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Atypical multi-drug resistance (MDR): low sensitivity of a P-glycoprotein-expressing human T lymphoblastoid MDR cell line to classical P-glycoprotein-directed resistance-modulating agents

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Verapamil, cyclosporin A (CsA), the cyclosporin derivative SDZ PSC 833 and the novel cyclopeptolide SDZ 280-446 were tested for their capacity to chemosensitize a P-glycoprotein (Pgp)-expressing multi-drug resistant (MDR) variant of the CEM human T lymphoblastoid cell subline (CCRF ACTD 400+). That MDR-CEM cell subline had been previously selected for MDR by actinomycin D and displayed a very high resistance phenotype: 3700-fold for actinomycin D, 3900-fold for vincristine, 1200-fold for taxol, 1000-fold for daunomycin (DAU) and 400-fold for colchicine. Interestingly, these MDR-CEM cells displayed little chemosensitization by resistance-modulating agents (RMA) which presumably work by inhibiting Pgp function. These MDR-CEM cells displayed virtually no chemosensitization by 1 μM verapamil or 1 μg/ml (about 0.8 μM) CsA, whereas their chemosensitization for different anti-cancer drugs (ACD) was rather stable (from 51- to 82-fold) with 1 μg/ml (about 0.8 μM) SDZ 280-446, while being very unbalanced (from 5- to 38-fold) with 1 μg/ml (about 0.8 μM) SDZ PSC 833. Exposure of the MDR-CEM cells to Pgp-directed RMAs, during their loading with DAU (DAU-loading phase), hardly restored DAU retention: SDZ 280-446 being as poorly active as SDZ PSC 833, and about only 3- and 4-fold more active than CsA and verapamil. In contrast, SDZ PSC 833 treatment of human MDR-KB and MDR-LoVo cell lines under the same conditions could restore most or all the DAU retention shown by the parental (Par) cells, in spite of their high level of resistance. By keeping the MDR-CEM cells in the presence of RMA throughout the experiment (both DAU-loading and DAU-efflux phases), a better DAU retention could be restored by the different RMAs used, their order of relative restoration activity being SDZ 280-446 3- to 4-fold > SDZ PSC 833 3- to 10-fold > CsA 2- to 4-fold > verapamil. Nevertheless, the level of DAU retention restored in the MDR-CEM cells reached a plateau at 50% of the Par-CEM cell level. Therefore, although the MDR-CEM cells expressed easily detectable membranous Pgp molecules and probably used them for DAU efflux, they displayed an additional efflux mechanism that was not sensitive to the Pgp inhibitors.

Key words: Anti-cancer drug efflux, atypical multi-drug resistance, P-glycoprotein, resistance-modulating agents.

Introduction

A major mechanism by which tumor cells can develop resistance to anti-cancer drugs (ACD) is by decreasing intracellular ACD bioavailability. Such multi-drug resistance (MDR) phenotype of the tumor cells is usually mediated by the overexpression of *mdr*1 gene-encoded P-glycoprotein (Pgp) molecules which pump drugs out of the cells by an ATP-dependent process.[1-3]

A major pharmacological approach consists of trying to inhibit the Pgp pump function sufficiently to restore sensitivity to the classical ACD within the range of dosages compatible with their *in vivo* therapeutic window.[2] A variety of resistance-modulating agents (RMA) now available could either inhibit MDR and/or interfere with the ACD binding to Pgp. The structures of such RMA are as widely different as the structures of the ACD which are Pgp substrates.[2] The earliest identified RMA were quinidine and verapamil, and the latest and most active ones appear to be the cyclosporin derivative SDZ PSC 833[4] and the novel semi-synthetic cyclopeptolide SDZ 280-446.[5]

While studying monoclonal antibodies against human Pgp epitopes, we used a new MDR-CEM cell line,[6] besides our previously studied human MDR-KB and MDR-LoVo cell lines.[4,5] The CCRF-CEM cell line, a commonly used human T lympho-

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blastoid cell line (ATCC CCL 119), displayed normal sensitivity to ACD. From this parental cell line (Par-CEM), a series of MDR-CEM sublines were derived by selection with vincristine, adriamycin or actinomycin D.[6] They exhibited distinct quantitative differences of cross-resistance profiles, but they all showed amplification and marked expression of the *mdr*1/Pgp gene.[7] Drs Volker Gekeler and Heyke Diddens (Tübingen) kindly provided us with the Par-CEM line and their MDR-CEM subline designated CCRF ACTD400+ which had been selected by growth in the presence of increasing doses of actinomycin D and eventually cultured in the presence of 400 ng/ml actinomycin D for several months. We controlled the expression of *mdr*1/Pgp on the membrane of the MDR-CEM cells (and its lack on the Par-CEM cells) by use of the mAb57 antibody which very specifically recognizes an extracellular epitope of Pgp.[8] The pair of Par-CEM and MDR-CEM cell lines was then assayed for resistance to various anti-proliferative agents as well as for susceptibility to drug resistance reversion by a few selected RMA. The present paper describes the peculiar features of this highly resistant MDR-CEM cell subline, which seems to use, besides *bona fide* Pgp, another resistance mechanism which presumably reduces intracellular ACD retention.

Materials and methods

Drugs

Colchicine (Sandoz Pharma, Basel, Switzerland), actinomycin D and vincristine (Serva, Heidelberg, Germany) and daunomycin (DAU; Sigma, St Louis, MO) were prepared as stock solutions in NaCl 0.9% at 1 mg/ml. Taxol from *Taxus brevifolia* (Calbiochem AG, Lucerne, Switzerland) was prepared as a 10 mg/ml stock solution in pure ethanol. Stock solutions of RMA were prepared in absolute ethanol at 10 mM for verapamil (Sigma) and at 10 mg/ml (about 8 mM) for cyclosporin A (CsA, molecular weight = 1206.6), SDZ PSC 833 (molecular weight = 1214.65) and SDZ 280-446 (molecular weight = 1182.6) (all from Sandoz).

Tumor cell lines

The Par and MDR cells were cultured in RPMI 1640 medium with 10% fetal calf serum and other supplements like P388 cells.[4] The Par and MDR-LoVo cells and the Par- and MDR-KB cells were maintained as described earlier.[4] For flow cytometry analyses, the cells were trypsinized the day before the experiment, scraped out of the flask and suspended by pipetting. The pair of Par-CEM and MDR-CEM cell lines was provided by Dr Volker Gekeler (Tübingen). The Par-CEM cell line was the normally drug-sensitive CCRF-CEM cell line (ATCC CCL 119), and the MDR-CEM subline, designated CCRF ACTD400+, had been selected by growth in the presence of increasing doses of actinomycin D and eventual culture in the presence of 400 ng/ml actinomycin D for several months.[6]

The MDR variants were continuously grown in the presence of the drug used for their selection: the MDR-LoVo cells with 0.1 μg/ml doxorubicin, the MDR-KB cells with 1.0 μg/ml vinblastine and the MDR-CEM cells with 0.4 μg/ml actinomycin D. The ACD were removed from the medium 3 days before the cell growth experiments.

Cell growth assays

Tumor cell growth and its drug-mediated inhibition were measured as described previously.[4,5,9,11] The growth levels obtained without RMA or ACD but with their solvents were taken as representing 100% growth. The resistance of the Par-CEM and MDR-CEM cells to various ACD was investigated by using the 'classical' MTT assay as described.[9,10] We used 3000 Par-CEM cells and 12 500 MDR-CEM cells per well, for a culture period of 4 days at 37 °C, in a humid atmosphere with 7% CO_2. The ACD and RMA IC_{50}s were calculated from the dose–response curves obtained by plotting the measured growth versus the ACD and RMA concentrations.[4,5,9,11] In chemosensitization assays, a complete (ACD dose-cell growth response) curve was constructed at each RMA concentration. An 'IC_{50}^{+}' value was thus obtained in the *presence* of each RMA concentration, the 'IC_{50}^{-}' value being obtained in the *absence* of RMA (but in the presence of its solvent). The increases of ACD sensitivity or 'gains' in sensitivity of the RMA-treated cells were given by the ratio IC_{50}^{-}/IC_{50}^{+}, a gain being calculated for a given RMA concentration.

Flow cytometry assays

The methods for measuring DAU retention were adapted for microculture plates from the method described earlier for P388 cells.[12] Briefly, DAU

accumulation was allowed by incubating 5×10^5 cells at 37 °C in a humidified atmosphere enriched with 7% CO_2 for 30 min, in the presence of 20 μM DAU ('DAU-loading phase') and of various concentrations of RMA or its solvent. After washes, DAU efflux was allowed by reincubating the cells for 15 min at 37 °C in culture medium only ('DAU-efflux phase'). The cells were then washed in PBS and fixed with PBS containing 3.7% formaldehyde, and their DAU-specific fluorescence was measured with a FACScan (Becton-Dickinson, Mountain View, CA). The intracellular DAU retention in MDR cells was expressed as a percentage of the retention obtained with similarly treated Par cells: mean fluorescence of RMA-treated MDR cells × 100/mean fluorescence of RMA-treated Par cells.

Two major protocols were used in this study depending on when the RMA was present and when not. In the protocol termed 'co-treatment', the RMA was present only for the DAU-loading phase and no longer after. In the protocol termed 'permanent treatment', the RMA was continuously present in the medium used for incubating and washing the cells.

Results

Resistance profile of MDR-CEM cells

The degree of resistance of the MDR-CEM cells versus the Par-CEM cells was measured using five different anti-proliferative agents: besides actinomycin D, the drug used for selecting this MDR variant of CEM cells, four other drugs, i.e. DAU, colchicine, taxol and vincristine, were also tested as they were previously studied in our laboratory on other pairs of Par and MDR cell lines. Under our conditions, the MDR-CEM cells displayed a nearly 3000-fold resistance to actinomycin D, in comparison with the Par-CEM cells, which fits with reported values.[6] Their relative resistance indices to colchicine, DAU, taxol and vincristine (Table 1) were found to be much higher than those of MDR-CHO cells,[9,11] MDR-P388 cells[4,11] and MDR-LoVo cells.[4] Actually, the resistance of this MDR-CEM (CCRF ACTD400+) cell subline[6] was of the same order of magnitude as the one of MDR-KB cells (KB-V1 subline)[4,13] for vincristine and colchicine. The degree of resistance of the MDR-CEM cell line to the fluorescent anthracycline antibiotic DAU was more than 2-fold higher than the one of our MDR-KB cell line (Table 1).

Table 1. Relative resistance indices obtained for CEM cells in comparison to KB, LoVo, CHO and P388 cells.

	DAU	Colchicine	Taxol	Vincristine
CEM.CCRF	961	390	1169	3853
KB	427	474	ND[a]	4423
LoVo	29	24	ND	28
CHO	93	54	36	28
P388	158	130	283	158

Values for CEM are calculated from the present work. Values for KB, LoVo, CHO and P388 are calculated from refs 4 and 11.
[a] ND, not determined.

As seen below, FACScan analyses indeed showed that the retention of DAU in the MDR-CEM cells was extremely low, reaching only 1% of that displayed by the Par-CEM cells.

Poor restoration of DAU retention in MDR-CEM cells by co-treatment with CsA or SDZ PSC 833

Since these MDR-CEM cells showed a particularly high level of DAU resistance, preliminary studies assayed the capacity of CsA to restore DAU retention. This was performed in 'co-treatment' conditions, i.e. by exposing the MDR-CEM cells to CsA during their 30 min exposure to DAU (DAU-loading phase), then washing and processing them through the DAU-efflux phase in RMA-free medium. The level of Pgp function inhibition brought by this RMA co-treatment was then measured by the DAU retention of the MDR cells expressed as the percentage of the Par cell DAU retention. The low restoration of DAU retention obtained was interpreted as being due to the relative weakness of CsA as Pgp-directed RMA and to a high level of Pgp expression by the MDR-CEM cells. CsA was then assayed in RMA co-treatment conditions, in comparison with the stronger RMA, SDZ PSC 833. Thus MDR-CEM cells, and Par-CEM cells as controls, were exposed to CsA or SDZ PSC 833 in co-treatment conditions and then analyzed by flow cytometry (Table 2). In contrast to our previous data with MDR-P388 cells,[12] little DAU retention was restored even at a high (50 μg ml) SDZ PSC 833 concentrations; CsA was less active at all concentrations.

Restoration of DAU retention by SDZ PSC 833 in MDR-LoVo and MDR-KB cells

Since the conditions used above were known to totally inhibit Pgp-mediated efflux in murine

MDR-P388 cells, another explanation for the limited restoration of DAU retention in human MDR-CEM cells could be either much higher levels of Pgp expression in the human MDR line than in the murine MDR line, or a peculiar species difference in murine and human Pgps. In order to find out whether an 'RMA co-treatment' procedure would be sufficient to restore the DAU retention of human tumor cells with an MDR phenotype substantially stronger than the one displayed by MDR-P388 cells, human MDR-LoVo cells (LoVo/Dx) and MDR-KB cells (KB-V1) were exposed to SDZ PSC 833 under co-treatment conditions. The level of Pgp function inhibition brought by this RMA co-treatment was then measured by the DAU retention of the MDR cells expressed as the percentage of the DAU retention of the respective Par cells in the presence of solvent alone (Table 3).

The results showed that a complete restoration of DAU retention could be obtained with MDR-LoVo cells treated with 5 μg/ml SDZ PSC 833 during the DAU-loading phase. A marked restoration of DAU retention was also obtained with the highly resistant MDR-KB cells by 10 μg/ml SDZ PSC 833, reaching 66% of the retention shown by Par-KB cells, but only 75% of the Par-KB cell DAU retention could be reached at 100 μg/ml SDZ PSC 833.

Nevertheless, as shown earlier for the murine MDR-P388 cells,[12] the human MDR-LoVo cells and MDR-KB cells could be strongly sensitized by exposure to SDZ PSC 833. Therefore, the very poor efficiency of SDZ PSC 833 in the DAU retention restoration for MDR-CEM cells was in sharp contrast with its high activity on two other human MDR tumor cell lines (Figure 1).

Table 3. DAU retention in human MDR-LoVo and MDR-KB cells treated with SDZ PSC 833 during the DAU-loading phase (co-treatment procedure)[a]

SDZ PSC 833 (μg/ml)	MDR-LoVo[b]	MDR-KB[c]
0	7.6 ± 1.1	7.6 ± 0.2
0.3	13.4 ± 4.5	8.9
1	32.7 ± 10.8	16.4 ± 1.8
1.5	52.5 ± 5.6	
3	70.1 ± 8.7	37.2
5	97.4 ± 10.3	
10	105.3 ± 10.1	67.3
50	111.1 ± 19.9	71.9
100		74.7

[a] Expressed as percentage of DAU retention in Par cells in the presence of the ethanol solvent alone; all samples contained 1% ethanol.
[b] Means ± SD values of two to five independent experiments.
[c] Means ± SD values of one or two independent experiments.
SDZ PSC 833 concentrations were as μg ml (1 μg/ml = about 0.8 μM).

Table 2. DAU retention in human MDR-CEM cells treated with SDZ PSC 833 or CsA during the DAU-loading phase (co-treatment procedure)[a]

RMA[a]	Par-CEM		MDR-CEM	
	SDZ PSC 833	CsA	SDZ PSC 833	CsA
0	100 ± 0		1.2 ± 0.4	
0.1	109 ± 19		1.3 ± 0.5	
0.3	102 ± 8		1.4 ± 0.6	
1	105 ± 3	106 ± 4	2.3 ± 0.8	1.4 ± 0.4
3	102 ± 6	106 ± 4	5.7 ± 2.0	1.5 ± 0.5
10	105 ± 6	103 ± 9	9.8 ± 5.5	2.6 ± 0.5
30		107 ± 8		3.1 ± 0.5
50	113 ± 16		16.9 ± 11.1	

[a] The DAU retention of RMA-treated Par cells is expressed as percentage of ethanol-treated Par cell retention. The MDR cell DAU retention is expressed as percentage of Par cell DAU retention treated by the same RMA concentrations. All samples contained 0.5% ethanol as solvent. Results are means ± SD values from six independent experiments.
[b] SDZ PSC 833 and CsA concentrations were as μg/ml (1 μg/ml = about 0.8 μM).

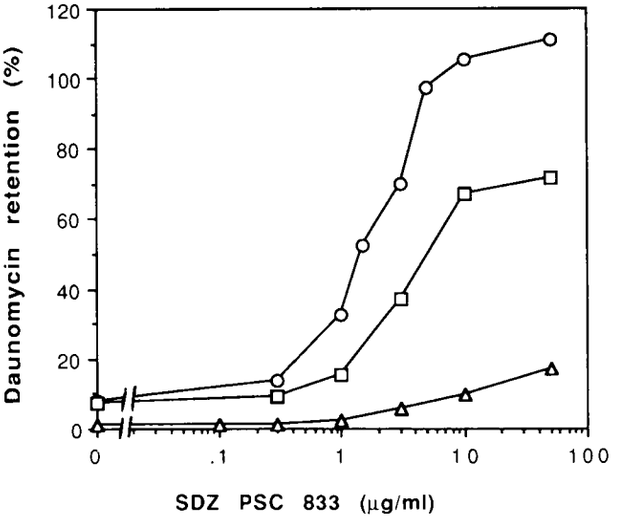

Figure 1. SDZ PSC 833 effect on DAU retention in three different human MDR cell lines. Mean values from independent experiments performed in co-treatment procedure conditions. (○) MDR-LoVo cells (two to five experiments), (□) MDR-KB cells (one or two experiments) and (△) MDR-CEM cells (six experiments).

Poor restoration of DAU retention in MDR-CEM cells by co-treatment with verapamil or SDZ 280-446

Since these MDR-CEM cells might represent a case of 'atypical MDR', these experiments were repeated several times and included two other RMA, verapamil, which is a classical Pgp-directed RMA but may work differently from CsA,[14] and the novel cyclopeptolide, SDZ 280-446, which was previously found to be only 3-fold less active than SDZ PSC 833 for DAU retention restoration in MDR-P388 cells.[5]

The data confirmed the poor activity of CsA and SDZ PSC 833 for restoring DAU retention, and further indicated that verapamil was even slightly less active than CsA, while SDZ 280-446 showed a level of activity virtually identical to that of SDZ PSC 833 (Table 4).

Higher DAU retention in MDR-CEM cells by RMA persistence

With MDR-P388 cells, a higher RMA efficacy in the restoration of the retention of a Pgp probe such as rhodamine-123[15] was obtained by exposing the cells to the RMA not only during the Pgp probe loading phase ('co-treatment' procedure), but also during the further washing steps and the Pgp probe efflux phase ('permanent treatment' procedure).

With the MDR-CEM cells and with all four tested RMA (verapamil, CsA, SDZ PSC 833 and SDZ 280-446), a stronger DAU retention was definitely restored when the RMA was present from the DAU loading to flow cytometry analyses

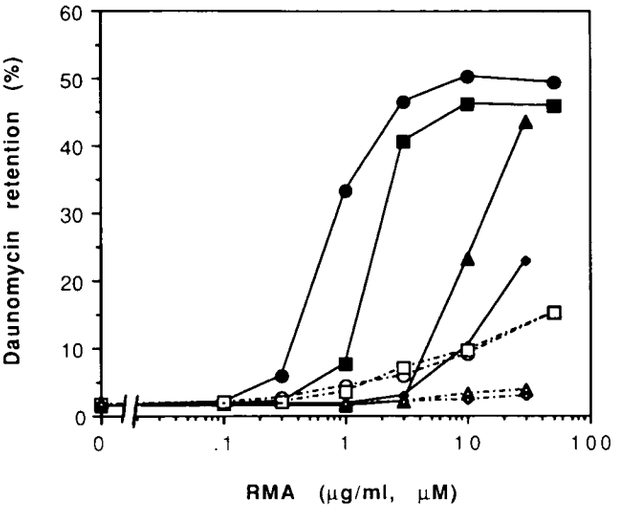

Figure 2. Comparison of the 'co-treatment' and 'permanent treatment' procedures for DAU retention by MDR-CEM cells. Mean values from six to eight experiments. Dotted lines and open symbols represent the co-treatment procedure (RMA present during the DAU-loading phase only); plain lines and closed symbols represent the permanent treatment procedure (RMA always present, during both the DAU loading and DAU efflux phases). (○, ●) SDZ 280-446, (□, ■) SDZ PSC 833, (△, ▲) CsA and (◇, ◆) verapamil.

('permanent treatment' procedure) than when the RMA was present only during the DAU-loading phase and not afterwards ('co-treatment' procedure) (Figure 2). In fact, in the latter 'co-treatment' procedure, verapamil and CsA had no significant activity, while SDZ 280-446 and SDZ PSC 833 had a similarly low activity. However, it did not seem possible to restore DAU retention completely in MDR-CEM cells: the level of DAU retention

Table 4. DAU retention in human MDR-CEM treated with RMA during the DAU-loading phase (co-treatment procedure)[a]

RMA[b]	SDZ PSC 833	SDZ 280-446	CsA	Verapamil
0		1.8 + 0.5		
0.1	2.0 ± 0.6	2.1 ± 0.4		
0.3	2.0 ± 0.4	2.7 ± 0.6		
1	3.5 ± 1.0	4.4 ± 0.9	1.9 ± 0.6	1.8 ± 0.5
3	7.2 ± 1.6	5.9 ± 1.3	2.1 ± 0.5	2.2 ± 0.5
10	9.8 ± 2.7	9.2 ± 4.0	3.3 ± 0.8	2.5 ± 0.6
30			3.9 ± 1.0	2.9 ± 0.7
50	15.4 ± 5.8	15.3 ± 7.4		

[a] The MDR cell DAU retention is expressed as percentage of Par cell DAU retention treated by the same RMA concentrations. All samples contained 0.5% ethanol as solvent. Results are means ± SD values from nine to 10 independent experiments.
[b] SDZ PSC 833. SDZ 280-446 and CsA concentrations were as μg/ml (1 μg/ml = about 0.8 μM), while verapamil concentrations were μM.

Table 5. Effect of the permanent exposure to the RMA (permanent treatment procedure) on the DAU retention in MDR-CEM cells[a]

RMA[b]	SDZ PSC 833	SDZ 280-446	CsA	Verapamil
0		1.6 ± 0.5		
0.1	1.9 ± 0.9	2.2 ± 0.8		
0.3	2.1 ± 0.9	5.9 ± 4.0		
1	7.6 ± 2.8	33.3 ± 7.4	1.6 ± 0.7	1.9 ± 0.7
3	40.5 ± 9.2	46.6 ± 5.6	2.1 ± 0.8	2.9 ± 0.5
10	46.3 ± 9.4	50.3 ± 7.2	23.1 ± 6.7	10.2 ± 3.9
30			43.4 ± 9.2	22.8 ± 12.9
50	45.9 ± 11.6	49.3 ± 9.9		

[a] The MDR cell DAU retention is expressed as percentage of Par cell DAU retention treated by the same RMA concentrations. All samples contained 0.5% ethanol as solvent. Results are means ± SD values from six to eight experiments.
[b] SDZ PSC 833, SDZ 280-446 and CsA concentrations were as μg/ml (1 μg/ml = about 0.8 μM), while verapamil concentrations were μM.

reached a plateau at about 50% of the Par-CEM cell level (permanent treatment, Figure 2).

Further calculations of the DAU retention data obtained with the 'permanent treatment' procedure (Table 5) showed the following. By comparing the levels of DAU retention restored at similar concentrations, verapamil looked to be half as active as CsA: 10% at 10 μM verapamil versus 23% at 10 μg/ml CsA; and 23% at 30 μM verapamil versus 43% at 30 μg/ml CsA. However, another way to compare the CsA/verapamil data was that the same low but substantial (23%) restoration of DAU retention was obtained by the permanent presence of 30 μM verapamil or 10 μg/ml (8 μM) CsA. Thus, depending on how the comparison was made, CsA was from 2- to 4-fold more active than verapamil. Similarly, to reach at least 40% of DAU retention required 30 μg/ml CsA but only 3 μg/ml SDZ PSC 833 and SDZ 280-446, suggesting that CsA might be an order of magnitude weaker than the other two RMA. A precise comparison of the latter two RMA with each other would require additional assays; however, at 1 μg/ml, SDZ 280-446 was substantially stronger than SDZ PSC 833. Thus, these DAU retention restorations showed a sharp RMA concentration dependence, particularly with SDZ 280-446 which was the most active RMA with this MDR cell-drug assay system. The clearest message was that even 50 μg/ml SDZ 280-446 could not restore more than 50% of the Par-CEM cell DAU retention.

Chemosensitization for MDR-CEM cell growth inhibition by ACD

The four different RMA, i.e. verapamil, CsA, SDZ PSC 833 and SDZ 280-446, were assayed for their chemosensitization activity. There was little inhibition of cell growth by the RMA alone at 1 μM for verapamil or 1 μg/ml (about 0.8 μM) for the cyclic peptides: the highest degree of inhibition (15%) being observed for CsA on the Par-CEM cells, but none of the four RMA showed significant toxicity for the MDR-CEM cells (Table 6).

Exposure to the RMA did not detectably sensitize the Par-CEM cells to the tested ACD, except possibly a 2.5-fold sensitization ('gain') to vincristine (Table 7), a degree of sensitization which might not be biologically interesting even when statistically significant. On the contrary, higher gains could be obtained with the MDR-CEM cells, although very variable depending on the RMA and ACD. There was virtually no detectable MDR-reversing activity of 1 μg/ml CsA or 1 μM verapamil for any ACD tested, whereas 1 μg/ml of either SDZ PSC 833 or SDZ 280-446 showed a biologically significant MDR-reversing activity with all ACD tested. Thus, 1 μg/ml (about 0.8 μM) SDZ PSC 833 and SDZ 280-446 increased the sensitivity of those MDR-CEM cells by, respec-

Table 6. Inhibition of cell growth induced by the RMA alone (%)[a]

	Par-CEM	MDR-CEM
SDZ PSC 833	6.9 ± 2.1	1.4 ± 2.4
SDZ 280-446	4.5 ± 4.7	1.0 ± 1.3
CsA	14.9 ± 1.8	0.9 ± 1.5
Verapamil	2.8 ± 4.5	5.8 ± 2.6

[a] 100 − (OD RMA treated cells × 100/OD solvent treated cells). The data represent the mean ± SD values calculated from three individual experiments. SDZ PSC 833, SDZ 280-446 and CsA were used at 1 μg/ml (about 0.8 μM), while verapamil was used at 1 μM.

Table 7. ACD IC_{50} obtained in the presence or absence of RMA and gain values

	IC_{50} (ng/ml)		Gain	
RMA	Par-CEM	MDR-CEM	Par-CEM	MDR-CEM
DAU				
0	5.8 ± 0.1	5575 ± 796		
SDZ PSC 833	5.5 ± 0.0	165 ± 58	1.0 ± 0.0	37.9 ± 10.1
SDZ 280-446	5.5 ± 0.1	75 ± 13	1.0 ± 0.0	76.2 ± 2.6
CsA	5.7 ± 0.4	4867 ± 910	1.0 ± 0.0	1.2 ± 0.1
verapamil	5.7 ± 0.1	4193 ± 1207	1.0 ± 0.0	1.3 ± 0.2
Colchicine				
0	2.2 ± 0.2	858 ± 43		
SDZ PSC 833	2.1 ± 0.0	29 ± 7	1.1 ± 0.1	30.2 ± 2.5
SDZ 280-446	2.1 ± 0.0	15 ± 1	1.1 ± 0.0	57.3 ± 7.9
CsA	2.0 ± 0.1	655 ± 29	1.1 ± 0.1	1.3 ± 0.1
verapamil	2.3 ± 0.1	790 ± 8	0.9 ± 0.1	1.1 ± 0.0
Taxol				
0	3.8 ± 2.3	4443 ± 2627		
SPZ PSC 833	2.5 ± 1.6	1258 ± 793	1.6 ± 0.2	3.9 ± 0.7
SDZ 280-446	2.4 ± 1.6	77 ± 1	1.6 ± 0.2	81.5 ± 1.0
CsA	2.6 ± 1.7	3787 ± 2305	1.5 ± 0.1	1.2 ± 0.1
verapamil	3.3 ± 2.0	3420 ± 2093	1.1 ± 0.0	1.3 ± 0.1
Vincristine				
0	1.7 ± 0.17	6550 ± 1400		
SPZ PSC 833	0.6 ± 0.01	1360 ± 490	2.4 ± 0.1	5.2 ± 1.0
SDZ 280-446	0.7 ± 0.03	141 ± 38	2.2 ± 0.1	51.0 ± 1.0
CsA	0.6 ± 0.02	6650 ± 2205	2.6 ± 0.1	1.1 ± 0.0
verapamil	1.1 ± 0.32	4317 ± 807	1.6 ± 0.5	1.8 ± 0.0
Actinomycin D				
0	0.7 ± 0.2	2565 ± 423		
SDZ PSC 833	0.6 ± 0.1	221 ± 12	1.1 ± 0.0	10.6 ± 0.7
SDZ 280-446	0.6 ± 0.1	60 ± 29	1.2 ± 0.0	61.1 ± 47.8
CsA	0.6 ± 0.1	2027 ± 168	1.1 ± 0.0	1.1 ± 0.1
verapamil	0.7 ± 0.2	2087 ± 77	1.1 ± 0.0	1.3 ± 0.3

The data represent the mean IC_{50} ± SD values from two to three individual experiments and the calculated gains. SDZ PSC 833, SDZ 280-446 and CsA were used at 1 μg/ml (about 0.8 μM), while verapamil was used at 1 μM.

tively, 38- and 76-fold for DAU, 30- and 57-fold for colchicine, 4- and 82-fold for taxol, 5- and 51-fold for vincristine, and 11- and 61-fold for actinomycin D (Table 7). Interestingly, SDZ 280-446 thus gave higher and rather homogeneous sensitivity gains (from 50- to 80-fold) for the different ACD, while SDZ PSC 833 chemosensitizing activity was very unbalanced with a 4-fold only for taxol and a maximum 38-fold for DAU (Table 7).

Discussion

This Pgp-expressing human T lymphoblastoid MDR-CEM cell line (designated CCRF ACTD400+), which had been selected for MDR by actinomycin D,[6] was found to display a very high resistance phenotype, in comparison with Par-CEM cells. Particularly, its 1000-fold DAU resistance could virtually not be reduced by verapamil (1 μM) or CsA (1 μg ml), while it was decreased 40 (±10)-fold by SDZ PSC 833 and 76 (±3)-fold by SDZ 280-446, both RMA being used at the 1 μg ml dosages which were not directly toxic for the cells in long-term assays (no significant inhibition of the cell growth in *in vitro* culture).

A short-term assay for the capacity of Pgp-directed RMA to neutralize Pgp function consisted in measuring their capacity to restore the intracellular DAU retention, by measuring the specific fluorescence of this anti-cancer anthracyc-

line. This assay allowed testing of inhibition of a membranous function using high concentrations of the RMA that would be quickly toxic for proliferating cells. When the cells were exposed to the RMA during their DAU-loading phase ('co-treatment' procedure), several RMA were shown to restore DAU retention in MDR-P388 cells to the Par-P388 cell levels, although with different efficiencies.[12]

With this MDR-CEM cell line, a marginal increase of DAU retention required 10 μg/ml CsA or verapamil, and even 10 μg/ml of our most potent RMA gave little increase of DAU retention in the MDR-CEM cells in comparison with the DAU retention shown by the Par-CEM cells. In fact, with this cell line and this read-out, SDZ PSC 833 and SDZ 280-446 appeared to be only slightly more active than CsA when applied on the cells only during the DAU-loading phase. Exposure of the MDR-CEM cells to 10 μg/ml SDZ 280-446, during the DAU-loading phase only, could restore their DAU retention to only 10% of the level shown by Par-CEM cells. This low restoration of DAU retention could not be simply explained by a too high Pgp expression, since 10 μg/ml SDZ PSC 833 could completely restore the DAU retention in the highly Pgp expressing MDR-LoVo cells of human origin. In the MDR-KB cells which highly overexpress Pgp, the retention of DAU was not fully restored (75% only) by 100 μg/ml SDZ PSC 833, but the activity of the RMA was already substantial at 1 and 3 μg/ml.

When this short-term assay of RMA activity was performed with MDR-P388 cells, higher levels of restoration could be obtained when the MDR cells were exposed to the RMA not only during the loading phase of the Pgp probe but also during the efflux phase.[15] When such a 'permanent treatment' procedure was assayed with the MDR-CEM cells, the activity of all RMA was indeed increased. Their capacity to restore the DAU retention emerged with the following order of potency: SDZ 280-446 (3- to 4-fold) > SDZ PSC 833 (3- to 10-fold) > CsA (2- to 4-fold) > verapamil. Yet, even the activity of SDZ 280-446 was still far from being high enough to completely reverse the MDR property. By exposing the MDR-CEM cells permanently to the RMA and by increasing the concentration of our best RMA up to 50 μg/ml, it was still not possible to fully restore the DAU retention. On the contrary, there was evidence that the level of DAU retention reached a plateau at about 50%. This could indicate that these MDR-CEM cells had a second mechanism for reducing DAU influx and/or increasing DAU efflux, which was not sensitive to our RMAs, i.e. a mechanism refractory to Pgp-directed RMAs.

Therefore, it is probable that this MDR-CEM cell line does not only use Pgp-mediated efflux to reduce its intracellular DAU accumulation. Whether this or these additional mechanism(s) is/are drug influx and/or efflux related is unknown.[16,21] Moreover, there is no need to postulate a new or totally different mechanism of efflux. Such an efflux might still be mediated by Pgp-*like* molecules or even by *mdr*1-encoded Pgp molecules made refractory to RMA-induced neutralization, by their interaction with other intracellular or membranous components of those MDR-CEM cells. For instance, phosphorylation of a given site on Pgp might increase Pgp function and this site might be (constitutively or not) hyperphosphorylated in MDR-CEM cells. If our RMA were not able to inhibit Pgp in its phosphorylated form, we would not be able to show a full restoration of DAU retention in the absence of phosphorylation inhibitors. Thus RMA-insensitive resistance mechanisms might be strictly based on *bona fide* Pgp and it is not really mandatory to call for paranormal ('atypical') efflux mechanisms. With MDR-P388 cells, several RMA were able to restore DAU retention completely,[12] although they could not do it for rhodamine-123,[15] suggesting the two Pgp probes might be handled by Pgp somewhat differently. Since DAU did not seem to be a stringent probe of Pgp function but could be also handled by an atypical pump,[20,21] it might be worth extending the study of this MDR-CEM cell line to other Pgp probes.

The unbalanced chemosensitization of MDR-CEM cells for different ACD by SDZ PSC 833 (from 5- to 38-fold) together with a rather stable chemosensitization power of SDZ 280-446 (from 51- to 82-fold) was intriguing, although previous studies comparing SDZ PSC 833 and SDZ 280-446 on other MDR cell lines showed that the levels of sensitization obtained by both of these RMAs could be rather unbalanced for different ACD.[4,5] At the molecular and membranous levels, the significance of such different degrees of chemosensitization is at present unknown.

However, the concept that different pharmacophores might be involved in the transport of different drugs by the *mdr*1-encoded Pgp is now emerging.[3,15] ACD used for selecting MDR variants of tumor cells are Pgp substrates and may lead to further somatic selection of mutants with altered profiles of resistance. At least *in vitro*, a series of

MDR-KB cell lines obtained by independent selection with colchicine, vinblastine or doxorubicin displayed a definitely preferential relative resistance to the ACD used for selection, although they showed a broad cross-resistance to the other drugs.[1] Single point mutations were shown to be sufficient to dramatically affect the profile of resistance of MDR-KB cells conferred by the germline Pgp, such as the marked alteration of the relative resistance to colchicine, vinblastine and doxorubicin, caused by a single glycine–valine interchange.[22] Similarly, mutations in the hamster *pgp*1 gene conferred a distinctive MDR profile to actinomycin D-selected sublines of MDR-DC/3F cells: highest resistance was now for actinomycin D instead of colchicine, as in the parental line.[23] Thus, while a mutation increased colchicine-resistance in one instance,[22] it decreased it in the other.[23]

Similarly, competition experiments also indicated the existence of distinct drug-binding sites. The colchicine binding/transport site on Pgp might be distinct from the Vinca alkaloid one: colchicine did not inhibit the *in vitro* photolabeling of Pgp by a vinblastine analog, whereas vinca alkaloids, verapamil and diltiazem, were able to do so.[24,25] Both calcium channel blockers were also able to inhibit Pgp photolabeling by a cyclosporin analog, whereas colchicine had no effect.[26]

Could such a selectivity be reflected at the level of RMA activity? SDZ PSC 833 was found more active than CsA, using four pairs of Par and MDR

Table 8. Comparisons of the chemosensitizing strengths of SDZ PSC 833 and SDZ 280-446 relative to CsA for five different MDR cell lines[a]

MDR tumor cells (*selected with*)	ACD	How many fold better than CsA?		Concentration of RMA at which it was measured (μg/ml)
		SDZ PSC 833	SDZ 280-446	
MDR-CHO cells (*colchicine*)	colchicine	50	65	0.1
	vincristine	35	48	
	etoposide	53	41	
	DAU	97	85	
MDR-P388 cells (*doxorubicin*)	colchicine	30	17	0.3
	vincristine	105	45	
	etoposide	7.4	3.6	
	DAU	12	6.8	
	doxorubicin	6.5	7.2	
MDR-LoVo cells (*doxorubicin*)	colchicine	64	53	1.0
	vincristine	154	20	
	etoposide	154	5.7	
	DAU	32	15	
MDR-KB cells (*vinblastine*)	colchicine	3.4	11	1.0
	vincristine	2.3	9.7	
	etoposide	2.3	4.9	
	DAU	4.9	6.1	
MDR-CEM cells (*actinomycin D*)	colchicine	23	44	1.0
	vincristine	4.7	46	
	DAU	32	64	
	taxol	3.3	68	
	actinomycin D	9.6	56	

[a] Such comparisons had to be made at *different* concentrations of the RMA for the different MDR cell lines. Indeed, the RMA concentrations had to fall within linear areas of the RMA dose–chemosensitizing response relations, i.e. to give a significant chemosensitization, but far enough from the plateau of response. Moreover, the RMA concentrations had also to be such that CsA, the reference RMA, was giving a significant response, i.e. far enough below the concentration at which it inhibited the cell growth by itself (e.g. 1 μg/ml could not be used for MDR-P388 cells because of direct CsA toxicity). Other ACD: not done or only preliminary/incomplete data.

cell lines from three different species [hamster (CHO), mouse (P388) and man (KB and LoVo)] and four different cellular origins [ovarian carcinoma (CHO), monocytic leukemia (P388), nasopharyngeal carcinoma (KB) and colon carcinoma (LoVo)].[4] The MDR cell sublines had been derived from the Par cell line by selective pressure for growth in the presence of three different ACD [colchicine (CHO), vinblastine (KB) and doxorubicin (LoVo and P388)]. That SDZ PSC 833 was a stronger RMA than CsA was also found with a variety of other MDR tumor cell lines, among which several other MDR sublines of CHO cells selected under colchicine pressure,[27] an MDR-L1210 murine leukemia selected under doxorubicin pressure,[28] a human small cell lung carcinoma NCI-HP69/LX4 cell line and a mouse EMT6/AR1.0 tumor cell line both selected under doxorubicin pressure,[29] as well as several other MDR cell lines where SDZ PSC 833 was compared with CsA (private communications).

Using the aforementioned four pairs of cell lines,[5] small differences of RMA powers between SDZ PSC 833 and SDZ 280-446 were commonly observed (Table 8). SDZ PSC 833 and SDZ 280-446 had rather similar strengths for restoring sensitivity of the MDR-CHO cells to *different classes of ACD*, i.e. they were not found to be ACD specific. Nevertheless, when SDZ PSC 833 and SDZ 280-446 were compared for their capacity to restore sensitivity to a given ACD in *different MDR cell lines*, SDZ PSC 833 was more active on the MDR-P388 and MDR-LoVo cell lines, both of which were selected to MDR by doxorubicin[5], whereas SDZ 280-446 was more active on the MDR-KB cell line selected with vinca alkaloids[5] and on the MDR-CEM cell line selected with actinomycin D (this paper).

It is thus interesting that, for the restoration of vincristine sensitivity in different MDR cell lines, SDZ PSC 833 somewhat differed from SDZ 280-446 (Table 8): both were about equal for MDR-CHO cells (colchicine selection), but SDZ PSC 833 was 2-fold better for MDR-P388 cells (doxorubicin selection), 7.5-fold better for MDR-LoVo cells (doxorubicin selection), 4-fold weaker for MDR-KB cells (vinblastine selection) and 10-fold weaker for MDR-CEM cells (actinomycin D selection). Therefore, SDZ PSC 833 might preferentially affect the pharmacophores on Pgp mutants selected under doxorubicin pressure, whereas SDZ 280-446 might do it better with Pgp mutants selected under vinca alkaloid or actinomycin pressure.

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