

Is rhodamine 123 an appropriate fluorescent probe to assess P-glycoprotein mediated multidrug resistance in vinblastine-resistant CHO cells?*

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Abstract. Cellular drug resistance, which involves several mechanisms such as P-glycoprotein (P-gp) overexpression, kinetic and metabolic quiescence, or the increase in the intracellular levels of glutathione, limits the effectiveness of cancer treatment. It has been reported that functional assessment of the cationic dye rhodamine 123 (Rho123) efflux reveals accurately the drug-resistant phenotype. To study cellular drug resistance, we have obtained a CHO-K1 derived cell line resistant to vinblastine by means of multistep selection. This cell line (CHOVBR) displays high reactivity with a monoclonal antibody (MAb) (C219) directed against an internal domain of P-gp, and an active Rho123 efflux, as shown by parallel flow cytometric and fluorometric assays. However, under similar experimental conditions, the drug-sensitive parental cell line CHO-K1 (as well as the myeloblastic KG1 and KG1a cell lines), was also able to pump Rho123 out. These parental CHO-K1 cells had a very low reactivity against the C219 Mab, as confirmed by Western blot analysis. Both vinblastine and verapamil inhibited Rho123 efflux in CHO-K1 cells, but had no effect on CHOVBR cultures. Also, deprivation of vinblastine for one month did not affect Rho123 efflux in these cells. Our results suggest that the activity of P-gp appears to be essential, but not sufficient to confer drug resistance, and that Rho123-based functional assays of drug resistance should be evaluated for each cellular experimental model.

Keywords: P-glycoprotein, rhodamine 123, drug resistance, MDR, CHO cells

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1. Introduction

One of the major drawbacks in cancer therapy is cellular drug resistance, which leads to a lack of response to cytotoxic drugs and worsens the outcome in cancer patients. Several mechanisms are involved in primary and secondary drug resistance [1]. P-glycoprotein [25], an ATP-dependent M_r 170,000 membrane protein encoded by the human MDR1 gene [34,42], reduces drug accumulation in resistant cells by actively extruding chemotoxic agents, including *Vinca* alkaloids, anthracyclines, taxol, epipodophyllotoxins, certain inhibitors of protein synthesis such as actinomycin D, and other structurally unrelated compounds. Expression of the multidrug resistance associated protein (MRP) gene, a membrane protein overexpressed in multidrug resistant cell lines not overexpressing P-gp [6], probably also plays an important role in human resistant tumors. Both glycoproteins are members of the ABC (ATP binding cassette) superfamily of transmembrane proteins acting as active transporters, which include several bacterial transporters [24] and the CFTR product of the cystic fibrosis gene [33].

Multidrug resistance (mdr) can be reversed *in vitro* by blocking the drug efflux, with P-gp modulators such as verapamil, cyclosporin, phenothiazines and dihydropyridines [4,40]. Clinical studies using these modulators in combination with chemotoxic agents have failed to demonstrate any therapeutic benefit, while at the same time, toxic effects were observed, nowadays leading to the use of non-immunosuppressive modulator analogues [26,43].

During the last few years several antibodies have been developed to better investigate the multidrug resistant phenotype. They can bind internal (C219, JSB1, mdr Ab-1) and external domains (MRK16, 4E3). It has been suggested that the clinical outcome may be related to the level of expression of P-gp; however, MDR1 gene does not seem to be functionally expressed in all malignant cells, probably due to variability in the glycosylation pattern. Thus, several drugs have been used in functional assays *in vitro* to determine its accumulation by tumor cells, and the modulatory effect of P-gp inhibitors on drug efflux. Anthracyclines and the cationic dye rhodamine 123 (Rho123) are P-gp substrates actively effluxed by multidrug resistant cells, allowing functional and phenotypic characterization of neoplastic cells by simultaneous staining with antibodies [21].

In this study we have used flow cytometry to determine the mdr phenotype *in vitro* in a CHO-K1-derived cell line (CHOVBR) made resistant to vinblastine in our laboratory by multistep selection. We analyzed P-gp expression by using the C219 MAbs, and we have performed functional assays by measuring the accumulation and efflux of Rho123 and the modulatory effect of verapamil. The concentration of Rho123 in the culture media was also assessed by fluorometric assays, showing that drug sensitive and drug resistant cells have reactivity to P-glycoprotein, and extrude Rho123. It is our belief that these results provide additional evidence to differentiate between the functional activity of P-gp and its mediated drug resistance, since other metabolic mechanisms may be involved in the mdr phenotype.

2. Material and methods

2.1. Cell lines and culture conditions

CHO-K1, KG1, and KG1a cells were obtained from the American Type Culture Collection (Rockville, MD, USA). KG1 and KG1a cells were grown as a suspension culture in RPMI 1640 medium (Gibco, Life Technologies Inc., Gaithersburg, MD, USA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units penicillin/ml, 100 mg/ml streptomycin (Biological Industries, Kibbutz Beth Haemek, Israel), and 10% FCS. CHO-K1 cells were grown in 92 × 17 mm

Petri dishes (Nunclon[®], Nunc, Roskilde, Denmark) and Ham's F-10 nutrient mixture (Imperial, Andover, UK), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units penicillin/ml, 100 mg/ml streptomycin, and 10% FCS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, and 95% air. Exponentially growing CHO cells were washed in cold PBS, and incubated with 1 ml of 0.25% trypsin (Biological Industries, Kibbutz Beth Haemek, Israel) for 5 min at 4°C. Suspensions with an optimal density of 5×10^5 cells per milliliter were prepared for immunofluorescence analysis.

2.2. Generation of drug resistant cells

We have generated a drug resistant cell line (CHOVBR) derived from its parental CHO-K1 cell line, that is highly resistant to Vbl (1280 ng/ml). Drug resistant CHOVBR cells were obtained by stepwise increasing concentrations of vinblastine, from 10 to 1280 ng/ml. Early colonies resistant to 10 ng/ml of Vbl appeared at day 7, and cultures were confluent at day 26. Colony counts at day 7 were only 300, out of 1.5×10^8 CHO-K1 cells initially plated. This selection procedure is shown in Fig. 1, where the drug concentration (Y) is expressed as a function of the establishment of drug-resistance (X), in days. Data distribution adjusted well to a potential equation ($Y = a \times X^b$). The regression equation, calculated as usual, $Y = 0.00165 \times X^{2.576111}$, is plotted in the same figure ($R^2 = 0.974$).

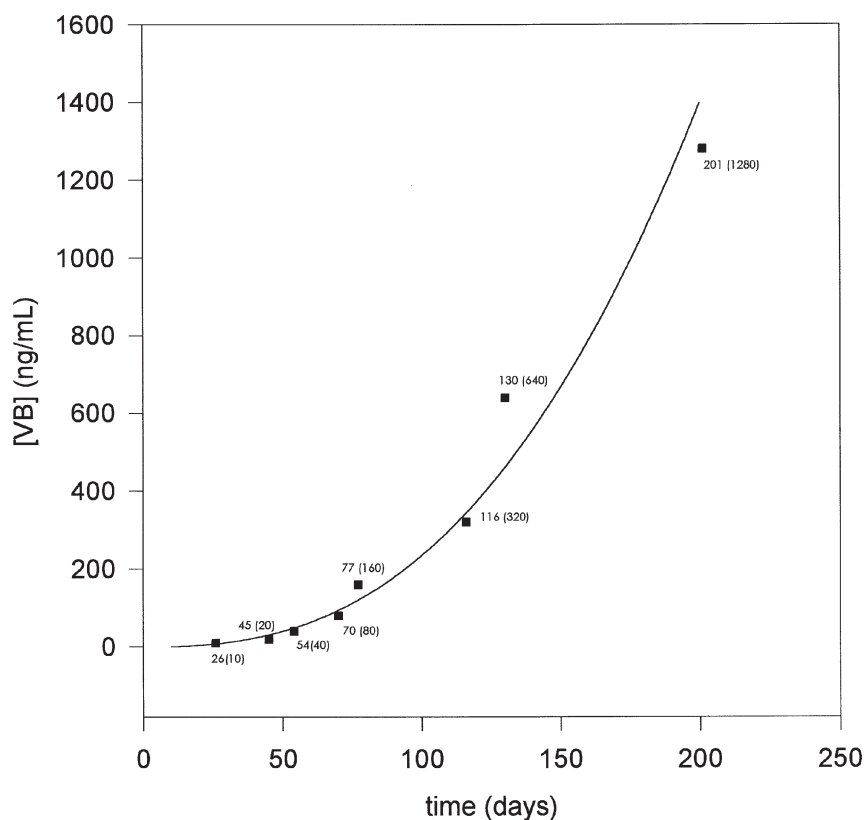


Fig. 1. Graph showing the selection procedure of CHO-K1 cells in phases of increased drug concentration from 10 to 1280 ng/ml of vinblastine. Each square represents the day of culture confluence or establishment of resistance, in days (numbers out of parenthesis), as a function of drug concentration (numbers in parenthesis).

2.3. Drugs and chemicals

Drugs and chemicals used in this study included: rhodamine 123 (Lambda Fluoreszenztechnologie, Graz, Austria), propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), vinblastine (Vbl) (Lilly S.A., Spain), and verapamil (Vpl) (Laboratorios Knoll, Spain).

2.4. Flow cytometry

Flow cytometry (FCM) analysis was performed using an EPICS Elite flow cytometer (Coulter Electronics, Inc., Hialeah, FL, USA) equipped with an argon ion laser tuned at 488 nm. Green fluorescence (FITC) was measured through a BP 525 nm filter. Acquisition was stopped when 10^4 gated events were collected in the FITC fluorescence-count histogram. Gating was based on forward scatter and side scatter (FS/SS) histograms, by encircling populations with amorphous regions. Data were stored as listmode files and analyzed using the Elite v. 4.01 Workstation Software.

2.5. Antibodies

C219 MAb recognizing a P-gp internal domain, and the 4E3 MAb which recognizes a human P-gp external domain, were purchased from Signet Laboratories, Inc. (Dedham, MA, USA). The mdr Ab-1 PAb raised against a P-gp cytoplasmic domain was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA, USA). Indirect immunofluorescence assays were performed using a fluorescein conjugated F(ab')₂ fragment antiserum (Kallestad, Austin, TX, USA). An isotypic control antibody (IgG2a, Coulter Corporation, Hialeah, FL, USA) was used to determine non-specific binding.

2.6. Western blot analysis

Cells were harvested and incubated on ice in a lysis buffer consisting of 10 mM Tris-Cl (pH 7.6), 140 mM NaCl, 1% Nonidet P-40, 0.4 mM sodium orthovanadate, 1 mM phenoxymethyl sulfofluoride, 50 mM sodium fluoride, and 10 mg/ml of aprotinin. The lysates were centrifugated at 14,000g for 8 min at 4°C. Membrane enriched preparations from drug sensitive (CHO-K1) and drug resistant cells (CHOVBR) were used to test the anti P-gp C219, mdr Ab-1, and 4E3 antibody reactivities. After 8% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysates, proteins were transferred to nitrocellulose membranes at 0.0025 amps/cm², for 50 min at room temperature, and stained for 1 h with the anti P-gp antibodies (1 µg/ml). Following washes, blots were incubated for 30 min with the specific peroxidase conjugate IgG (Dako) diluted 1 : 8000. Then, and after washes, detection of primary antibodies was accomplished by using the enhanced chemiluminescence assay (Amersham, Arlington Heights, IL, USA).

2.7. Cell fixation and immunofluorescence labelling

Exponentially growing cells were rinsed twice in cold PBS supplemented with 0.1% NaN₃ (E. Merck, Darmstad, Germany) and 1% bovine serum albumine (BSA) (Sigma Chemical Co., St. Louis, MO, USA), and resuspended at a final concentration of 5×10^5 cells/ml. Cell fixation was performed for 30 min at -20°C (on duplicates) in 70% methanol.

Unfixed cells were used for external epitopic domain analysis. In order to prevent low P-gp epitopic reactivity due to trypsin treatment, cells were rinsed twice in cold PBS-BSA, and seeded in Petri dishes

in a Hank's balanced salt solution (Gibco, Life Technologies Inc., Gaithersburg, MD, USA) at a final concentration of 5×10^5 cells/ml, for 30 min in a cell incubator, to allow epitope recovery. Plated exponentially growing cells were prepared for fluorescence microscopy analysis to better evaluate the 4E3 MAb reactivity, and to compare with flow cytometry results.

All incubations were carried out for 30 min at 4°C. Cells were rinsed twice in cold PBS-BSA by pelleting at 1500 rpm for 5 min, prior to analysis.

2.8. Rhodamine 123 uptake/retention assays

KG1, KG1a, CHO-K1 and CHOVR cells, its derived cell line resistant to Vbl, were seeded in 6-well tissue culture testplates at a final concentration of 10^5 cells/ml, 3 ml total volume, 12 h before the Rho123 uptake/retention experiments, to achieve conditions which allow optimal growth. The rhodamine 123 uptake was determined by adding 200 ng/ml of this dye (stock solution 1 mg/ml H₂O) to the culture medium, for 1 h, in the presence or absence of Vbl 1 µg/ml, Vpl 10 µg/ml, and Vbl plus Vpl at the same concentrations, in separate wells. After 1 h of incubation, cells were washed and resuspended in Rho123-free culture medium, while maintaining the other drugs to evaluate their effect on Rho123 retention. Dead cells were excluded by simultaneous staining with propidium iodide 5 µg/ml (stock solution 1 mg/ml H₂O).

2.9. Fluorometric assays

To assess Rho123 efflux from CHO-K1 and CHOVR cells, comparative experiments were carried out by using a luminescence spectrofluorometer, model LS50 (Perkin-Elmer Corporation, Norwalk, CT, USA). Following Rho123 treatment as previously described, cells were washed and resuspended in Rho123-free culture medium. Cell-free culture medium was immediately measured for Rho123 fluorescence with excitation at 460 nm and emission at 520 nm, at 10 min intervals for 1 h.

3. Results

3.1. P-glycoprotein expression

Drug sensitive CHO-K1 cells, and its vinblastine derived CHOVR subline were assayed for P-gp expression by Western blot with the C219, mdr Ab-1, and 4E3 antibodies. C219 MAb displayed a reactive band with a relative molecular weight of 170,000, characteristic of P-gp, also showing the overexpression of a polypeptide between Mr 160,000 and 180,000 in the case of vinblastine-resistant CHOVR cells. Both mdr Ab-1 and 4E3 antibodies were unreactives with membrane cell preparations from cell lysates (Fig. 2). Flow cytometric results for P-gp expression showed that 4E3 MAb had very little reactivity in both CHO-K1 and CHOVR cell lines (2–4%), and had undetectable levels by fluorescence microscopy analysis. In addition, flow cytometry analysis of mdr Ab-1 PAb showed stronger staining than C219 MAb, but had crossreactivity with specific chinese hamster proteins. C219 MAb had very low reactivity in CHO-K1 cells (8%), while CHOVR cells display an increased reactivity to this antibody (35–37%) as shown in Fig. 3.

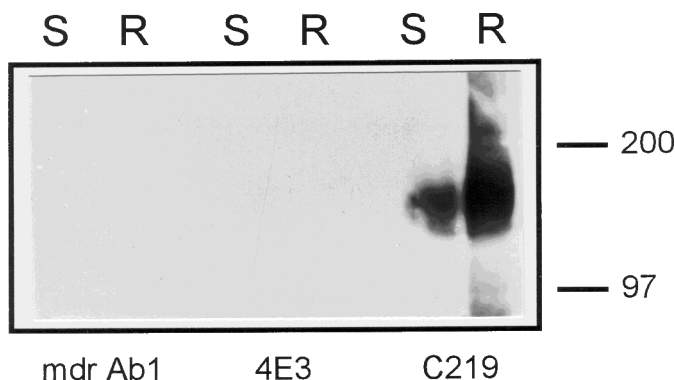


Fig. 2. Purified membrane fractions isolated from CHO-K1 (S: vinblastine sensitive) and CHOVR (R: vinblastine resistant) cells were analyzed by Western blotting. The immunoblot was incubated with the anti-P-gp mdr Ab-1, 4E3, and C219 antibodies. Note that specific antigen-antibody complexes were only visualized when C219 MAb was used.

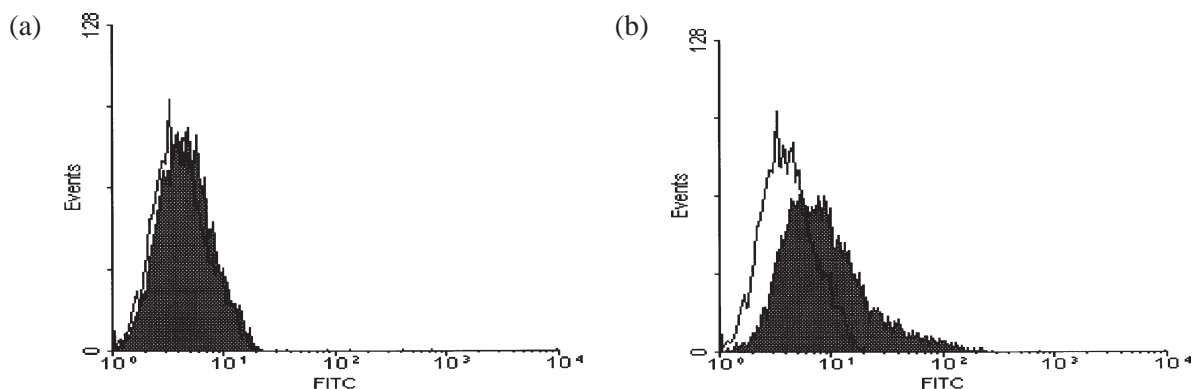


Fig. 3. Uniparametric histograms showing P-glycoprotein expression in CHO-K1 drug sensitive cells and in CHOVR drug resistant cells. White areas represent the immunofluorescence distribution of the isotopic control. Reactive cells (shaded areas) were quantified by histogram subtraction. (a) Fluorescence profile of CHO-K1 cells indicating C219 minimal staining (8%). (b) Detection of P-glycoprotein positive cells in CHOVR (35%).

3.2. Functional assessment of P-glycoprotein

Rho123 experiments were addressed to study P-gp active efflux in KG1 and in KG1a cells, as well as in CHO-K1 drug sensitive cells, CHOVR cells resistant to Vbl (1280 ng/ml), and in CHOVR cells which had been deprived of Vbl for one month to investigate a possible loss of drug resistance. Rhodamine 123, a dye considered a good indicator of mdr activity, was effluxed by KG1 and KG1a cells, and this efflux was blocked by verapamil (data not shown). At $t = 0$ min, Rho123 was retained by CHO-K1 cells about 2.73-fold more effectively than CHOVR cells, and the time that P-gp needed to efflux the 50% of Rho123 out of the cells was 8.4 min. However, under the same conditions, CHOVR cells were able to efflux the 50% of Rho123 in 3.5 min, hence, showing differences in the velocity of the transporter (Fig. 4). This efflux activity of CHO-K1 cells was inhibited when Vbl or Vpl were present in the culture medium. Both Vpl and Vbl, together, showed a more potent inhibitory effect of Rho123 transport than either of them alone, but Vpl alone had a stronger effect than Vbl. In addition, CHOVR cells showed non-significant efflux differences in relation to P-gp inhibitors, and Vbl deprived CHOVR cells had similar Rho123 transport activity than that of CHOVR cells

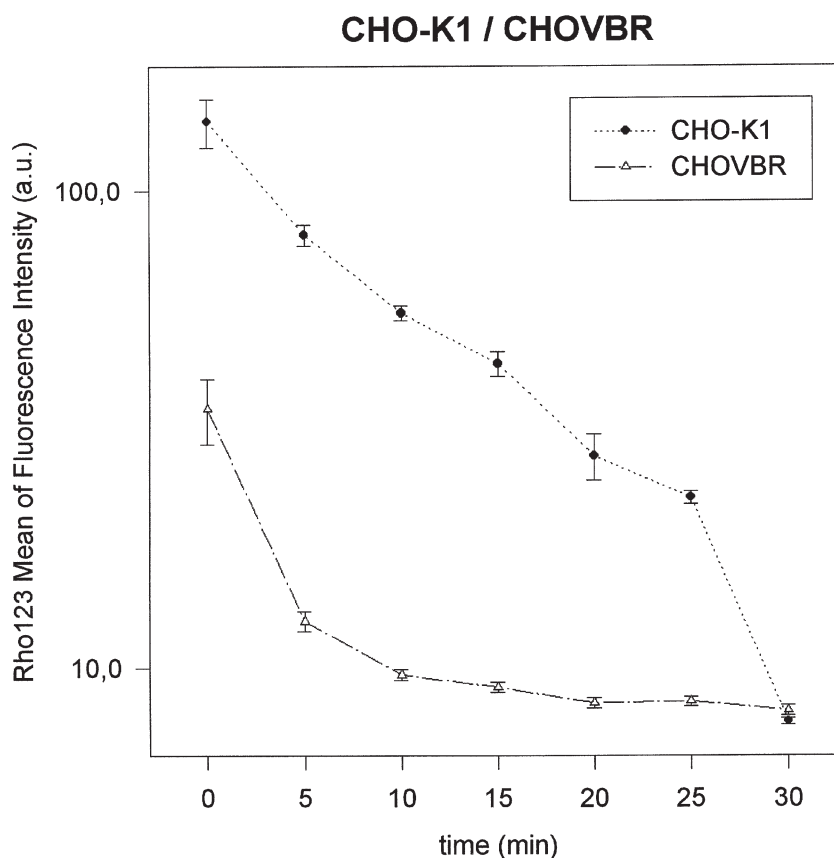


Fig. 4. Flow cytometry assessment of P-glycoprotein activity with chinese hamster ovary CHO-K1 (●) and CHOVR (Δ) cell lines. Cells were precharged with 200 ng/ml of rhodamine 123 in a cell incubator. After 1 h incubation, cells were washed and resuspended in Rho123 free medium, for 30 min at 37°C. Fluorescence arbitrary units (a.u.) were calculated in terms of mean of fluorescence intensity.

(Fig. 5). Drug sensitive, drug resistant, and drug-deprived resistant cells were characterized taking into account that forward scatter versus Rho123 fluorescence histograms can indicate heterogeneity on Rho123 retention, and the effect of blockers or mdr indicator dyes on scatter and viability. Neither evidence of heterogeneity in Rho123 retention nor scatter/viability changes were observed in drug resistant cells. Only Vbl-exposed CHO-K1 cultures showed small forward scatter changes.

3.3. Fluorometric measurements

The assessment of the dye efflux was undertaken to validate flow cytometry experiments. Fresh medium cultures from CHO-K1 and CHOVR were tested for Rho123 fluorescence following Rho123 uptake experiments and after washing as described in the material and methods section. All fluorescence measurements were performed with excitation at 460 nm and emission at 520 nm, as a result of the fluorescence excitation scan test. Culture medium supplemented with Rho123 was used as a reference sample. Although both drug sensitive and drug resistant cells displayed different curve patterns, fluorescence measurements at different intervals showed an increase of Rho123 in culture medium, indicating active Rho123 efflux.

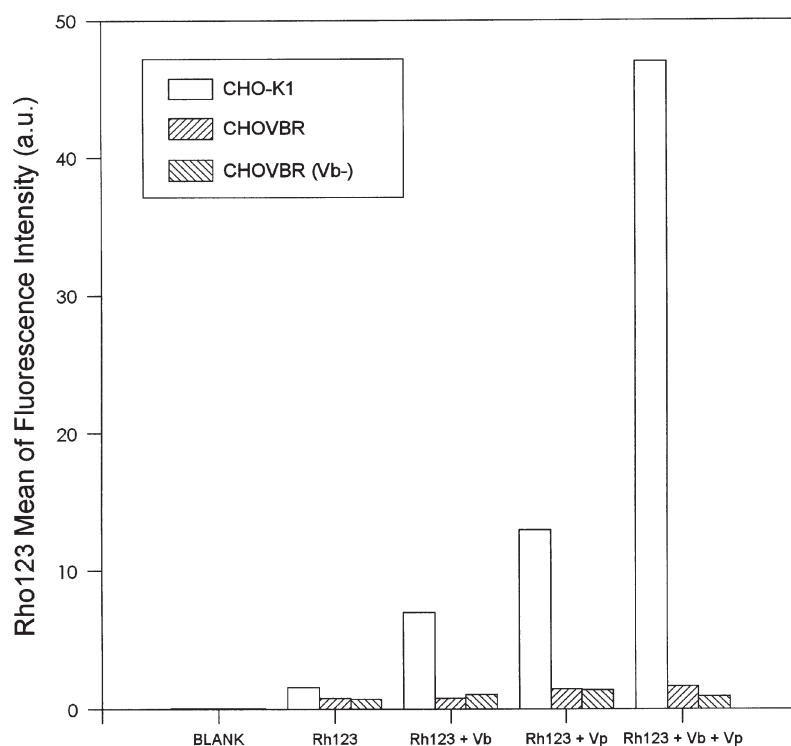


Fig. 5. Experiments of rhodamine 123 retention with CHO-K1 and CHOVR cells. Cells were incubated in the absence or in the presence of verapamil and vinblastine. Drug sensitive cells (CHO-K1) had P-gp inhibition, but CHOVR drug resistant cells, even one month vinblastine deprived CHOVR (VB-) cells, did not had efflux inhibition. Data shown are representative of three separate experiments.

4. Discussion

Rhodamine 123 has been widely used to assess the P-gp function [10,27], since it has been demonstrated to be a substrate for this transporter [15]. A variety of unrelated lipophilic compounds used as anticancer drugs are also substrates for P-gp. Thus, an increased expression of the MDR1 gene might be associated with multidrug resistance and with poor clinical outcome in patients with malignancies. By using leukemic cells, Bailly et al. [3] reported that functional assays reproduce multidrug resistant phenotypes with high accuracy, as compared to analysis of active membrane transporters such as P-gp, or multidrug resistant associated proteins. It has been suggested that bifunctional activities of P-gp can be related to two different configurations of the protein [18] which may also report different reactivities to commercially available antibodies, making the determination of a baseline for clinical resistance difficult. However, a simple and specific drug-resistance screening test has not been developed yet.

P-gp is expressed in normal cells from a wide variety of tissues, such as the adrenal gland, specialized secretory epithelial surfaces, the capillary endothelium of blood vessels in the brain, skin, testes [2,8, 17,38,39], and hematopoietic stem cells, which show reduced staining of Rho123 as a consequence of the active P-gp pumping action [10]. Certain drug sensitive cells such as the leukemic KG1 and KG1a expressing significant levels of P-gp also have mdr phenotype by means of Rho123 efflux assessment. Furthermore, since either normal cells and drug sensitive tumor cells can demonstrate active Rho123 efflux, interpretation of Rho123 efflux data in terms of drug resistance mechanisms must be cautious.

The present study suggests that both drug sensitive and drug resistant cells are able to actively efflux Rho123, as demonstrated by P-gp functional assessment. Besides, when drug sensitive cells were tested, P-gp inhibitors such as verapamil and vinblastine blocked Rho123 efflux, whereas no effect was observed when drug resistant cells were used.

We believe that certain tumor cells are able to achieve drug resistance not only by single point mutations, taking into account that a stress metabolism is also needed: a massive mitotic arrest is observed following the addition of vinblastine to drug sensitive CHO cultures (data not shown). This situation could involve cellular events such as those that reestablish microtubule function and mitotic spindle formation [40] in order to overcome the effect of drug uptake on cells with abnormal patterns of charged membrane phospholipids [16], or to restore the P-gp phosphorylation and kinase regulation [5,22,29]. Furthermore, it has been reported that specific P-gp mutations affect the substrate specificity of the transporter [9,13,14,19]. In this way, Rho123 might be transported also by non mutated P-gp as occurs in drug sensitive cells, whereas for the transport of anticancer drugs more specific mutations could be involved. Mutation of a glycine to valine at residue 185 in a colchicine selected cell line changes the pattern of resistance by improving the ability of the mutant to pump colchicine and etoposide while decreasing its ability to pump vinblastine and actinomycin D [13,37]. Although drug resistance is commonly associated with P-gp overexpression, other mechanisms can be involved. Chin et al. [12] demonstrated that p53 and *ras* gene cotransfection can activate *mdr1* promoter. Recently, Zastawny et al. [44] reported that the p53 protein interacts with *mdr1* core promoter sequences, suggesting a mechanism by which P-gp may be overexpressed in human cancers that also express mutant p53.

In terms of toxicity, Rho123 is a cationic dye that produces no detectable effect on cellular function when used at concentrations up to 10 $\mu\text{g/ml}$ for 10 min at 37°C, although in isolated mitochondria, ATP synthesis is inhibited by 90% even with low concentrations of Rho123 (5 $\mu\text{g/ml}$) (for a review see [11]). Singer et al. [36] suggested that when mitochondrial ATP production is inhibited in cancer cells, cytoplasmic glycolysis is stimulated in order to maintain energy requirements. Our fluorometric results suggest that the low Rho123 toxicity levels allow cell efflux with minimal metabolic stress, and only high Rho123 concentrations endanger long term ATP availability. On the contrary, anticancer drugs usually have different metabolic effects and higher toxicities. Vinblastine acts by blocking cell division and interfering amino acid metabolism. In fact, 50% of cell death is observed when 50–70 ng/ml of drug are added to CHO-K1 cultures within the first 24 hours (data not shown).

In conclusion, these data demonstrate that vinblastine sensitive CHO-K1 cells, which show low reactivity to P-gp, are able to transport actively the fluorescent dye Rho123, and that this efflux can be inhibited by vinblastine and verapamil. Efflux from the parental line is probably due to the expression of very low, but detectable, levels of P-gp, but the presence of other mechanisms such as MRP cannot be ruled out. MRP expression has been classically characterized by means of specific antibodies, RT-PCR, and efflux of fluorescent anthracyclines. However, few authors describe the use of Rho123-efflux measurements using these transporters [41]. MRP-mediated resistance is characterized by low levels of resistance to lipophilic xenobiotics, and the pharmacological characterization of multidrug resistant MRP-transfected human tumor cells demonstrate low levels of resistance to taxol, vinblastine, and colchicine [7]. In addition, cells overexpressing MRP could not be labeled with photoactivatable analogues of vinblastine [28].

Finally, resistant cells with increased P-gp expression also show a Rho123 efflux which is not blocked by P-gp inhibitors, even in CHO-VBR vinblastine deprived cells. Hence, the effect of P-gp

blockers deserves special consideration, since the non-blocking activity might be related not only to the intrinsic specificity of the blocker, but also, very possibly to non P-gp transporters or to P-gp mutants, which may have similar efflux ability. Therefore, drug resistant phenotype might be better defined by the non-blocking effect, because CHOVR resistant cells are not affected by classical P-gp modulators. Since drug sensitive cells can also show Rho123 efflux as the drug resistant sublines, a contradiction arises: P-gp expression could be necessary, but not sufficient to determine drug resistance in tumor cells. For this reason, interpretation of Rho123 efflux data in terms of drug resistance mechanisms must be careful.

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