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Decreased uptake of cyclosporin A by P-glycoprotein (Pgp) expressing CEM leukemic cells and restoration of normal retention by Pgp blockers

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The P-glycoprotein (Pgp) molecules which are expressed on multidrug resistant (MDR) tumor cells can efflux a variety of cytostatics. In both normal and tumoral epitheliums, Pgp molecules are selectively expressed on the apical surface of the epithelial cells. Such a distribution seems to be responsible for the transcellular transport of Pgp substrates, including cyclosporin A (CsA), from the basal to the apical side. Some normal lymphoid cells also express small amounts of Pgp molecules, for as yet unknown functions. Nevertheless, the sensitivity of their mitogen-induced proliferation to cytostatics, including doxorubicin and CsA, could be increased by the Pgp blockers. Using isotopically-labeled CsA and tumoral lymphoid cell lines, we now show a higher CsA retention in Pgp-lacking parental ('Par') cells than in Pgp-expressing MDR cells. The Pgp blockers can restore the CsA retention in the MDR cells to its level in the Par cells.

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

Introduction

Class I mdr P-glycoprotein (Pgp) molecules are members of a family of the ATP binding cassette (ABC) transporters.[1] Unlike other ABC family members, Pgp molecules possess a very broad substrate specificity, the transported compounds, including anti-cancer drugs, showing neither structural nor functional relationships besides their high lipophilicity.[2] When Pgp molecules are overexpressed by various tumor cells, this leads to the emergence of a multidrug resistance (MDR) phenotype, which is often responsible for the failure of chemotherapy.[3] A variety of chemosensitizers or 'resistance modulating (RM) agents', among which the most commonly used are verapamil and Cyclosporin A (CsA), can substantially inhibit the function of the *class I mdr* Pgp 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 non-immunosuppressive cyclosporin SDZ PSC 833 or cyclopeptolide SDZ 280-446.[4] This may be due to the fact that these RM agents are real 'Pgp blockers', as they can achieve a virtually irreversible inhibition of Pgp function. At variance, other RM agents, including CsA, inhibit Pgp function in a reversible fashion, presumably because they are themselves substrates, therefore being only competitive inhibitors for the transport of other Pgp substrates.[5]

Pgp molecules were found to be expressed and functionally active not only in tumoral cells but also in normal ones. In epithelial tissues, Pgp molecules are selectively expressed on the apical surface of the cells.[6,7] Such a distribution seems to be responsible for the transcellular transport of Pgp substrates, including CsA, from the basal to the apical side of both normal and tumor epithelial cell monolayers in *in vitro* culture.[8–10] In the mouse, the Pgp blockers SDZ 280-446 and SDZ PSC 833 could increase the sensitivity of mice to CsA or to ivermectin *in vivo*,[11] presumably by altering their overall bioavailability as well as their selective tissue distribution. Pgp molecules were also found to be active not only in tumoral leukocytes but also in normal ones; in particular macrophages and most $CD8^+$ lymphocytes, but also some $CD4^+$ ones, were found to express *class I mdr* gene encoded Pgp

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molecules. Though such Pgp expression occurs for as yet unknown normal physiological functions, lymphoid cell Pgp molecules could at least efflux a Pgp substrate (rhodamine 123).[12,13] Furthermore, when mouse spleen cells were exposed to a mitogen, the presence of the Pgp blockers increased the sensitivity of their proliferation to doxorubicin and CsA, suggesting the Pgp molecules could confer some resistance to the cytostatics.[14,15]

Using a novel pair of CEM T cell leukemia lines, the Pgp-lacking parental (Par-CEM) and Pgp-expressing (MDR-CEM) cells, we found a higher uptake of isotopically-labeled CsA by Par-CEM than by MDR-CEM cells. Moreover, the Pgp blockers SDZ 280-446 and SDZ PSC 833 restored the CsA retention in the MDR-CEM cells to their levels in the Par-CEM cells.

Materials and methods

Drugs

The anti-cancer drugs were doxorubicin (Adriamycin–HCl; Serva, Heidelberg, Germany) and vinblastine (vinblastine sulfate; Lilly, Giessen, Germany). The Pgp 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 were 5 μg/ml (13.16 μM) for R-123 and 20 μM (11.3 μg/ml) for DAU. The radiolabeled CsA was [^{3}H]CsA (specific activity: 7.2 mCi/mg; Amersham, Buckinghamshire, UK). 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 Pharma, Basel, Switzerland). The final RM-agent concentrations in culture medium depended on the assay, though being most often 1.0 and 3.0 μg/ml for verapamil, and 0.03, 0.1, 0.3, 1.0 and 3 μg/ml for the cyclosporins (CsA, SDZ PSC 833) and the cyclopeptolide (SDZ 280-446). For the RM agents, 1 μg/ml is equal to around 2 μM for verapamil and around 0.8 μM for CsA, SDZ PSC 833 and SDZ 280-446.

Cells

Two pairs of parental (Par) and multidrug resistant (MDR) tumor cell lines were used: the earlier described murine monocytic leukemia Par-P388 and MDR-P388 (obtained through doxorubicin-resistance selection), and the newly studied 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[16] and for another CEM cell line pair.[17] 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.

Normal mouse spleen cells were prepared from B6D2F1 mice (7–12 week old females; Iffa-Credo, l'Arbresle, France). The mouse spleens were gently teased through a stainless-steel screen, washed and resuspended in RPMI 1640 culture medium with 10% fetal calf serum and other supplements as described earlier.[15]

Cell growth inhibition assays of Pgp function

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 used in this study[16] and for a different CEM cell line pair[17] to that studied here. The cell numbers put in the culture (cells/well: 2×10^3 Par-P388, 5×10^3 MDR-P388, 3×10^3 Par-CEM, 122×10^3MDR-CEM) and the culture duration (three days for P388 cells or 4 days for CEM cells) were chosen such that the cells would still be in the exponential growth phase at the time of MTT addition. Briefly, the cells were cultured (37°C, 7.5% CO_2) in the presence of a range of cytostatic (doxorubicin or vinblastine) concentrations and a range of RM agent concentrations. Then, an MTT assay was performed and the absorbances were read at 540 nm. The control growth levels, obtained in the presence of the drug solvent but in the absence of RM agent and of cytostatic, were routinely taken as 100% growth. Earlier assays showed that the solvent itself did not change the cell growth. The cytostatic IC_{50}s were calculated from the dose–response curves obtained by plotting the measured growth versus the cytostatic concentration as described previously. The cytostatic IC_{50}^{+} values were determined in the presence of RM agent, while the IC_{50}^{-} values

were obtained only in the presence of the RM agent solvent. The increased cytostatic sensitivity was expressed as 'resistance decrease factors' (RDF) which was calculated by the ratio: IC_{50}^{-}/IC_{50}^{+} (formerly called 'sensitivity gain'[18]).

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

For fluorescence cytometry, the cells were treated by the RM agents and the fluorescent Pgp probes (R-123 or DAU), in conditions described elsewhere as co-incubation conditions.[19] Cell exposure to the Pgp probes was done in U-bottom 96-well microplates (Costar 3799), which were kept at 4°C unless indicated otherwise. For convenience, earlier DAU retention studies by flow cytometry[5] had been done with fixed cells. Experiments comparing live cells and fixed cells (1% paraformaldehyde in phosphate-buffered saline) showed the results of DAU retention to be equivalent. In view of its typical transmembrane potential-related retention in the mitochondrial compartment, R-123 retention required live cells. For microplate fluorometry, the treatments with the Pgp probe and the RM agent were thus adapted from earlier described flow cytometry procedure.[19–21] 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 Pgp probe uptake (influx phase) was allowed at 37°C (water bath) for 15 min in the case of R-123 or 30 min in the case of DAU. The excess of fluorescent Pgp probe and RM agent was washed away by two cycles of centrifugation (5 min at 200 *g*) and resuspension in culture medium. The Pgp-mediated efflux of Pgp probe (efflux phase) was allowed at 37°C (water bath) in 200 μl of Pgp probe- and RM agent-free medium. The duration of the efflux phase was 15 min for both Pgp probes. 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). Since all microplates could be read within a short time, only live cells were used both for DAU and R-123. The microtiter plates were then analyzed with a fluorescent plate reader (Cytofluor™ 2350; Millipore, Saint-Quentin, France). The following filters were used for excitation (485 ± 20 nm) and for emission of R-123 (530 ± 25 nm) or of DAU (590 ± 35 nm). Results are shown as 'relative retention' of the fluorescent probes of Pgp function, i.e. MDR-cell fluorescence versus Par cell fluorescence measured at the various RMA concentrations.

For testing the effect of Pgp blockers or of weaker RM agents on the retention of the Pgp probes by the MDR and Par cells, the procedure was the same for DAU and R-123. Briefly, the cells (5×10^5 cells in a final volume of 200 μl medium per well) were pre-incubated with the chemosensitizers or their solvent only (controls) in co-incubation conditions. The Pgp probes were then added to the cells (37°C, water bath) at the different concentrations and for the different influx phase durations specific for each Pgp probe, washed twice, resuspended in chemosensitizer and Pgp probe-free medium and incubated again (37°C, water bath) for the efflux phase. The restoration of the retention of the Pgp probes DAU and R-123 in the MDR cells could be shown simply as the percentage of the Pgp probe retention by similarly treated Par cells, as no effect of RM agent treatment was found on the latter.

Radiolabeled CsA assays

The assays were performed on duplicate 75 μl samples of 10^6 cells (freshly removed from bulk cultures and washed with medium) in ice-cooled 96-well microplates (Costar 3799). For testing the effects of strong Pgp blockers or weaker RM agents, the cells were preincubated with the chemosensitizers or their solvent only (controls) for 150 min in culture conditions. The cells were first treated in a 37°C, 7.5% CO_2 humidified incubator and then cooled down for 30 min at 4°C (in the fridge). The ice-cooled radiolabeled CsA solution was then added. This handling preserved 100% cell viability (detected by Trypan blue), being thus less rough than the transfer from the 37°C water bath to an ice bath, in which case less than 75% viability was found.

All chemosensitizers were prepared as stock solutions at 8, 0.8 and 0.08 mM in absolute ethanol and diluted 1000 times with culture medium to reach the final concentrations (8, 0.8 and 0.08 μM) for pre-incubation with the cells. Ethanol contents were thus identical in all flasks, solvent controls being achieved with drug-free ethanol as starting solution. Assays with 80 μM SDZ 280-446 were also performed, but required a 100-fold only dilution of the 8 mM stock solution; this led to excess ethanol content, which caused some cell death over the 150 min preincubation at 37°C in the incubator and might thus alter the results of short-term CsA uptake studies as well.

Before each experiment, the stock solution of radiolabeled [^{3}H]CsA (0.12 mM; specific activity: 7.2 mCi/mg) was first 10-fold diluted in culture

medium, and then isotopically 10-fold diluted with 8 μM unlabeled CsA. In assays, the final CsA concentration of 2 μM (precisely 2.16 μM) was obtained by setting 25 μl of this [^{3}H]CsA solution in a final 100 μl culture medium per well. [^{3}H]CsA uptake was studied by incubating cell samples at 37°C in the water bath, for a pulse exposure ('influx phase') of 5 min in most studies. After this short uptake phase, the [^{3}H]CsA excess, not taken up or not retained by the cells, was rapidly removed as follows: the cells were centrifuged twice at 200 *g* at 4°C, resuspended in 100 μl of ice-cooled CsA-free and RM agent-free medium, and reincubated ('efflux phase') for 5 min at 37°C (water bath). After three further fast washes by centrifugation at 4°C and resuspension in 200 μl of ice-cooled CsA-free and RM agent-free medium, the cell suspensions were directly transferred in 3 ml of scintillator liquid and the radioactivity was measured in a Beckman LS 6500 scintillation counter.

There might be some potential difficulties in setting up or using the method as measuring specific [^{3}H]CsA binding to cells is known to be difficult because the molecule is highly hydrophobic. Unspecific cyclosporin adsorption to plastic is a well known problem: about 60% of [^{3}H]CsC added in solution was reported as adhering to the plastic materials; as the equilibrium would be reached after 30 min, it was recommended to preincubate all materials for 60 min at 37°C with cold cyclosporin.[22] In order to avoid excessive [^{3}H]CsA adsorption to the wells, we performed comparative experiments where all relevant materials were preincubated or not with CsA, but without detectable difference. Why preincubation of plastic materials was not necessary might come from our assay conditions: exposure of the cell to the [^{3}H]CsA pulse at 37°C was short (5 min); any other cell handling being performed at 4°C.

Results

Modulation of MDR-CEM cell resistance to cytostatics

The cell line pair most extensively used by our laboratory was the Par-P388 and MDR-P388 pair;[5,20] unfortunately, in culture, it showed an excessive sensitivity to several chemosensitizers (particularly CsA) as they could directly interfere with cell growth. Studies with human cell lines were preferred (see Discussion) but an earlier studied pair of Par-CEM and MDR-CEM cell lines showed an unknown drug efflux mechanism in addition to the Pgp-mediated one,[17] making it unsuitable for studies of Pgp-mediated drug efflux. Therefore, here we used another pair of Par-CEM and MDR-CEM cells whose drug-sensitivity properties will be shortly described first in reference to those of the pair of Par-P388 and MDR-P388 cells. The resistance level of MDR-CEM cells (e.g. about 1000-fold more vinblastine resistant than Par-CEM cells) was higher than the MDR-P388 cell one (67-fold more vinblastine resistant and 220-fold more doxorubicin resistant than Par-P388 cells).

When used at concentrations where they showed no growth inhibitory effects *per se*, the Pgp blockers SDZ PSC 833 and SDZ 280-446, as well as CsA (used here as RM agent), could substantially decrease the MDR-P388 cell resistance to doxorubicin and could completely reverse their vinblastine resistance. The usual RM agent verapamil was only marginally (at 1.0 μg/ml) to weakly (at 3.0 μg/ml) effective in restoring the sensitivity of the MDR-P388 cells to both tested anti-cancer drugs (Table 1).

In the case of the CEM cell line pair, there was no sensitization of the Par-CEM cells when exposed to the chemosensitizers. When the MDR-CEM cells were treated with the chemosensitizers at 1.0 μg/ml, a concentration giving negligible cell growth, there were large differences of their capacity to decrease the high vinblastine IC_{50} of the MDR-CEM cells, measured as 'RDF'; while the usual RM agents, CsA (RDF = 1.6 ± 0.4) and verapamil (RDF = 1.6 ± 0.7), were hardly active, the Pgp blockers SDZ 280-446 and SDZ PSC 833 definitely increased the vinblastine sensitivity of the MDR-CEM cells (Table 2). When compared over a 0.03–3.0 μg/ml range, the Pgp blockers provided distinct RDF profiles as a function of the Pgp blocker concentration (Figure 1).

The Pgp blockers thus caused a significant increase of vinblastine sensitivity at dosages as low as 0.1 μg/ml SDZ 280-446 or 0.3 μg/ml SDZ PSC 833, a maximal sensitization of more than 200-fold being found for 3.0 μg/ml of both Pgp-blockers. SDZ 280-446 was nearly three to 10 times more potent than SDZ PSC 833 over a certain concentration range (Table 2). Thus, the RDF values were similar with 0.3 μg/ml SDZ PSC 833 (3.7 ± 0.4) and 0.1 μg/ml SDZ 280-446 (3.9 ± 0.7); when compared at the 0.3 μg/ml concentration, the RDF was about 8-fold higher with SDZ 280-446 (33 ± 5) than with SDZ PSC 833 (3.7 ± 0.4). Interestingly, the RDF were similar for both Pgp blockers at 1.0 μg/ml (± 120) and 3.0 μg/ml (± 230). Though high, such RDF did not correspond to a complete abrogation of the vin-

Table 1. Restoration of MDR-P388 cell growth sensitivity to doxorubicin and vinblastine[a]

Cell line[b] and RM agent (μg/ml)	Doxorubicin RDF[c]				Vinblastine RDF[c]			
	0.1	0.3	1.0	3.0	0.1	0.3	1.0	3.0
Par-P388								
verapamil	ND	ND	0.8	0.9	ND	ND	1.1	1.4
CsA	1.3	1.0***	1.1***	1.0***	ND	ND	1.0***	0.8***
SDZ PSC 833	0.9	0.9	1.0*	1.5***	1.1	1.1	1.0*	1.1***
SDZ 280-446	0.9	0.8	1.2*	1.1***	1.0	1.0	1.1*	0.9***
MDR-P388								
verapamil	ND	ND	2	7	ND	ND	1.9	7
CsA	2.6	18*	97**	155***	ND	ND	67***	67***
SDZ PSC 833	75	99	101*	116**	67	67	67*	67**
SDZ 280- 446	36	86	108	122**	67	67	67*	67**

[a]For doxorubicin, the mean IC_{50} were 16 ± 1 ng/ml for Par-P388 cells and 3550 ± 520 ng/ml for MDR-P388 cells; for vinblastine, the mean IC_{50} values were 1.57 ± 0.14 ng/ml for Par-P388 cells and 105 ± 10 ng/ml for MDR-P388 cells. The resistance degrees of the MDR-P388 cells relative to Par-P388 cells were 220-fold for doxorubicin and 67-fold for vinblastine. 1 μg/ml is equal to about 2 μM for verapamil and about 0.8 μM for CsA, SDZ PSC 833 or SDZ 280-446.
[b]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).
[c]The results are shown as RDF (see text). Means of triplicate determinations within a single control experiment, the variability from one experiment to another being generally less than 20%. ND = not determined.

Table 2. Restoration of MDR-CEM cell growth sensitivity to vinblastine[a]

Cell line[b] and RM agent (μg/ml)	Vinblastine RDF[c]				
	0.03	0.1	0.3	1.0	3.0
Par-CEM					
verapamil	ND	ND	ND	1.4 ± 0.3	ND
CsA	ND	ND	ND	1.1 ± 0.3*	ND
SDZ PSC 833	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.1*
SDZ 280-446	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0*
MDR-CEM					
verapamil	ND	ND	ND	1.6 ± 0.7*	ND
CsA	ND	ND	ND	1.6 ± 0.4	ND
SDZ PSC 833	1.1 ± 0.0	1.4 ± 0.1	3.7 ± 0.4	129 ± 26	235 ± 23
SDZ 280-446	1.3 ± 0.1	3.9 ± 0.7	33 ± 5	114 ± 19	224 ± 18

[a]The mean vinblastine IC_{50} values were 0.5 ± 0.1 ng/ml for Par-CEM cells and 490 ± 30 ng/ml for MDR-CEM cells. Thus, the resistance degree of the MDR-CEM cells relative to Par-CEM cells was 1000-fold for vinblastine. 1 μg/ml is equal to about 2 μM for verapamil and about 0.8 μM for CsA, SDZ PSC 833 or SDZ 280-446.
[b]Cell growth inhibition by the chemosensitizer alone was * negligible (10–20% maximum in case labeled).
[c]The results are shown as RDF (see text). Means of two to four individual determinations (each in triplicates) ± a variability index calculated as a standard deviation of the mean. ND = not determined.

blastine resistance of the MDR-CEM cell line ($IC_{50} = 490 \pm 30$ ng/ml), which was nearly 1000-fold more resistant than its Par-CEM control cell line ($IC_{50} = 0.5 \pm 0.1$ ng/ml). Higher Pgp blocker concentrations did not give meaningful data due to the growth inhibitory properties of the Pgp blockers alone for the control Par-CEM cells.

Restoration of DAU and R-123 retention in MDR-CEM cells

The restoration of the retention of various Pgp probes is a short-term assay of Pgp function which does not require cell growth. Therefore, assaying its inhibition could also be performed with higher RM agent concentrations than those used in chemosen-

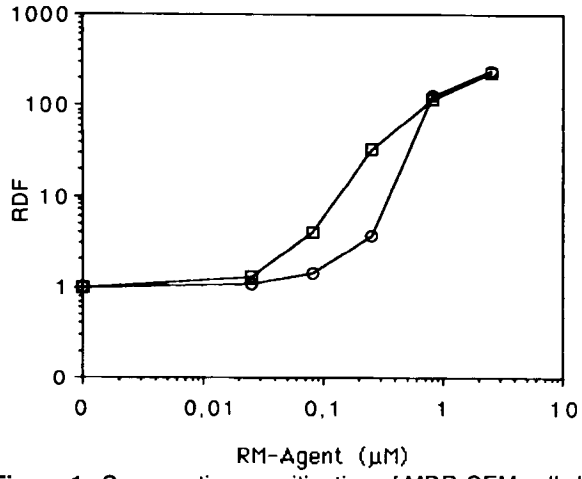

Figure 1. Comparative sensitization of MDR-CEM cells by SDZ PSC 833 and SDZ 280-446. Y-axis, RDF; X-axis, RM agent concentration. Though the RDF are similar for SDZ PSC 833 (○) and SDZ 280-446 (□) at 0.8 and 2.5 μM, at the lower 0.08 and 0.25 μM concentrations, SDZ 280-446 gave higher RDF values.

sitizing assays. The effects of a co-incubation with the RM agents or solvent controls (ethanol) were tested independently for the restoration of DAU (Table 3) and R-123 retention (Table 4) in bulk culture fluorometry assays in microplates. When performed as parallel experiments (as done for some of the data of Tables 3 and 4), there were differences of retention of the Pgp probes by MDR-P388 and MDR-CEM with regard to their Par-P388 and Par-CEM controls, as well as differences in the capacities of the RM agents to restore the Pgp probe retention in the MDR cells, but globally the conclusions were similar.

DAU (Table 3). In the absence of RM agent treatment (± the ethanol solvent), the DAU retention levels shown by MDR-P388 and MDR-CEM cell microcultures were about 5 and 12% of the Par-P388 and Par-CEM cell microcultures, respectively. When the four RM agents were assayed for their capacity to restore DAU retention in the MDR-P388 cells, their ranking was similar to an earlier one obtained with the same P388 cell line pair and flow cytometry analyses.[5,18] The tested RM agents could dose-dependently restore DAU retention in the MDR-P388 cells, though verapamil was poorly active and CsA was much less active than SDZ PSC 833 or SDZ 280-446. The DAU retention in MDR-P388 cells was largely restored with the highest tested concentrations (30 μg/ml) of several RM agents (60% or above with CsA and 90% or above with SDZ PSC 833 or SDZ 280-446) but not of verapamil (some 20% at best). Fluorometry analyses of microcultures of the CEM cell line pair showed similar features. A complete restoration (95% or above) of DAU retention was obtained with 30 μg/ml SDZ PSC 833, SDZ 280-446 or CsA, but verapamil was less active.

R-123 (Table 4). Fluorometry analyses of cell microcultures showed that, in the absence of RM-agent treatment (± ethanol solvent), the R-123 retention levels shown by MDR-P388 and MDR-CEM cells were about 5 and 6% of the Par-P388 and Par-CEM cells, respectively. When verapamil and CsA were assayed for their capacity to restore R-123 retention in the MDR-P388 cells, their differences of potency were confirmed. The data were similar to earlier

Table 3. DAU retention in MDR cells and RM agent effects[a]

	Pgp probe retention in MDR cells as percent of Par cells						
RM agent (μg/ml)	0.03	0.1	0.3	1.0	3.0	10	30
P388 cells							
verapamil				15	ND	15 ± 8	17 ± 1
CsA				9	ND	51 ± 21	64 ± 17
SDZ PSC 833	16 ± 4	51 ± 16	67 ± 10	77 ± 0	75	90 ± 2	90 ± 5
SDZ 280-446	12 ± 6	29 ± 4	46 ± 22	55 ± 3	57	80 ± 5	90 ± 4
CEM cells							
verapamil				37	38 ± 17	51	67 ± 1
CsA				38	59 ± 10	89 ± 19	119 ± 10
SDZ PSC 833	13	56 ± 2	57 ± 3	77 ± 12	86 ± 18	87 ± 6	98 ± 25
SDZ 280-446	35	41 ± 11	69 ± 24	85 ± 10	92 ± 25	91 ± 14	112 ± 38

[a]The restoration of the DAU retention was measured by fluorometry using a Cytofluor (Millipore) with live cells. It is shown as percentage of the Pgp probe retention by similarly treated Par cells. No effect of RM agent treatment on the latter was shown, unless indicated in the text. No effect of the RM agent solvent alone (ethanol 2.5%) was found. In the absence of RM agent treatment or with its ethanol solvent only, DAU retention in the MDR cells as percentage of the respective Par cells reached 5 ± 7% in the P388 cell case and 12 ± 13% in the CEM cell case. The results are shown as means ± SD of independent experiments [two to three with P388 cells, and two to four with CEM cells (single values for single assays; ND = not done)], each assay being performed in duplicate. 1 μg/ml is equal to about 2 μM for verapamil and about 0.8 μM for CsA, SDZ PSC 833 and SDZ 280-446.

Table 4. R-123 retention in MDR cells and RM agent effects[a]

	Pgp probe retention in MDR cells as percent of Par cells					
RM agent (μg/ml)	0.3	1	3	10	30	100
P388 cells						
CsA	6 ± 2	6 ± 3	10 ± 7	14 ± 8	28 ± 15	50 ± 31
SDZ PSC 833	37 ± 25	71 ± 35	84 ± 15	90 ± 12	93 ± 13	98 ± 29
CEM cells						
verapamil	ND	ND	12 ± 9	10 ± 6	8 ± 3	ND
CsA	ND	ND	12 ± 5	19 ± 4	43 ± 1	ND
SDZ PSC 833	ND	22 ± 5	49 ± 2	76 ± 17	76 ± 1	ND
SDZ 280-446	ND	33 ± 1	63 ± 14	76 ± 19	86 ± 14	ND

[a]The restoration of R-123 retention was measured by fluorometry using a Cytofluor (Millipore) with live cells. It is shown as percentage of the Pgp probe retention by similarly treated Par cells. No effect of RM agent treatment on the latter was shown, unless indicated in the text. No effect of the RM agent solvent alone (ethanol 2.5%) was found. In the absence of RM agent treatment or with its ethanol solvent only, R-123 retention in the MDR cells as percentage of the respective Par cells reached 5 ± 2% in the P388 cell case and 6 ± 1% in the CEM cell case. The results are shown as means (± SD) of independent experiments (two to four for P388 cells and two for CEM cells; ND = not done), each assay being performed in duplicate. 1 μg/ml is equal to about 2 μM for verapamil and about 0.8 μM for CsA, SDZ PSC 833 and SDZ 280-446.

ones obtained in flow cytometry studies of the same P388 cell line pair.[20] The R-123 retention in MDR-P388 cells was nearly restored by 3–10 μg/ml SDZ PSC 833, whereas this could not be achieved by the highest tested CsA concentration (100 μg/ml). Similar features were found with the CEM cell line pair. The restoration of R-123 retention by the MDR-CEM cells was virtually complete, 90% of the R-123 retention shown by Par-CEM cells being reached at the high (30 μg/ml) concentrations of SDZ PSC 833 or SDZ 280-446; a lower R-123 retention was achieved with CsA (some 40% at best) and no substantial restoration was seen with verapamil over the dose range tested.

Comparative uptake of [^{3}H]CsA by Par and MDR cells

The Par and MDR cells were only pulse-exposed to [^{3}H]CsA as it was thought that the differences would be more evident: the MDR cell-expressed Pgp molecules would be unable to completely interfere with [^{3}H]CsA influx, thus being only able to slow down its accumulation in the cytosol and its further binding to its high-affinity receptor, cyclophilin. To show [^{3}H]CsA retention differences between MDR-P388 and Par-P388 cells required lower [^{3}H]CsA concentrations than the roughly 2 μM one used with the MDR-CEM and Par-CEM cells: after a 5 min exposure to [^{3}H]CsA, its relative retention in MDR-P388 cells (as a percentage of the Par-P388 cell one) was 116% (with 2.16 μM [^{3}H]CsA), 54% (1.08 μM), 27% (0.54 μM) and 18% (both 0.27 and 0.135 μM). Most experiments were performed with the human CEM cell line pair, whose Par and MDR line differed by the highest resistance index.

When exposed for 5 min to 2.16 μM [^{3}H]CsA in the absence of any chemosensitizer or solvent, the MDR-CEM cells definitely took up less CsA than the Par-CEM cells. The [^{3}H]CsA retention shown by MDR-CEM cells reached only a mean of 22% (± 9) of its retention in simultaneously studied Par-CEM cells ($n = 14$; individual percentages: 7, 8, 13, 14, 19, 22, 22, 23, 23, 26, 27, 29, 36, 39). The level of the decreased [^{3}H]CsA retention shown by MDR-CEM cells thus showed a high variability, one cause of which might be the variable physiological status of the cells at the time of the assay. The difference of [^{3}H]CsA retention between Par-CEM and MDR-CEM cells was also evident when their [^{3}H]CsA exposure time was reduced to 3 min (relative retention of 19% (± 12; $n = 3$); in one experiment, a marked difference of CsA retention was still observed for longer [^{3}H]CsA exposure times, such as 60 min (relative retention of 5.5%; $n = 1$).

Restoration of a normal [^{3}H]CsA retention in MDR cells by Pgp blockers

All experiments performed with SDZ 280-446 ($n = 10$) and SDZ PSC 833 ($n = 2$) and the CEM cell line pair showed that the Pgp blockers could dose-dependently increase the retention of [^{3}H]CsA in the MDR cells up to the levels shown by the Par cells.

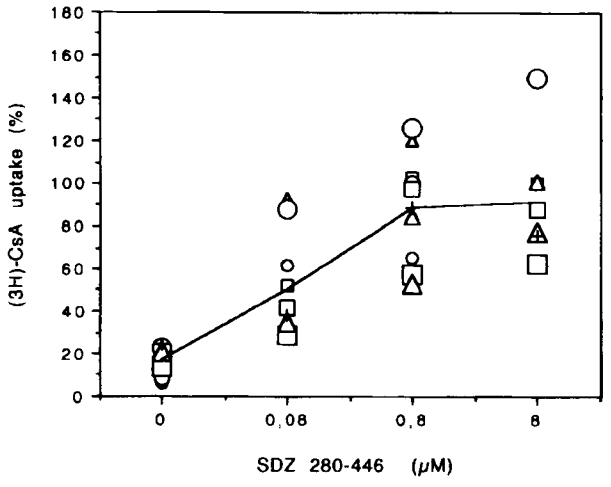

Figure 2. Retention of [^{3}H]CsA in MDR-CEM cells pretreated with SDZ 280-446. *Y*-axis, [^{3}H]CsA retention in MDR-CEM cells as percentage of its retention in Par-CEM cells; *X*-axis, SDZ 280-446 concentration. The 10 different symbols represent the values obtained in 10 independent experiments using a 5 min [^{3}H]CsA pulse exposure; the line representing the mean of the individual values shows a virtually complete restoration of [^{3}H]CsA retention in the MDR-CEM cells pretreated with 0.8 μM SDZ 280-446.

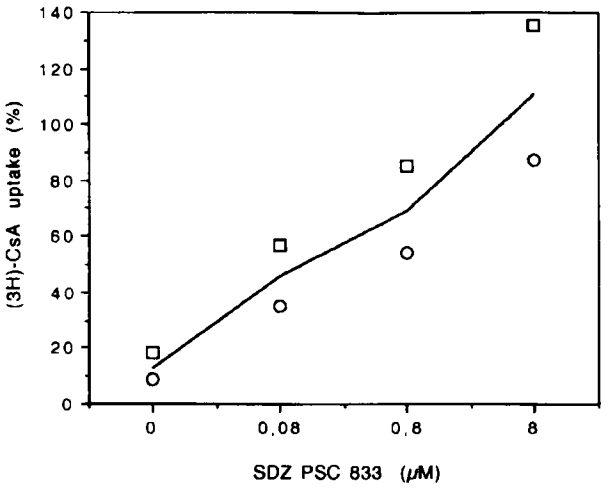

Figure 3. [^{3}H]CsA retention in MDR-CEM cells pretreated with SDZ PSC 833. *Y*-axis, [^{3}H]CsA retention in MDR-CEM cells as percentage of its retention in Par-CEM cells; *X*-axis, SDZ PSC 833 concentration. The two different symbols represent the values obtained in two independent experiments and the line represents the mean value. After a 5 min [^{3}H]CsA pulse exposure, a large restoration of [^{3}H]CsA retention (80–90%) in the MDR-CEM cells by their pretreatment with SDZ PSC 833 requires 8 μM in one assay and only 0.8 μM in the other.

Though there was a high experimental variability in the levels of [^{3}H]CsA retention shown by the MDR-CEM cells (as a percentage of those of Par cells), the same general dose-activity profile was observed (Figure 2): the lowest SDZ 280-446 concentrations (0.08 μM), known to decrease vinblastine-resistance in the long-term cell growth assay, were always able to restore a substantial [^{3}H]CsA retention (50%; ±23; $n=10$).

After their pretreatment with 0.8 μM of SDZ 280-446, the [^{3}H]CsA retention of the MDR-CEM cells was close (89%; ±25; $n=10$) to the one shown by Par-CEM cells. That a virtually complete inhibition of Pgp function was actually reached was suggested by the fact that no stronger restoration of [^{3}H]CsA retention was found when the MDR-CEM cells were pretreated with 8 μM SDZ 280-446 (91%; ±25; $n=9$). All those SDZ 280-446 concentrations shared the same 1% ethanol content, whose lack of cellular toxicity was shown by their lack of effect on the [^{3}H]CsA retention by the Par-CEM cells. Assays performed with 80 μM SDZ 280-446 were impaired by the larger solvent volume (50 μl) (1% ethanol) brought with this dose, which showed early cell toxicity. In two experiments performed with the same range of concentrations, SDZ PSC 833 appeared equipotent to SDZ 280-446, bringing a partial restoration of [^{3}H]CsA retention at 0.08 μM and a

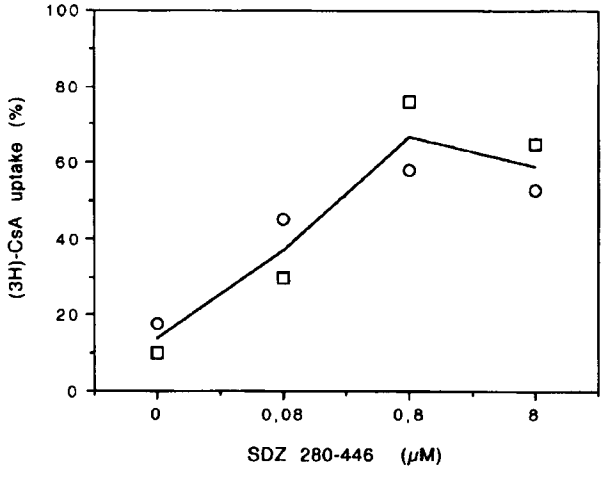

Figure 4. Retention of [^{3}H]CsA (3 min pulse only) in SDZ 280-446-pretreated MDR-CEM cells. *Y*-axis, [^{3}H]CsA retention in MDR-CEM cells as percentage of its retention in Par-CEM cells; *X*-axis, SDZ 280-446 concentration. The two different symbols represent the values obtained in two independent experiments and the line represents the mean value, using a 3 min [^{3}H]CsA pulse exposure only; no complete restoration of [^{3}H]CsA retention in the MDR-CEM cells was seen even after pretreatment with 8 μM SDZ 280-446.

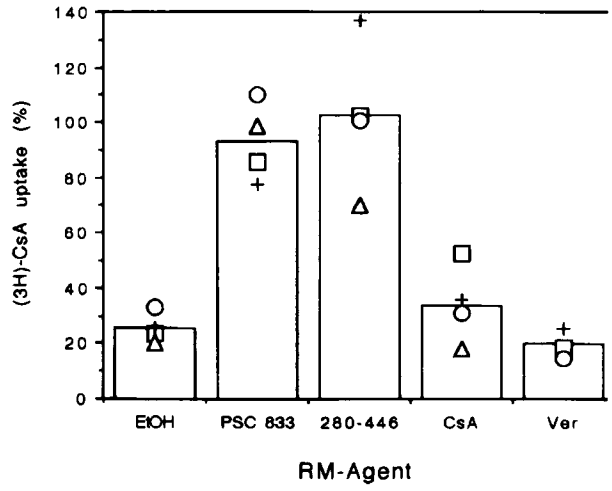

Figure 5. Comparison of SDZ PSC 833, SDZ 280-446, CsA and verapamil pretreatments for the restoration of [^{3}H]CsA retention. *Y*-axis, [^{3}H]CsA retention in MDR-CEM cells as percentage of its retention in Par-CEM cells; *X*-axis, RM agents. The [^{3}H]CsA pulse exposure lasted 5 min and the RM agent concentration during the 150 min pretreatment was 0.8 μM everywhere. The different symbols correspond to different experiments: four for SDZ PSC 833, SDZ 280-446, CsA and the ethanol solvent control (EtOH), and three for verapamil (Ver). The capacity of SDZ PSC 833 and SDZ 280-446 to restore the [^{3}H]CsA retention was largely superior to that of verapamil or cold CsA.

complete one from 0.8 μM (Figure 3). In the case of [^{3}H]CsA exposure times restricted to 3 min only, SDZ 280-446, even at 8 μM, did not permit us to restore complete CsA accumulation in MDR cells (Figure 4).

Four individual experiments studied whether cold CsA and verapamil might also increase [^{3}H]CsA retention by the MDR-CEM cells, in comparison with SDZ 280-446 and SDZ PSC 833, all chemosensitizers being used at the 0.8 μM concentration (Figure 5). While both Pgp blockers gave a complete restoration of [^{3}H]CsA retention in the MDR-CEM cells, verapamil was found totally inefficient. After pretreatment with 0.8 μM CsA, the MDR-CEM cells showed a slightly increased retention of the [^{3}H]CsA (used at 2 μM during the 5 min pulse exposure of the cells), suggesting that a slight competition might indeed take place. Nevertheless, in two out of four experiments, the Par-CEM cells displayed a slightly decreased [^{3}H]CsA retention which complicates the interpretation of assaying cold CsA preincubation.

Other assays, using higher concentrations of cold CsA (8 μM and higher) and the 150 min long preincubation, showed a decreased [^{3}H]CsA retention in the Par-CEM cells as well as in the MDR-CEM cells (data not shown). This suggested that a substantial fraction of the CsA-binding sites in the cell cytosol might become saturated by the unlabeled CsA. Such binding to cyclophilin being very stable, availability of further cyclophilin or other cytosolic CsA-binding sites might have been much reduced for any further added [^{3}H]CsA. Similarly, the use of higher verapamil concentrations did not allow us to study its capacity to restore the retention of [^{3}H]CsA in MDR-CEM cells (data not shown). Indeed, moderate increases of [^{3}H]CsA retention were obtained after their preincubation with 8 or 25 μM verapamil, concentrations which did not alter the uptake of [^{3}H]CsA by Par-CEM cells. This could suggest that such verapamil dosages could sufficiently inhibit Pgp function to favor [^{3}H]CsA uptake in the MDR-CEM cell cytosol. Unfortunately, at higher (80 μM) verapamil concentrations, not only did the MDR-CEM cells not retain more [^{3}H]CsA, but the similarly treated Par-CEM cells showed an abnormally low [^{3}H]CsA uptake, making such assays meaningless.

Discussion

As found early in studies on Pgp function,[23] cellular accumulation of CsA may thus be controlled, at least in part, by its interaction with Pgp molecules. Indeed, the accumulation of CsA by a MDR-subline of tumoral CHO cells was reduced relative to that of the parental cells, but increased by treatment with verapamil. *In vitro* cultures of cell monolayers from both normal and tumoral epithelial tissues recently provided evidence for the transcellular transport of CsA, from the basal to the apical side; this was shown with the human colon carcinoma Caco-2 cells,[8] the porcine kidney proximal tubule LLC-PK_1 tumor cells[9] and normal capillary endothelial cells of mouse brain origin.[10] This may provide a mechanistic explanation for the increased toxicity in the mouse of CsA, when used in combination with strong Pgp blockers such as SDZ PSC 833.[11] Similarly, when mouse spleen cells were exposed to a mitogen, the sensitivity of their proliferation to CsA was increased by the presence of Pgp blockers,[15] presumably through the recruitment, within the pool of CsA-sensitive lymphoid cells, of a small fraction of less sensitive Pgp-expressing macrophages, $CD8^+$ lymphocytes and 'preactivated' $CD4^+$ lymphocytes.[12,13]

In view of the latter finding, our specific problem

was to compare the uptake of CsA by cells which have a CsA-inhibitable efflux mechanism and cells which are lacking it. In the present paper, using human CEM T lymphocyte leukemia cells, we showed a higher uptake of [^{3}H]CsA by Par-CEM than by their MDR-CEM variants. Moreover, the Pgp blockers SDZ 280-446 and SDZ PSC 833 could restore the [^{3}H]CsA retention in the MDR cells to nearly their levels in the Par cells. SDZ 280-446 looked to be slightly more active than SDZ PSC 833. Though this was in line with the larger RDF obtained with SDZ 280-446 with the MDR-CEM cells, the large inter-experimental variability seen with SDZ 280-446 and the limited number of assays with SDZ PSC 833 do not allow us to conclude this was a significant difference.

Though Pgp-expressing cells represent a small fraction only of normal spleen cells and the level of Pgp expression on their surface is low, their proportions were reported to increase after mitogenic stimulation.[12] Some [^{3}H]CsA retention assays were thus performed with normal spleen cell suspensions, 3 days after their exposure to concanavalin A. However, no increased [^{3}H]CsA retention could be detected on the whole cell population whether or not the cells were treated with SDZ 280-446 (data not shown). Possibly, our assay may not be sensitive enough to detect changes of [^{3}H]CsA retention in normal mouse lymphoid cells, perhaps because of their cellular heterogeneity and/or the much lower Pgp expression levels of normal leukocytes in comparison with MDR lymphoid tumor cells.

While it is rather easy to compare MDR cells and Par cells for their retention of various Pgp substrates, such as radiolabeled anti-cancer drugs (vinca alkaloids, doxorubicin) or fluorescent probes (DAU, R-123), this was not the case with CsA. Since Pgp expression should only cause a delay in the time needed for CsA reaching its cytosolic receptor, it was speculated that too long a period of exposure to CsA might mask the delayed uptake of [^{3}H]CsA by MDR cells and make less obvious any Pgp effect on CsA retention. Though the 5 min pulse exposure [^{3}H]CsA was retained for the final procedure, longer incubation times gave significant results, but shorter pulses were not satisfactory because the effects of Pgp blockers on [^{3}H]CsA retention by MDR cells were not so marked.

Definitely, in view of the high affinity of CsA for its cytosolic receptor cyclophilin, any [^{3}H]CsA which had crossed the plasma membrane would become irreversibly bound to the cytosolic cyclophilin and be no longer available for active efflux by Pgp or passive diffusion from the cell. Therefore, in order to show a difference of [^{3}H]CsA retention by MDR cell lines, [^{3}H]CsA had to be used at sufficiently low concentrations to avoid saturation of the Pgp pump capacity. With the CEM cell line pair, 2.16 μM [^{3}H]CsA was found suitable, whereas assays performed with a 10-fold higher [^{3}H]CsA concentration (21.6 μM) no longer allows us to discriminate between Pgp-expressing tumor cells and Pgp-lacking ones. With the P388 cell line pair, lower than 2 μM [^{3}H]CsA concentrations had to be used to differentiate Par cells and MDR cells, possibly because the Pgp function was substantially lower in our MDR-P388 line (about 100- to 200-fold more resistant than the Par-P388 line) than in our MDR-CEM line (about 1000-fold more resistant than the Par-CEM line), thus because their effluxing capacity was more rapidly overwhelmed.

Competititon for cyclophilin-binding sites might also be involved in the decreased [^{3}H]CsA retention by Par cells exposed to cold CsA, a feature which did not permit us to measure the competition of cold CsA for [^{3}H]CsA uptake at the Pgp level. Since reduced [^{3}H]CsA retention was also found for the MDR-CEM cells, there might be enough leakage of cold CsA into the cytosol, through the Pgp-containing membrane barrier, and its binding to cyclophilin. Thus, irrespective of whether Pgp was totally blocked or not by the cold CsA, the fraction of cold CsA which reached the cytosol was high enough to efficiently compete for [^{3}H]CsA binding to the cytosolic cyclophilin. The presence of a high-affinity cytosolic receptor for the weakly inhibitory RM agent thus impaired competition assays using cold CsA excess. Similarly, cell damage caused by the calcium channel antagonist might explain why a preincubation with excess verapamil (80 μM) decreased the [^{3}H]CsA retention of the Par-CEM cells. Indeed, the presence of cyclophilin in the extracellular culture medium, e.g. released from dead or leaky cells, should also alter [^{3}H]CsA uptake, as cyclophilin would absorb part of the added [^{3}H]CsA and decrease its availability for influx in the cell by passive diffusion.

The use of a novel pair of human lymphocytic leukemia CEM cells and their necessary characterization before use for CsA uptake studies calls for some comments, since in our earlier studies in the MDR field we used several other pairs of Par and MDR cells of various origins. From earlier flow cytometry studies at the single cell level,[5,10,18] the MDR-P388 cells were known to retain much less DAU or R-123 than Par-P388 cells. For several reasons, however, it was useful to extend such results obtained with a murine monocytic leukemia to hu-

man cells such as the CEM lymphocytic leukemia. In all species, cancer MDR is mediated by *class I mdr* gene expression but in the mouse there are two different Pgp isotypes (encoded by the *mdr1a* and *mdr1b* genes) which show differences of expression in selected tissues and of function with regard to different substrates. At variance, there is a single kind of Pgp in the human, which is encoded by a single *MDR1* gene, and which cumulates the distributions and functions of both murine Pgp isotypes.[2] Using MDR cells of human origin offered the possibility for testing potential effects of anti-Pgp mAb on the Pgp function. A further advantage of using the CEM cells was that non-adherent cells are more suitable for *in vitro* and *in vivo* studies. Thus, the formerly used hamster CHO and murine P388 sublines were excluded, as well as the strongly adherent human KB and Lovo sublines,[16] and the novel CEM cell line pair was characterized.

Interestingly, SDZ 280-446 was a stronger chemosensitizer than SDZ PSC 833 for the MDR-CEM cell line used in this study, similar to what was observed earlier with the other pair of Par-CEM and MDR-CEM cell lines;[17] this might be a feature specific for CEM cells. The formerly used CEM cell pair was unfortunately not suitable for drug efflux studies as its Pgp-expressing MDR variant (selected for MDR character by actynomycin D) showed an additional efflux-mediated resistance mechanism: the highest testable concentrations of the strongest Pgp blockers could at best restore 50% of the DAU retention in the MDR cells.[17] In contrast, a more complete restoration of the retention of two fluorescent probes of Pgp function (DAU and R-123) was obtained with the novel MDR-CEM cells (selected for MDR by vinblastine). DAU retention could be completely restored at low Pgp blocker concentrations (95% or higher restoration), showing they did not display another efflux-mediated resistance mechanism than the classically RM agent sensitive Pgp-mediated one. The restoration of R-123 retention in MDR-CEM cells reached 90% only of Par-CEM cell retention, but there is no need for another R-123 effluxing mechanism to explain the data. Indeed, the intracellular accumulation and retention of the cationic hydrophobic R-123 depends not only on the presence of Pgp, but also on the mitochondrial activity and contents, on the proportions of cells in the different cell cycle phases, as well as on the plasma membrane composition and potential; such differences might thus account for the slight difference of absolute R-123 retention capacity by Par-CEM cells and by MDR-CEM cells, even when Pgp function is totally blocked in the latter.

Conclusion

The retention of [^{3}H]CsA was thus lower in MDR-CEM cells than in Par-CEM cells, but it could be restored by Pgp blockers. Though CsA can inhibit Pgp function, our data confirm that it is itself a slow substrate of *class I mdr* Pgp.[8–10] Being both an RM agent and a substrate, CsA might inhibit its own efflux, or it might simply be a slow substrate. Comparing CsA uptake by Par cells and MDR cells was not an easy task, the presence of membranous Pgp being unable to completely interfere with [^{3}H]CsA influx, thus being only able to slow down the cytosolic accumulation of [^{3}H]CsA on its abundant high-affinity receptor, cyclophilin. If CsA actually inhibits the function of the pump only in a competitive and reversible way, this might explain why CsA is a much less efficient Pgp inhibitor than SDZ PSC 833 or SDZ 280-446.

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