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Short communication

Reversal of typical multidrug resistance by cyclosporin and its non-immunosuppressive analogue SDZ PSC 833 in Chinese hamster ovary cells expressing the *mdr*1 phenotype

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Summary. The new non-immunosuppressive cyclosporin derivative SDZ PSC 833 (PSC) is a potent agent used to overcome typical multidrug resistance (MDR) associated with overexpression of the *mdr*1 gene encoding for a P-170 glycoprotein. In the present study, the efficacy of PSC as compared with cyclosporin was determined in Chinese hamster ovary cell lines exhibiting different levels of resistance to colchicine (0, 0.1, 0.2 and 10 µg/ml, respectively). Low concentrations of PSC (8.2 nM) increased the cytotoxicity of colchicine in cell lines expressing low levels of drug resistance. The concentration resulting in 50% cell survival (LC_{50} value) found for colchicine alone or in combination with PSC in the CHO-A3 cell line that was resistant to 100 ng colchicine/ml decreased from >500 to 200 ng/ml at 8.2 nM PSC and to <100 ng/ml at 82 and 820 nM PSC. In the CHO-A3 cell line that was resistant to 200 ng colchicine/ml, the LC_{50} values decreased from >500 ng/ml for colchicine alone to 500 ng/ml for colchicine used in combination with 8.2 nM PSC and to <100 ng/ml for colchicine combined with 82 or 820 nM PSC. At a concentration of 82 nM PSC, the maximal effect in MDR reversal was observed in the cell lines exhibiting moderate resistance. In the highly resistant cell line, PSC (820 nM) also reversed colchicine resistance. In drug-accumulation experiments, we obtained a 4-fold increase in intracellular doxorubicin accumulation using 820 nM PSC. A comparison of PSC with cyclosporin revealed that a cyclosporin concentration 20-fold that of PSC was required to obtain the same sensitising effect. On the basis of these data, it may be concluded that PSC is a most promising chemosensitiser.

Introduction

Multidrug resistance (MDR) characterised by reduced drug accumulation in resistant cells as compared with their drug-sensitive parental cells is often associated with increased expression of a 170-kDa transmembrane glycoprotein (P-glycoprotein, Pgp) [3, 10]. The level of Pgp expression is correlated with the level of drug resistance and reflects the decreased accumulation of anthracyclines and vinca alkaloids [3, 11, 14]. In a variety of human malignancies, the *mdr*1 gene that encodes for Pgp is expressed at increased levels [8]. In vitro, the lowered drug accumulation can be reversed by numerous agents, including calcium antagonists such as verapamil, calmodulin inhibitors and cyclosporin A (CsA) [4, 9, 13–15]. Some of these agents have recently entered clinical trials [5, 16, 18]. Recently, SDZ PSC 833 (PSC; mol. wt., 1,214.65 Da), a new cyclosporin D analogue, was introduced as a chemosensitiser. PSC is described as being more potent than CsA [2, 7]. It lacks the nephrotoxic and haemodynamic side effects of CsA and has been purported to be non-immunosuppressive [1].

In the present study, we investigated the sensitisation of Chinese hamster ovary (CHO) cell lines expressing different levels of Pgp in association with the MDR phenotype using various PSC concentrations, and we compared these findings with the sensitisation obtained using CsA. In addition, the effect of PSC preincubation on the intracellular accumulation of the Pgp-associated drug doxorubicin was evaluated in a Pgp-expressing CHO MDR cell line.

Materials and methods

Drugs and chemicals. Doxorubicin (DOX; Farmitalia, Milan, Italy) and daunorubicin (DNR; Rhone-Poulenc Pharma, France) were dissolved in sterile distilled water to a concentration of 1 mg/ml and were stored as aliquots at –80°C. Dilutions were made in either sterile water or 1 M TRIS buffer (pH 8.8) immediately before use. Colchicine (COL; Sigma, St. Louis, Mo., USA) was dissolved in dimethylsulphoxide (DMSO; Merck, Darmstadt, FRG) at 200 mg/ml and stored at –80°C. PSC (Sandoz, Basel, Switzerland) and CsA (Sandoz) were dissolved in absolute ethanol at 5 mg/ml and stored at 4°C. Dilutions were made in medium immediately before use. Methanol and chloroform were obtained from Rathburn (Walkerburn, Scotland) and glacial acetic acid was supplied by Merck; all were of analytic grade. Desipramine was obtained from Sigma.

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Cells and medium. The CHO cell line grows as an attached monolayer on plastic. The parent cell line, CHO-AUX B1, was cultured in α-minimum essential medium (α-MEM; Gibco, Paisley, UK) supplemented with gentamycin (60 μg/ml, Gibco) and 10% foetal calf serum (FCS, Gibco). MDR sublines were derived by in vitro growth of the parent line in increasing concentrations of COL. The sublines were routinely cultured in COL at concentrations of 100, 200 (CHO-A3) or 10,000 ng/ml (CHO-C5). The cell lines were maintained as stock cultures in 162-cm^2 flasks incubated at 37° C in a fully humidified atmosphere comprising 5% CO_2 in air. A single-cell suspension was produced by removal of the adherent cells by scraping followed by their incubation with 0.1% (w/v) collagenase (Sigma) at 37° C for 30 min. Finally, the collagenase-treated cells were washed twice in medium.

Immunohistochemistry studies. For the determination of Pgp expression, the monoclonal antibody C219 (Centocor, Malvern, Pa., USA) was used. Cytospin preparations of the CHO cell lines were prepared and air-dried for 18 h. They were fixed in methanol/acetone (50/50%), soaked in 50 mM TRIS in 0.02% Tween-20 for 5 min and incubated with 10% rabbit +1% goat serum for 30 min. Undiluted C219 monoclonal antibody or mouse IgG_{2a} (Coulter, USA) was added and the preparations were incubated overnight at 4° C. Antimouse immunoglobulin was added to each slide, the slides were washed, and the cells were then incubated with alkaline phosphatase substrate (APAAP) for 60 min at 37° C and washed three times.

MTT assay. The in vitro sensitivity of the cell lines to COL in the absence or presence of various concentrations of PSC was determined using a modification of the assay originally described by Mosmann [12]. Briefly, a single-cell suspension of 5×10^4 cells/ml medium supplemented with 10% FCS was prepared for all cell lines. Then, 200-μl aliquots of the suspension were dispensed into 90-well flat-bottomed microtiter plates (Costar, Cambridge, Mass.). After 2 h incubation, the appropriate doses of PSC, CsA or solvent control (ethanol, 0.02% v/v) were added to the wells in a volume of 10 μl. After 1 additional h incubation, COL or solvent control (DMSO, 0.005% v/v) was added in a further volume of 10 μl. In these experiments four replicate wells were used at each point. The plates were incubated for 68 h. Next, 30 μl of a 5-mg/ml solution of MTT (Sigma) was added to each well and the plates were incubated for another 4 h. The plates were then centrifuged for 10 min at 500 *g*. After removal of the supernatants, the formazan crystals were dissolved with 100 μl DMSO. The optical density (OD) of the wells was measured using a Titertek multiscan plate reader (Flow) at 540 nm. The percentage of cell survival was defined as the mean OD of treated wells divided by the mean OD of untreated control wells $\times 100\%$. The LC_{50} value, defined as the drug concentration resulting in 50% cell survival, was derived by calculating the point at which the dose-response curve crossed the point of 50% cell survival. The CHO-A3 (100 ng) subline was used for purposes of comparing PSC with CsA.

Drug accumulation. The effect of PSC on intracellular drug accumulation was studied in the CHO-A3 (100 ng) cell line using DOX. After 2 h incubation of 10^7 cells in 75-cm^2 flasks, PSC was added (final concentration, 0, 8.2 or 820 nM). The cells were incubated for 1 additional h prior to the addition of DOX (final concentrations, 50, 250 or 1,000 ng/ml). After an incubation period of 30, 60 or 120 min, the monolayers were washed twice with phosphate-buffered saline (PBS, Gibco). Next, the cells were collected by scraping; after centrifugation, the cell pellets were stored at –80° C until their analysis by high-performance liquid chromatography (HPLC) using a procedure described elsewhere [17].

Extraction procedure and HPLC analysis for DOX in cells. The thawed cell pellets, supplemented with 250 μl PBS and internal standard (DNM), were sonicated at 120 W for 2 min (Eurosonic 44, FRG). The suspension was extracted twice with 4 ml chloroform/methanol (9 : 1, v/v). Following centrifugation at 1,000 *g* for 10 min, the two chloroform phases were collected and evaporated in one step at 37° C under nitrogen. The dry residue was dissolved in 1 ml chloroform/methanol (9 : 1, v/v), the solution was centrifuged for 10 min at 1,000 *g* and 500 μl was injected onto the chromatographic column.

The chromatographic system consisted of a Dupont Instruments 870 pump module and a sampling valve equipped with a sampling loop of 500 μl. The analytic column (200 mm × 3 mm inside diameter) was packed with spherical silica gel Lichrosorb Si-60 (partical size, 7 μm; Chrompack). Fluorescence detection (Shimadzu spectrofluorometer RF-510) was carried out using an excitation wavelength of 488 nm and an emission wavelength of 550 nm. The column was eluted with chloroform/methanol/glacial acetic acid/water (720 : 210 : 40 : 30, by vol.) supplemented with 0.3 mM $MgCl_2$ and 10 μg desipramine/ml at a flow rate of 0.5 ml/min. The integration of the peaks was performed using a Spectra-physics SP 4270 integrator. Quantification of the cellular drug concentration was achieved by measuring the peak-height ratio of DOX and the internal standard and was calculated from a calibration curve for peak height versus concentration.

Results

The Pgp expression was determined by immunocytochemical staining using the C219 monoclonal antibody for each CHO cell line tested during the experiments. The parental CHO-AUX B1 cells showed no staining, whereas the CHO-A3 (100 ng) subline showed a faint but distinct membrane staining. The staining of the CHO-A3 (200 ng) cells was more distinct than that of the CHO-A3 (100 ng) cells. The highly resistant CHO-C5 subline showed a strongly positive staining. The positivity of the staining reaction was clearly associated with the level of COL resistance of the cell lines.

The in vitro sensitivities of the various CHO cell lines to COL in combination with PSC are shown in Fig. 1. The addition of different PSC concentrations or of solvent controls without COL to the cultures produced no significant cytotoxic effects on the cell lines tested (data not shown). At the lowest dose (8.2 nM), PSC increased the cytotoxic effects of COL on the CHO parent line. With increasing drug resistance of the cell lines, higher PSC concentrations were needed to reverse the drug resistance. The LC_{50} values for the CHO-A3 (100 ng) subline decreased from >500 ng COL/ml alone to about 200 ng COL/ml at 8.2 nM PSC and to <100 ng COL/ml at 82 and 820 nM PSC. For the CHO-A3 (200 ng) subline, the same results were obtained except for the 8.2 nM PSC concentration, at which the LC_{50} value was 500 ng COL/ml. The LC_{50} values for the CHO-C5 cell line decreased to 500 ng COL/ml at 820 nM PSC.

For HPLC analysis of cellular drug accumulation, the detection limit of the method used was 10 ng DOX/ 5×10^6 cells. After 2 h incubation with DOX at 250 or 1,000 ng/ml, a 4-fold increase in cellular DOX accumulation could be achieved using 820 nM PSC in comparison with solvent alone. Cellular DOX-accumulation data are summarised in Table 1.

The studies in the CHO-A3 (100 ng) subline using CsA instead of PSC showed that a concentration of 820 nM CsA could effectively increase COL cytotoxicity. The LC_{50} values were >500 ng COL/ml at a CsA concentration of 8.2 nM, about 500 ng COL/ml at 82 nM CsA and <100 ng COL/ml at 820 nM CsA. The relative sensitisation of the CHO-A3 (100 ng) resistant subline using CsA and PSC is

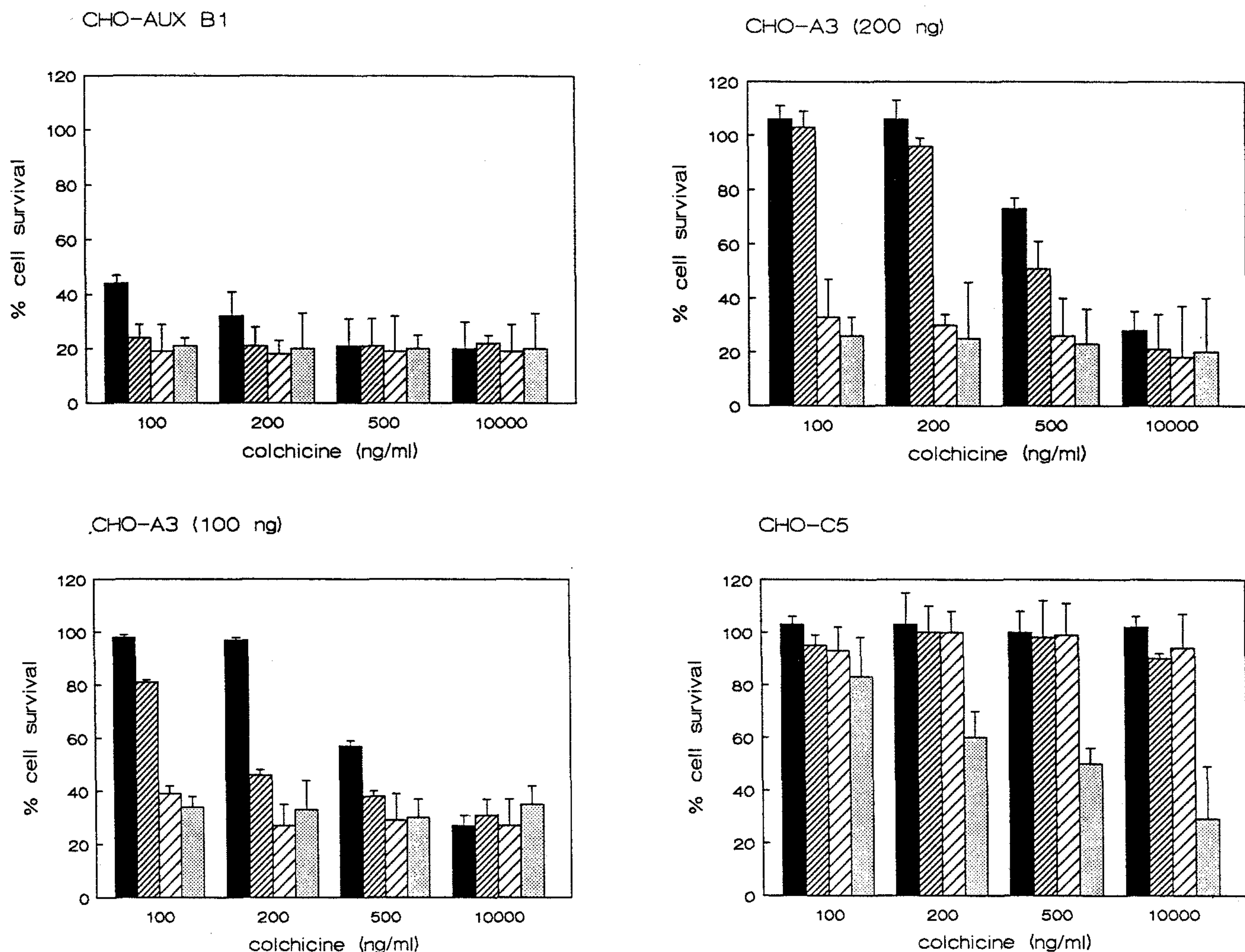

Fig. 1. Effect of colchicine in the presence or absence of different PSC concentrations on the survival of CHO cells. ▬, 0 nM; ▨, 8.2 nM; ▨, 82 nM; ▩, 820 nM. Data represent mean values ± SD for four replicate wells

Table 1. Cellular DOX accumulation in the CHO-A3 (100 ng) subline exposed to DOX combined with PSC

Incubation concentration	Incubation period		
	30 min	60 min	120 min
DOX 50 ng/ml:			
PSC 0 nM	<10	<10	<10
PSC 8.2 nM	<10	<10	<10
PSC 820 nM	<10	<10	15
DOX 250 ng/ml:			
PSC 0 nM	11	14	16
PSC 8.2 nM	15	20	22
PSC 820 nM	20	53	62
DOX 1000 ng/ml:			
PSC 0 nM	33	50	73
PSC 8.2 nM	48	45	98
PSC 820 nM	97	147	307

Values represent the cellular accumulation of DOX in ng/5 × 10^6 cells

shown in Fig. 2. The results of this experiment indicate that PSC is about 20 times more effective than CsA in modulating drug resistance.

Discussion

In the present study, we evaluated the MDR modulatory effects of PSC on CHO cell lines expressing different levels of drug resistance. The parental, non-drug-resistant CHO-AUX B1 cell line used in this study expresses low physiological levels of Pgp [10] that cannot be detected by staining with the C219 monoclonal antibody. In agreement with this observation, we observed a minor increase in cytotoxicity using PSC at a concentration as low as 8.2 nM. This low dose of PSC also increased cytotoxicity in the CHO-A3 (100 ng) subline. The LC_{50} values decreased from >500 ng/ml for COL alone to about 200 ng/ml for COL with 8.2 nM PSC. However, for complete reversal of COL resistance, 82 nM PSC had to be used (LC_{50}, <100 ng COL/ml). This PSC concentration also reversed drug resistance in the CHO-A3 (200 ng) subline (LC_{50}, <100 ng COL/ml). The highly resistant CHO-C5 subline showed a

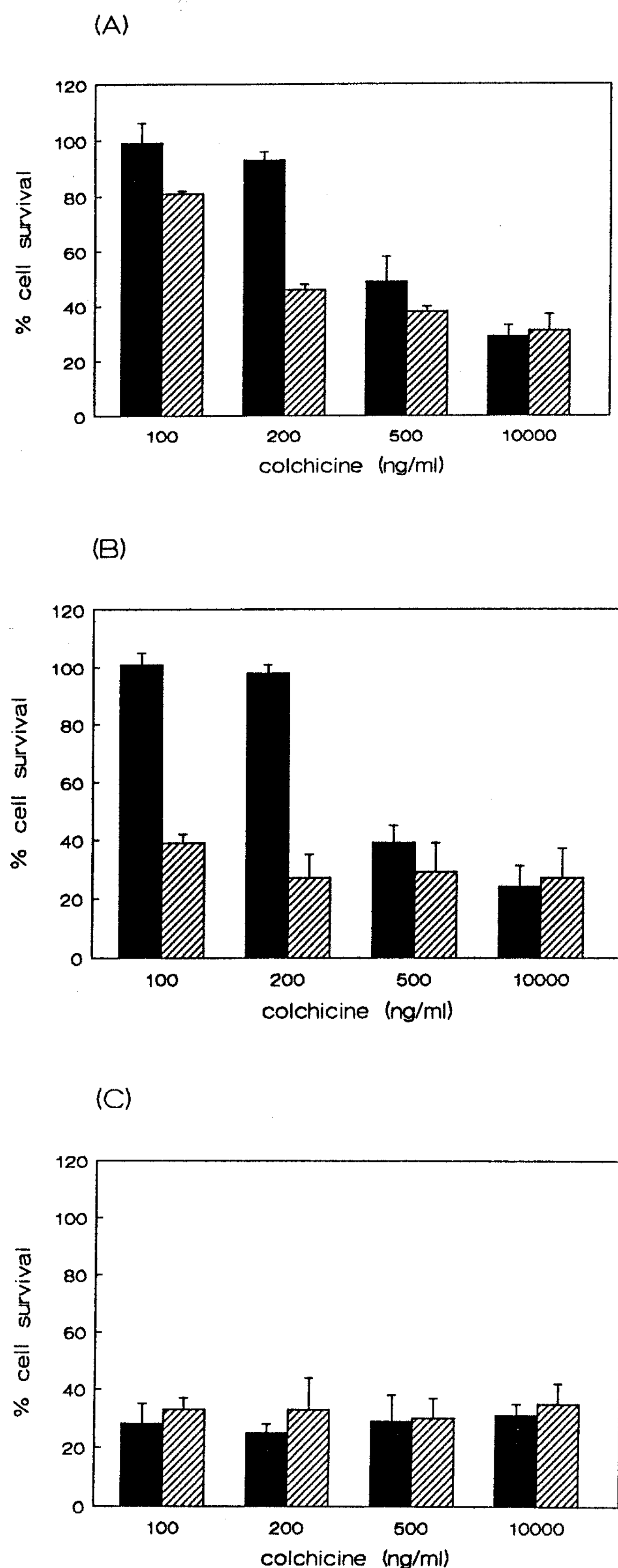

Fig. 2 A – C. Effect of concentrations of **A** 8.2 nM, **B** 82 nM and **C** 820 nM CsA (■) and PSC (▨) in the presence of colchicine on the survival of the CHO-A3 (100 ng) subline. Data represent mean values ± SD for four replicate wells

response in the form of increased cytotoxicity at 820 nM PSC (LC_{50}, 500 ng COL/ml). These results indicate that with increased expression of Pgp in the cell lines [10], higher PSC concentrations were required to obtain effective reversal of the drug resistance. Because Pgp expression in many human malignancies is lower than that in the drug-resistant CHO sublines used in the present study, [8] the effective PSC concentrations needed to reverse drug resistance in vivo in humans may well be below 82 nM. In contrast, the effective CsA concentrations should be near 820 nM [Sonneveld et al., submitted for publication].

Prior studies by Gavériaux et al. [6] have demonstrated a significant Pgp modulation effect by CsA at 840 nM in the CHO parental and weakly resistant cell lines, which is comparable with our results. Using the weakly resistant CHO-A3 (100 ng) subline, which was chosen because its level of resistance is clinically relevant [Sonneveld et al., submitted for publication], we compared the efficacy of PSC and CsA. The results indicated that a CsA concentration about 20-fold that of PSC was required to obtain the same sensitising effect.

To exclude a direct effect of PSC on cell survival in these cell lines, we investigated the effect of PSC on drug accumulation. Previoulsy, a considerable, 3-fold enhancement of DNR accumulation by 500 nM CsA was reported in a CHO-A3 (100 ng) cell line [14]. In our drug-accumulation experiments, we obtained a minor increase in DOX accumulation using 8.2 nM PSC, whereas a 4-fold increase in cellular DOX acumulation was achieved using 820 nM PSC.

Clinical studies have indicated that CsA can be an effective Pgp-modulating agent [16, 18]. Because PSC, in contrast to CsA, produces neither toxic side effects such as nephrotoxicity nor haemodynamic side effects [1], higher doses can presumably be used to reverse or prevent the development of multidrug resistance. From the present data, we conclude that PSC is capable of reversing drug resistance caused by overexpression of Pgp. PSC was shown to be a much more potent sensitiser in comparison with CsA. These observations and the results of previous in vivo experiments [1] indicate that PSC can be of important clinical use in reversing drug resistance.

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