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Detection of P-glycoprotein expression by tumoral cells with NBDL-CsA, a fluorescent derivative of cyclosporin A

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The P-glycoprotein (P-gp) molecules which are expressed on multidrug-resistant (MDR) tumor cells efflux a variety of anti-cancer drugs, such as doxorubicin. Though first described as an inhibitor of P-gp function, cyclosporin A (CsA) was more recently shown to behave as a substrate of the P-gp pump. The retention of [^{3}H]CsA was reduced in MDR cells of the human leukemic CEM cell subline, in comparison with the drug-sensitive parental (Par) subline. MDR-CEM cell treatment by the P-gp blockers restored the [^{3}H]CsA retention to the control Par-CEM cell levels. Using a novel fluorescent CsA derivative, [N-ε-(4-nitrobenzofurazan-7-yl)-D-Lys^8] cyclosporin (NBDL-CsA), we now show that MDR cells can be distinguished from Par cells both at the cell population level (in microculture) and at the single cell level (by use of flow cytometry).

Key words: Cyclosporin A efflux, multidrug resistance, P-glycoprotein, SDZ PSC 833.

Introduction

Class I *mdr* P-glycoprotein (P-gp) molecules are members of a family of the ATP-binding cassette (ABC) transporters.[1] Unlike other ABC family members, P-gp molecules possess a very broad substrate specificity, the transported compounds, among which anti-cancer drugs, showing neither structural nor functional relationships besides their high lipophilicity.[2] When P-gp molecules are overexpressed by various tumor cells, this leads to the emergence of a multidrug resistance (MDR) phenotype, which is often responsible for failure of chemotherapy.[3] A variety of chemosensitizers or 'resistance modulating agents' (RM agents), among which the most commonly used are verapamil and cyclosporin A (CsA), can substantially inhibit the function of the class I *mdr*-encoded P-gp molecules which are expressed on the plasma membrane of the tumor cells and which are responsible for the efficient removal of anti-cancer drugs while traversing the tumor cell membrane.

The most efficient way to achieve a complete pharmacological reversal of tumor cell MDR is to perform cytostatics-based chemotherapy in combination with the little or non-immunosuppressive cyclosporin SDZ PSC 833 or cyclopeptolide SDZ 280-446,[4] due to the fact that both the latter RM agents are real P-gp blockers as they can achieve a virtually irreversible inhibition of Pgp function.[5]

Using isotopically labeled [^{3}H]CsA, we recently showed its higher uptake by P-gp-lacking parental (Par) cells of the human CEM T cell leukemia than by cells of the P-gp-expressing MDR cell subline. Furthermore, the P-gp blockers could restore [^{3}H]CsA retention in the MDR-CEM cells to its level in the Par-CEM cells, a finding which was compatible with the slow P-gp-substrate character of CsA.[6]

Using a novel fluorescent CsA derivative, we now show that MDR cells could be similarly distinguished from Par cells both at the cell population level (in microculture) and at the single cell level (by use of flow cytometry). This opens a way to approaching the characterization of CsA-retaining cells and CsA-effluxing ones at the single cell level.

Materials and methods

Drugs

The anti-cancer drugs were doxorubicin (adriamycin–HCl; Serva, Heidelberg, Germany) and vinblastine (vinblastine sulfate; Lilly, Giessen, Germany). The P-gp probes, Rhodamine-123 (R-123 [MW = 380], Eastman Kodak, Rochester, NY) and daunomycin (DAU [MW = 564], Sigma, St Louis, MO) were prepared as stock solutions at 1 mg/ml, respectively, in bidistilled water and 0.9% NaCl. Their final concentrations in the culture medium

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were 5 μg/ml (13.16 μM) for R-123 and 20 μM (11.3 μg/ml) for DAU. NBDL-CsA, the fluorescent cyclosporin derivative [*N*-ε-(4-nitrobenzofurazan-7-yl)-D-Lys[8]] cyclosporin (SDZ 217-881; MW = 1422.83; Sandoz Pharma, Basel, Switzerland), was made as described earlier.[7] It was dissolved in absolute ethanol as a stock solution at 1 mg/ml and diluted with culture medium to various concentrations just before use.

The RM agents were prepared as stock solutions in absolute ethanol at 10 mM for verapamil (MW = 491; Sigma) and at 10 mg/ml (about 8 mM) for CsA (MW = 1206.6), SDZ PSC 833 (MW = 1214.65) and SDZ 280-446 (MW = 1182.6) (all from Sandoz). The final RM agent concentrations in culture medium depended on the assay, though being most often 1.0, 3.0, 10 and 30 μg/ml. For the RM agents, 1 μg/ml = ± 2 μM for verapamil, ± 0.8 μM for CsA, SDZ PSC 833 and SDZ 280-446, and ± 0.7 μM for NBDL-CsA.

Cells

Two pairs of Par and MDR tumor cell lines were used: the murine monocytic leukemia Par-P388 and MDR-P388 (obtained through doxorubicin-resistance selection), and the human T leukemia Par-CEM (CEM 1-3) and MDR-CEM (obtained through vinblastine-resistance selection); those cell line pairs were kindly provided by, respectively, Dr M Grandi (Farmitalia, C Erba Research Center, Milano, Italy) and Professor M Cianfriglia (Istituto di Sanita, Roma, Italy). The culture conditions were as described earlier for the P388 cell line pair[8] and for the CEM cell line pair.[6] Both MDR tumor lines were continuously grown in the presence of the drug used for their selection: 0.25 μg/ml doxorubicin for MDR-P388 cells and 0.1 μg/ml vinblastine for MDR-CEM. The MDR-P388 cells were about 200-fold more resistant to doxorubicin than the Par-P388 cells,[8] whereas the MDR-CEM cells were about 1000-fold more resistant to vinblastine than the Par-CEM cells.[6]

Measurements of P-gp function by a cell growth inhibition assay

Tumor cell growth and its drug-mediated inhibition were measured as described previously, using the MTT assay for quantitating live cell mass in each well of 96-well microplates (Costar 3599). The methods were similar to those described elsewhere for the P388 cell line pair[8] and for the CEM cell line pair.[6] The increased cytostatic sensitivity was expressed as 'resistance decrease factors' (RDF) which were calculated by the ratio: IC_{50^-}/IC_{50^+}, the cytostatic IC_{50^+} and IC_{50^-} values being determined in the presence of, respectively, the RM agent and its solvent only.[6]

Fluorescence cytometry assays of P-gp function by DAU or R-123 retention

For fluorescence cytometry, the cells were treated by the RM agents and the fluorescent P-gp probes (R-123 or DAU) in microculture plates as described previously.[6] Briefly, 5×10^5 cells in 200 μl medium per well were exposed to R-123 [5 μg/ml (13.16 μM)] or DAU [20 μM (11.3 μg/ml)] and a RM agent concentration range. The P-gp probe uptake (influx phase) was allowed at 37°C (water bath) for 15 min in the R-123 case or 30 min in the DAU case. The excess of fluorescent P-gp probe and of RM agent were washed away by two cycles of centrifugation (200 *g*, 5 min at 4°C) and resuspension in culture medium. The P-gp-mediated efflux of P-gp probe (efflux phase) was allowed for 15 min (for both DAU and R-123) at 37°C (water bath) in 200 μl of P-gp probe and RM agent-free medium.

Besides bulk culture fluorometry,[6] single cell flow cytometry was used to study the retention of the P-gp probe. The duration of the efflux phase was 3 h in the DAU case and 15 or 60 min in the R-123 case. Following further washes (one in medium, two in PBS), the cell pellets were resuspended at 4°C in 200 μl PBS for the R-123-stained samples or in PBS–1% paraformaldehyde for the DAU-stained samples. The flow cytometry analyses were performed with a FACScan cell analyzer (Becton Dickinson, Mountain View, CA) equipped with an argon ion laser (15 mW) tuned at 488 nm. Dead cells and debris were excluded by setting a gate on the basis of their decreased forward light scatter. In the fluorescence histograms, the *x*-axis was a logarithmic scale for the fluorescence level and the *y*-axis was an arithmetic scale for the number of cells recorded in each channel. The effects of P-gp blockers or weaker RM agents on the retention of the P-gp probes by the MDR and Par cells were assayed as described earlier.[6]

Fluorescence cytometry assays of P-gp function by NBDL-CsA retention

The principles of the fluorescence cytometry assays with NBDL-CsA were essentially similar to those for DAU or R-123, but there were differences for the

durations of the influx and efflux phases as well as the P-gp probe concentrations. Indeed, for studying the retention of NBDL-CsA as P-gp probe by the MDR and Par cells, and for testing the effect of P-gp blockers or weaker RM agents, the procedure was adapted from similar assays using [^{3}H]CsA as P-gp probe.[6]

The NBDL-CsA stock solution in ethanol was first diluted with culture medium just before use to reach 4-fold higher concentrations than the final ones in the assay (25 µl were added to 10^6 cells in 75 µl). A series of preliminary exploratory assays indicated that in order to obtain a substantial fluorescence of the Par-P388 and Par-CEM cells, the NBDL-CsA concentration had to be higher than 2 µM (as used for [^{3}H]CsA studies);[6] most assays were performed with 6 or 8 µM NBDL-CsA and the CEM cell line pair. In all assays, the cells were first exposed to the NBDL-CsA (with or without RM agent) at 37°C (water bath) for a short NBDL-CsA influx phase which was limited to 5 min only, as was done with [^{3}H]CsA as probe.[6] The cells were then washed by two cycles of centrifugation (5 min at 200 *g*, 4°C) and resuspension in culture medium (200 µl).

For bulk culture fluorometry, the NBDL-CsA efflux phase was limited to 5 min only at 37°C (water bath), as was done with [^{3}H]CsA as probe.[6] After three additional washes, the cell pellets were resuspended in 200 µl of culture medium and the samples transferred into other microtiter plates (Nunc Maxisorp). The microtiter plates were then analyzed with a fluorescent plate reader (Cytofluor™ 2350; Millipore, Saint-Quentin, France). For single cell flow cytometry, the efflux phase was extended to 3 h.

For the fluorescence cytometry analyses of NBDL-CsA retention (excitation: 450–490 nm; emission: 520 nm), we used the same settings as for R-123, both for bulk culture fluorometry with the Cytofluor™ 2350 (485 ± 20 nm excitation and 530 ± 25 nm emission filters) and for single cell flow with the FACScan (Filter FL1). The results of the bulk culture fluorometry assays were expressed as 'Retention increase factors' (RIF), i.e. factors of increased NBDL-CsA-specific fluorescence shown by chemosensitizer-treated cells in comparison with their untreated (solvent control treated) cells.

Results

Assay of NBDL-CsA as a chemosensitizer for anti-cancer drugs

Comparisons of CsA and NBDL-CsA for their capacity to chemosensitize MDR cells suggested that NBDL-CsA could not inhibit P-gp mediated resistance. Indeed, when tested at 1 µg/ml on the pair of P388 cell lines for their capacity to increase doxorubicin sensitivity, CsA and NBDL-CsA gave RDF values of, respectively, 94 and 2.0 with MDR-P388 cells, the Par-P388 cells being little affected (with RDF values of about 1.2).

Further comparisons of a small dose range (0.1–3.0 µg/ml) of NBDL-CsA and CsA for their RM activity and for their intrinsic cytotoxicities (Table 1) showed that the fluorescent derivative was not totally inactive but significantly less RM-active than CsA: at the 3 µg/ml concentrations, a more than 120-fold doxorubicin sensitization was obtained

Table 1. Restoration of MDR-P388 and MDR-CEM cell growth sensitivity (RDF)[a] to doxorubicin and vinblastine, respectively[b]

	RM agent (µg/ml)							
	Par				MDR			
Cell line	0.1	0.3	1	3	0.1	0.3	1	3
P388								
CsA	1.1 ± 0.2	1.0 ± 0.1**	1.2 ± 0.3***	1.2 ± 0.5***	2.4 ± 0.3	15 ± 4*	94 ± 11**	129***
NBDL-CsA	1.0 ± 0.0*	1.2 ± 0.1***	1.2 ± 0.5***	0.7 ± 0.5***	1.3 ± 0.4	1.2 ± 0.3	2.0 ± 1.0	10 ± 9*
CEM								
CsA	1.0 ± 0.0	1.0 ± 0.1*	0.9 ± 0.0*	0.8 ± 0.1***	1.0 ± 0.0	1.0 ± 0.1	2.0 ± 0.5	36
NBDL-CsA	1.0 ± 0.0	1.0 ± 0.1*	0.8 ± 0.1*	0.8 ± 0.1***	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1

[a] The results are shown as RDF. Means of two independent experiments (in triplicate), the variability from one experiment to another being generally less than 20%. Cell growth inhibition by the chemosensitizer alone was either *negligible (10–20% maximum), **medium (20–30%) or ***excessive (higher than 30% making the significance of the RDF value subject to caution).

[b] With doxorubicin, the mean IC_{50} values were 16 ± 5 ng/ml for Par-P388 cells and 3946 ± 1542 ng/ml for MDR-P388 cells. The resistance degree of the MDR-P388 cells relative to Par-P388 cells was 246-fold for doxorubicin. With vinblastine, the mean IC_{50} values were 0.6 ± 0.1 ng/ml for Par-CEM cells and 554 ± 89 ng/ml for MDR-CEM cells. The resistance degree of the MDR-CEM cells relative to Par-CEM cells was 895-fold for vinblastine; 1 µg/ml is equal to about 0.8 µM for CsA and about 0.7 µM for NBDL-CsA.

with CsA (though at the cost of excessive intrinsic toxicity), while NBDL-CsA gave a much lower 10-fold RDF. With MDR-CEM cells, which show a higher resistance level than MDR-P388 cells, no sensitization to vinblastine was found with 3 μg/ml NBDL-CsA (RDF of about 1.0) in comparison with a 36-fold one with CsA, CsA itself being little active with a RDF of 2.0 at 1 μg/ml.

This low RM activity of NBDL-CsA could be interpreted as showing that either it did not penetrate the cell membrane or it was an easier/faster substrate of the P-gp pump than CsA, being somewhat like vinblastine, which is a weak competitive inhibitor for still easier P-gp substrates. These two alternatives could be discriminated by analyses of the direct toxicity data. At low concentrations, both CsA and NBDL-CsA caused a stronger cell growth inhibition for Par cells than for MDR cells, which did not prove but seemed to be compatible with the interpretation that NBDL-CsA could actually enter the cells. Therefore, the lower RM activity of NBDL-CsA for MDR-P388 cells might be attributed to it being a faster P-gp substrate than CsA.

Restoration of P-gp probe retention in MDR cells by RM agents

Bulk culture fluorometry studies. The restoration of the retention of various P-gp probes is a short-term assay of P-gp function which does not require cell growth. Therefore, assaying its inhibition could also be performed with high chemosensitizer concentrations which interfered by themselves with the cell growth required for chemosensitizing assays. The fluorescent CsA derivative NBDL-CsA was assayed with the pairs of tumor leukocyte lines, in order to determine whether, just like R-123 or DAU, it could be used to differentiate MDR cells and Par cells at the cell population level by their fluorescence level. Earlier experiments showed that in the absence of chemosensitizer treatment, the P-gp probe retention levels shown by MDR-P388 and MDR-CEM cell microcultures were about, in the case of DAU, 5 and 12 ± 13% and in the case of R-123, 5 and 6%, respectively, of the Par-P388 and Par-CEM cell microcultures.[6]

In a series of exploratory experiments, microcultures of MDR cells and Par cells were pulse-exposed to various concentrations of NBDL-CsA and its retention measured by fluorometry. Exposure of Par cells to NBDL-CsA concentrations 2 μM or less (such as those used for [^{3}H]CsA retention studies) led to fluorescence signals in the Par cells which were too low to reliably monitor a potential modulation by RM agents of the still lower NBDL-CsA retention levels shown by the MDR cells (not shown). Higher NBDL-CsA concentrations allowed a better distinction of the MDR-CEM and Par-CEM cells, the former cells retaining 14–25% of the NBDL-CsA retained by the latter cells (Table 2). With the P388 cell line pair, the NBDL-CsA dose-dependence of the distinction was not obvious, so that besides a few pilot experiments with P388 cells, most of our assays of restoration of NBDL-CsA retention by Pgp blockers were performed with the CEM cell line pair and 6 μM NBDL-CsA.

When RM agent concentrations (ranging from 0.03 to 30 μg/ml) were assayed for their capacity to restore P-gp probe retention in MDR-CEM cells, 0.3–1.0 μg/ml SDZ PSC 833 and SDZ 280-446 gave substantial levels of restoration of the retention of DAU and R-123, while higher concentrations of CsA were required, verapamil being little (DAU) or not (R-123) active.[6] The four RM agents were then assayed for their capacity to restore NBDL-CsA retention in MDR-CEM cells to their levels in Par-CEM (Table 3).

When pulse-exposed to 6 μM NBDL-CsA in the absence of any treatment with a chemosensitizer, the MDR-CEM cells retained about 17% of the NBDL-CsA retained by the Par-CEM cells. At least for concentrations below 30 μg/ml, all four RM agents did not affect the NBDL-CsA retention by the Par-CEM cells, the RIF remaining close to unity. In contrast, they all substantially increased the retention of NBDL-CsA in MDR cells. While CsA and verapamil concentrations of 1–3 μg/ml or greater were required for 2-fold RIF, lower concentrations of SDZ PSC 833 or SDZ 280-446 could achieve such significant increases of NBDL-CsA retention and only SDZ 280-446 regularly allowed

Table 2. NBDL-CsA retention of MDR cells as percentage of its retention by Par cells[a]

Molarity (μM)	Retention by MDR cells as percent of retention by Par cells[a]	
	CEM	P388
2	49 ± 43	27 ± 5
4	25 ± 16	32 ± 21
6	18 ± 5	20 ± 6
8	17 ± 3	17 ± 7
10	14 ± 4	24 ± 2

[a] The exposure time of the cells to the CsA probe was 5 min everywhere. The results are shown as the means ± SD of three independent experiments.

Table 3. NBDL-CsA retention (RIF) in CEM cells and RM agent effects[a]

	RM agent (μg/ml)						
Cell line	0.03	0.1	0.3	1	3	10	30
Par-CEM							
Verapamil	1.1 ± 0.1	1.1 ± 0.4	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.5
CsA	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.2
SDZ PSC 833	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.2
SDZ 280-446	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.6 ± 0.1
MDR-CEM							
Verapamil	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.5 ± 0.8	1.6 ± 0.4	2.0 ± 1.0	2.4 ± 1.2
CsA	0.8 ± 0.1	1.2 ± 0.5	1.2 ± 0.2	2.1 ± 0.9	2.1 ± 0.7	2.3 ± 0.6	3.0 ± 1.6
SDZ PSC 833	0.8 ± 0.2	1.1 ± 0.1	1.6 ± 0.3	2.2 ± 0.3	3.0 ± 0.2	4.6 ± 1.4	4.2 ± 0.6
SDZ 280-446	1.5 ± 0.6	2.0 ± 0.9	2.9 ± 1.8	4.4 ± 1.4	5.8 ± 2.2	5.9 ± 1.5	5.1 ± 1.2

[a] The retention is measured by the NBDL-CsA specific fluorescence; the change of NBDL-CsA retention is given as RIF. These RIF are shown as the means ± SD (three independent experiments), of the respective control solvent (ethanol)-treated Par-CEM cells and MDR-CEM cells. NBDL-CsA exposure concentration was 6 μM for a 5 min influx and efflux was allowed for 5 min. In culture medium (no solvent), the NBDL-CsA retention by MDR-CEM reached 14 ± 9% of the Par-CEM one; in the presence of the ethanol solvent, the NBDL-CsA retention by MDR-CEM reached 17 ± 9% of the Par-CEM one.

its nearly complete restoration to the Par-CEM cell levels (RIF ≥ 5), suggesting that only that one RM agent could completely inhibit the capacity of P-gp to reduce NBDL-CsA accumulation in MDR-CEM cells.

Single cell flow cytometry studies. The MDR-P388 and MDR-CEM tumor cells were compared with their respective Par-P388 and Par-CEM cells (Figures 1–4). The cells were briefly pre-treated with 10 μg/ml SDZ PSC 833 or its solvent only (controls). They were then exposed to the P-gp probes, DAU, R-123 or NBDL-CsA, in conditions allowing a clear distinction of the P-gp probe retention by Par cells and MDR cells. A long efflux phase might be needed to efficiently discriminate P-gp-lacking normal lymphoid cells from P-gp-expressing ones.[9] The tumoral leukocytes were thus analyzed by single cell flow cytometry, after 3 h long efflux phases in both DAU and NBDL-CsA cases; with the R-123 probe, a 3 h efflux could not be used for practical reasons, but a 60 min long efflux was compared to the 15 min one used in the bulk culture fluorometry assays.[6]

DAU (Figure 1). In the absence of SDZ PSC 833 treatment (solvent controls), the MDR-CEM and MDR-P388 cells displayed much lower DAU fluorescence levels than their respective Par cell controls. As shown by the population fluorescence profiles in a typical experiment (Figure 1), all MDR cells had the same very low DAU retention while the Par cells had higher but more heterogeneous DAU retention levels. When P-gp function was inhibited by 10 μg/ml SDZ PSC 833, DAU retention did not substantially change in Par cells, while it became

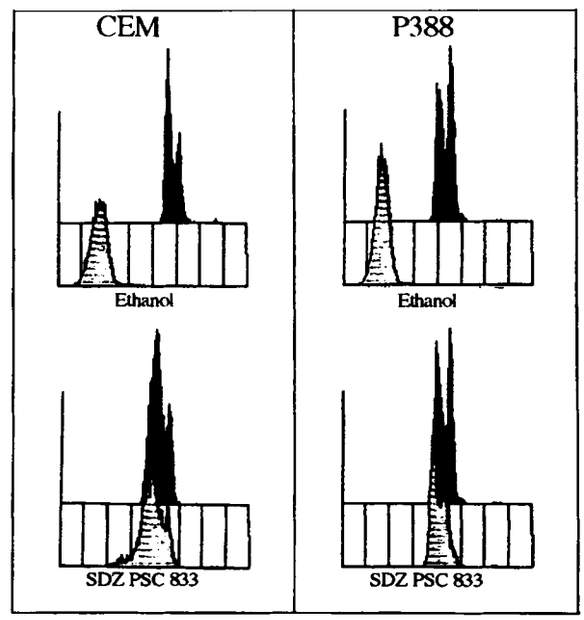

Figure 1. Single cell flow cytometry analyses of DAU retention by pairs of MDR and Par cells of the CEM and P388 cell lines. The *y*-axes are arithmetic scales showing the numbers of cells per fluorescent channels and the *x*-axes are logarithmic scales with 10-fold fluorescence levels increments per shown division. The dark (black) profiles represent the Par cells and the light (grey) profiles represent the MDR cells. The cells were exposed to 20 μM DAU for 30 min at 37°C (influx phase); after washing the DAU excess away, the cells were reincubated for 3 h at 37°C for DAU efflux. The P-gp probe influx occurred either in the absence (ethanol) or the presence of 10 μg/ml SDZ PSC 833; the P-gp probe efflux occurred in SDZ PSC 833 and P-gp probe-free culture medium. The cells were washed and analyzed by flow cytometry to construct cell population profiles.

less homogeneous in MDR cells and closer to the DAU retention shown by Par cells. A small fraction of the MDR-CEM cell population remained substantially less fluorescent than Par-CEM cells, whereas the restoration of DAU retention in the MDR-P388 cells was virtually complete.

R-123 (Figure 2). In the absence of SDZ PSC 833 treatment (solvent controls), the MDR-CEM and MDR-P388 cells retained much lower levels of R-123 than their respective Par cell controls. Representative data from the same experiment allowed us to compare the effect of the shorter (15 min) or longer (60 min) duration of the efflux phase between all diagrams (Figure 2). The population fluorescence profiles showed that both MDR cell and Par cell populations were rather homogeneous with regards to R-123 retention levels. A small fraction of the Par-CEM population retained definitely less R-123, though more than the MDR-CEM cell line selected for use in this study; that fraction was less obvious with the 60 min efflux phase than with the 15 min one. While both efflux phase durations gave similar R-123 retention for MDR-P388 cells, the more resistant MDR-CEM cells showed a lower R-123 retention in the 60 min efflux case than in the 15 min one.

When the P-gp function was inhibited by 10 μg/ml SDZ PSC 833, the R-123 retention levels shown by the Par tumor cells did not substantially change and those of MDR tumor cells became closer to, though still lower than, the R-123 retention levels of Par tumor cells. Moreover, although the MDR-P388 cell population acquired a rather high and homogeneous R-123 retention profile, the MDR-CEM cells showed a very high heterogeneity of R-123 retention levels compared with the Par-CEM cells: not only did most cells not retain as much R-123 as most Par CEM cells, but a number of them looked to remain rather unhibited by the RM agent. This feature was more obvious for the 60 min duration of R-123 efflux than for the 15 min one, suggesting that a substantial fraction of the SDZ PSC 833-treated MDR-CEM cells did not show a stable and/or complete inhibition of P-gp function and started to recover partial P-gp function during that additional 45 min period of time.

NBDL-CsA (Figure 3). In the absence of SDZ PSC 833 treatment (solvent controls), the MDR-CEM and MDR-P388 cells retained less NBDL-CsA (two orders of magnitude) than their respective Par cell controls, as shown in a typical experiment (Figure 3). The population fluorescence profiles showed that both MDR cell and Par cell populations were rather homogeneous with regard to NBDL-CsA retention

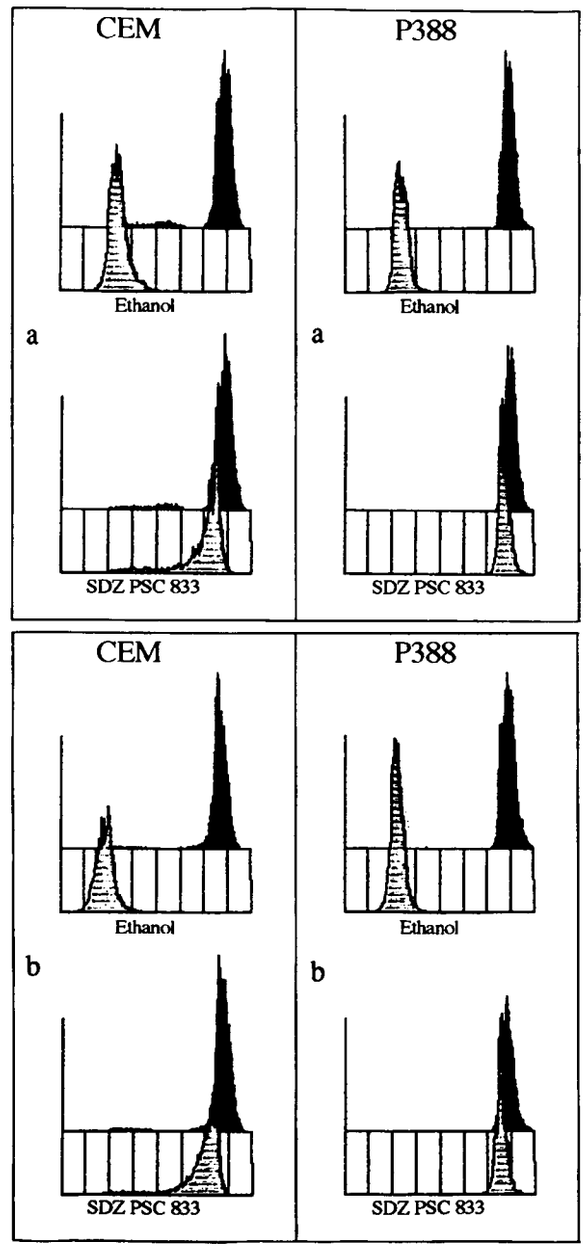

Figure 2. Single cell flow cytometry analyses of R-123 retention by pairs of MDR and Par cells of the CEM and P388 cell lines. Representation as for Figure 1. The cells were exposed to 5 μg/ml R-123 for 15 min at 37°C (influx phase); after washing the R-123 excess away, the cells were reincubated in drug and P-gp probe-free culture medium, for either 15 min (a) or 60 min (b) at 37°C, washed and then analyzed by flow cytometry.

levels. A small fraction of the Par-CEM cell population retained definitely less NBDL-CsA, though more than the MDR-CEM cell line. When the P-gp function was inhibited by 10 μg/ml SDZ PSC 833,

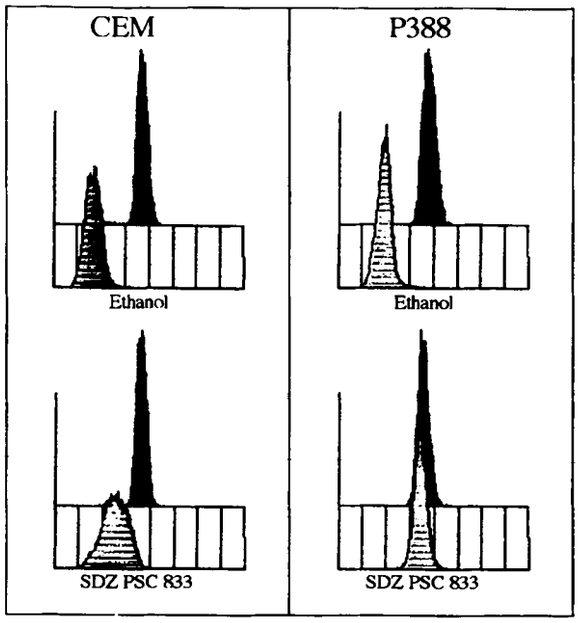

Figure 3. Single cell flow cytometry analyses of NBDL-CsA retention by pairs of MDR and Par cells of the CEM and P388 cell lines. Representation as for Figure 1. The cells were exposed to 8 μM NBDL-CsA for 5 min at 37°C (influx phase); after washing the NBDL-CsA excess away, the cells were reincubated for 3 h at 37°C in drug and P-gp probe-free culture medium, washed and then analyzed by flow cytometry.

the NBDL-CsA retention levels shown by the Par cells did not change and remained homogeneous (very narrow profiles). There was a nearly complete restoration of the NBDL-CsA retention by MDR-P388 cells to the levels shown by Par-P388 cells. In contrast, this was far from the case with the MDR-CEM cells: there was a definite increase of the NBDL-CsA retention levels of the MDR-CEM cells, but most MDR-CEM cells showed NBDL-CsA retention levels which were substantially lower (one order of magnitude) than those of Par-CEM cells. Furthermore, a substantial fraction of the MDR-CEM cells remained refractory to P-gp inhibition by SDZ PSC 833 or had already recovered their full P-gp function by the end of the 3 h efflux phase in the SDZ PSC 833-free medium.

The actual capacity to discriminate P-gp-expressing cells from P-gp-lacking ones with NBDL-CsA was probed by preparing various mixtures of Par-CEM and MDR-CEM cells (75/25%, 50/50% and 25/75%) just before the experiment. The NBDL-CsA retention levels shown by the Par-CEM and MDR-CEM cell mixtures remarkably fitted the expectations as they resolved the whole cell population in two well separated populations whose proportions corresponded to the Par-CEM and MDR-CEM cells in the mixtures. After SDZ PSC 833 treatment, it was obvious that recovery of NBDL-CsA retention by the MDR-CEM cells was fairly incomplete.

Discussion

P-gp molecules are expressed and known to be functionally active on a variety of cells of both tumor and normal tissue origin.[9–17] P-gp is definitely used as a means by MDR tumor cells to efflux anticancer agents, but the physiological function of P-gp on normal cells remains poorly understood. P-gp expression on the apical surface of specific epithelial and endothelial cell linings has the most likely function of excluding toxic compounds of exogenous and endogenous origin from the whole body or some sanctuaries (brain), or might be involved in steroid hormone transport (adrenals,

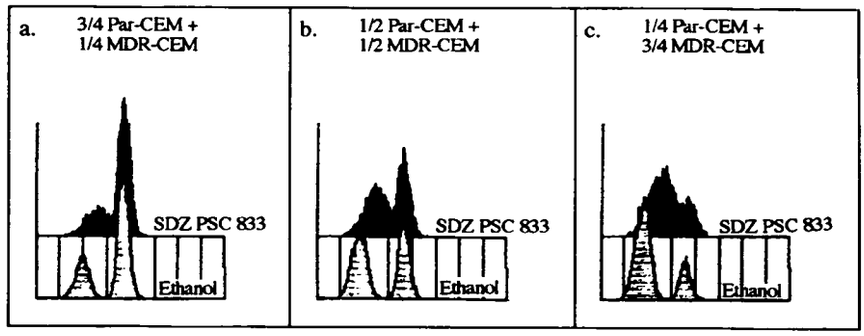

Figure 4. Single cell flow cytometry analyses of NBDL-CsA retention by mixtures of MDR CEM and Par-CEM cells. Representation as for Figure 1. From left to right, the MDR/Par cell ratio was 25/75, 50/50 and 75/25. NBDL-CsA influx occurred either in the absence (ethanol, grey profiles) or the presence of 10 μg/ml P-gp blocker (SDZ PSC 833, black profiles).

endometrium). As a matter of fact, P-gp molecules are involved in the transcellular transport of such xenobiotics, among which CsA, from the basal to the apical side of both normal and tumoral epithelial cell monolayers in *in vitro* culture.[18–20] That CsA was a P-gp substrate was confirmed by the restoration of its intracellular accumulation in MDR cells by P-gp blockers.[6] Furthermore, *in vivo* inhibition of the P-gp function of the blood–brain barrier also resulted in neurotoxicity of CsA and ivermectin, two slow P-gp substrates.[21]

The occurrence of P-gp molecules on specific subsets of leukocytes,[9–12] as well as the modulation of their expression as a function of the differentiated status of such cells is most intriguing. Therefore, we were looking at methods aimed at studying P-gp function on leukocytes. Most of our efforts thus converged towards setting up methodologies that might be suitable to study P-gp expression and function on normal cells, in particular leukocytes. Pairs of Par and MDR P388 and CEM cell lines were studied as representative leukemias of murine monocytic and human lymphocytic origin. P-gp seemed more effective in MDR-CEM cells than in MDR-P388 cells since their relative resistances to the drug used for their MDR selection were, respectively, about 1000 and 200 in comparison with their fully drug-sensitive Par cell lines, the MDR-CEM cells being also more difficult to sensitize than the MDR-P388 cells. The Par and MDR P388 and CEM cells were also compared for their capacity to be discriminated by their retention of the usual fluorescent P-gp probes, DAU and R-123, and the novel one, NBDL-CsA. From earlier studies,[5,22] the MDR-P388 cells were known to retain much less DAU or R-123 than Par-P388 cells. With the MDR-CEM cells, P-gp blockers could restore the retention of DAU and of R-123 close to the Par-CEM levels ($\geq$95% for DAU and 90% for R-123) when measured by bulk culture fluorometry.[6] When NBDL-CsA was used as P-gp probe, microcultures of MDR and Par cells were also readily discriminated in spite of the fact that the background retention of NBDL-CsA in the MDR cells reached a fifth of the one in Par cells; the restoration of NBDL-CsA retention by P-gp blockers was obvious.

When analyzed by single cell flow cytometry, the MDR-P388 and MDR-CEM cells were easily discriminated from their respective Par cells, by largely distinct fluorescence profiles at the cell population level; this was also obvious with NBDL-CsA, the novel P-gp probe. However, while the difference of P-gp probe retention between MDR and Par cells, as measured in terms of $\log_{10}$ differences of mean fluorescent channels, was nearly 3-fold in the case of DAU retention and 4- to 5-fold in the case of R-123 retention, it reached only 2-fold in the case of NBDL-CsA retention.

The restoration of P-gp probe retention by SDZ PSC 833 also depended on the cell line and on the P-gp probe used. DAU retention was virtually restored in both types of MDR lines, though better in the P388 cells than in the CEM ones; R-123 retention was not fully restored in all MDR-CEM cells while it was in MDR-P388 cells; and NBDL-CsA retention was restored in a minor fraction of the MDR-CEM cells while it was in all MDR-P388 cells.

In the case of R-123 retention, the comparison of the SDZ PSC 833-treated samples submitted to 15 or 60 min R-123 efflux provided a key for this different behavior: the fraction of MDR-CEM cells which recovered a low (less than parental) R-123 retention was larger after 60 min efflux than after 15 min efflux. This suggested that there was a large overload of P-gp function during the influx phase, but also that the P-gp function was recovering rapidly in such cells and the intracellularly retained R-123 was available for efflux. Since no such reappearance of low R-123 retaining cells occurred in the MDR-P388 cell case, this would suggest that their P-gp function remained totally inhibited. The simplest interpretation might be that MDR-P388 P-gp function would be lower than MDR-CEM P-gp function (different numbers of P-gp molecules per cell), but another one is that the P-gp molecules of murine MDR-P388 cells were more sensitive to SDZ PSC 833 inhibition than the P-gp molecules of human MDR-CEM cells.

In the case of DAU retention, only very few SDZ PSC 833-treated MDR-CEM cells showed lower fluorescence than the Par cells; since this occurred in spite of a 3 h long post-treatment incubation, during which a substantial recovery of P-gp function should have occurred, it suggested that the intracellularly retained DAU was not or very poorly exchangeable with the cell plasma membrane for being effluxed by P-gp molecules freed from P-gp inhibition. Presumably, after overload of P-gp function during the DAU influx phase, a large fraction of DAU molecules remained within the cell, either because of a poorly reversible binding to cytosolic targets or because of a sequestration within some intracellular compartments.

In the case of NBDL-CsA retention, the major fraction of the SDZ PSC 833-treated MDR-CEM cell population effluxed the P-gp probe, presumably because NBDL-CsA might stay in the cell plasma membrane or was free in the cell as a rapidly exchangeable form. Although this was not specifi-

cally studied, the NBDL-CsA is unlikely to show cyclophilin binding, but the chemical modification might confer the molecule binding properties to an unknown cellular component, reducing its exchangeability with the membrane and its effluxability by P-gp or other plasma membrane-located mechanisms. Its presently known features would, however, suggest that, after membrane penetration, NBDL-CsA might either stay there or go free in cytosol but can easily partition back to the plasma membrane, so that it can be quickly P-gp-effluxed.

Conclusion

Since after mixing various proportions of tumoral MDR-CEM and Par-CEM cells and exposing them to NBDL-CsA, the MDR cells could still be discriminated from the Par cells, this NBDL-CsA detection method might be also suitable for the discrimination of P-gp-expressing cells and P-gp-lacking ones in normal cell suspensions. Preliminary studies with cells from mouse lymphoid organs, using the cell lines as controls, would suggest that NBDL-CsA, like R-123, but not DAU, might allow us to discriminate P-gp-expressing cells from P-gp-lacking cells in the mouse spleen and lymph node cell populations.

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