, possibly implying the megachannel and/or P-gp for accumulation and efflux of the drug. In view of this hypothesis, it was necessary to clarify the possible involvement of mitochondria as a drug sequestration compartment. This property could be related to mitochondrial expression of P-gp.

In this purpose, we analyzed simultaneously by confocal microscopy, after immunostaining with monoclonal antibodies (MoAbs), the expression of intracellular P-gp in the Golgi apparatus and in mitochondria. In addition, Western blot and immunocytometric assays were carried out on isolated whole mitochondria from K562-sensitive and doxorubicin-resistant cell lines, with different anti-P-gp monoclonal antibodies. These analyses demonstrated the presence of P-gp in mitochondria of K562-resistant cell lines. Functional assays were performed on isolated whole mitochondria to assess their capacity for doxorubicin sequestration.

## 2. Materials and methods

### 2.1. Cell line and culture conditions

Doxorubicin-sensitive K562 cells and their doxorubicin-resistant counterparts [5,6] were grown in suspension in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (Eurobio) and 2 mM L-glutamine (Eurobio, France). Cells were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$ The resistant cell lines were obtained by progressive adaptation of the parental sensitive cells (K562/S) to 0.2 μg/ml of doxorubicin for K562/0.2R cells, and then further to 0.5 μg/ml of doxorubicin for K562/0.5R cells [6]. Before the experiments, the resistant cell lines were maintained without drug for at least 8 weeks for a complete elimination of doxorubicin. In this way, all the modifications observed were not due to a direct effect of doxorubicin to cells, but were related to the cell phenotype.

### 2.2. Monoclonal antibodies

For immunostaining studies of P-gp, we have used several monoclonal antibodies, reacting specifically with different epitopes of human P-gp. Two MoAbs recognize P-pg external epitopes. The MoAb named UIC2 [15], an $IgG_{2a}$ which recognizes a conformational epitope, was purchased from Beckman-Coulter (Marseille, France) and was used either uncoupled or directly coupled to phycoerythrin (PE). The F4 MoAb [16], an $IgG_1$ which binds to an epitope located in the N-terminal extracellular loop, was purchased from Sigma–Aldrich Chimie (Saint-Quentin-Fallavier, France). A third MoAb, C494 [17], an $IgG_{2a}$ reacting with a P-pg internal epitope, was purchased from Dako (Trappes, France). Immunostaining of mitochondria was performed with M117, an $IgG_1$ obtained from Leinco Technologies (Saint-Louis, USA), which recognizes a 60 kDa non-glycosylated human mitochondrial protein [18]. Immunostaining of the Golgi apparatus was performed with an anti-Golgi antibody, 58 K, an $IgG_1$ recognizing a 58 kDa protein (Sigma–Aldrich Chimie). A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Pharmingen, San Diego, CA, USA) was used as secondary antibody.

### 2.3. Expression of external and total P-gp by flow cytometry

Immunofluorescent staining of doxorubicin-sensitive and -resistant K562 cells was first achieved to determine the concentration of UIC2 necessary to saturate all P-gp sites. For the measurement of total P-gp (that accessible on the plasma membrane and the intracellular one), cell fixation and permeabilization were performed before staining with the Intrastain kit (Dako) according to manufacturer's instructions. This was not done for the study of the plasma membrane-associated P-gp since immunostaining was carried out on

intact living cells. In both cases, before staining, cells were washed twice in phosphate-buffered saline (PBS). Then, $7 \times 10^5$ cells/sample were incubated for 30 min at 4 °C with different concentrations of the primary MoAb (UIC2), ranging from 0.5 to 5 μg. This was done before fixation for plasma membrane P-gp and after cell fixation and permeabilization for total P-gp. Cells were then washed twice in PBS containing 1% bovine serum albumin (PBS–BSA) and the pellet was resuspended in the presence of 4 μg of the secondary antibody diluted in PBS–BSA. After 30 min incubation at 4 °C, cells were washed twice in PBS–BSA and resuspended in 500 ml of PBS–paraformaldehyde (PFA) 1%. An isotypic control ($IgG_{2a}$, Beckman-Coulter) was processed to determine non-specific binding. Cell fluorescence was analyzed with a FACS Vantage flow cytometer (Becton-Dickinson, Grenoble, France). For each sample, $10^4$ cells were analyzed after elimination of dead cells, debris and aggregates, with a flow rate of 1000 cells/s. For each immunostaining, the mean fluorescence signal of the corresponding negative control was subtracted from the mean fluorescence signal of the assay.

### 2.4. *In situ analysis of P-gp expression by confocal laser scanning microscopy*

For single immunostaining of doxorubicin-sensitive and -resistant K562 cells, two staining protocols were used: (i) for P-gp expressed on the plasma membrane, $2.5 \times 10^5$ cells/sample were stained with UIC2-FITC (3 μg/assay); after washing, cells were fixed with PBS–PFA, and counter-staining of the nucleus was realized with propidium iodide (PI) (5 μg/assay) and (ii) for P-gp expressed intracellularly and on the plasma membrane (total P-gp), cells were first fixed and permeabilized with the Intrastain kit, and P-gp was then stained with UIC2-FITC (3 μg/assay); after washing, a counter-staining of nucleus was realized with PI (5 μg/assay).

For the co-localization experiments, cells were first fixed and permeabilized with the Intrastain kit. Mitochondria and the Golgi apparatus were, respectively, stained with M117 (25 μl/assay) and 58 K (2 μg/assay) MoAbs, which were revealed by a 4 μg of the secondary antibody diluted in PBS–BSA. Immunostaining of P-gp was revealed with PE-associated UIC2 (3 μg/assay).

Two confocal imaging analyzers were used. In a first set of experiments, we employed an ACAS 570 (Meridian, Okemos, MI, USA), located in Institut Pasteur, Paris, consisting of a 200 mW argon ion laser (Coherent 92-S, Palo Alto, CA, USA), coupled with an attenuator 7% filter, and emitting at 488 nm. An optical microscope (Olympus, Tokyo, Japan) was equipped with an oil immersion objective lens (×63). Collection of the FITC green fluorescence of the Golgi apparatus or mitochondria was carried out through a band-pass filter (530 ± 15 nm) and PE orange-red fluorescence of P-gp was collected through a high-pass filter (575 nm). In a second set of experiments, samples were analyzed using a fluorescence microscope (Axiovert 200M, Zeiss), equipped with laser scanning confocal imaging system (LSM 510 Meta, Zeiss), comprising one 30 mW argon and two He–Neon lasers. After immunostaining of P-gp with a FITC-conjugated MoAb and counter-staining of nucleus with PI, green and red fluorescence signals were collected, respectively, with BP 505–530 and 560–615 nm emission filters. For the double immunostaining of mitochondria and P-gp (FITC green and PE orange-red fluorescences), the Lambda mode was used; images were collected to each 10.7 nm after excitation at 488 nm, in order to reduce fluorescence overlapping. Image processing was obtained with a LSM 510 computer software (Zeiss).

### 2.5. *Preparation of isolated mitochondria from K562 cells*

Mitochondria were prepared according to Bourgeron et al. [19] as modified by Denis-Gay et al. [20]. The purification degree of mitochondria obtained by this method was previously checked, especially concerning the absence of debris originating from other cellular membrane structures [21]. Further purification of mitochondria was avoided since it has been shown that it increased the risk of undesirable selection of a particular fraction of the mitochondrial population, potentially leading to the loss of altered mitochondria [22]. All steps of the purification were performed at 4 °C. Cells ($5 \times 10^6$) were collected by centrifugation (600 × *g* for 10 min at 4 °C) and were then washed twice in Tris–HCl 10 mM, pH 7.4, containing 100 mM sucrose, 1 mM EGTA, 20 mM MOPS and 1 mg/ml BSA. The pellet was resuspended in the buffer solution supplemented with 10 mM triethanolamine, 5% (v/v) Percoll and 0.1 mg/ml digitonin, for 5 min at 4 °C. Finally, the mixture was homogenized in a Potter homogenizer (10 strokes, 1000 rpm) before being diluted at the ratio of 1/5 in Tris–HCl 10 mM, pH 7.4, containing 300 mM sucrose, 1 mM EGTA, 20 mM MOPS and 1 mg/ml BSA, and centrifuged at 2500 × *g* for 5 min at 4 °C. The supernatant, containing mitochondria, was collected, whereas the pellet was resuspended in the last buffer and re-centrifuged. The supernatants were combined and centrifuged at 10,000 × *g* for 10 min at 4 °C in order to collect mitochondria in the pellet. Isolated mitochondria were washed twice in the same conditions before being resuspended in hypertonic Tris–HCl 10 mM, pH 7.4, containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM $KH_2PO_4$, 1 mg/ml BSA and 10 mM succinate. Mitochondrial integrity was assessed by a respiratory functional test according to Bourgeron et al. [19]. The mitochondrial fraction could be then used for the different analyses described below.

### 2.6. *Western blot analysis of P-gp expression in whole cells and mitochondria*

Detection and analysis of P-gp expression from cells and mitochondrial fractions isolated from parental and MDR variant cell lines were performed by immunoblotting. Equivalent amounts of cell proteins (80 μg) were suspended in a denaturing buffer (Tris–HCl 10 mM, pH 8, containing 5% glycerol, 2.5% SDS, 5% β-mercaptoethanol and 0.005% bromophenol blue). Proteins from cells or mitochondria were resolved by SDS-PAGE (8%) electrophoresis gels, and electroblotted onto PVDF membranes (Amersham). After 2 h incubation at 4 °C with 1% blocking reagent, the membranes were incubated overnight at room temperature with the F4 MoAb for K562 whole cell lysates. Mtochondrial proteins were resolved under the same conditions as whole cell lysates using either 60 μg of proteins and the F4 MoAb (15.6 μg/ml). Blots were revealed by chemoluminescence using a rabbit

anti-mouse peroxidase-labeled secondary antibody (1.3 μg/ml) purchased from Dako, with the chemiluminescence blotting substrate kit (Roche, Meylan, France) according to manufacturer's instructions, and evaluated with the Image station 440 CF from Kodak (Boston, MA, USA).

### 2.7. P-gp expression in isolated mitochondria by flow cytometry

For each assay, an aliquot of isolated whole mitochondria (50 μg proteins) from sensitive and resistant K562 cells was resuspended in 500 ml hypertonic buffer. Mitochondria were incubated for 1 h at 4 °C with different concentrations of the primary MoAb (ranging from 0.5 to 6 μg for UIC2, 1 to 8 μg for F4 and 0.5 to 12 μg for C494). Optimal labeling of organelles was obtained with 6 μg of UIC2, 8 μg of F4 and 6 μg of the FITC-coupled secondary antibody, as described above for intact cells. For labeling of mitochondria with C494, a particular protocol was used. This MoAb cross-reacts with pyruvate carboxylase (PC), an abundant mitochondrial enzyme [23]. The non-specific immunoreactivity was abolished by pre-incubating C494 in the presence of an excess of the synthetic-specific peptide (TLEG). Briefly, C494 (12 μg) was pre-incubated for 1 h at 4 °C in distilled water without (control C494) or in presence (TLEG C494) of 10–100-fold molar excess of PC epitope-specific peptide. Then, the mitochondria samples were stained either with control C494 or TLEG C494 as described. All washes were performed in the hypertonic buffer. An isotypic control (Beckman-Coulter) was processed to determine non-specific binding. To present P-glycoprotein expression, we applied the consensus method for P-gp detection in human hematological malignancies [24,25]. Thus, the mean fluorescence was expressed as the ratio of the fluorescence signal of the assay versus the fluorescence signal of the corresponding negative control.

### 2.8. Functional assay of mitochondrial P-gp by flow cytometry

Whole isolated mitochondria (50 μg proteins in hypertonic buffer) from sensitive and resistant lines were divided in test tubes to evaluate mitochondrial autofluorescence as well as the uptake and efflux of doxorubicin into and out of organelles, either without any other drug, or in the presence of specific MoAbs (4 μg of UIC2 or 1.5 μg of F4), or in the presence of inhibitors (5 μM cyclosporine A or 10 μM dexverapamil or 50 μM quinine). Pre-incubations were performed at 20 °C for 1 h (MoAbs) or 30 min (inhibitors). Then, doxorubicin (10 μM final) was added to all samples, which were further incubated for 1 h at 20 °C, avoiding light exposure. After incubation, the samples were kept on ice for a few minutes until flow cytometric analysis. Concomitantly, in order to estimate doxorubicin efflux from mitochondria, 2 ml of hypertonic buffer were added in appropriate samples. Mitochondria were centrifuged for 5 min at 450 × *g* at 4 °C and washed once more with 2 ml of buffer, then diluted in 500 μl of buffer at 20 °C. All tubes were incubated for 1 h at 20 °C. The fluorescence of doxorubicin was measured in each sample (for uptake and retention evaluation), on 10,000 isolated mitochondria with a flow rate of 500 events/s on a FACS Vantage flow cytometer (Becton-Dickinson), after elimination of debris and aggregates.

### 2.9. Statistics

An unpaired Student's *t*-test of the Statview™ software was used for all experiments. Coefficients of variation (for cytometric or microscopic analyses) corresponded to standard deviation/mean and were expressed in percentage.

## 3. Results

### 3.1. Studies in whole cells

We first investigated the intracellular expression of P-gp in doxorubicin-sensitive and MDR K562 cells by flow cytometry, using UIC2. P-gp expression was estimated on the plasma membrane in comparison with its total expression in whole cells (Fig. 1 and Table 1). On plasma membranes (mb-P-gp), the protein was undetectable in K562-sensitive cells and strongly expressed in resistant cells. Despite a lower level of resistance, P-gp expression at the membrane level was 2.35-fold higher in K562/0.2R than in K562/0.5R ($p < 0.05$). In whole cells, P-gp was barely detected in sensitive K562 cells, and strongly expressed in both K562-resistant lines. It was not possible to determine the saturating concentration of UIC2 in K562-sensitive cells, even with high quantities of MoAb (up to 10 μg), because fluorescence was always parallel to that of the isotypic control. As a consequence, it is likely that UIC2 binding was not specific in K562-sensitive cells. In resistant cells, total P-gp expression was 1.97-fold higher in K562/0.2R than K562/0.5R ($p < 0.05$). The proportion between external and internal P-gp was slightly and not significantly different in the two variants: about 57% of total P-gp was associated to the plasma membrane of K562/0.2R cells and 48% for K562/0.5R cells. Similar results were obtained with F4 MoAb.

The expression of membrane-associated P-gp and total P-gp was also studied by confocal microscopy with UIC2. In order to obtain a better discrimination of P-gp expression, a counter-staining of the nucleus was realized with addition of propidium iodide just before microscopic analyses. As expected, no expression was observed on the plasma membrane of sensitive cells (Fig. 2A), whereas, in both MDR variants, membrane-associated P-gp gave a strong signal (Fig. 2B and C). Total P-gp expression was then evaluated in whole cells after permeabilization and staining with UIC2 MoAb. In sensitive cells, P-gp gave a faint signal in the cytoplasm (Fig. 2D). In both MDR variants, P-gp was strongly expressed at the membrane level and in the cytoplasm of the cells (Fig. 2E and F). The total P-gp label was lower in the K562/0.5R variant than in the K562/0.2R.

In order to test the hypothesis of the presence of mitochondrial P-gp in K562 MDR cells, various experiments were carried out. The expression of P-gp at the mitochondrial level was sought by confocal microscopy in whole cells. After fixation and permeabilization, P-gp was stained with PE-conjugated UIC2 and mitochondria were stained with M117 and revealed by a FITC-coupled secondary antibody. Non-specific binding in the three K562 cell lines, estimated with

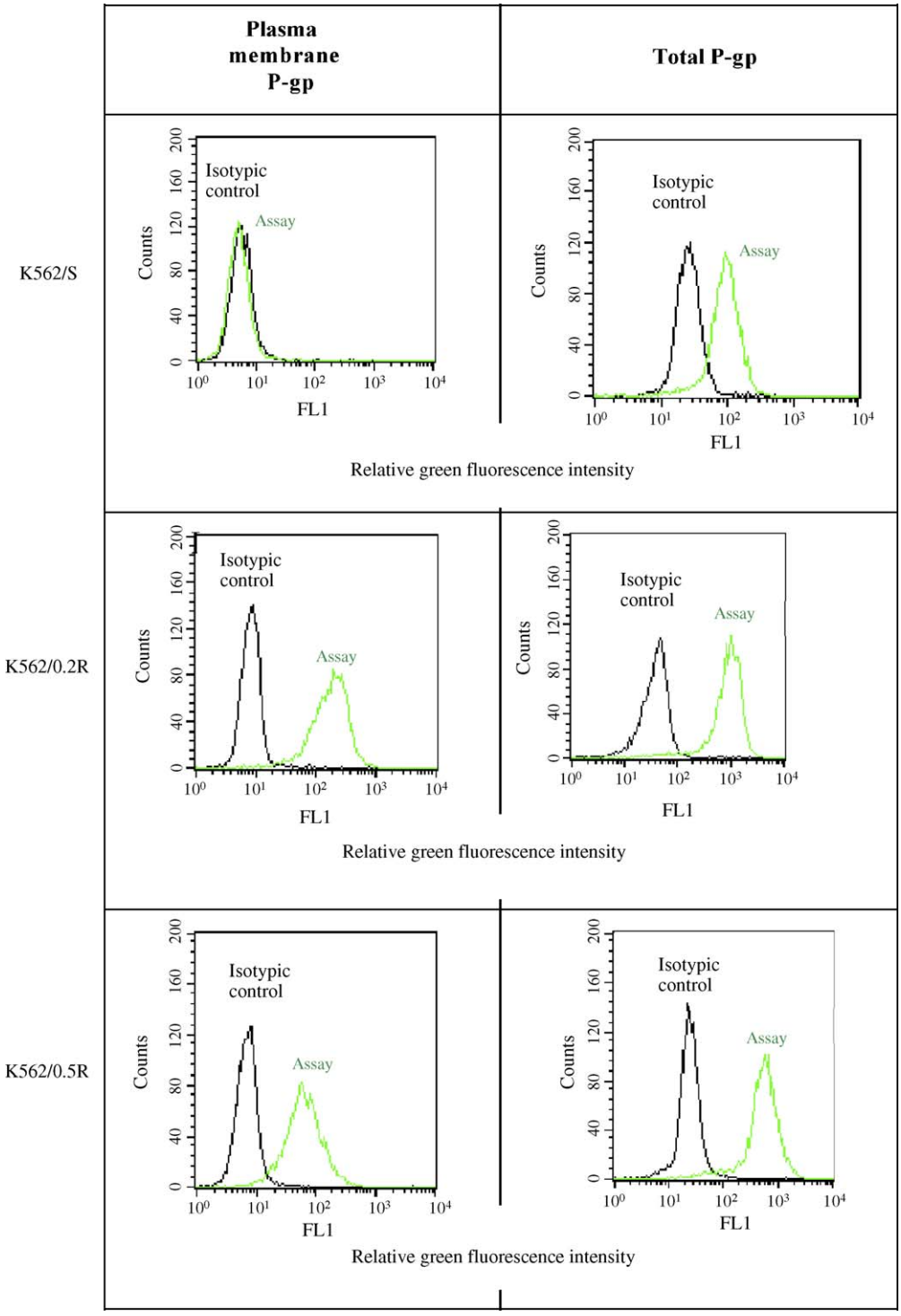


**Fig. 1 – Example of representative histograms obtained by flow cytometry with K562 cell lines (sensitive and resistant variants). For each staining of plasma membrane P-gp (mb-P-gp) (living cells) or total P-gp (fixed cells), $7 \times 10^5$ cells were incubated for 30 min at 4 °C with 3 μg UIC2 MoAb. This was done before fixation for plasma membrane P-gp and after cell fixation and permeabilization with the Intrastain kit (Dako) for total P-gp. After washing, the pellet was resuspended with 4 μg of the secondary antibody coupled to FITC. After 30 min incubation at 4 °C, cells were washed twice and resuspended in 500 μl of PBS–PFA 1%. An isotypic control ($IgG_{2a}$) was processed to determine non-specific binding. The relative mean of green fluorescence was evaluated on $10^4$ cells for each sample after elimination of dead cells, debris and aggregates. Cumulative data are shown in Table 1. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)**

**Table 1 – Flow cytometric analyses of plasma membrane and whole cell P-gp expression in K562 cell lines**

| Cell lines | K562/S | K562/0.2R | K562/0.5R |
|---|---|---|---|
| mb-P-gp expression (UIC2) | $0.95 \pm 0.05$ | $150 \pm 16^{***}$ | $63.7 \pm 6.0^{***,\dagger}$ |
| Total P-gp expression (UIC2) | $37.7 \pm 8.2$ | $262 \pm 20^{***}$ | $133 \pm 14^{*,\dagger}$ |
| mb-P-gp (mean value) (%) | 2.5 | 58 | 48 |
| Intracellular P-gp (mean value) (%) | 97.5 | 42 | 52 |

For each staining of plasma membrane P-gp (mb-P-gp) (living cells) or total P-gp (fixed cells), $7 \times 10^5$ cells/sample were washed twice in PBS. For the measurement of total P-gp, cell fixation and permeabilization were performed with the Intrastain kit (Dako) before staining with MoAbs. In all cases, cells were incubated for 1 h at 4 °C with the UIC2 MoAb (3 μg/assay). Cells were then washed twice and the pellet was resuspended with 4 μg of the secondary antibody coupled to FITC. After 30 min incubation at 4 °C, cells were washed twice and resuspended in 500 μl of PBS–PFA 1%. An isotypic control ($IgG_{2a}$, Beckman-Coulter) was processed to determine non-specific binding. The degree of P-gp expression was evaluated as arithmetic mean fluorescence intensity for each cell line, relative to the isotypic control. Each assay was done at least on four independent experiments.

* Significance of resistant cells vs. sensitive cells, $p < 0.05$.

*** Significance of resistant cells vs. sensitive cells, $p < 0.001$.

† Significance of 0.5R cells vs. 0.2R cells, $p < 0.05$.

irrelevant MoAbs, produced a very faint fluorescence (data not shown). In sensitive K562/S cells, a bright green fluorescence was detected, characteristics for mitochondria, while a very faint orange-red signal appeared throughout the cytoplasm (Fig. 3A). In contrast, in K562-resistant cells (Fig. 3B and C), a bright green fluorescence and a bright orange-red fluorescence appeared localized in the cytoplasm. In the merge, it was possible to discriminate between the orange-red fluorescence of P-gp (PE), the green fluorescence of mitochondria (FITC) and the yellow fluorescence corresponding to an overlapping of FITC and PE, indicated by red arrows, and which can be interpreted as P-gp expression at the mitochondrial level. The intracellular green and red fluorescence localization appeared variable in both K562-resistant sublines. Nevertheless, it was possible to observe a similar overlapping of FITC and PE fluorescence signals in both resistant K562 cells, as shown in histograms of green or orange-red fluorescence (line profiles).

For the evaluation of the percentage of total P-glycoprotein, which appeared localized in mitochondria, we analyzed at least 10 cells, each at least on 10 slices. This percentage was $22 \pm 7\%$ in K562/0.2R and $15 \pm 5\%$ in K562/0.5R, respectively. The dispersion of the values was confirmed by the orange-red fluorescence semi-quantification of P-gp, with the Meridian confocal imaging analyzer, and was achieved on at least 100 cells for each staining. The relative intensity was $1435 \pm 355$ (CV = 25%) and $750 \pm 399$ (CV = 53%), respectively, for K562/R0.2 and K562/R0.5 cells. These values are analogous to those obtained with cytometric analyses of P-gp expression (CV = 44 and 51% for K562/R0.2 and K562/R0.5 cells, respectively). Nevertheless, the coefficient of correlation obtained when plotting P-gp versus mitochondria was highly significant for the two resistant cell lines (0.96451 and 0.93148 for K562/R0.2 and K562/R0.5 cells, respectively). This demonstrates a correlation between the expression of P-gp and the quantity of mitochondria in a given cell.

In order to evaluate the relative importance of the expression of P-gp at the mitochondrial level, we also analyzed the localization of P-gp in the Golgi apparatus. In sensitive

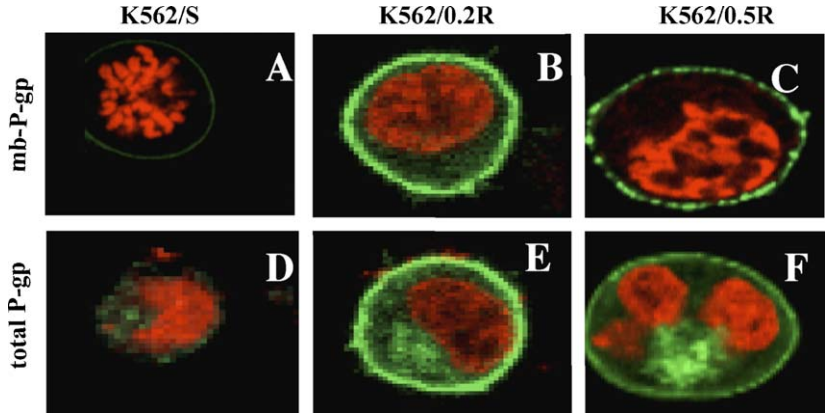


**Fig. 2 – Detection of membrane and total P-gp in doxorubicin-resistant K562 cells by confocal laser scanning microscopy. Plasma membrane P-gp in K562/S (A), K562/0.2R (B) and K562/0.5R (C) cells was detected by staining of $7 \times 10^5$ cells/sample with UIC2-FITC (3 μg/assay). After washing and cell fixation, propidium iodide (5 μg/assay) was added and produced a red fluorescence of the nucleus. Total P-gp was detected after cell fixation and permeabilization before staining with UIC2-FITC (3 μg/assay) in K562/S (D), K562/0.2R (E) and K562/0.5R (F). After washing, a counter-staining of the nucleus was realized with PI (5 μg/assay). In both cases, immunofluorescence was analyzed with the confocal microscope LSM 510 Meta (Zeiss). The P-gp green and the nucleus red fluorescence signals were collected, respectively, with BP 505–530 nm and LP 560 nm emission filters, on confocal optical sections of 0.45 μm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)**

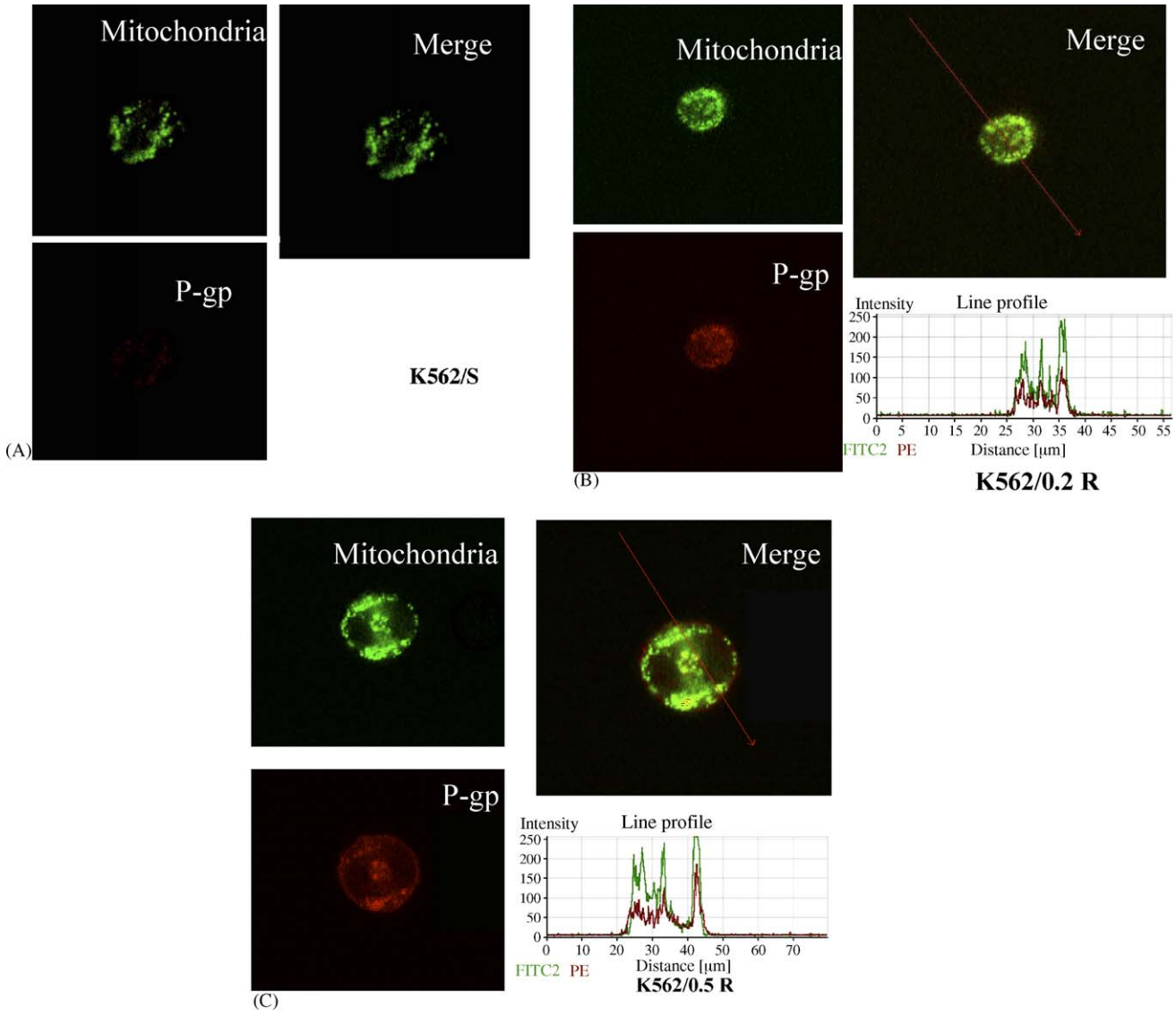


**Fig. 3 – Expression of P-gp in mitochondria of K562 MDR cells as determined by confocal laser scanning microscopy. Cells ($2.5 \times 10^5$ per sample) were first fixed and permeabilized with the Intrastain kit. Mitochondria were stained with 25 μl/assay of M117 antibody and revealed with a FITC-conjugated goat anti-mouse $IgG_1$ antibody (4 μg). Immunostaining of P-gp was obtained with PE-associated UIC2 (3 μg/assay). Samples were analyzed with a confocal laser scanning microscope LSM 510 Meta (Zeiss), equipped with laser ion argon emitting at 488 nm. The Lambda mode was used, images were carried out each 10.7 nm in order to reduce fluorescence overlapping. The green and red fluorescences, respectively, of mitochondria and P-gp were collected with BP 505–530 nm and LP 560 nm emission filters, on confocal optical sections of 0.45 μm. (A) K562/S, (B) K562/0.2R and (C) K562/0.5R cells. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)**

K562/S cells, only a bright green fluorescence was detected, corresponding to Golgi apparatus (Fig. 4A). The orange fluorescence of P-gp and the green fluorescence of the Golgi apparatus were observed in resistant K562 cells (Fig. 4B and C), and distinct yellow fluorescence signals were detected, corresponding to P-gp expression at the level of the Golgi apparatus. The percentage of total P-glycoprotein present the Golgi apparatus was highly variable according to the cell and the slice analyzed (at least 14 slices for each cell). Its mean value was 27% in K562/0.2R (range: 18–56%) and 39% in K562/0.5R (range: 10–62%). Nevertheless, the coefficient of correlation obtained when plotting P-gp versus Golgi apparatus was highly significant for both resistant cell lines (0.90813 and 0.89365 for K562/R0.2 and K562/R0.5 cells, respectively). Therefore, there is also a correlation between the expression of P-gp and the amount of Golgi-specific structures.

### 3.2. *Studies in isolated mitochondria*

We have then researched the presence and functionality of P-gp in isolated mitochondria from sensitive and doxorubicin-resistant K562 cells. P-gp expression was first evidenced by

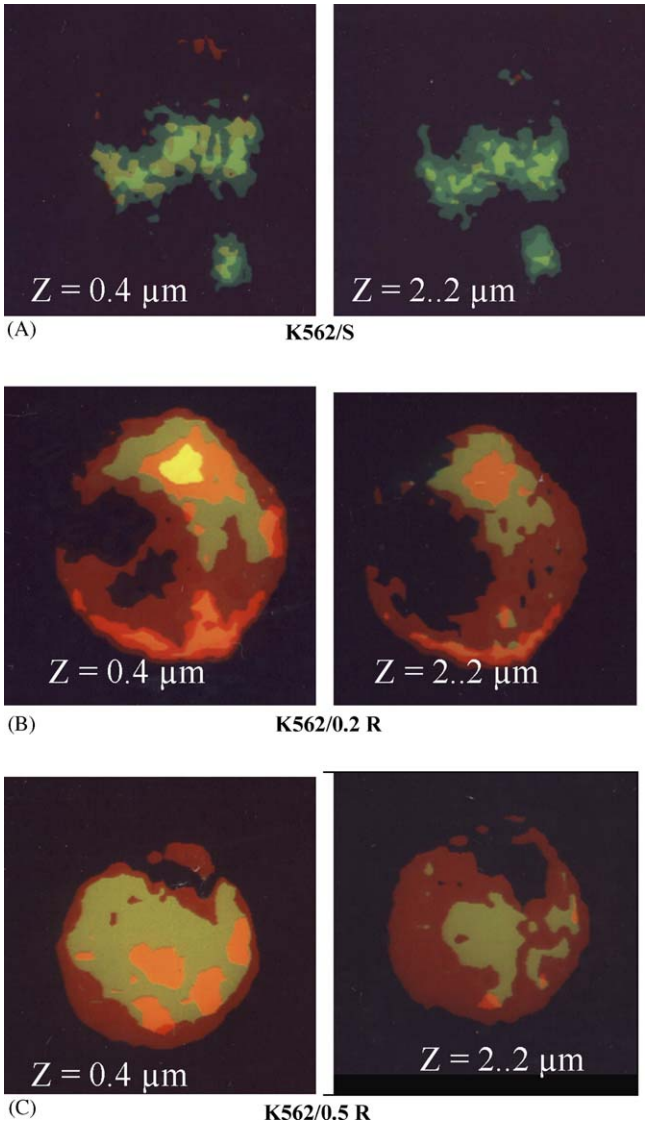


**Fig. 4 – Expression of P-gp in Golgi apparatus, as determined by confocal laser scanning microscopy. Cells ($2.5 \times 10^5$ per sample) were first fixed and permeabilized with the Intrastain kit. The Golgi apparatus was stained with 2 μg/assay of anti-Golgi 58 K antibody and revealed with a FITC-conjugated goat anti-mouse $IgG_1$ antibody (4 μg). Immunostaining of P-gp was obtained with PE-associated UIC2 (3 μg/assay). Samples were analyzed with a confocal laser scanning microscope ACAS 570 (Meridian) equipped with laser ion argon emitting at 488 nm. The green fluorescence of FITC was collected through a band-pass filter (530 ± 15 nm) and the orange fluorescence of PE was collected through a high-pass filter (575 nm). Several optical sections (0.2 μm) of MDR K562 cells are shown. (A) K562/S, (B) K562/0.2R and (C) K562/0.5R cells. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)**

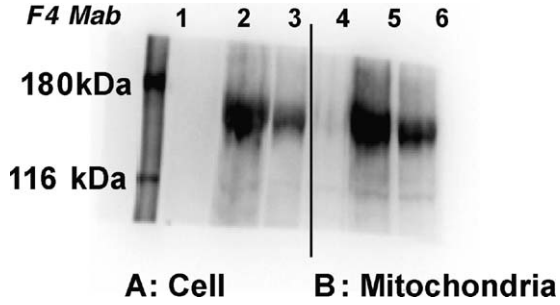


**Fig. 5 – Western blot analysis of P-gp expression in cells and mitochondria from K562 cell lines. Proteins from whole cell or mitochondrial lysates were resolved by SDS-PAGE (8%) P-gp expression was detected with two different primary antibodies, then revealed with a rabbit anti-mouse peroxidase-labeled secondary antibody. Detection with F4 (15.6 μg/ml) in: (A) whole cells (lane 1: K562/S, lane 2: K562/0.2R and lane 3: K562/0.5R) and (B) mitochondria isolated from K562/S (lane 4), K562/0.2R (lane 5) and K562/0.5R (lane6).**

Western blotting and revelation with F4, which specifically recognizes an extracellular epitope of P-gp (Fig. 5A). P-gp was not present in mitochondria isolated from K562-sensitive cells, but a significant expression was detected in mitochondria isolated from K562/0.2R and K562/0.5R (Fig. 5B), with a $M_r$ of 170 kDa, identical to that observed in whole cell lysates. P-gp expression in whole isolated mitochondria was also studied by flow cytometry, with three MoAbs recognizing different external (UIC2 and F4) and internal (C494) epitopes (Fig. 6 and Table 2). Since C494 cross-reacts with pyruvate carboxylase, a mitochondrial enzyme, it was necessary to eliminate non-specific binding. The problem has been solved by pre-incubating the MoAb with an excess of TLEG peptide. The process significantly reduced the organelle staining by 38.9 ± 6.2% for mitochondria from K562-sensitive cells, 45.9 ± 8.1 and 55.6 ± 10.2%, respectively, for mitochondria from K562/0.2R and K562/0.5R cells. For the three antibodies, the fluorescence intensity of isolated mitochondria from parental sensitive K562 cells was very low and was similar to that obtained with irrelevant MoAbs (ratio close to 1). In contrast, with all MoAbs, P-gp expression was detected in mitochondria isolated from the two resistant cell lines. As plasma membrane P-gp, mitochondrial P-gp expression was always higher for K562/0.2R than for K562/0.5R. Moreover, for both resistant lines, mitochondrial P-gp staining was similar with the two MoAbs recognizing external epitopes (UIC2 and F4), the specific TLEG-C494 MoAb giving the highest relative fluorescence signal.

The functionality of P-gp expressed in mitochondria, from whole organelles isolated from K562 cells, has been evaluated by flow cytometric analyses of doxorubicin accumulation and efflux. In addition, the effects of modulators were studied, using either specific MoAbs (UIC2 and F4), or small molecule inhibitors (cyclosporine A, dex-verapamil and quinine).

Doxorubicin accumulation in mitochondria isolated from the sensitive parental cell line (K562/S) significantly increased ($p < 0.05$) when incubated in the presence of anti-P-gp MoAbs or of inhibitors (Table 3). Prior incubation with antibodies did not modify subsequent drug accumulation. Cyclosporine A, dex-verapamil and quinine induced an increase of about 40% in doxorubicin uptake. A similar change was also observed for

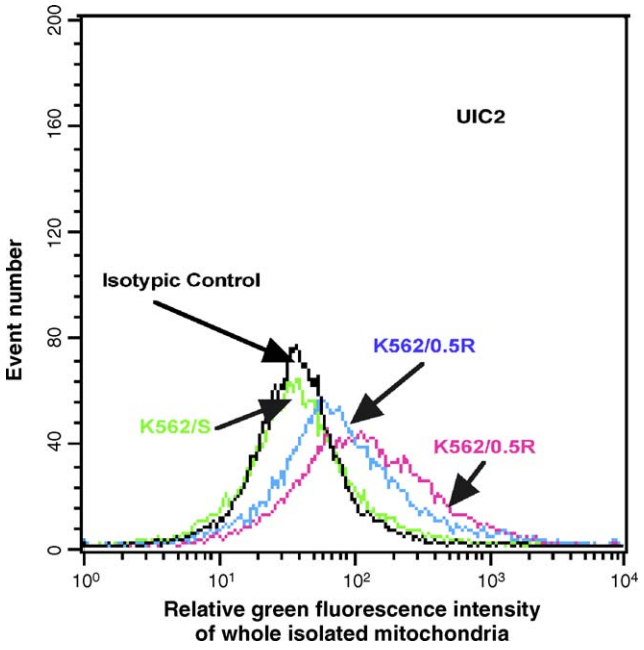


**Fig. 6 – Example of representative histograms obtained by flow cytometry with whole mitochondria isolated from K562 cell lines (sensitive and resistant variants). An aliquot of 50 μg of mitochondrial proteins was stained with 6 μg UIC2 ($IgG_{2a}$) or identical quantity of isotypic control ($IgG_{2a}$). After two washes, the primary MoAbs were revealed with a FITC-conjugated secondary antibody. The relative mean of green fluorescence was evaluated on $10^4$ whole mitochondria (1500 events/s) isolated from K562/S, K562/0.2R and K562/0.5R cells. Cumulative data are shown in Table 2. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)**

**Table 2 – Flow cytometric detection of P-gp with three different antibodies in whole mitochondria isolated from K562 cell lines**

| Antibody | UIC2 | F4 | C494 |
|---|---|---|---|
| K562/S | 1.08 ± 0.07 | 1.12 ± 0.16 | 1.23 ± 0.24 |
| K562/0.2R | 3.29 ± 0.13*** | 3.77 ± 0.70* | 4.82 ± 0.62*** |
| K562/0.5R | 2.39 ± 0.13***,† | 2.75 ± 0.55***,† | 4.25 ± 0.74*** |

An aliquot of 50 μg of mitochondrial proteins was stained with MoAbs: 6 μg UIC2, or 8 μg F4 or 12 μg C494. These MoAbs recognise different P-pg epitopes: UIC2 ($IgG_{2a}$) recognises a conformational external epitope of P-gp, F4 ($IgG_1$) binds an extracellular N-terminal loop epitope, C494 ($IgG_{2a}$) recognises intracellular epitopes. The non-specific immunoreactivity of pyruvate carboxylase with C494 was abolished by its pre-incubation in the presence of an excess (10–100-fold molar excess) of the synthetic-specific peptide (TLEG). After two washes, the primary MoAbs were revealed with a FITC-conjugated secondary antibody. Arithmetic mean fluorescence intensities were determined on $10^4$ whole mitochondria (1500 events/s) isolated from K562/S, K562/0.2R and K562/0.5R cells, relatively to the isotypic control. Each assay was done at least on four independent experiments.

* Significance of resistant cells vs. sensitive cells, $p < 0.05$.

*** Significance of resistant cells vs. sensitive cells, $p < 0.001$.

† Significance of 0.5R cells vs. 0.2R cells, $p < 0.05$.

nigericin- and oligomycin-treated mitochondria (results not shown). Doxorubicin accumulation was significantly higher in mitochondria from K562-resistant cell lines than in those from sensitive cells. None of the two MoAbs, UIC2 or F4, was able to alter doxorubicin accumulation in the mitochondria from resistant cells. In contrast, the three small molecule inhibitors significantly decreased ($p < 0.05$) doxorubicin uptake in mitochondria from MDR cells. The mitochondria isolated from both resistant cell lines sequestered similarly the drug. Other compounds, such as atractyloside or oligomycin, which are inhibitors of adeninenucleotide translocase and ATP synthase, respectively, had similar effects on doxorubicin uptake (results not shown). Moreover, doxorubicin fluorescence in mitochondria from resistant K562 cells became similar to that of the parental K562 cells after treatment with these inhibitors.

After doxorubicin accumulation, mitochondria were washed and centrifuged, resuspended in drug-free medium and analyzed by flow cytometry. There was a significant efflux of doxorubicin from mitochondria (>70%). In all cases, the

**Table 3 – Functional analysis of P-gp in mitochondria isolated from sensitive and resistant K562 cell lines, as analyzed by flow cytometry**

| Mitochondria from | K562/S | K562/0.2R | K562/0.5R |
|---|---|---|---|
| Doxorubicin alone | 100 | 173 ± 12 | 157 ± 8 |
| Doxorubicin + UIC2 | 122 ± 6* | 168 ± 11 | 141 ± 7 |
| Doxorubicin + F4 | 143 ± 9* | 171 ± 8 | 163 ± 10 |
| Doxorubicin + cyclosporine A | 132 ± 7* | 126 ± 13* | 124 ± 6* |
| Doxorubicin + quinine | 137 ± 4* | 130 ± 1* | 130 ± 3* |
| Doxorubicin + dex-verapamil | 138 ± 15* | 125 ± 4* | 128 ± 6* |

The analyses were performed with whole mitochondria (50 μg proteins/sample) isolated from K562/S, K562/0.2R and K562/0.5R cells; we studied the accumulation and efflux of doxorubicin (1 h at 10 μM, 20 °C). Prior incubations of mitochondria were carried out with UIC2 (4 μg) or F4 (1.5 μg) MoAbs for 1 h at 20 °C, or with cyclosporine A (5 μM), dex-verapamil (10 μM) or quinine (50 μM), for 30 min at 20 °C. Mean fluorescence of doxorubicin (drug accumulation) was then measured by flow cytometry. Drug efflux was estimated after washing and resuspending mitochondria in drug-free buffer (1 h at 20 °C). Cytometric analyses were performed on $10^4$ mitochondria (1500 events/s). The results correspond to the ratio (≥200) of the mean fluorescence intensity of each sample relative to the autofluorescence intensity of isolated mitochondria. The fluorescence was estimated relatively to fluorescence intensity normalized to 100 for doxorubicin accumulation in mitochondria isolated from sensitive K562 cells. At least three independent experiments were realized for each assay.

* Significance of doxorubicin + MoAb or modulator vs. doxorubicin alone, $p < 0.05$.

residual relative fluorescence of doxorubicin was comparable in mitochondria isolated from K562/S cells (34.0 ± 3.4%), from K562/0.2R cells (34.8 ± 4.7%) and from K562/0.5R cells (30.5 ± 2.9%), and the addition of MoAbs or drugs did not modify this phenomenon.

## 4. Discussion

Several mechanisms can explain MDR phenotypes, often involving drug detoxification by means of the overexpression of plasma membrane ABC proteins, such as P-gp, MRP1 or BCRP [1–3]. These proteins, and possibly other proteins such as LRP, may also be responsible for drug redistribution within the cells [7]. Doxorubicin-resistant K562 cells are commonly used as a model for MDR studies, particularly via P-gp overexpression. We have already shown that inhibition of P-gp activity by modulators does not completely restore sensitivity of K562 MDR cells to doxorubicin and a redistribution of this drug has been observed in the K562/0.2R variant [5]. These results suggest the involvement of other resistance mechanisms in these cells, possibly through intracellular drug redistribution.

Drug localization in cytoplasmic compartments of MDR cells has been observed by several authors by confocal laser scanning microscopy, using mainly doxorubicin because of its fluorescent properties [5,7–11]. Some studies on intracellular expression of P-gp have been reported, aiming at the clarification of the relationship between cytoplasmic localization and the multidrug resistance phenotype [8–12]. In different cell lines, P-gp has been detected in the nucleus [8], in the Golgi apparatus [9] as well as in unidentified vesicles, possibly lysosomes or recycling endosomes [10–12]. In cells transfected with the *MDR1* gene, P-gp was detected in vesicles located around the periphery of nucleus [26], suggesting a mitochondrial pattern. Gong et al. [14] have shown that accumulation of daunorubicin occurred in mitochondria-like organelles in K562-resistant cells and suggested a role for P-gp in drug intracellular transport. Shapiro et al. [12] and Ferrao et al. [27] demonstrated the involvement of P-gp in drug compartmentalization in leukemic cell lines and patient samples. It appears, therefore, that cytoplasmic localization could be involved in the sequestration of doxorubicin in organelles, preventing it to reach its nuclear targets. It could also correspond to a storage pool of P-gp for maintaining a steady-state level of surface P-gp for a novel mechanism of drug sequestration in K562 myeloid leukemic cells.

In favor of a role for mitochondria in doxorubicin sequestration in MDR cells, in addition to the works already mentioned [14,27], is the fact that some MDR modulators, such as cyclosporine A or quinine, which are able to mediate doxorubicin redistribution in MDR cells are also known as antagonists of the mitochondrial megachannel [13]. As a consequence, mitochondria may appear as a good candidate for doxorubicin sequestration in K562 MDR cells. In view of this hypothesis, we have studied in detail the possible mitochondrial location of P-gp and the involvement of mitochondria as a drug sequestration compartment.

Using flow cytometry, we first verified that P-gp was expressed on the plasma membrane and in whole cells of the different K562 variants. We noticed a low-level, non-saturable binding of UIC2 in sensitive K562 whole cells, parallel to the binding of an isotypic antibody, and which is irrelevant for this reason. We also noticed, using three different MoAbs, that the variant with the lowest degree of resistance (K562/0.2R) contained a higher amount of P-gp than the variant with the highest degree of resistance, both at the plasma membrane level and in whole cells. Such a discrepancy between the level of resistance and the amount of P-gp has been already mentioned in various cell lines and is probably related to the multifactorial nature of MDR [28].

Using confocal microscopy, a strong P-gp staining appeared on the plasma membrane of the two resistant cell lines, with more intensity for K562/0.2R cells, confirming cytometric analyses. In addition, intracellular expression of P-gp was also noticed, preferentially accumulated in restricted areas of the cytoplasm. Such a distribution has already been observed in MDR cellular models and attributed to the Golgi apparatus [9] or unknown vesicles [12,26].

Dual fluorescence study allowed a positive approach of P-gp localization. In K562-sensitive cells, the simultaneous labeling of P-gp and mitochondria provided neither the orange-red fluorescence signal corresponding to P-gp, nor the yellow fluorescence signal corresponding to P-gp and mitochondrial co-expression. In contrast, P-gp appeared localized in mitochondria of K562-resistant cells and accounted for about 20% of the total P-gp signal in the two sublines. A significant orange-red signal, corresponding to intracellular P-gp alone, indicated that P-gp was also present in other intracellular compartments of resistant cells, such as lysosomes and recycling endosomes. With simultaneous labeling of P-gp and the Golgi apparatus, a marked yellow fluorescence was detected in both K562-resistant variants, corresponding to 27–39% of intracellular P-gp, but was not observed in sensitive cells, confirming the observations of Molinari et al. [9]. The localization of P-gp in the Golgi apparatus may reflect both a processing and transport compartment of P-gp from the endoplasmic reticulum to the plasma membrane and an intracellular sequestration compartment [29]. The important variability in the proportion of Golgi apparatus or mitochondria-associated P-gp can be attributed to the asynchronous growth of the cells and their study at different steps of the cell cycle. It has been shown that the level of expression of P-gp and its distribution changed during the cell cycle [30,31]. We observed that, in cells undergoing mitosis, no P-gp was detected at the plasma membrane level of K562-resistant cells (results not shown).

The presence of P-gp was assessed by Western blotting of mitochondria isolated from resistant cell lines with two different antibodies, able to stain a 170 kDa protein. Five mammalian ABC proteins have been identified in mitochondria [32]. They correspond to ‘‘half-transporters’’ and have a size (65–94 kDa) much lower than that of P-gp (170 kDa). The conventional SDS-PAGE technique we used allows to exclude the detection of a dimeric mammalian mitochondrial ABC transporter corresponding its functional structure [33]. In addition, the homology of mitochondrial ABC transporters with P-gp is relatively low (less than 50%), and is important only in the ATP-binding domain, whereas the epitopes recognized by all MoAbs used in this study are localized in other domains [15–17]. These features exclude the possibility

that the mitochondrial P-gp evidenced in this study could be the mitochondrial ABC transporters. The purification of mitochondria by differential centrifugation is a widely used technique [19,20], which generally provides organelles with a high degree of purity (≥90%), rendering unlikely a significant contamination of our preparations by P-gp originating from another cellular localization [21,22]. However, one cannot exclude a slight difference between plasma membrane P-gp and mitochondrial P-gp, especially at the level of the glycanic moiety.

P-gp expression in whole isolated mitochondria was investigated by flow cytometry with several MoAbs. For staining with C494, a specific pre-treatment allows to avoid reactivity with pyruvate carboxylase, a mitochondrial protein not related to P-gp [23]. Mitochondria from K562-sensitive cells do not contain detectable P-gp. In contrast, in mitochondria from both K562-resistant cell lines, P-gp expression was observed whatever the MoAb used. From the flow cytometry data, we cannot conclude about the relative amounts of mitochondrial P-gp in the two K562-resistant cell lines because the fluorescence signals were similar excepted with UIC2. In addition, in mitochondria from both K562-resistant cell lines, fluorescence intensity was similar with F4 and UIC2 and higher with C494, which was not the case for plasma membrane P-gp. This could be due to a difference in epitope accessibility by the MoAbs and suggests an opposite topology in mitochondria and in the plasma membrane.

Mitochondrial P-gp functionality was investigated in the presence of several MoAbs and inhibitors. Anti-P-gp MoAbs and inhibitors enhanced doxorubicin uptake in mitochondria from sensitive cells, a phenomenon that cannot be attributed to P-gp inhibition, since it is absent in these mitochondria. As shown by several authors, doxorubicin spontaneously binds numerous mitochondrial targets: DNA, cardiolipin and respiratory chain complexes (see for review [34]). The effect of MoAbs and small inhibitors can involve an alteration of membrane conformation leading to a decrease in the fixation of doxorubicin to its mitochondrial targets. In mitochondria isolated from K562-resistant cells, in contrast, which accumulate more doxorubicin than those from sensitive cells, the presence of inhibitors decreased this accumulation while the antibodies had no significant effect. These results are important because both tend to suggest that mitochondrial P-gp has a topology inverse to that observed in plasma membrane: (i) it provides an increased accumulation of the drug in the organelle; (ii) ''extracellular'' MoAbs have no effect on this accumulation, probably because they cannot reach any binding site; (iii) small inhibitors, which can cross the mitochondrial membrane, reduce drug accumulation in the organelle; (iv) oligomycin, which blocks ATP synthesis, also reduces mitochondrial drug accumulation. The residual fluorescence after washing was similar in mitochondria isolated from all three cell lines, even in the presence of P-gp inhibitors. Drug retention appeared, therefore, independent from P-gp expression.

One can propose, as a general conclusion, that mitochondrial P-gp expressed in K562-resistant cells behaves as a unidirectional pump, oriented in a direction opposite to that of the plasma membrane. This orientation is compatible with that of another mitochondrial ABC transporters, M-ABC1, which is embedded in the inner mitochondrial membrane and exposes its ATP-binding domain in the mitochondrial intermembrane space [35]. At the level of the plasma membrane, doxorubicin uptake is mostly passive and extrusion is active through P-gp action [36]. In mitochondria, doxorubicin uptake would be P-gp-dependent and specifically inhibited by P-gp reverters, whereas its exit would be essentially passive. A potential role for mitochondrial P-gp in MDR cells would be to protect the nucleus from doxorubicin. Nevertheless, the drug uptake in mitochondria from MDR K562 cell lines could lead to oxidative damage in relation with its interaction with mitochondrial components. In fact, due to low capacities of organelles to repair oxidative insults, the free oxygen radicals

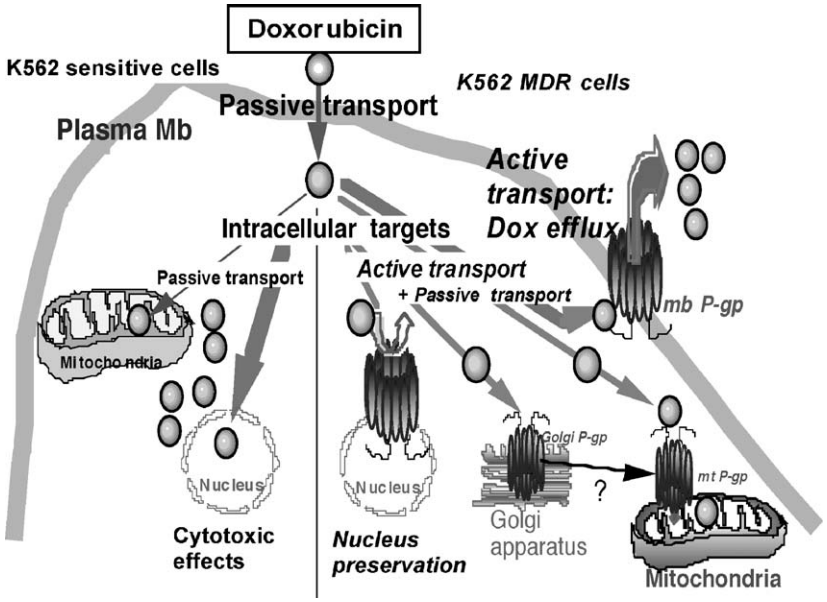


**Fig. 7 – A hypothetic scheme for mitochondrial topology and functionality. A hypothesis was proposed in agreement with the results obtained concerning mitochondrial P-gp expression and function.**

can induce lipid peroxidations, bioenergetic failure, DNA mutations, membrane disorders and mitochondrial dysfonctions [34]. Drug detoxification would then be the usual purpose of vesicular traffic and secretion/and or autophagy of damaged organelles such as mitochondria [37]. An increase in the number of autophagic vacuoles, favoring the removal of damaged intracellular macro molecules, is observed in cancer cells in response to drug treatment [38].

A model of P-gp function at the plasma membrane and at the mitochondrial levels is proposed in Fig. 7. Sequestration of anticancer drugs by an active process in this organelle could contribute to the multidrug resistance phenotype and explain several features often observed, such as intracellular drug tolerance [24] or drug redistribution upon the action of modulators like quinine [5]. Such drug sequestration has already been observed in cytoplasmic vesicles of K562-resistant cells, although the compartment involved in this process was not identified [12,26], despite the fact that the Golgi apparatus and lysosomes clearly contribute to drug sequestration [39]. The fact that cyclosporine A and quinine are not pure inhibitors of P-gp but can also interact with the mitochondrial permeability transition pore may be confounding. One cannot exclude that the effects observed with these compounds are due to their interference with the pore, or that mitochondrial P-gp is bound to this macromolecular complex. This would explain the differential effects of verapamil and quinine, which remained unexplained [5].

Our results raise different questions to be solved in the future about the intracellular trafficking of P-gp and the import of P-gp in mitochondria. Other points remain to be understood, such as the possible relationship between the P-gp expression, its localization, and its function with other proteins of multidrug resistance family. The sequential expression of different proteins involved in MDR phenotype varies according to the degree of resistance of K562 cells [6]. Moreover, Larsen et al. [7] suggested that the degree of P-gp over expression could influence the organization and function of other membrane proteins such as ion transporters. Such features are now to be taken in consideration for a complete understanding of the role of P-gp in the MDR phenotype.

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