Activity of cyclosporins as resistance modifiers in primary cultures of human haematological and solid tumours

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Summary The semiautomated fluorimetric microculture cytotoxicity assay (FMCA) was used for evaluation of the ability of cyclosporin A (CsA) and its novel non-immunosuppressive derivative SDZ PSC 833 (PSC) to modify the response to doxorubicin or vincristine in vitro in different haematological and solid human tumour types. Primary cultures of 322 tumour samples were analysed. Both cyclosporins showed resistance-modifying activity in all haematological tumours tested, and in solid tumours activity was observed in ovarian carcinoma and childhood tumours. Little or no effect was found in the remaining tumour types, including breast, renal and adrenal cortical carcinomas and adult sarcomas. In most of the responsive cases the interaction between the modifier and the cytotoxic drug was synergistic. There was a tendency to higher activity in samples from previously treated patients, and an inverse relationship between degree of cytotoxic drug resistance and resistance-modifying activity was noted. No difference in potency between CsA and PSC could be discerned. The results indicate differential in vitro resistance-modifying activity of the cyclosporins depending on tumour type. The results also suggest that treatment with resistance modifiers should be considered also for primary therapy of drug-sensitive tumours. Drug resistance assays such as the FMCA may become useful in preclinical evaluation of resistance modifiers.

Multidrug resistance (MDR) defines a cellular phenotype in which the development of resistance in vitro to one cytotoxic drug confers cross-resistance to other structurally and functionally dissimilar drugs (Borst, 1991; van Kalken et al., 1991). The resistance pattern of the classical MDR phenotype often includes the anthracyclines and the vinca alkaloids and is often associated with increased expression of the membrane-bound 170 kDa P-glycoprotein (Pgp), coded for by the MDR1 gene. Pgp is believed to function as an ATP-dependent efflux protein that actively extrudes the drugs from the tumour cell (Borst, 1991; van Kalken et al., 1991). For at least some tumour types, Pgp has been implicated in clinical drug resistance and prognosis (van Kalken et al., 1991).

In vitro, MDR may be circumvented by a variety of non-cytotoxic agents, including calcium channel blockers, cyclosporins, phenothiazines, neuroleptics and cephalosporins (Ford & Hait, 1990). The prospect of reversing MDR in the clinical setting has therefore attracted considerable interest, and some clinical pilot studies using verapamil have been promising (Dalton et al., 1989; Miller et al., 1991). Although verapamil is probably the most well-studied resistance modifier (RM), cardiovascular side-effects preclude its clinical use at in vitro active concentrations (Pennock et al., 1991). Cyclosporin A (CsA), on the other hand, has been shown to reverse resistance in vitro at concentrations readily achievable in vivo without serious toxic effects (Twentyman, 1988; Sonneveld et al., 1992). Furthermore, a non-immunosuppressive cyclosporin analogue denoted SDZ PSC 833 (PSC) was recently found to be 10-fold more potent than CsA as an RM in MDR cell lines (Gaveriaux et al., 1991). The cyclosporins may thus be well suited for testing the concept of resistance modification in the clinic. Indeed, some preliminary studies have indicated clinical resistance-modifying activity of CsA in myeloma (Sonneveld et al., 1992) and acute myelocytic leukaemia (AML; List et al., 1993), and phase II trials of both CsA and PSC are ongoing.

Recently some preliminary evidence was published suggesting that diagnosis-specific activity could be accurately detected in accordance with clinical experience by non-clonogenic drug resistance assays based on measurement of cell kill in the whole tumour cell population (Weisenthal, 1991; Weisenthal et al., 1991). Corroborating these findings, we have recently found that the FMCA retrospectively can detect diagnosis-specific drug activity for a series of standard drugs (Nygren et al., 1993) and prospectively for 2-chlorodeoxyadenosine (CdA) (Larsson et al., 1994) and taxol (submitted for publication). The present study was therefore undertaken to investigate the relative tumour type-specific resistance-modifying activity of CsA and PSC in combination with doxorubicin (Dox) or vincristine (Vcr) in vitro, using a broad spectrum of tumour diagnoses.

Materials and methods

Tumour samples and cell preparation

A total of 322 tumour samples from patients with AML, acute lymphocytic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), chronic myelocytic leukaemia (CML), myeloma, non-Hodgkin's lymphoma (NHL) or different types of solid tumours were included in the study. The samples were obtained from routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. In some cases tumour sampling was performed for in vitro drug sensitivity testing only, which was approved of by the research ethical committee at the Uppsala University Hospital. The number and characteristics of the samples are detailed in Table I. Tumour tissue from solid tumours was minced into 1 mm³ pieces and tumour cells were then isolated by collagenase dispersion and Percoll (Kabi-Pharmacia, Uppsala, Sweden) density gradient centrifugation as described previously (Nygren & Larsson, 1991). Leukaemic cells were obtained from bone marrow or peripheral blood by 1.077 g ml⁻¹ Ficoll-Paque (Kabi-Pharmacia) density gradient centrifugation (Larsson et al., 1992a). Viability was determined by trypan blue exclusion test and the proportion of tumour cells was judged by inspection of May–Grünwald–Giemsa-stained cytocentrifuge preparations by a trained cytopathologist.

Culture medium RPMI-1640 (Northumbria Biologicals, Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Northumbria), 2 mM glutamine, 50 μg ml⁻¹ streptomycin and 60 μg ml⁻¹ penicillin (Northumbria) was used throughout. Cells were cryopreserved in culture medium containing 10% dimethyl sulphoxide (DMSO;

Table 1 Number and treatment characteristics of the samples investigated for the effect of resistance modifiers

Prior chemotherapy	Diagnosis											
	ALL	AML	CML/ myeloma	CLL	NHL	Sarcoma	Breast cancer	Ovarian cancer	Renal cancer	ACC	Paediatric solid tumours	Assorted solid tumours
No	24	43	1	7	8	6	5	9	10	4	1	12
Yes	23	50	9	17	9	1	6	9	0	0	6	5
Unknown	13	7	1	3	18	1	1	4	0	0	1	8
Total evaluable	60	100	11	27	35	8	12	22	10	4	8	25

The table shows the number of successfully analysed samples for the effect of resistance modifiers. In the CML/myeloma group were four CMLs and seven myelomas. The sarcoma group included samples from adult patients only. Paediatric solid tumours included sarcoma (1), Ewing sarcoma (3), Wilms tumour (2), neuroblastoma (1) and phaeochromocytoma (1). The assorted solid tumour group included adenocarcinomas of the lung (3), parotid (2) and medullary thyroid (2), small-cell lung cancer (4), carcinoid (3), colon carcinoma (4), urinary bladder cancer (1), cholangiocarcinoma (1), mesothelioma (1), plasmacytoma (1), malignant insulinoma (1), squamous epithelial cancer (1) and teratoma (1). Abbreviations: ALL, acute lymphocytic leukaemia; AML, acute myelocytic leukaemia; CML, chronic myelocytic leukaemia; CLL, chronic lymphocytic leukaemia; NHL, Non-Hodgkin's lymphoma; ACC, adrenocortical carcinoma.

Sigma, St Louis, MO, USA) and 50% FCS by initial freezing for 24 h at −70°C followed by storage in liquid nitrogen. Both fresh and cryopreserved samples were used in this study.

Reagents and drugs

Fluorescein diacetate (FDA; Sigma) was dissolved in DMSO and kept frozen (−20°C) as a stock solution (10 mg ml⁻¹) protected from light. CsA and PSC, provided by P. Anderson (Sandoz, Basle, Switzerland), were dissolved in absolute ethanol–phosphate-buffered saline (PBS) and further diluted in PBS. The final concentration of ethanol never exceeded 0.1%, which had no effect on cell survival. Dox was obtained from Farmitalia Carlo Erba (Milan, Italy) and Vcr from Lilly (Giessen, Germany). CsA and PSC were generally tested at two concentrations, 1 and 3 µg ml⁻¹, whereas Dox and Vcr were tested at 0.5 and 0.1 µg ml⁻¹ respectively, cut-off concentrations empirically derived (EDCCs) as previously described (Larsson et al., 1992a). Experimental plates were prepared with 20 µl per well of drug solution at ten times the desired final concentration with a programmable pipetting robot (Pro/Pette; Perkin Elmer, Norwalk, CT, USA). The plates were stored frozen at −70°C until further use. Drug stability during storage conditions was estimated by repeated testing of sensitive cell lines (to be published). CsA and PSC could be stored in this way for at least 2 months. The experiments were performed with continuous drug exposure.

Equipment

The 96-well scanning fluorometer (Fluoroscan 2; Flow, Herts, UK) is equipped with a xenon lamp and broadband interference filters exciting fluorescence at 485 nm for FDA. The emitted light from a vertical light path of each well is sequentially read at 538 nm. One plate is read in approximately 1 min. In most experiments, cells, medium and drugs were added to the wells by the pipetting robot, Pro/Pette. Addition of buffer and fluorochrome was performed with an automatised 96-well dispenser (Multidrop, Flow).

Cytotoxicity assay procedure

The principal steps of the FMCA procedure have been described previously (Larsson et al., 1992a). The assay is based on measurement of fluorescence generated from hydrolysis of FDA to fluorescein by cells with intact plasma membranes. On day 1 180 µl per well of the tumour cell preparation (0.5–5 × 10⁵ cells per ml of culture medium) was seeded into the wells of V-shaped 96-well microtitre plates (Nunc, Roskilde, Denmark) prepared as described above. Six blank wells received only culture medium and six wells with cells, but without drugs, served as control. The plates were then incubated at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide. At the end of the incubation period (72 h), the medium was removed and the cells washed with PBS. PBS at 100 µl per well containing FDA (10 µg ml⁻¹) was then added columnwise to control, experimental and blank wells and the plates were incubated for 1 h before reading the fluorescence in the Fluoroscan 2. The fluorimeter was blanked against wells containing PBS including the dye but without cells.

Quality control

Quality criteria for a successful assay included >70% tumour cells in the cell preparation prior to incubation, a fluorescence signal in control cultures of more than five times mean blank values and a coefficient of variation (CV) in control cultures of <30%. The overall success rate of the assay was approximately 85% for haematological tumours and 60% for solid tumours with too low a proportion of tumour cells in the cell preparation being the most common cause of assay failure. Other causes were low yield of cells

after separation or a low fluorescence signal in controls. Only successfully analysed samples are reported here.

Quantification of FMCA results

The results obtained by the indicator FDA are presented as survival index (SI), defined as the fluorescence of experimental cultures expressed as a percentage of that of control cultures with blank values subtracted. Since for each sample the single drugs and the combinations were tested on the same microtitre plate, and since the CV in test wells is independent of the SI level, the mean intra-assay CV (CV in controls within a plate) was used to define response to RMs. The RM response rate was thus defined as the proportion of samples showing a >25% (2–3 times mean intra-assay CV in controls) decrease in SI for Dox or Vcr after addition of RM at the EDCC in a particular sample, and was divided into two groups, a 25–50% (+) and a >50% (4–5 times mean control CV) decrease (+ +). Drug interaction analysis was performed using the multiplicative concept (Valeriote & Lin, 1975), in which the effect of an additive combination is expected to be equal to the product of the effect of its constituents, whereas synergy is observed when the effect is greater than this product. Thus, for a two-drug additive combination composed of single agents with SI values of 50% and 40%, the combination would be expected to result in an SI value of 20% (0.5 × 0.4). The ratio of observed SI values and those expected according to an additive interaction model was then calculated and observed/expected ratios <0.8 were defined as synergistic interactions (Ying Tan *et al.*, 1988). In some analyses the cytotoxicity data for Dox were divided into three groups based on the median SI value and standard deviation (s.d.) (Weisenthal *et al.*, 1991). Values less than the median were termed low drug resistance (LDR), values greater than the median but less than the median + 1 s.d. intermediate drug resistance (IDR) and values greater than the median + 1 s.d. were termed extreme drug resistance (EDR). Previous studies relating *in vitro* assay results to clinical outcome have shown that when the individual SIs for a particular cytotoxic drug in a large number of samples are divided into three statistically defined categories as above, different clinical response probabilities for these groups are observed. The LDR category has a probability of clinical response to the particular drug higher than expected, IDR a lower and EDR an extremely low probability of response (Bosanquet, 1991; Larsson & Nygren, 1993).

Statistical analysis

SI values were compared using the Student's *t*-test. Correlations were performed using Pearson's correlation coefficient and differences in proportions were analysed by the approximate normal test for comparison of proportions (Colton, 1974). The level of significance was set to *P*<0.05.

Results

Effect of single drugs

The effects of Dox, Vcr, CsA and PSC on SI for haematological and solid tumours are shown in Figure 1. For Dox and Vcr mean values ± standard error of the mean (s.e.m.) for all samples were 53 ± 1.7 and 70 ± 1.5 respectively. The group of haematological samples showed lower mean SI values than the solid tumour group in response to Dox (42 ± 1.7 and 83 ± 2.8 respectively) and Vcr (66 ± 1.7 and 87 ± 2.5) and these differences were highly significant (*P*<0.001). At 1 µg ml⁻¹ CsA or PSC little or no effect was discernible for any of the tumour groups. At 3 µg ml⁻¹, CsA showed increased activity in NHL/CLL and AML/CML/ myeloma with mean SI values of 79 ± 8.3 and 82 ± 3.4 respectively. At this concentration 22% of the NHL/CLL samples had an SI <50%, which should be compared with the 0% and 10% for the solid and AML groups respectively
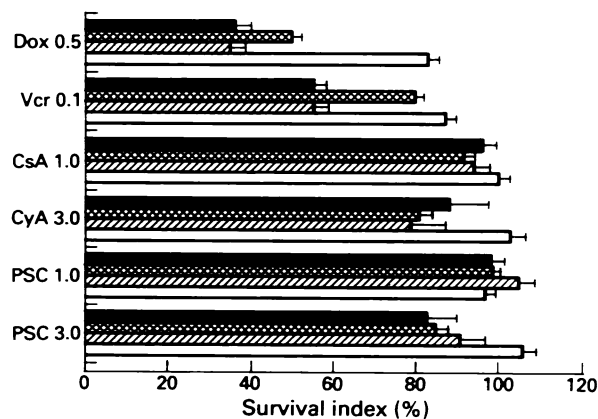

Figure 1 Effect of CsA, PSC, Dox and Vcr in samples from patients with ALL (■ ; *n* = 15–76), AML/CML/myeloma (▨ ; *n* = 50–111), NHL/CLL (▨ ; *n* = 18–62) and solid tumours (□ ; *n* = 48–95). The results are presented as mean values ± s.e.m.

(not shown). PSC at 3 µg ml⁻¹ had effects similar to CsA in the AML and ALL groups, but slightly weaker effect in NHL/CLL.

Activity of CsA and PSC as RMs

Haematological tumours showed high and concentration-dependent response rates to addition of RM to both Dox and Vcr. The concentration dependence of CsA and PSC was especially notable for NHL with 86–100% response at the highest concentration. Solid tumours were generally less responsive, with the exception of paediatric solid tumours and to a lesser extent also ovarian carcinoma, in which response rates of 19% or more were observed at the lowest concentration of CsA or PSC in combination with Dox. In contrast, samples of renal carcinoma and adrenocortical carcinoma showed no responses at any concentration or combination. In the solid tumour groups, CsA and PSC appeared less effective in combination with Vcr than with Dox (Tables II and III). The differences between the group of haematological tumours and the solid tumour group with respect to *in vitro* response rates to addition of RM were statistically significant for all combinations (*P*<0.001–0.05). When all diagnoses were ranked for the resistance-modifying activity of CsA and PSC, an overall correspondence between CsA and PSC activity was observed. The top six ranked diagnoses in repsonse to CsA thus corresponded to those most responsive to PSC in all cases for Dox, and in five out of six cases for Vcr. Renal and adrenocortical carcinoma showed the lowest rank order for both CsA and PSC in combination with either Dox or Vcr. The most notable difference between CsA and PSC was the higher rank order for paediatric solid tumours for the resistance-modifying activity of PSC compared with that of CsA.

Type of interaction

There was a good correspondence between the RM response rates and the proportion of samples showing a synergistic interaction between the RM and Dox or Vcr (Table IV). The percentage decrease in cytotoxic drug-induced SI after addition of RM and the observed/expected ratios, calculated on the basis of the multiplicative concept for drug interactions, showed a strong and significant negative correlation. The highest correlation coefficients were observed for PSC at both concentrations and for CsA at the lower concentration. Slightly weaker correlations were noted for CsA at 3 µg ml⁻¹ (Table IV).

Relationship to previous treatment

In Figure 2 tumours were grouped according to treatment status and analysed with respect to response rates to the

Table II *In vitro* response rates to cyclosporin A (CsA) and SDZ PSC 833 (PSC) as resistance modifiers in combination with doxorubicin

(Cytotoxic drug: Doxorubicin 0.5 μg ml⁻¹)

Diagnosis	CsA 1 μg ml⁻¹ +	CsA 1 μg ml⁻¹ Total (%)	CsA 1 μg ml⁻¹ ++	CsA 3 μg ml⁻¹ +	CsA 3 μg ml⁻¹ Total (%)	CsA 3 μg ml⁻¹ ++	PSC 1 μg ml⁻¹ +	PSC 1 μg ml⁻¹ Total (%)	PSC 1 μg ml⁻¹ ++	PSC 3 μg ml⁻¹ +	PSC 3 μg ml⁻¹ Total (%)	PSC 3 μg ml⁻¹ ++
ALL	6/60 (10)	**20**	6/60 (10)	6/15 (40)	**60**	3/15 (20)	10/54 (19)	**25**	3/54 (6)	5/15 (33)	**60**	4/15 (27)
AML	27/100 (27)	**41**	14/100 (14)	17/47 (36)	**64**	13/47 (28)	17/87 (20)	**26**	5/87 (6)	14/47 (30)	**64**	16/47 (34)
CML/myeloma	3/11 (27)	**45**	2/11 (18)	3/4 (75)	**75**	0/4 (0)	1/9 (11)	**22**	1/9 (11)	2/4 (50)	**75**	1/4 (25)
CLL	10/27 (37)	**44**	2/27 (7)	3/10 (30)	**70**	4/10 (40)	3/19 (16)	**27**	2/19 (11)	3/10 (30)	**60**	3/10 (30)
NHL	8/35 (23)	**29**	2/35 (6)	7/9 (78)	**100**	2/9 (22)	5/32 (16)	**19**	1/32 (3)	6/8 (75)	**100**	2/8 (25)
Breast	0/9 (0)	**0**	0/9 (0)	1/7 (14)	**14**	0/7 (0)	0/6 (0)	**0**	0/6 (0)	1/3 (33)	**33**	0/3 (0)
Sarcoma	1/8 (12)	**12**	0/8 (0)	2/4 (50)	**50**	0/4 (0)	0/8 (0)	**0**	0/8 (0)	1/4 (25)	**25**	0/4 (0)
Ovary	3/22 (14)	**19**	1/22 (5)	2/15 (13)	**20**	1/15 (7)	4/22 (18)	**23**	1/22 (5)	3/15 (20)	**20**	0/15 (0)
Renal	0/10 (0)	**0**	0/10 (0)	0/5 (0)	**0**	0/5 (0)	0/10 (0)	**0**	0/10 (0)	0/5 (0)	**0**	0/5 (0)
ACC	0/4 (0)	**0**	0/4 (0)	0/1 (0)	**0**	0/1 (0)	0/4 (0)	**0**	0/4 (0)	0/1 (0)	**0**	0/1 (0)
Paediatric solid	1/8 (12)	**24**	1/8 (12)	1/7 (14)	**28**	1/7 (14)	1/6 (17)	**34**	1/6 (17)	2/6 (33)	**50**	1/6 (17)
Assorted solid	3/25 (12)	**12**	0/25 (0)	3/18 (17)	**17**	0/18 (0)	0/20 (0)	**0**	0/20 (0)	3/17 (18)	**18**	0/17 (0)
Total	62/319 (19)	**28**	28/319 (9)	45/142 (32)	**49**	24/142 (17)	41/277 (15)	**20**	14/277 (5)	40/135 (30)	**50**	27/135 (20)

The table shows the number of samples with 25–50% (+) or >50% (++) decrease in doxorubicin-induced survival index in the presence of modifier/number of successfully analysed samples (%). The total response rate (+ and ++) is indicated in per cent in bold numbers. Abbreviations as in Table I.

Table III *In vitro* response rates to cyclosporin A (CsA) and SDZ PSC 833 (PSC) as resistance modifiers in combination with vincristine

(Cytotoxic drug: Vincristine 0.1 μg ml⁻¹)

Diagnosis	CsA 1 μg ml⁻¹ +	CsA 1 μg ml⁻¹ Total (%)	CsA 1 μg ml⁻¹ ++	CsA 3 μg ml⁻¹ +	CsA 3 μg ml⁻¹ Total (%)	CsA 3 μg ml⁻¹ ++	PSC 1 μg ml⁻¹ +	PSC 1 μg ml⁻¹ Total (%)	PSC 1 μg ml⁻¹ ++	PSC 3 μg ml⁻¹ +	PSC 3 μg ml⁻¹ Total (%)	PSC 3 μg ml⁻¹ ++
ALL	13/48 (27)	**37**	5/48 (10)	2/6 (33)	**66**	2/6 (33)	7/44 (16)	**25**	4/44 (9)	3/6 (50)	**83**	2/6 (33)
AML	20/78 (26)	**38**	9/78 (12)	11/38 (29)	**61**	12/38 (32)	9/78 (12)	**21**	7/78 (9)	8/38 (21)	**53**	12/38 (32)
CML/myeloma	2/6 (33)	**50**	1/6 (17)	1/1 (100)	**100**	0/1 (0)	1/6 (17)	**34**	1/6 (17)	1/1 (100)	**100**	0/1 (0)
CLL	6/22 (27)	**59**	7/22 (32)	1/6 (17)	**84**	4/6 (67)	5/15 (33)	**66**	5/15 (33)	1/6 (17)	**84**	4/6 (67)
NHL	10/32 (31)	**43**	4/32 (12)	4/7 (57)	**100**	3/7 (43)	5/31 (16)	**16**	0/31 (0)	4/7 (57)	**86**	2/7 (29)
Breast	0/5 (0)	**0**	0/5 (0)	0/2 (0)	**0**	0/2 (0)	0/5 (0)	**0**	0/5 (0)	0/2 (0)	**0**	0/2 (0)
Sarcoma	0/7 (0)	**14**	1/7 (14)	0/3 (0)	**0**	0/3 (0)	0/7 (0)	**0**	0/7 (0)	0/3 (0)	**0**	0/3 (0)
Ovary	1/16 (6)	**18**	2/16 (12)	1/9 (11)	**11**	0/9 (0)	1/16 (6)	**6**	0/16 (0)	1/9 (11)	**11**	0/9 (0)
Renal	0/10 (0)	**0**	0/10 (0)	0/1 (0)	**0**	0/1 (0)	0/10 (0)	**0**	0/10 (0)	0/1 (0)	**0**	0/1 (0)
ACC	0/4 (0)	**0**	0/4 (0)	ND	**ND**	ND	0/4 (0)	**0**	0/4 (0)	ND	**ND**	ND
Paediatric solid	0/3 (0)	**0**	0/3 (0)	0/2 (0)	**0**	0/2 (0)	1/3 (33)	**33**	0/3 (0)	0/2 (0)	**0**	0/2 (0)
Assorted solid	0/13 (0)	**0**	0/13 (0)	2/5 (40)	**40**	0/5 (0)	1/12 (8)	**8**	0/12 (0)	1/4 (25)	**25**	0/4 (0)
Total	52/244 (21)	**33**	29/244 (12)	22/80 (28)	**54**	21/80 (26)	30/231 (13)	**20**	17/231 (7)	19/79 (24)	**49**	20/79 (26)

The table shows the number of samples with 25–50% (+) or >50% (++) decrease in vincristine-induced survival index in the presence of modifier/number of successfully analysed samples (%). The total response rate (+ and ++) is indicated in per cent in bold numbers; ND, not done. Abbreviations as in Table I.

Table IV Relationship between resistance modifier response rates and the percentage of samples showing synergistic interactions, for cyclosporin A (CsA) or SDZ PSC 833 (PSC) in combination with doxorubicin (Dox) or vincristine (Vcr)

	Responders (%)[a]	Synergy (%)[b]	Correlation O/E vs change in SI[c]	n	P-value
Dox 0.5 µg ml⁻¹					
+ CsA 1 µg ml⁻¹	28	33	− 0.75	296	<0.001
+ CsA 3 µg ml⁻¹	49	41	− 0.51	132	<0.001
+ PSC 1 µg ml⁻¹	20	26	− 0.77	269	<0.001
+ PSC 3 µg ml⁻¹	50	47	− 0.75	129	<0.001
Vcr 0.1 µg ml⁻¹					
+ CsA 1 µg ml⁻¹	33	38	− 0.67	229	<0.001
+ CsA 3 µg ml⁻¹	53	41	− 0.54	81	<0.001
+ PSC 1 µg ml⁻¹	20	29	− 0.82	225	<0.001
+ PSC 3 µg ml⁻¹	49	40	− 0.82	78	<0.001

[a]Percentage of samples showing $>25\%$ decrease in cytotoxic drug-induced survival index (SI) in presence of modifier, Range of s.d. $= 2-6\%$. [b]Percentage of samples with observed/expected ratio (O/E) <0.8 (see Materials and methods). Range of s.d. $= 3-5\%$. [c]Change in SI refers to the percentage change in cytotoxic drug-induced SI caused by the cyclosporin.

Table V Relationship between resistance modifier response rates for cyclosporin A (CsA) and SDZ PSC 833 (PSC) in combination with doxorubicin (Dox), and Dox sensitivity

Dox 0.5 µg ml⁻¹	LDR[a] +	+ +	Total	n	IDR +	+ +	Total	n	EDR +	+ +	Total	n
+ CsA 1 µg ml⁻¹	25	11	**36**	174	18	8	**26**	85	7	3	**10**[b]	60
+ CsA 3 µg ml⁻¹	42	24	**66**	67	33	15	**48**	39	11	6	**17**[b]	36
+ PSC 1 µg ml⁻¹	20	5	**25**	154	7	4	**11**[b]	68	9	5	**14**	55
+ PSC 3 µg ml⁻¹	40	28	**68**	65	28	21	**49**	39	10	3	**13**[b]	31

The table shows the percentage of samples showing $25-50\%$ (+) or $>50\%$ (+ +) decrease in Dox-induced survival index (SI) in the presence of modifier. The total response rate (+ and + +) is indicated in bold numbers. [a]LDR (low drug resistance) indicates samples showing SI values $<$median, IDR (intermediate drug resistance) those showing SI values between median and median + 1 s.d. and EDR (extreme drug resistance) those showing SI values$>$median + 1 s.d. The median and s.d. were calculated from the Dox responses in all samples. [b]The response rates in the EDR group were significantly lower than in both the LDR group and the IDR group for 1 and 3 µg ml⁻¹ CSA and for 3 µg ml⁻¹ PSC. The difference between the LDR and IDR group was significant for 1 µg ml⁻¹ PSC.
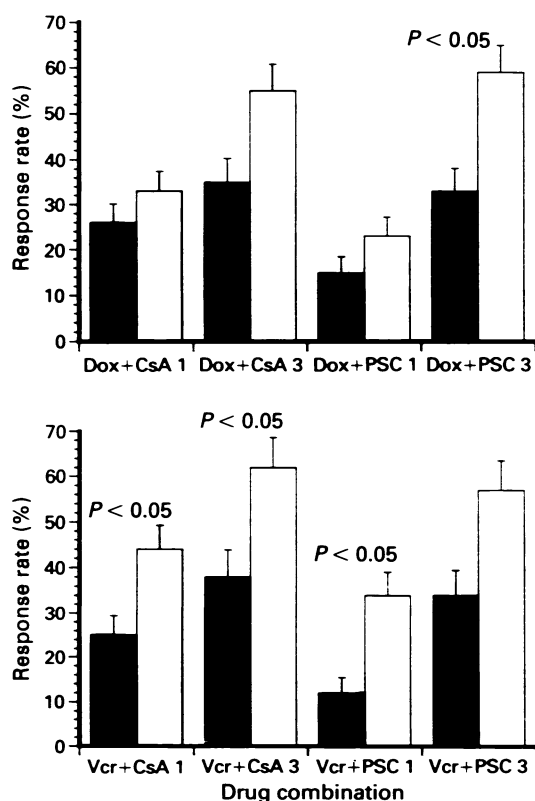
Figure 2 Response rates to CsA and PSC in combination with Dox or Vcr for previously untreated (■) or treated (□) patients, presented as mean values ± s.e.m. Statistically significant differences are indicated.

RMs. A tendency to increasing response rates in the treated category was observed, and the differences were statistically significant for the combinations Dox + PSC 3 µg ml⁻¹ and Vcr + 1 or 3 µg ml⁻¹ CsA or 1 µg ml⁻¹ PSC ($P<0.05$).

Relationship to cytotoxic drug sensitivity

In Table V the samples were divided into LDR, IDR and EDR groups with regard to Dox sensitivity. The highest RM response rates were found in the LDR category followed by IDR and EDR. In the EDR group significantly lower resistance-modifying activity than in both the LDR group ($P<0.001$) and the IDR group ($P<0.05$) was observed for Dox + CsA 1 and 3 µg ml⁻¹ and Dox + PSC 3 µg ml⁻¹. Between the LDR and IDR groups statistical difference was observed for Dox + PSC 1 µg ml⁻¹ ($P<0.05$).

Discussion

In the present study the RM effects were measured at fixed concentrations of the chemotherapeutic drugs instead of measuring changes in the concentration required to achieve a fixed effect (i.e. IC₅₀), a procedure often used in experiments on drug-resistant cell lines. One reason was that from many of the samples not enough tumour cells were obtained to permit detailed dose–response analysis. Measuring variable effects at a fixed dose in highly heterogeneous patient populations also resembles the clinical phase II setting. The concentrations used were carefully chosen to produce maximal scatter of SI values in a large panel of samples in order to have the highest probability to distinguish sensitive from resistant samples (Larsson *et al.*, 1992a). Furthermore, the individual intra-assay variation (CV) was used to statistically define the

response criteria. Since the primary objective was to investigate the relative subpanel specificity of RM effects under identical in vitro conditions, we find the present approach adequate for that purpose.

At 1 µg ml^{-1} CsA and PSC, the cyclosporins alone had little effect, whereas at 3 µg ml^{-1} a small cytotoxic effect was noted in the haematological tumours. For CsA this was most apparent in the CLL/NHL group, which is in accordance with a previous study (Larsson et al., 1992b). In the majority of cases the effect of the cyclosporins alone could not explain the effect observed for the combinations since there was a close correspondence between the percentage decrease in cytotoxic drug-induced SI after addition of RM and the calculated observed/expected ratios according to the multiplicative concept for drug interactions. The proportion of samples with synergistic interactions also closely paralleled the response rate to addition of RM. However, 3 µg ml^{-1} CsA in combination with Dox or Vcr showed a slightly weaker correlation between the RM-mediated percentage decrease in cytotoxic drug-induced SI and observed/expected ratios compared with the other RM concentrations tested. This may be due to some effect of CsA alone, and indicates that, for at least some tumours and at higher concentrations, the effect of CsA alone may contribute to the cytotoxic efficacy of a CsA and cytotoxic drug combination.

The present results demonstrate that CsA and PSC show high in vitro resistance-modifying activity against haematological tumours, in which Pgp has been implicated in drug resistance (Hall & Cattan, 1991; Nooter & Herweijer, 1991). The solid tumours were mostly considerably less responsive. However, both paediatric solid tumours and ovarian carcinoma showed substantial response to both CsA and PSC at the lower concentration. Included in the paediatric solid group were four sarcomas and one neuroblastoma, which are tumour types in which Pgp has been shown to be linked to the outcome of therapy (Chan et al., 1990, 1991). However, this observation should be interpreted with caution because of the limited number of samples tested so far.

Not all diagnoses showed responses in correspondence with known patterns of Pgp expression. Renal and adrenocortical carcinoma showed no responses at all despite reported high intrinsic expression of Pgp (Goldstein et al., 1989). Also, adult sarcomas and breast carcinoma have been reported to express intermediate levels of Pgp (Nooter & Herweijer, 1991), but showed little response to RM addition in the present study. In contrast, ALL, dominated by paediatric samples, showed high response rates to CsA and PSC but has been reported to express the Pgp in only a small fraction of cases (Pieters et al., 1992). However, the role of Pgp in childhood ALL still appears controversial (Gosauguen et al., 1993).

Clinical documentation on the RM efficacy of cyclosporins is sparse. In a study of CsA + vinblastine given to 22 patients with renal cell carcinoma, no responses were observed (Rodenburg et al., 1991). In a trial of 23 patients with metastatic colon carcinoma only 1 out of 23 responded with a partial remission to an epirubicin + CsA combination (Murren et al., 1991). In AML, on the other hand, good responses were reported for patients resistant and refractory to AraC + daunorubicin after addition of CsA (List et al., 1993). Promising tumour responses were also obtained in myeloma patients using VAD (Vcr + Dox + prednisolone) + CsA (Sonneveld et al., 1992). Furthermore, in a recent literature review of clinical investigations of MDR-reversing agents, presenting results from more than 350 treated patients, many leukaemias, lymphomas and myelomas were considered to be potentially responsive whereas, in solid tumour types, only a few patients appeared to benefit from the RM treatment (Raderer & Scheithauer, 1993). Although these observations are in accordance with the present in vitro results, the true value of the present in vitro predictions will have to await the completion of phase II trials representing a broader array of diagnoses with careful monitoring of

systemic exposure of both the cytotoxic drugs and the RMs.

Interestingly, we found no increased potency of PSC compared with CsA, despite previous reports of a 10- to 20-fold difference in favour of PSC observed in some (Gaveriaux et al., 1991; Jonsson et al., 1992), but not all (Friche et al., 1992), MDR cell lines. There is no difference in storage stability of the stock solutions or in stability under the FMCA culture conditions between the cyclosporins (not shown). Moreover, Pgp-expressing MDR cell lines, analysed regularly at our laboratory under identical conditions, are more susceptible to PSC than to CsA (Jonsson et al., 1992). These facts, together with the high resistance-modifying activity noted in ALL, indicate the presence of additional cyclosporin-sensitive mechanisms of resistance other than Pgp in the clinical specimens. The non-Pgp-mediated MDR phenotype has been shown to be sensitive, at least partly, to RMs (Baas et al., 1990; van Kalken et al., 1991), and alternative target proteins have recently been identified (Slovak et al., 1993). The fact that some of the tumour types originating from Pgp-expressing tissues, and which are known to express high Pgp levels, do not show any response to PSC and CsA clearly indicates that RM-insensitive mechanisms of resistance are also important, at least for these types of tumours. In this context, one should note that clinically achievable steady-state concentrations of CsA correspond to the in vitro concentrations tested (1-3 µg ml^{-1}) (Sonneveld et al., 1992). The Dox and Vcr concentrations used (EDCCs) correspond roughly to the clinically achievable peak plasma concentration (Alberts & Chen, 1980). Using bioassay techniques under the FMCA conditions these concentrations were shown to give an in vitro exposure > 5-fold higher than what is clinically achievable (to be published). Consequently, the lack of response in some of the solid tumour types does not seem to be related to insufficient exposure to the RM or the cytotoxic drug.

Although a tendency to an increased resistance-modifying activity was observed in samples from previously treated patients, the response rate of those from untreated patients was also substantial. Interestingly, the highest response rates to RMs were found in the most drug-sensitive group of samples, the LDR group, whereas the EDR group showed significantly lower response rates. The low frequency of responders to RMs in the EDR population thus further underlines the virulent nature of this in vitro-defined type of resistance (Weisenthal et al., 1991). These observations suggest that RM treatment may be especially advantageous as adjunct to up-front therapy in tumour types showing some degree of cytotoxic drug sensitivity.

In summary, the present results indicate differential in vitro activity of cyclosporins among the tumour types tested with regard to the potentiating effect on the activity of two cytotoxic drugs. If this tumour type-specific activity is substantiated.in clinical trials, non-clonogenic assays like the FMCA may be useful in evaluation of new RMs and in targeting specific diagnoses and patients for initial clinical trials of RMs.

Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; RM, resistance modifier; CsA, cyclosporin A; PSC, SDZ PSC 833; AML, acute myelocytic leukaemia; ALL, acute lympocytic leukaemia; CML, chronic myelocytic leukaemia; CLL, chronic lymphocytic leukaemia; NHL, non-Hodgkin's lymphoma; LDR, low drug resistance; IDR, intermediate drug resistance; EDR, extreme drug resistance; CV, coefficient of variation; s.d., standard deviation; s.e.m., standard error of the mean.

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