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Resistance Modification by PSC-833, a Novel Non-immunosuppressive Cyclosporin A

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A novel non-immunosuppressive cyclosporin A, PSC-833, has been tested for its ability to circumvent resistance to doxorubicin, vincristine and colchicine in human and murine multidrug resistantant (MDR) cell lines. This compound is shown to be a highly potent resistance modifier, being 7–10-fold more potent than the parent compound, cyclosporin A, whilst approximately equal to cyclosporin A in the growth inhibitory effects of compound alone. Reversal of the P-glycoprotein-associated MDR drug accumulation defect is a major component of resistance reversal for PSC-833, as it is for cyclosporin A.
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INTRODUCTION

AGENTS THAT can reverse multidrug resistance (MDR) have entered clinical trial over the last few years [1–4]. The lead compound, verapamil, although dose-limited by its cardiac toxicity, has been reported to produce positive effects in drug-resistant myeloma patients [1]. A search for improved resistance-modifiers has resulted in the identification of several compounds which are more potent than verapamil. Among these is the immunosuppressive agent, cyclosporin A [5] which has a particularly high binding affinity to P-glycoprotein, the drug efflux "pump" molecule frequently overexpressed in MDR cells [6]. The immunosuppression brought about by cyclosporin A is a disadvantage for its use as a resistance modifier and we examined the relationship between immunosuppression and resistance modification for a number of cyclosporin A analogues [7, 8]. Among naturally-occurring cyclosporins, we found a close relationship between the two properties [7], but we found that chemically-modified cyclosporins could be non-immunosuppressive whilst being highly effective resistance modifiers [8]. One non-immunosuppressive compound, O-acetyl cyclosporin A (B3-243) was approximately 4-fold more potent that cyclosporin A in the human small cell lung cancer MDR subline H69/LX4 [8]. The effectiveness of O-acetyl cyclosporin A as a resistance modifier was subsequently confirmed by others [9]. We subsequently found, however, that in mouse cell lines with low P-glycoprotein expression, the potencies of O-acetyl cyclosporin A and cyclosporin A were equal and that, in lines with high P-glycoprotein expression, cyclosporin A was superior [10].

Studies carried out at Sandoz have now led to the availability of PSC-833, a novel cyclosporin A analogue, which is a lead compound for clinical trial as a resistance modifier [11]. In this paper we describe our initial findings regarding the effectiveness of PSC-833 in a variety of *in vitro* model systems.

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MATERIALS AND METHODS

Cell lines

We used two pairs of parent and MDR cell lines in these studies. The human small cell lung cancer line NCI-H69/P was originally obtained from Drs D. Carney and A. Gazdar of the NCI/Navy Medical Oncology Branch, Bethesda, Maryland, USA. The MDR subline NCI-H69/LX4 was derived in this laboratory by *in vitro* growth in doxorubicin and maintained in 0.4 μg/ml of this agent [12, 13]. Subline H69/LX4 expressed

high levels of P-glycoprotein whereas this was not detectable in the parent line [13]. Mouse tumour cell line EMT6 was originally described by Rockwell *et al.* [14] and our parent cell line EMT6/Ca/VJAC (hereafter referred to as EMT6/P) is a subline thereof. The MDR subline EMT6/AR1.0 was also derived by *in vitro* growth in doxorubicin and maintained at 1 μg/ml [15]. The parent cell line expressed very low levels of P-glycoprotein whereas high levels were seen in EMT6/AR1.0 [15]. Both MDR sublines showed reduced ability to accumulate drugs involved in the MDR phenotype compared to their respective parent lines [12, 15]. Routine tests for mycoplasma were carried out on all cell lines and results were negative throughout the period of these studies.

Culture conditions

The H69 cell lines were grown in RPMI 1640 medium plus 10% fetal calf serum whilst the EMT6 lines were grown in Eagle's minimal essential medium (MEM) with Earles' salts plus 20% newborn calf serum (all from Gibco Biocult). Both media were supplemented with penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively). Cell stocks were maintained in 75 cm^2 tissue culture flasks (Falcon) in humidified atmosphere of 8% CO_2 + 92% air at 37°C. Single cell suspensions of EMT6 cells were achieved by 15 min incubation in 0.1% trypsin, whilst suspensions of H69 cells combining single cells and small clumps of cells (<10 cells) were produced by pipetting.

Drugs

The cytotoxic drugs used in these studies were doxorubicin (Farmitalia), colchicine (Sigma) and vincristine (David Bull Laboratories). These were dissolved in sterile water at 500 μg/ml, sterilised by filtration, and aliquots stored at −20°C (doxorubicin, colchicine) or at 4°C (vincristine). Dilutions for use in experiments were made in phosphate-buffered saline (PBS) immediately before use. Cyclosporin A (MW = 1203) and PSC-833 (3'-keto-Bmt^1)-[Val^2]-cyclosporin A) (MW = 1215) were kindly supplied by Sandoz, Basel, Switzerland. These resistance modifiers were dissolved in absolute ethanol at 5 μg/ml and stored at 4°C. Dilutions were made in medium immediately before use. Appropriate solvent controls were used in all experiments.

Cell response

The responses of the various cell lines to combinations of cytotoxic drugs and cyclosporins were measured using the MTT colorimetric assay [16]. Our adaptations of this assay as used for the H69 and EMT6 cell lines have been previously described in detail [8, 15, 17]. Basically, cell suspensions were inoculated into wells on 96-well tissue culture plates at cell numbers previously determined to produce an optical density of around 1.0 after 3 days (EMT6 lines) or 6 days (H69 lines) growth. These numbers were 6×10^2, 1.6×10^3, 6×10^3 and 1×10^4 cells/well in 200 μl of medium for EMT6/P, EMT6/AR1.0, H69/P and H69/LX4, respectively. Cyclosporins were added after 2 h of cell incubation and cytotoxics after a further 1 h in volumes of 10 μl and 20 μl, respectively.

After the appropriate incubation period, in which control cell numbers increased by 10–20 fold, 20 μl of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT, Sigma) in PBS was added to each well and the plates incubated for a further 5 h. At the end of this time, the medium was aspirated from each well and 200 μl DMSO added. (For the H69 cell lines, it was necessary to centrifuge the plates, 200 *g* for 5 min, in order to pack the cells to the base of the wells before medium aspiration.) The plates were then shaken on a plate shaker for 10 min and the optical density of each well read on Titertek Multiskan MCC plate reader at 540 nm and a reference wavelength of 690 nm. For each cytotoxic drug dose response curve, seven 2-fold drug dilutions were used plus a control, with three replicate wells at each point. From plots of the data the ID_{50} values (i.e. the dose of drug to reduce control optical density by 50%) were determined as previously described [15].

Drug accumulation

Effects of the cyclosporins on MDR drug accumulation were measured using tritium-labelled daunomycin. Use of this compound, an analogue of doxorubicin, allows accumulation to be studied at molar drug concentrations similar to those used in the cytotoxicity experiments. The labelled compound (^3H daunorubicin) (51.8 GBq/mmol; New England Nuclear) was stored at −70°C in methanol. EMT6/P and EMT6/AR1.0 cells were inoculated into 3 cm diameter wells on 6-well multiplates (Sterilin) 48 h before experiments were carried out. Initial numbers of cells per well were adjusted so that the numbers 48 h later would be equal for the two cell types (EMT6/P = 4×10^4/well; EMT6/AR1.0 = 6×10^4/well). EMT6/AR1.0 cells were grown in the absence of drug over this period. To commence experiments, the medium was aspirated from each well and replaced with 2 ml of medium at 37°C containing the labelled compound (3.7 kBq/ml) together with carrier daunorubicin to a final concentration of 1 μmol/l. After 2 h, the medium was again aspirated from each well and the cell monolayer rinsed three times with ice-cold PBS. 1 ml distilled water was then added to each well and the wells left for 2 h for cell lysis to occur. The contents of each well were then pipetted several times, 0.5 ml transferred to a liquid scintillation vial and 5 ml Aquasol (Dupont) added. The vials were counted the following day on a liquid scintillation counter. A cell count was carried out on three replicate wells of each cell type and this allowed the determined values of isotope uptake per well to be corrected to uptake per cell.

RESULTS

Human cells

The changes in ID_{50} of doxorubicin, vincristine and colchicine in H69/LX4 cells in the presence of various concentrations of

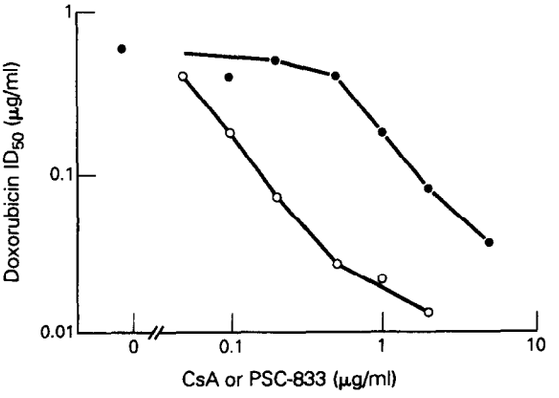

Fig. 1. Effects of various concentrations of cyclosporin A (●) or PSC-833 (○) on the ID_{50} of doxorubicin in the H69/LX4 cell line. Values were determined from dose response curves with three replicate wells/point.

Table 1. Sensitisation to vincristine and colchicine in cell line H69/LX4

Cytotoxic drug	Cyclosporin A (μg/ml)				PSC-833 (μg/ml)			
	0	1	2	5	0.1	0.2	0.5	1.0
Vincristine	0.80	0.13* (6.2)	0.013 (62)	0.0018 (444)	0.15 (5.3)	0.027 (30)	0.0029 (276)	0.0015 (533)
Colchicine	0.080	0.021 (3.8)	0.0037 (22)	0.0025 (32)	0.020 (4.0)	0.0056 (15)	0.0028 (29)	0.0023 (35)

*ID_{50} in μg/ml. Figures in parentheses are sensitisation ratio = ratio of ID_{50}s in absence/presence of modifier. Independent repeat experiments gave similar results.

cyclosporin A or PSC-833 are shown in Fig. 1 and Table 1. It may be seen from Fig. 1 that both cyclosporin A and PSC-833 were able to overcome almost completely resistance to doxorubicin in this MDR cell line, the ID_{50} of the corresponding H69/P parent line being, in this experiment, 0.007 μg/ml. However, the dose of PSC-833 to effect a 10-fold reduction in ID_{50} was 10.8-fold lower than the dose of cyclosporin A to produce the same effect. In a repeat experiment, the ratio obtained was 8.3. Similar data for the effects of cyclosporin A and PSC-833 on the sensitivity of H69/LX4 cells to vincristine and colchicine (Table 1) give cyclosporin A/PSC-833 ratios for reduction of ID_{50} by 10-fold of 10.0 and 8.8, respectively.

In the H69/P parent cell line, no sensitisation to doxorubicin or colchicine was produced by either cyclosporin A at doses up to 5 μg/ml (data not shown). No sensitisation to vincristine was seen by either sensitiser at doses up to 1 μg/ml, but a 2-fold sensitisation was produced by 5 μg/ml cyclosporin A.

No effects of either cyclosporin A alone on cell growth were seen at doses of up to 2 μg/ml. At 5 μg/ml, each produced a 20–30% reduction in final optical density.

Mouse cells

Results of an experiment in which EMT6/P and EMT6/AR1.0 cells were treated with doxorubicin in combination with cyclosporin A or PSC-833 are shown in Fig. 2. Parent and MDR cells were each sensitised by both cyclosporins, but, again, PSC-833 was considerably more potent that cyclosporin A. In EMT6/AR1.0, the ratio of CsA/PSC-833 concentrations for reduction of ID_{50} by 10-fold was 6.4, whilst, in EMT6/P, the ratio of concentrations to reduce ID_{50} by 2-fold was 7.0. Repeat experiments gave values of 8.0 and 13.5, respectively.

Daunorubicin accumulation

Results of an experiment to determine the effects of cyclosporins on daunorubicin accumulation in EMT6/P and EMT6/AR1.0 cells are shown in Fig. 3. It may be seen that both cyclosporins increase DNR accumulation, that PSC-833 is clearly more potent than cyclosporin A and that lower cyclosporin A doses are required for optimal enhancement of accumulation in EMT6/P than in EMT6/AR1.0.

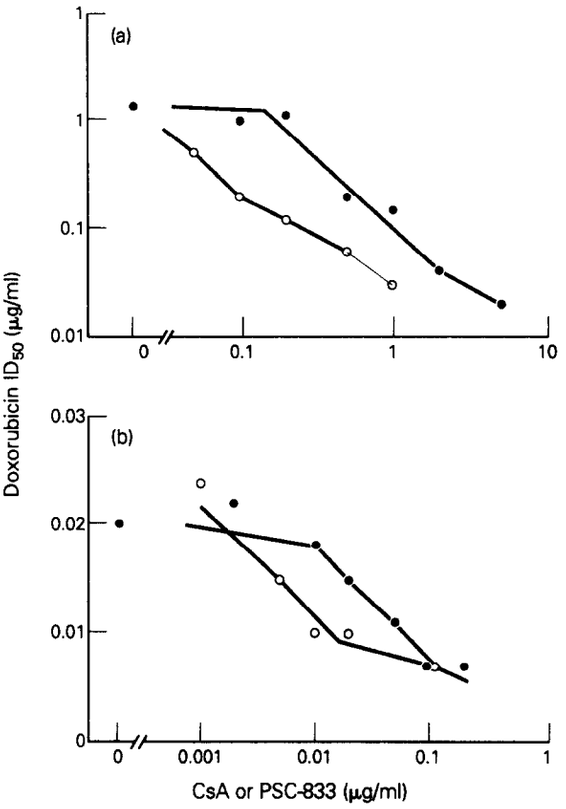

Fig. 2. Effects of various concentrations of cyclosporin A (●) or PSC-833 (○) on the ID_{50} of doxorubicin in the (a) EMT6/AR1.0 or (b) EMT6/P cell lines. Note that the ordinate scale is logarithmic in (a) and linear in (b).

DISCUSSION

The data presented in this paper indicate PSC-833 to be a highly potent resistance modifier. In the various experiments carried out in human and mouse cell lines which express P-glycoprotein, PSC-833 was between 7 and 10-fold more potent than cyclosporin A in potentiating the growth inhibitory effects of doxorubicin, vincristine and colchicine. These data are in agreement with those of Gaveriaux *et al.* [11] obtained in other model systems.

In a previous report [8], we described a non-immunosuppressive cyclosporin analogue, O-acetyl cyclosporin A (B3-243) which we found to be approximately 4-fold more potent than cyclosporin A in reversal of doxorubicin resistance in the H69/LX4 line. In subsequent experiments with a range of EMT6 cell lines, however, we found O-acetyl cyclosporin A and cyclosporin A to be of approximately equal effect in lines with low P-glycoprotein expression and cyclosporin A to be a more efficient sensitiser than O-acetyl cyclosporin A in lines with high P-glycoprotein expression [10]. The consistently high potency seen with PSC-833 in the current studies clearly represents a major improvement over the more variable effects of O-acetyl

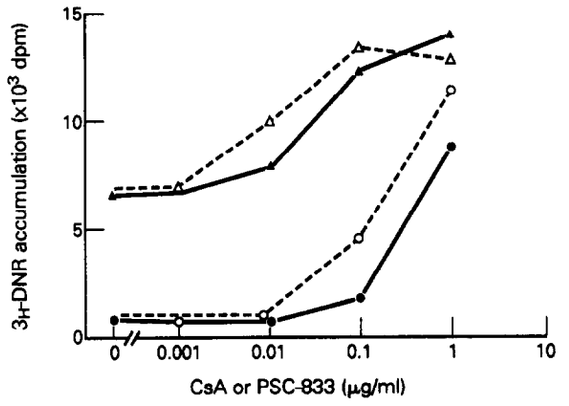

Fig. 3. Effects of various concentrations of cyclosporin A (closed symbols) or PSC-833 (open symbols) on the 2 h accumulation of ^{3}H-daunorubicin by EMT/AR1.0 (circles) and EMT6/P cells (triangles). Data points ($\times 10^3$ dpm per 2 $\times$ 10^5 cells) are means of three replicate wells. S.E. < 6% of the mean values. Similar data were obtained in an independent repeat experiment.

cyclosporin A. Our data indicating that PSC-833 is more potent than cyclosporin A in increasing cellular accumulation of ^{3}H-DNR provide at least in part a mechanistic basis for the sensitisation observed in the MTT assay. It is not possible, because of the wide spacing of cyclosporin doses used in the accumulation study, to deduce precise relative potencies. The data are, however, compatible with a 3–5-fold greater potency for PSC-833 than for cyclosporin A. The published data on cyclosporin A are rather conflicting with regard to the precise role of increased cytotoxic drug accumulation in chemosensitisation of parent and MDR cells. In our EMT6 cell lines, however, it seems clear that increased drug accumulation is a significant component of sensitisation by both cyclosporin A and PSC-833.

Encouraging results with cyclosporin A in experimental systems led to its entry into clinical trial as a resistance modifier in a number of centres. Although not to date reported as a major problem in such studies, the immunosuppressive properties of cyclosporin A are certainly a matter of some concern in this context. Furthermore, it seems unlikely that clinically achievable plasma levels of cyclosporin A will reach those indicated as necessary in *in vitro* studies to produce optimal sensitisation of cells expressing high levels of P-glycoprotein [5, 7]. Preliminary data indicate that plasma levels of around 1 μg/ml cyclosporin A can be achieved in patients although some toxicity was also seen in these early clinical studies [18, 19]. If preclinical toxicology of PSC-833 is satisfactorily completed, entry of this compound into clinical trial may be anticipated with interest. It should certainly provide a test of the hypothesis that compounds which inhibit P-glycoprotein-mediated MDR are likely to be useful in clinical chemotherapy.

Note added in proof. Whilst this paper was in press, additional papers have been published by the group at Sandoz showing that PSC-833 can restore cellular accumulation of daunorubicin *in vitro* in an MDR mouse P388 leukaemia cell line [20] and that it can act as an effective modifier of resistance in this same cell line *in vivo* [21].

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