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Comparative effect of verapamil, cyclosporin A and SDZ PSC 833 on rhodamine 123 transport and cell cycle in vinblastine-resistant Chinese hamster ovary cells overexpressing P-glycoprotein

Jordi Pétriz, Joaquín Sánchez, Joan Bertrán and Joan García-López
Departament de Criobiologia i Teràpia Cellular, Centre de Referència de Citometria Izasa-Coulter, Institut de Recerca Oncològica, Hospital Duran i Reynals, Gran Via, Km 2.7, L'Hospitalet de Llobregat, 08907 Barcelona, Spain. Tel: (+34) 3 3357652; Fax: (+34) 3 2632251.

The product of the *mdr1* gene, P-glycoprotein (P-gp), represents a common mechanism of cellular resistance to a wide variety of structurally and functionally unrelated drugs. A range of structurally different P-gp inhibitors, such as verapamil, cyclosporin A and SDZ PSC 833, have been shown to modify multidrug resistance (MDR). We used flow cytometry to investigate *in vitro* modulation of P-gp-dependent efflux of rhodamine 123 (Rh123). The capacity to modulate the MDR phenotype of vinblastine-resistant Chinese hamster ovary (CHO) cells was assessed by analyzing the concentration of modulator needed to decrease the Rh123 mean fluorescence intensity by 50%. We found that the cyclosporin derivative SDZ PSC 833 was significantly more effective than cyclosporin A and verapamil, either in the presence or absence of fetal calf serum-supplemented media. This study indicates that analysis of Rh123 efflux modulation can be used to determine the optimal doses of MDR inhibitors *in vitro* and suggests that more than one modulator is needed to measure P-gp function, since verapamil had no effect on Rh123 modulation when MDR cells were used.

Key words: CHO cells, drug resistance, multidrug resistance, P-glycoprotein, rhodamine 123.

Introduction

Progressive exposure of cell lines to anticancer agents (i.e. anthracyclines) has been used to obtain drug-resistant sublines *in vitro*.[1] To better understand the mechanisms involved in multidrug resistance (MDR), cell lines have been generated displaying increased levels of drug resistance.[2-5] Drug resistance can be achieved through specific membrane transporters that pump cytotoxic agents out the cell to reduce the intracellular concentration of drugs. The *mdr1* and the *mrp* gene products, respectively, P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP),[7] depend on adenosine triphosphate (ATP) to pump certain anticancer drugs out of the cell. These molecules belong to the superfamily of ATP binding cassette (ABC) transporters,[6,7] which also includes the *pfmdr1* gene product of *Plasmodium falciparum* associated with chloroquine resistance,[8] the cystic fibrosis gene product (CFTR)[9] and the yeast STE6 gene product.[10] P-gp is a 170–180 kDa transmembrane molecule protein that is normally expressed in secretory tissues, liver, colon, kidney, some endothelia, peripheral blood lymphocytes and hemopoietic precursors.[11-16] Tumors derived from such cells can overexpress P-gp, resulting in MDR phenotypes.[17] However, after chemotherapy, tumor cells derived from tissues not expressing P-gp can also show MDR phenotypes.[18]

It has been reported that P-gp expression at diagnosis appears to be a prognostic factor that predicts tumor drug resistance *in vivo*.[19-20] Modulation of MDR with non-cytotoxic agents such as verapamil, phenotiazines, quinidine, reserpine, cyclosporin A[21] or its derivatives may serve to sensitize tumor resistant cells. Therefore, immunological, molecular and functional analysis of P-gp may have a predictive value for treatment.[22] Measurement of the MDR reversing efficacy *in vitro* indicates that (i) structurally non-related modulators show specific patterns of reversal against P-gps,[23] (ii) sensitivity of multidrug transport to a reversing agent depends on the agent, the cytotoxic drug and the presence or absence of mutations which can modify substrate

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Correspondence to J Pétriz

specificity,[24] and (iii) the clinical potential of MDR modulators is limited because of strong binding to plasma proteins.[25] In this study we have used flow cytometry techniques to study the reversal efficacy of three different P-gp inhibitors (verapamil, cyclosporin A and SDZ PSC 833) to inhibit the transport of a well known P-gp substrate [rhodamine 123 (Rh123)] in *mdr1*$^+$ vinblastine-resistant Chinese hamster ovary (CHO)-derived sublines, both in the presence and in the absence of fetal calf serum (FCS).

Material and methods

Cell lines and culture conditions

CHO-K1 cells were obtained from the ATCC (Rockville, MD), and 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 U/ml penicillin, 100 mg/ml streptomycin (Biological Industries, Kibbutz Beth Haemek, Israel) and 10% FCS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. CHO resistant sublines (CHOVBR) were derived from the parental CHO-K1 cell line and obtained by stepwise increasing concentrations of vinblastine, ranging from 10 to 1280 ng/ml. $CHOVBR_{640}$ and $CHOVBR_{1280}$ cells were cultured under the same conditions as CHO-K1 cells, but maintained in the presence of 640 and 1280 ng/ml of vinblastine, respectively. P-gp levels were assayed by Western blot and RT-PCR.[26]

Drugs and Chemicals

Drugs and chemicals used in this study included: Rh123 (Lambda Fluoreszenztechnologie, Graz, Austria), propidium iodide (Sigma, St. Louis, MO), vinblastine (Lilly, Barcelona, Spain), verapamil (Laboratorios Knoll, Barcelona, Spain), cyclosporin A and SDZ PSC 833 (Novartis Farmaceutica, Barcelona, Spain).

Flow cytometry (FCM)

FCM analysis was performed using an EPICS XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon ion laser tuned at 488 nm. Green fluorescence (Rh123) was measured through a 525 nm bandpass 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 WinMDI Software (version 2.1.3).

Rh123 uptake/retention assays

Cell lines resistant to vinblastine derived from CHO-K1, $CHOVBR_{640}$ and $CHOVBR_{1280}$, were seeded in 24-well tissue culture test plates (Costar, Cambridge, MA) to a final concentration of 10^5 cells/ml, 2 ml total volume, 12 h before the Rh123 uptake/retention experiments in the presence of 10% FCS, to achieve optimal growth conditions. The Rh123 uptake was determined by adding 200 ng/ml of this dye (stock solution 1 mg/ml H_2O) to the culture medium, with and without FCS, and incubating for 1 h, in the presence or absence of either verapamil (0.2–20 μM), cyclosporin A (0.8 nM–8 μM) or SDZ PSC 833 (0.8 nM–8 μM), in separate wells. After 1 h of incubation, cells were washed and resuspended in Rh123-free culture medium, with or without FCS, while maintaining the other drugs to evaluate their effect on Rh123 retention. Dead cells were excluded by simultaneous staining with 1 μg/ml PI (stock solution 1 mg/ml H_2O). All experiments were carried out in triplicates.

Cell fixation and DNA staining

Treated cells were rinsed twice in cold PBS supplemented with 0.1% NaN_3 (Merck, Darmstad, Germany) and 1% bovine serum albumin (PBS-BSA) (Sigma), and resuspended to a final concentration of 2×10^5 cells/ml. Cell fixation was performed for 30 min at −20°C (in triplicates) in 70% methanol. DNA was stained with PI. Briefly, cells were rinsed twice in PBS-BSA, RNA was digested with Ribonuclease A (Sigma) for 30 min and then 20 μg/ml PI, from a stock solution of 1 mg/ml, was added 10 min prior to the analysis.

Statistical analysis

Differences in the mean of fluorescence intensity under the different serum and inhibitor conditions were analyzed by Student's *t*-test and the Wilcoxon signed-rank test.

Results

Pattern of Rh123 retention in parental CHO-K1, $CHOVBR_{640}$ and $CHOVBR_{1280}$ cell lines

Rh123 retention was evaluated both in sensitive and resistant CHO sublines, in the presence of FCS as described in Materials and methods, for 30 min. At $t=0$, Rh123, which is considered an indicator of MDR activity, was retained by CHO-K1 cells about 2.73-fold more effectively than $CHOVBR_{640}$ cells and about 3.99-fold more effectively than $CHOVBR_{1280}$ cells. After 30 min, Rh123 efflux in CHO-K1 cells fitted well to a linear model ($r^2=0.95$; $p=0.002$), requiring an average of 7.3 min to decrease to 50% of Rh123 mean fluorescence intensity, whereas both $CHOVBR_{640}$ and $CHOVBR_{1280}$ cells fitted to non-linear models, reaching the same level of Rh123 fluorescence after 3.5 and 3.9 min, respectively. The changes in Rh123 mean fluorescence intensity in CHO cells are summarized in Table 1.

Table 1. Pattern of retention of Rh123 in CHO cells

Time (min)	Decrease of Rh123 MFI (a.u.)		
	CHO-K1	$CHOVBR_{640}$	$CHOVBR_{1280}$
0	139.40±16.22	51.13±3.15	34.90±5.47
5	80.70±4.11	14.33±1.07	12.53±0.60
10	55.47±2.04	12.37±0.21	9.69±0.24
15	43.47±2.61	11.23±0.23	9.14±0.23
20	27.83±3.02	10.87±0.21	8.46±0.19
25	22.80±0.71	9.43±1.77	8.54±0.19
30	7.76±0.13	10.47±0.23	8.18±0.19

Values represent the mean fluorescence intensity of three different assays.

Pattern of inhibition of reversing agents in parental CHO-K1, $CHOVBR_{640}$ and $CHOVBR_{1280}$ cell lines

The differential effects of verapamil, cyclosporin A and SDZ PSC 833 on Rh123 in CHO-K1 and their derived vinblastine-resistant sublines were evaluated. Optimal concentrations of MDR modifiers were studied in the presence and in the absence of FCS. The concentration of inhibitor (μM) needed to produce a retention of 50% in Rh123 mean fluorescence intensity was calculated under the different serum conditions by the functional Rh123 assay, showing that for CHO-K1 cells, the most effective agent was the cyclosporin derivative SDZ PSC 833, that achieved 50% Rh123 retention at 4.34×10^{-3} μM. This effect was 67-fold (0.29 μM) stronger than the immunosuppressive agent cyclosporin A and 1339-fold (5.81 μM) stronger than the calcium antagonist verapamil. However, both drug-resistant cells $CHOVBR_{640}$ and $CHOVBR_{1280}$ were insensitive to verapamil treatment at concentrations ranging from 0.2 to 20 μM. Similarly, as observed with CHO-K1 cells, SDZ PSC 833 was the most effective inhibitor, bringing to 50% the Rh123 retention at 0.35 μM with $CHOVBR_{640}$ cells and at 0.57 μM with $CHOVBR_{1280}$ cells, whereas the concentrations of cyclosporin A needed to achieve the same effect on Rh123 retention were 16-fold (5.55 μM) and 12-fold higher (6.83 μM), respectively.

The potency of these MDR modulators was also evaluated in the presence and in the absence of 10% FCS. The efficacy of verapamil, cyclosporin A and SDZ PSC 833 to inhibit Rh123 efflux under different serum conditions is summarized in Table 2. The presence of serum proteins had no significant effect on the 50% Rh123 retention when verapamil or SDZ PSC 833 were added to CHO-K1 cultures. However, when serum was present, the amount of cyclosporin A needed to reach the same level of Rh123 retention was 1.22-fold higher (0.29 μM) than under FCS-free conditions (0.237 μM). Likewise, when using $CHOVBR_{640}$ cells, cyclosporin A had less inhibitory activity under serum conditions (5.55 μM) than under serum-free conditions (4.11 μM). Verapamil, cyclosporin A and SDZ PSC 833 showed a reduced MDR reversing efficacy when FCS was present. SDZ PSC 833 had an enhanced Rh123 retention ability under serum-free conditions (0.39 μM) than under the presence of serum

Table 2. Differential reversing efficacy of P-gp inhibitors on 50%/t_{50} Rh123 retention of CHO cells

Inhibitor (μM)	CHO-K1		$CHOVBR_{640}$		$CHOVBR_{1280}$	
	+FCS	−FCS	+FCS	−FCS	+FCS	−FCS
Verapamil	5.811	5.952	–	–	–	–
Cyclosporin A	0.290[a]	0.237[a]	5.552[b]	4.108[b]	6.835	6.617
SDZ PSC 833	4.34×10^{-3}	4.16×10^{-3}	0.346	0.234	0.567[c]	0.393[c]

Values represents the mean of three different assays. [a]$p=0.0001$; [b]$p<0.0001$; [c]$p<0.0001$.

(0.57 μM) in $CHOVBR_{1280}$ cells. The results of three representative experiments are shown in Figure 1.

Cell cycle effect of cyclosporin A and SDZ PSC 833 on $CHOVBR_{640}$ and $CHOVBR_{1280}$ vinblastine-resistant sublines

The effects of cyclosporin A and SDZ PSC 833 on cell cycle were determined on the vinblastine-resistant sublines. Both $CHOVBR_{640}$ and $CHOVBR_{1280}$ cells (grown in the presence of 640 and 1280 ng/ml of vinblastine, respectively) were exposed to these P-gp inhibitors for 72 h. The amount of inhibitor used under serum conditions was calculated by means of functional Rh123 assays, at a concentration which produced 50% of Rh123 retention. Both $CHOVBR_{640}$ and $CHOVBR_{1280}$ control cells showed normal cell

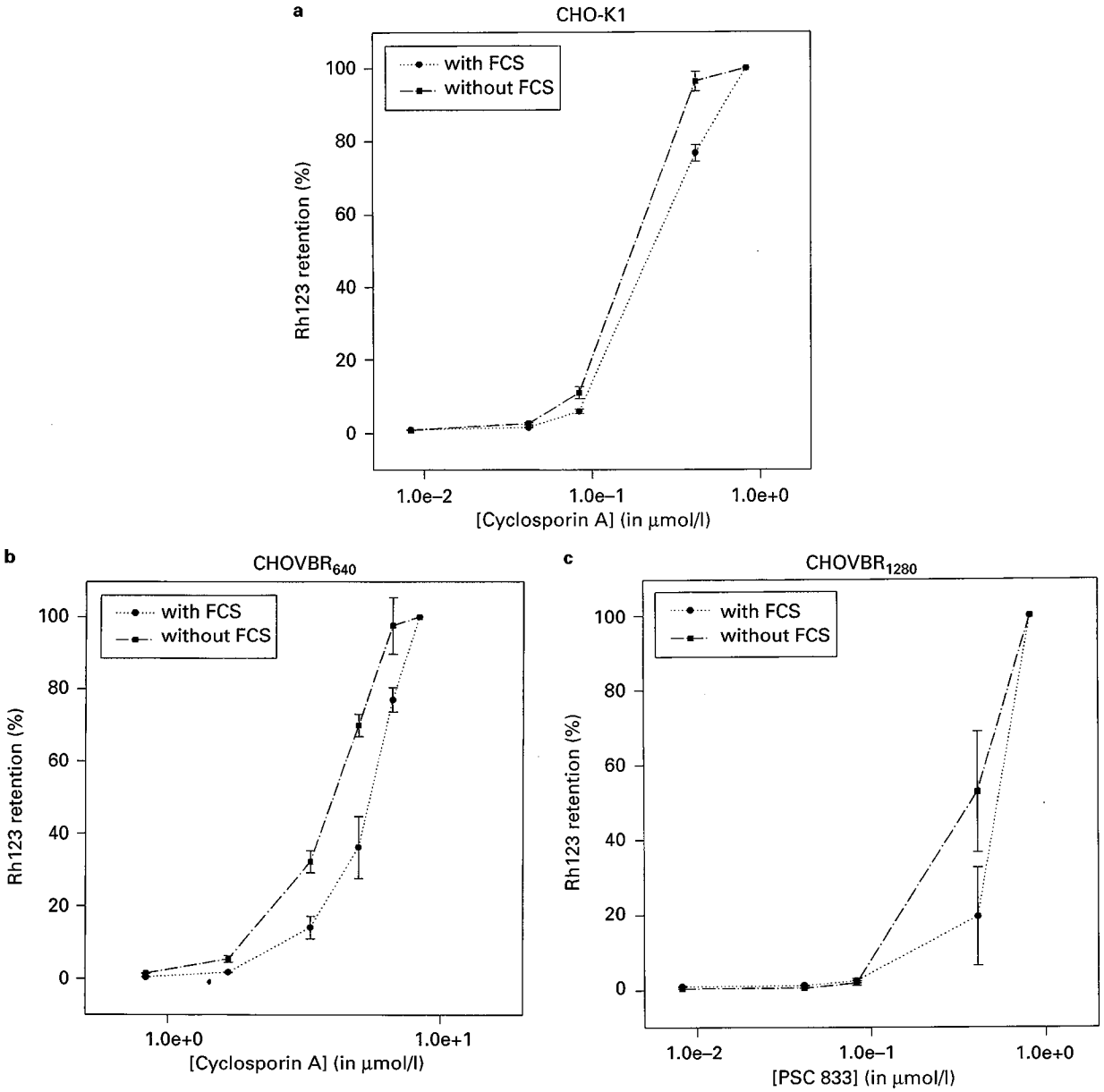

Figure 1. Effects of different serum conditions on Rh123 retention. Reversing efficacy was determined by the 50% average rate of Rh123 retention tabulated in Table 2. CHO cells were exposed to increasing concentrations of P-gp inhibitors in the presence and absence of FCS. Experiments showing statistically significant differences are plotted.

cycle phases, with constant $[(S+G_2/M)/(G_0G_1)]$ ratios throughout time. Following incubation with cyclosporin A, CHOVBR cells showed a rapid increase of the G_2/M phase values, from 8.7 ± 0.1 at 0 h, to 43.8 ± 16.1, to 80.0 ± 8.3 and to $70.5\pm2.4\%$ of $CHOVBR_{640}$ cells at 24, 48 and 72 h, respectively. Likewise, $CHOVBR_{1280}$ cells also showed increased G_2/M phase values, ranging from 40.4 ± 0.7 to 88.4 ± 0.9 and to 70.9 ± 2.3 at the same time intervals. Mitotic accumulation was also observed after exposure of CHOVBR cells to SDZ PSC 833, showing similar G_2/M phase values than under cyclosporin A treatment; from 13.6 ± 2.9 at 0 h to 44.2 ± 1.2 to 87.0 ± 1.1 and to $70.3\pm3.9\%$ of $CHOVBR_{640}$ cells, and to 41.5 ± 11.7, 69.2 ± 16.4 and $66.1\pm1.5\%$ of $CHOVBR_{1280}$ at 24, 48 and 72 h, respectively (Figure 2). Statistical analysis of $[(S+G_2/M)/(G_0G_1)]$ percent of CHOVBR cells did not show significant differences between cells treated with cyclosporin A or SDZ PSC 833.

Discussion

Both *in vitro* and *in vivo* administration of anticancer agents together with specific pharmacological compounds, such as verapamil, cyclosporin A and the more recent cyclosporin derivative SDZ PSC 833, can decrease drug resistance levels in cancer cells.[27] In this study, we demonstrate by means of Rh123 functional FCM assays if a significantly different reversal efficacy of these three P-gp inhibitors could be detected. For this purpose, we used CHO cells expressing low (CHO-K1) and high levels (CHOVBR) of the *mdr1* gene product.

Efflux and retention of Rh123 was measured in CHO cells. CHO sublines showed a heterogeneous pattern of Rh123 retention, probably due to the different levels of P-gp expression.[26] Particularly, CHO-K1 cells retained Rh123 more efficiently, whereas both $CHOVBR_{640}$ and $CHOVBR_{1280}$ cells, which overexpress P-gp, showed 3- to 4-fold reduced Rh123 staining. These results are consistent with previous studies showing that P-gp prevents intracellular drug accumulation[28] and that P-gp handles influx of its substrates separately.[29] In addition, both CHO-K1 and CHOVBR cells were able to actively efflux this dye, indicating different transport velocities; CHO-K1 cells displayed a linear efflux of Rh123, whereas the kinetics of Rh123 efflux of CHOVBR cells fitted to a non-linear model. This different pattern of Rh123 efflux could be explained by the enrichment of P-gp molecules in CHOVBR cells, either involving alternative mechanisms of transcriptional regulation,[30] *mdr1* gene amplification[31] or post-translational modifications that can alter P-gp function.[32]

Determination of *in vitro* MDR inhibitor potency showed that the cyclosporin analog SDZ PSC 833 had the highest reversing efficacy, followed by the

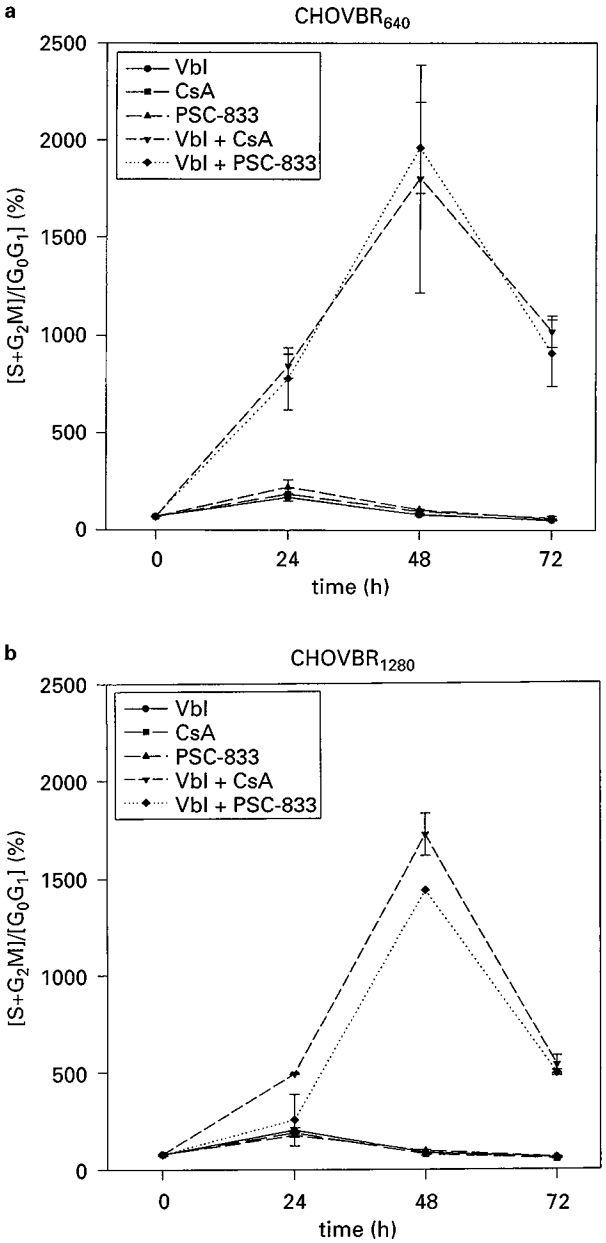

Figure 2. Effects of cyclosporin A and SDZ PSC 833 on vinblastine cytotoxicity in CHO resistant sublines (CHOVBR). $CHOVBR_{640}$ and $CHOVBR_{1280}$ cells were cultured at the same conditions as parental cells, but maintained in the presence of 640 and 1280 ng/ml vinblastine, respectively. Cells were incubated at 37°C with the amount of inhibitor needed to achieve the 50% average rate of Rh123 retention.

immunosuppressive agent cyclosporin A and by verapamil. Although the mechanism of action of these modulators is not completely understood, differences in the dose of modulator required to produce 50% Rh123 retention could be attributed, on the one hand, to the different P-gp competing specificity of these inhibitors,[24] and, on the other hand, to P-gp isoforms with enhanced substrate specificity,[23] thus explaining the loss of reversal efficacy of verapamil on CHOVBR cells. In addition, the bioactive role of MDR inhibitors may affect Rh123 retention in P-gp$^+$ samples. A significantly decreased activity of MDR modulators has been reported in serum conditions when compared with serum-free conditions.[25] Previously, Lehnert[33] showed that increased concentrations of serum protein had strong effects on P-gp reversal. Since we also wanted to analyze the potency of MDR modulators, either in the presence or in absence of FCS, Rh123 efflux experiments were carried out comparing the concentration of modulator needed to achieve 50% Rh123 retention. After cyclosporin A treatment, P-gp function was significantly decreased under serum conditions in both CHO-K1 and $CHOVBR_{640}$ cells, whereas SDZ PSC 833 exhibited a significantly reduced activity under serum conditions when $CHOVBR_{1280}$ cells were used. Hence, it is conceivable that this discrepancy in the action of the MDR modulators in both drug-sensitive and drug-resistant CHO cells is associated with a competitive binding to drug-recognition sites, rather than a different inhibitory pattern due to serum conditions.

Previous studies on cell lines showed that reduced Rh123 retention, together with increased resistance to this dye, and reversal by verapamil are related to the MDR phenotype.[34] We found that verapamil did not block Rh123 efflux in CHOVBR cells. Therefore, the use of only one P-gp modulator would not be an accurate indication of P-gp activity for MDR assessment. Moreover, analysis of P-gp isoforms reveals major differences in the range of 5- to 60-fold in the modulator dose to increase vinblastine cytotoxicity.[35]

The antimitotic alkaloid vinblastine is commonly used for the treatment of testis cancer and advanced Hodgkin's disease. The cytotoxic effect of antimitotic drugs alters microtubule dynamics and mitotic progression at metaphase, resulting in abnormal mitotic exit and apoptotic death of cancer cells,[36] or blocking the efflux of Rh123 from CHOVBR cells.[26] In our studies, vinblastine resistance was reversed in CHOVBR cells after cyclosporin and SDZ PSC 833 treatments. Analysis of cellular DNA content showed a mitotic block at 48 h (Figure 2), followed by a massive cell depletion from cultures, suggesting a similar efficacy for both treatments.

Conclusion

In summary, we have used drugs recognized by the MDR efflux pump to demonstrate a different P-gp reversal pattern of three common MDR modulators. In addition, our data also suggest a different substrate specificity in cells overexpressing P-gp, that could be explained by the existence of distinct interaction sites on P-gp, conferring enhanced ability to pump out certain drugs. Furthermore, *in vitro* testing of MDR modulators in CHOVBR cultures suggest that 50% Rh123 retention value determination may help to adjust the optimal dose of inhibitor required to increase *in vitro* antineoplastic drug cytotoxicity. Further ongoing studies in resistant neoplastic cells will help to elucidate the significance of Rh123 retention as a prognostic value to assess multidrug resistance.

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