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RESTORING UPTAKE AND RETENTION OF DAUNORUBICIN AND IDARUBICIN IN P170-RELATED MULTIDRUG RESISTANCE CELLS BY LOW CONCENTRATION D-VERAPAMIL, CYCLOSPORIN-A AND SDZ PSC 833

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ABSTRACT

Background. **The overexpression of the mdr-1 gene that codes for a 170 Kd transmembrane glycoprotein (P170) is a factor accounting for decreased cell sensitivity to anthracyclines and other drugs and is related with treatment failure in acute leukemia and in other tumors. Several agents, including verapamil and cyclosporin derivatives, can modify P170-related resistance in vitro and can be proposed as adjuvant treatment for leukemia and cancer.**

Materials and Methods. **To investigate the optimal conditions for adjuvant treatment, D-verapamil (D-VRP), cyclosporin-A (CyA) and the cyclosporin derivative SDZ PSC 833 (PSC) were used alone and in combinations, at drug concentrations that can be achieved and maintained in vivo. The drugs were tested for their capacity of restoring intracellular daunorubicin (DNR) and idarubicin (IDA) accumulation in high-resistant (CEM VLB 300) and in intermediate-resistant (CEM VLB 100) cells, by comparison with the non-resistant, parental cell line (CCRF CEM).**

Results. **In intermediate-resistant cells, IDA alone was more convenient than DNR plus modifiers, and full restoration of intracellular IDA concentration to the level of non-resistant cells was obtained with low dose D-VRP (1 μM), CyA (0.8 μM) and PSC (0.4 μM). In high-resistant cells no modifier or modifier combination was able to restore intracellular DNR concentration to the value of non-resistant cells. Intracellular IDA concentration was nearly completely restored only with D-VRP (2-3 μM) and CyA (0.8-1.6 μM) in combination or with PSC alone (0.4 μM).**

Conclusions. **These data suggest that as it concerns P170-related resistance, IDA alone is as efficient as or even more efficient than DNR plus modifiers, and that residual resistance to IDA can be overcome with a combination of D-VRP and Cy-A at a clinical achievable concentration, or with a more powerful modifier like SDZ PSC 833.**

Key words: anthracyclines, multidrug resistance, multidrug resistance modifiers

In vitro established tumor and leukemia cell lines can become resistant to antitumor agents by amplification or by overexpression of a gene that is called mdr-1 and that codes for a 170 Kd transmembrane glycoprotein (P170) that acts as an ATP-related efflux pump.[1-5] By that pump, the intracellular concentration of a number of cytotoxic drugs, including anthracyclines, anthracenedione and epipodophylline derivatives as well as Vinca alkaloids and others, is lowered, and cells become resistant (pleiotropic or multidrug resistance, MDR).

In vitro, it was well shown that the degree of

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MDR was related with P170 expression and that a number of different agents were able to interfere with P170 and to restore cell sensitivity to cytotoxic drugs.[6-11] These agents are usually referred to as MDR revertants or modifiers. *In vivo*, several independent studies showed that P170 overexpression in tumor cells was related with tumor treatment failure,[12-18] providing a rationale for the clinical or therapeutic application of MDR modifiers. However, the application is not simple because for many MDR modifiers the concentrations that are required to restore the sensitivity *in vitro* cannot be achieved or maintained *in vivo*.[6-11, 19-22]

Based on these considerations, we investigated the effects of three MDR modifiers alone and in combination in an *in vitro* model including sensitive, intermediate-resistant and high-resistant cells, testing for daunorubicin (DNR), that is the first-line anthracycline for leukemia treatment, and for its 4-demethoxy derivative idarubicin (IDA), that was already shown to be less influenced by P170 action mechanism.[10, 23-27]

The aim of this study was to find out if it was possible to restore full sensitivity to anthracyclines in intermediate-resistant and in high-resistant cells, by using MDR-modifiers at a clinically acceptable concentration.

Materials and Methods

Drugs

Daunorubicin (DNR) and idarubicin (IDA) were purchased from Farmitalia-Carlo Erba, Italy. Cyclosporin-A (Cy-A) and SDZ-PSC 833 (PSC) were a gift from Sandoz, CH. D-verapamil (D-VRP) was a gift from Knoll, Italy. Anthracyclines were dissolved in distilled water at 100 μg/mL. PSC and Cy-A were dissolved in ethanol and D-VRP was dissolved in methanol. All the drugs were aliquoted and stored at –20°C.

Cell lines

For this study, we used the CCRF-CEM (CEM) cell line that was obtained from a case of human T-cell acute lymphocytic leukemia and its MDR sublines that were named CEM 100 and CEM 300 because they were selected and maintained with vinblastine at a concentration of 100 and 300 ng/ml respectively.[10,25,28-29] The relevant features of these cells are shown in Table 1. Parental CEM cells did not express detectable amounts of mdr-1 mRNA, reacted very weakly with the P170-directed MRK-16 monoclonal antibody, and by the MTT assay[25-30] were inhibited by low anthracycline doses. CEM 100 (intermediate-resistant cells) and CEM 300 (high-resistant cells) were different as to mdr-1 mRNA, P170 expression and in vitro sensitivity to DNR and to IDA (Table 1)[31-32]

Intracellular anthracyclines uptake studies

Intracellular anthracyclines concentration was determined by flow cytometry with a FACScan (Becton Dickinson, BD) equipped with an argon laser tuned at 488 nm. The Lysis II software package (BD) was used to generate FL2 histograms to calculate the means of the linear fluorescence intensity distribution and to generate the forward scatter (FCS) histograms used to calculate the relative cell volumes. Results were expressed as the normalized mean fluorescence index (NMFI), according to Luk.[33] For DNR and IDA uptake studies, exponentially growing cells were collected, washed twice in RPMI, checked for vitality with the tripan blue exclusion test and counted; 2.5×10^6 cells/mL were resuspended in medium alone, and in a medium containing increasing doses of DNR or IDA (range 100-3000 ng/mL) and incubated for

Table 1. P170 expression (mean fluorescence index with MRK-16) and anthracyclines sensitivity (inhibition dose 50 by MTT assay) in sensitive CEM cells, in intermediate-resistant CEM 100 cells and in high-resistant CEM 300 cells.

cells	*P170 expression (MFI)*	*inhibition dose 50 (ng/mL) DNR*	*IDA*
CEM	4	4	2.5
CEM 100	16	75	7.0
CEM 300	26	220	10.0

two hours at 37°C with 5% CO_2. After two washes in ice-cold phosphate buffered saline (PBS), the cells were kept on ice and immediately analysed with the flow cytometer. Because a significant linear correlation was found between drug dose exposure and fluorescence intensity (NMFI) (Figure 1), in the experiments with D-VRP, Cy-A and PSC, anthracycline concentration was standardized at 1000 ng/mL. Controls consisted in cells plus modifiers, cells plus modifier solvents, or cells plus anthracyclines as appropriate.

Results

The effects of D-VRP, Cy-A and PSC on anthracycline cell content is shown in Table 2 and in Figure 2. In sensitive CEM cells the increase in anthracycline content was small, ranging between 5 and 20%. In MDR cells the increase in anthracycline content was much greater, but it varied according to the degree of the resistance of the cells (CEM 100 and CEM 300), to the drugs (DNR and IDA) and to the modifiers. In intermediate-resistant CEM 100 cells, DNR content was increased several folds

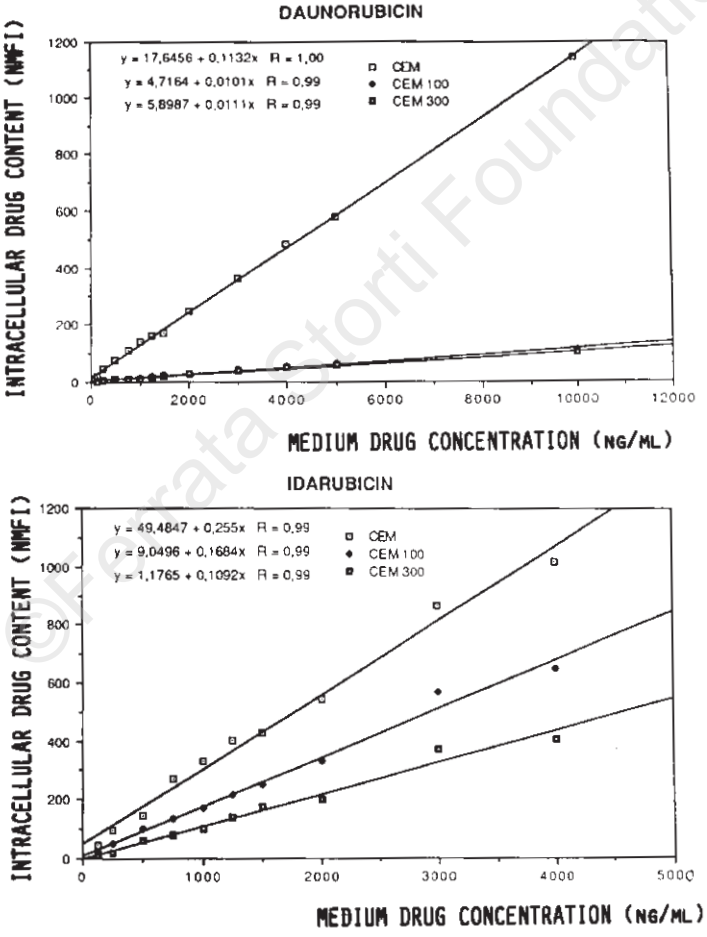

Figure 1. Relationship between medium drug concentration and intracellular drug content (normalized mean fluorescence index or NMFI) after 2-hour drug exposure. The relationship was linear for both anthracyclines, either in the parental, non-MDR line (CEM) or in the two MDR lines (CEM 100 and CEM 300).

Table 2: Anthracyclines cell content without (control) and with D-VRP, Cy-A or PSC. The content was expressed as the normalized mean fluorescence index, as described in Methods. CEM, CEM 100 and CEM 300 are the sensitive, intermediate-resistant, and the high resistant cell lines.

		daunorubicin			*idarubicin*		
		CEM	CEM 100	CEM 300	CEM	CEM 100	CEM 300
CONTROL (no modifiers)		147	16	12	334	170	100
D-VRP	1 μM	161 (+9%)	33 (+106%)	16 (+33%)	362 (+8%)	353 (+108%)	142 (+42%)
	2 μM	163 (+11%)	64 (+300%)	18 (+50%)	385 (+15%)	357 (+110%)	139 (+39%)
	3 μM	151 (+3%)	80 (+400%)	24 (+100%)	400 (+20%)	350 (+106%)	168 (+68%)
Cy-A	0.8 μM	168 (+14%)	55 (+244%)	16 (+33%)	392 (+17%)	284 (+67%)	156 (+56%)
	1.6 μM	167 (+14%)	99 (+519%)	18 (+50%)	400 (+20%)	364 (+114%)	190 (+90%)
PSC	0.4 μM	154 (+5%)	109 (+581%)	33 (+175%)	389 (+16%)	311 (+83%)	200(+100%)
	0.8 μM	163 (+11%)	118 (+637%)	65 (+442%)	390 (+17%)	340 (+100%)	238(+138%)
	1.6 μM	163 (+11%)	125 (+681%)	69 (+475%)	391 (+17%)	341 (+100%)	249(+149%)

by all modifiers, and IDA content was approximately doubled. In high-resistant CEM 300 cells, DNR content was doubled only by D-VRP 3 μM and was more than doubled only by PSC. In the same CEM 300 cells, IDA content was doubled only with PSC.

Table 3 shows the ratio between the anthracycline content of sensitive CEM cells and that of intermediate-resistant CEM 100 cells. A ratio close or equal to one, that would define full neutralization of P170-mediated cell resistance, was obtained for IDA with all modifiers at all

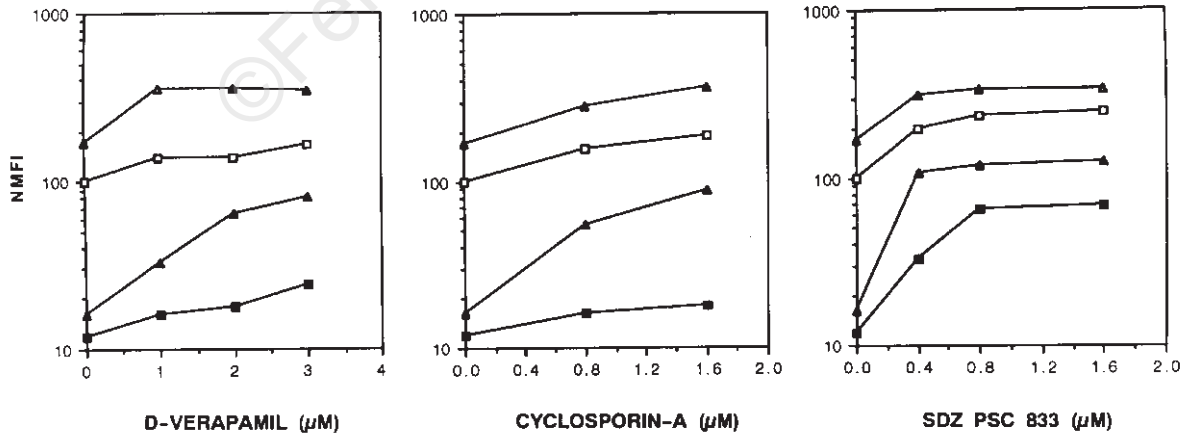

Figure 2. The effect of D-Verapamil, Cyclosprin-A and SDZ PSC 833 on the intracellular content of Daunorubicin (lower lines) and of Idarubicin (upper lines) high-resistant CEM 300 cells (squares) and in intermediate-resistant CEM 100 cells (triangles).

Table 3. Ratio between anthracycline cell content of sensitive CEM cells and of intermediate-resistant CEM 100 cells. A ratio close or equal to one is required to show that P170-associated resistance was completely overcome.

		daunorubicin		*idarubicin*	
		CEM/CEM 100	ratio	CEM/CEM 100	ratio
CONTROL (no modifiers)		147/16	9.2	334/170	2.0
D-VRP	1 μM	161/33	4.9	362/353	1.0
D-VRP	2 μM	163/64	2.5	385/357	1.1
D-VRP	3 μM	151/80	1.9	400/350	1.1
Cy-A	0.8 μM	168/55	3.0	392/284	1.4
Cy-A	1.6 μM	167/99	1.7	400/364	1.1
PSC	0.4 μM	154/109	1.4	389/311	1.2
PSC	0.8 μM	163/118	1.4	390/340	1.1
PSC	1.6 μM	163/125	1.3	391/341	1.1

Table 4. Ratio between anthracycline cell content of sensitive CEM cells and of high-resistant CEM 300 cells. A ratio close to one indicating full neutralization of P170-associated resistance, was approached only for IDA and only with PSC.

		daunorubicin		*idarubicin*	
		CEM/CEM 300	ratio	CEM/CEM 300	ratio
CONTROL (no modifiers)		147/12	12.2	334/100	3.3
D-VRP	1 μM	161/16	10.1	362/142	2.5
D-VRP	2 μM	163/18	9.0	385/139	2.8
D-VRP	3 μM	151/24	6.3	400/168	2.4
Cy-A	0.8 μM	168/16	10.5	392/156	2.5
Cy-A	1.6 μM	167/18	9.3	400/190	2.1
PSC	0.4 μM	154/33	4.7	389/200	1.9
PSC	0.8 μM	163/65	2.5	390/238	1.6
PSC	1.6 μM	163/69	2.4	391/249	1.6

tested concentrations. For DNR, neutralization was less complete and required higher concentration of D-VRP and Cy-A. Therefore Table 3 data show that intermediate MDR could be completely overcome with IDA and low dose modifiers.

Table 4 shows the ratio between the anthracycline content of sensitive CEM cells and that of high-resistant CEM 300 cells. A ratio close to 1 was obtained only for IDA and only with PSC. Resistance to DNR could not be completely overcome with any tested modifiers. With the aim of achieving full neutralization, CEM 300 cells were treated with a combination of D-VRP and Cy-A or PSC (Table 5).

For DNR the combination of D-VRP 2 to 3 μM with Cy-A 0.8 to 1.6 μM yielded better results than either modifiers alone but the lowest ratio were clearly unsatisfactory ranging between 4.8 and 5.2. The combination of PSC with D-VRP was also slightly better than PSC alone, and the lowest ratios ranged between 1.8 and 2.1.

For IDA, the combination of Cy-A with D-VRP led to a small improvement and the combination of PSC with DVRP was not better than PSC alone. However, by several combinations as well as by PSC alone, the ratio was lower than 2 and was always lower than for DNR.

Discussion

The mechanisms that cause cell sensitivity to antitumor agents and even more the factors that account for success or for failure of cancer chemotherapy are complex and multifactorial.[34-36] The application of the knowledge that was recently accumulated in this area, requires a stepwise process based on the identification of agents and procedures which have a substantial potential of affecting significantly a specific resistance mechanism. If one shares the basic concept that under the same conditions the difference between a MDR and a non-MDR cell lies in P170 function, the first specific target should be to identify the conditions by which P170-related resistance can be completely abolished. For that purpose, it is necessary to realize that conditions may be different depending on the properties of target cells and on the type of cytotoxic drugs, which are tested or used.

In this study, we tested DNR, that is a standard reference for first-line treatment of leukemia, and its derivative IDA, which is more expensive and more toxic, and was introduced only recently in the treatment of leukemia.[37-40] The choice of testing IDA was due not only to the studies reporting that IDA was as effective as or even more effective than DNR,[37-40] but more to the finding that cellular pharmacoki-

Table 5. Ratio between anthracycline content of sensitive CEM cells and of high-resistant CEM 300 cells, with D-VRP, Cy-A and PSC alone and in combination. DNR data are reported in the upper part of the table, showing that resistance to DNR remained substantial with D-VRP and Cy-A and was not completely overcome with PSC and D-VRP. IDA data are reported in the lower part of the table. Combinations were not much better than modifiers alone, and full restauration of sensitivity could not be achieved, but ratios became closer to one and were always lower than for DNR.

daunorubicin

PSC		//	//	//	0.8 μM	1.6 μM
Cy-A		//	0.8 μM	1.6 μM	//	//
D-VRP	0	12.2	10.0	9.3	2.5	2.4
	1 μM	10.0	9.4	9.1	2.5	2.0
	2 μM	9.0	6.5	5.2	2.3	2.0
	3 μM	6.9	5.2	4.8	2.1	1.8

idarubicin

PSC		//	//	//	0.8 μM	1.6 μM
Cy-A		//	0.8 μM	1.6 μM	//	//
D-VRP	0	3.3	2.5	2.1	1.6	1.5
	1 μM	2.5	2.1	1.7	1.8	1.4
	2 μM	2.8	1.7	1.5	1.6	1.4
	3 μM	2.4	1.7	1.4	1.5	1.4

netic of IDA is less P170-dependent than that of DNR, so that MDR cells are more sensitive to IDA than to DNR.[10, 23-27] To investigate the best procedure of restoring full sensitivity to these anthracyclines, we tested comparatively two cell sublines which shared the same origin but which differed significantly for mdr-1 expression, P170 content and drug sensitivity (Table 1). MDR modifiers included two agents that are used in current investigations, namely D-VRP and Cy-A at clinically achievable concentrations.[20, 41-45] The third modifier was a powerful cyclosporin derivative that was not yet available for clinical investigation.[10, 46-49]

This study confirmed that in MDR cells treatment with all modifiers, and expecially with PSC, was relatively more efficient in increasing intracellular DNR concentration than IDA concentration.[10, 23-27] However, the purpose of the study was not just increasing anthracycline cell concentration by comparison with untreated MDR cells, but bringing anthracycline cell concentration back to the same value of parental non-MDR cells. Achieving this result requires that the ratio between sensitive CEM cells and MDR cells becomes very close or equal to one. In intermediate-resistant CEM 100 cells IDA alone had a better ratio than DNR plus D-VRP 1-2 μM and DNR plus Cy-A 0.8 μM. Furthermore, IDA ratio was lowered to 1.0-1.4 by coincubation with each of all three modifiers at the lowest tested concentrations. In high-resistant CEM 300 cells, a DNR ratio of one could not be obtained with any modifiers, either alone or in combination. In contrast, an IDA ratio lower than 2 could be obtained both with PSC alone and with D-VRP and Cy-A in combination.

The application of these data to the treatment of cancer and leukemia is not straightforward. In any case, as far as P170-related MDR is concerned, IDA alone is likely to be always better than DNR. Moreover, low grade resistance to IDA but not to DNR can be overcome by exposure to relatively low concentration of D-VRP (1 μM) and of Cy-A (0.8 μM) alone. Higher concentration of D-VRP (2-3 μM) in combination with Cy-A (0.8-1.6 μM), or the use of more powerful modifiers like PSC, are required in cases of high grade resistance. Since IDA is more expensive and more toxic than DNR, in vitro testing of leukemic cells may be helpful prior to deciding the treatment, and it can be performed in real time. However, in vitro testing results would depend on the average expression of P170 in leukemic cells and could not pick up the cases where high-expression P170 cells are rare. In prior studies using immunocytochemistry to detect MDR leukemic cells, we found that also a proportion of less than 1% of these cells could predict for early relapse after a complete remission was achieved, hence for treatment failure.[15, 18]

Finally, it should not be overlooked that substitution of DNR with IDA may increase hematologic and non-hematologic toxicity. It was at least reassuring the finding that no modifiers, either alone (Table 2) or in combination (data not shown), was able to increase intracellular

anthracycline content of non-MDR cells of more than 20%.

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