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SDZ PSC-833—a novel potent *in vitro* chemosensitizer in multiple myeloma

Bertil Jonsson,[CA] Kenneth Nilsson, Peter Nygren and Rolf Larsson

B Jonsson and R Larsson are at the Division of Clinical Pharmacology, K Nilsson is at the Department of Pathology, and P Nygren is at the Department of Oncology, University Hospital, Uppsala University, S-751 85 Uppsala, Sweden. Fax: +46-18-519237. Tel: +46-18-664260.

Multiple myeloma cell lines and patient tumor samples with and without the expression of the classical multidrug resistance (MDR) phenotype were investigated *in vitro* for drug induced cytotoxicity and modulation of drug resistance. Overall there was a good correlation in the cell lines between MDR expression, as measured by immunocytochemistry with monoclonal antibodies against P-glycoprotein 170 (Pgp), and *in vitro* resistance to doxorubicin (dox) and vincristin (vcr). Drug resistance in the cell line RPMI 8226 dox 40, expressing a high level of Pgp, was almost completely reversed by the novel non-immunosuppressive cyclosporin A (CsA) analog SDZ PSC-833 (PSC), while the chemosensitizers verapamil, CsA and quinine, in clinically achievable concentrations, were much less effective. In cell lines with low Pgp expression, PSC and the other chemosensitizers seem equally effective. The patient tumor samples were selected to represent different combinations of Pgp expression, drug resistance and effects of chemosensitizers. PSC and CsA appeared equally potent and resistance modulation was detected not only in Pgp positive, but also in Pgp negative tumor samples. Furthermore, in one case of a Pgp expression myeloma, chemosensitizers were without effect. These findings indicate the need to incorporate *in vitro* chemosensitivity assays with Pgp determination when the effects of MDR modulating chemosensitizers are to be studied in the clinic.

Key words: Drug resistance, drug sensitivity assay, multiple myeloma, resistance modifiers, SDZ PSC-833.

Introduction

Classical type multidrug resistance (MDR) is mediated by the expression of an ATP dependent efflux protein, the transmembrane P-glycoprotein 170 (Pgp).[1] In patients with relapsing or refractory multiple myeloma, Pgp is commonly recognized together with clinical resistance to doxorubicin (dox) and vincristine (vcr), drugs normally highly active and of considerable therapeutic importance in this disease.[2] A variety of drugs have been shown to decrease or reverse MDR *in vitro*, among them verapamil, cyclosporin A (CsA), quinine, reserpin and erythromycin. These drugs all seem to act by blocking the drug efflux function of Pgp, thereby restoring intracellular concentrations of anticancer drugs.[3]

In a clinical study, Salmon *et al.*[4] showed that among 10 patients with refractory, Pgp positive multiple myeloma, four responded clinically to the addition of verapamil to the base line treatment with dox and vcr. There are several possible reasons for therapeutic failure when a chemosensitizer is used to overcome drug resistance. For instance, the side effects of the sensitizer, cardiotoxicity in the case of verapamil, may prevent the achievement of serum concentrations necessary to overcome resistance. The availability of chemosensitizers with a better therapeutic index would thus be of considerable importance.

The immunosuppressive drug cyclosporin A (CsA) reverses MDR equally well or more efficiently than verapamil, the most recognized and best tested resistance modulator.[5] Recently, Sandoz PSC-833 (PSC), a non-immunosuppressive CsA analog, was found to be a far more active resistance modifier than CsA, when tested on cell lines selected *in vitro* for drug resistance.[6] Furthermore, PSC seems to have a favorable toxicological profile as compared with CsA.

We and others have previously found a good overall correlation between *in vitro* drug cytotoxicity as measured in various assays and clinical

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[CA] Corresponding Author

outcome, especially with regard to drug resistance.[7] The use of such assays to detect MDR and to measure resistance modulation therefore seems attractive.

In this paper we describe the capacity of the new chemosensitizer PSC to modulate drug resistance in myeloma cell lines, representing parental as well as *in vitro* induced resistance, and in myeloma patients, selected to represent different combinations of Pgp expression, drug resistance and effects of chemosensitizers.

Materials and methods

Reagents and drugs

Fluorescein diacetate (FDA; Sigma, St Louis, MO) was dissolved in dimethyl sulfoxide (Sigma). CsA and PSC (kindly provided by Sandoz, Basel, Switzerland) were first dissolved in ethanol and then diluted in phosphate-buffered saline (PBS, final ethanol concentration below 0.5%). Quinine (Sigma) was diluted in PBS. The other drugs were standard pharmaceuticals obtained commercially and dissolved as described elsewhere.[8]

Cell lines

The myeloma cell line RPMI 8226 and two sublines selected for doxorubicin resistance, 8226 dox 6 and 8226 dox 40, were kindly provided by Dr WS Dalton (Tucson, AZ).[9] The U-266 1970 cell line was isolated from a myeloma patient with resistant disease[10] and the early passages of this cell line used in the present study express *in vitro* drug resistance. Cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum (Flow, Irvine, UK), glutamine, streptomycin and penicillin. The interleukin-6 (IL-6) dependent U-266 1970 cell line was grown with support of IL-6 producing fibroblasts.

Patient tumor samples

Heparinized bone marrow aspirates were subject to Ficoll-Isopaque density separation (SD 1.077 g/ml). An aliquot of the separated cells was used to prepare cytospin slides for immunocytochemical and May Grünewald Giemsa (MGG) staining. The remaining cells were used for drug sensitivity testing. In the presented patients more than 70% of the cells after Ficoll separation were tumor cells, as determined morphologically in the MGG stained slides. The drug sensitivity testing was performed with a combination of the two methods described below.

In vitro sensitivity testing

The fluorometric microculture cytotoxicity assay (FMCA) has been detailed elsewhere.[11] The method is based on the capacity of viable cells to hydrolyze non-fluorescent FDA to fluorescent fluorescein. In brief, cells from cell lines (20 000/well) and patients (50 000/well) were incubated in triplicate in 96-well microtiter plates (Nunc, Roskilde, Denmark) for 72 h under standard culture conditions with or without added drugs. After culture the medium was removed and after one wash in PBS, 100 μl of this buffer containing FDA was added. After a 1 h incubation, fluorescence was read in a Fluoroscan II (Flow). Results for the cell lines are presented as the mean of three experiments. Data are shown as survival index (SI), defined as the fluorescence of experimental as percent of control wells, with blank values subtracted.

A dye exclusion test modified after Weisenthal[12] was subsequently performed as described elsewhere.[8] Briefly, 100 μl of a solution containing formaldehyde fixed chicken red blood cells (CRBCs, 75 000/ml) in 0.9% NaCl with 0.25% Nigrosine and 0.5% Fast Green was added to selected wells. After incubation for 10 min, cytospin slides were prepared for immunocytochemical or MGG staining. CRBCs served as an internal standard. The ratio of viable tumor cells:CRBCs for experimental wells was then expressed as a percent of that of control wells.

The cytotoxic drug sensitivities are presented as SI values versus drug concentrations or, due to small sample size, as SI values at the empirically defined cut off concentration (EDCC) of each cytotoxic drug. The EDCC is the drug concentration where the difference was most apparent between sensitive and resistant subsets of patient samples in a pilot series.[8]

Resistance modifiers were tested at concentrations achievable in the clinic: 1 μg/ml for CsA, 2.5 μg/ml for verapamil and 10 μg/ml for quinine. PSC was tested at the same concentration as CsA. At these concentrations the modifiers alone did not effect cell survival.

Immunocytochemistry

For determination of Pgp, the specific monoclonal antibodies JSB-1 (Sanbio, Amsterdam, The Netherlands) and C219 (Centocor, Malvern, PA) were used with a standard ABC kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Briefly, cytocentrifuge preparations were fixed in acetone, endogenous peroxidase was blocked with 0.3% H_2O_2 and unspecific binding inhibited with rabbit blocking serum (Vector Laboratories). The C219 and the JSB-1 antibodies were diluted 1:20 and 1:30, respectively. The parental RPMI 8226 cell line and the Pgp positive sublines served as negative and positive controls for grading of the staining in the patient tumor samples. The FMCA procedure did not influence the quality of subsequent immunostaining in the cell lines.

Results

Cell lines

Immunostaining showed expression of Pgp in the resistant sublines of RPMI 8226 and in U-266 1970. Staining was strong (+++) and medium (++) in the sublines RPMI 8226 dox 40 and RPMI 8226 dox 6, respectively, while the U-266 1970 cell line stained weakly (+) (Table 1). The relative intensity of the staining between the cell lines was the same for the JSB-1 and the C219 antibodies.

Overall there was a good agreement between the staining reaction and the resistance to dox and vcr (Figure 1). PSC almost completely reversed the resistance to vcr and dox in the RPMI 8226 dox 40 subline where the other chemosensitizers were much less active (Figure 1, Table 1). However, this difference diminished in parallel with declining Pgp expression and no obvious difference in modulating potency was thus noted in the cell line U-266 1970, expressing low levels of Pgp.

Patients

Three patients, selected to represent different myeloma cell phenotypes with respect to Pgp expression, drug sensitivity and resistance modulation *in vitro* will be described.

Patient 1 was on second line treatment with a dox–vcr containing regimen, 'VAD'.[13] After initial response there were signs of progression. The percentage of myeloma cells after separation was 80%, with 60% of them expressing Pgp weakly (+). *In vitro* testing with the FMCA indicated reversible drug resistance (Figure 2, Table 2). The dye exclusion test after combined dox and CsA incubation supported the effective resistance modulation. After two courses with VAD + CsA with clinical response, a new *in vitro* evaluation was done. At this time the percentage of myeloma cells in the marrow sample had decreased and there were approximately 40% myeloma cells after Ficoll separation. The tumor cells were expressing Pgp with an unaltered staining intensity. The dye exclusion test was compatible with reversible resistance, but not as pronounced as in previous testing (not shown). Cytospin preparations after a single dox incubation *in vitro* showed an enrichment of Pgp positive cells among the surviving cells, in contrast to slides made after addition of chemosensitizers, where no such phenomenon was detected.

Patient 2 was on intermittant high dose corticosteroid treatment due to primary clinical resistance to alkylating drugs. The percentage of myeloma cells was approximately 70% and staining with the C219 antibody was negative in all cells. However, chemosensitivity testing with the FMCA and the dye exclusion test displayed moderate resistance to dox with the same degree of reversibility with CsA and PSC (Table 2).

Patient 3 was tested at diagnosis. The marrow contained 70% myeloma cells after Ficoll separation and there was a distinct but weak (+) Pgp staining

Table 1. SI, Pgp 170 expression and chemosensitizing in cell lines[a]

Cell line	Pgp 170	SI (%) dox	SI (%) dox + CsA	SI (%) dox + PSC
RPMI 8226	–	41	31	28
RPMI 8226 dox 6	++	102	75	34
RPMI 8226 dox 40	+++	102	100	51
U-266 1970	+	69	43	40

[a] SI for cell lines in response to dox at EDCC (0.5 μg/ml) ± CsA (1 μg/ml) or PSC (1 μg/ml).

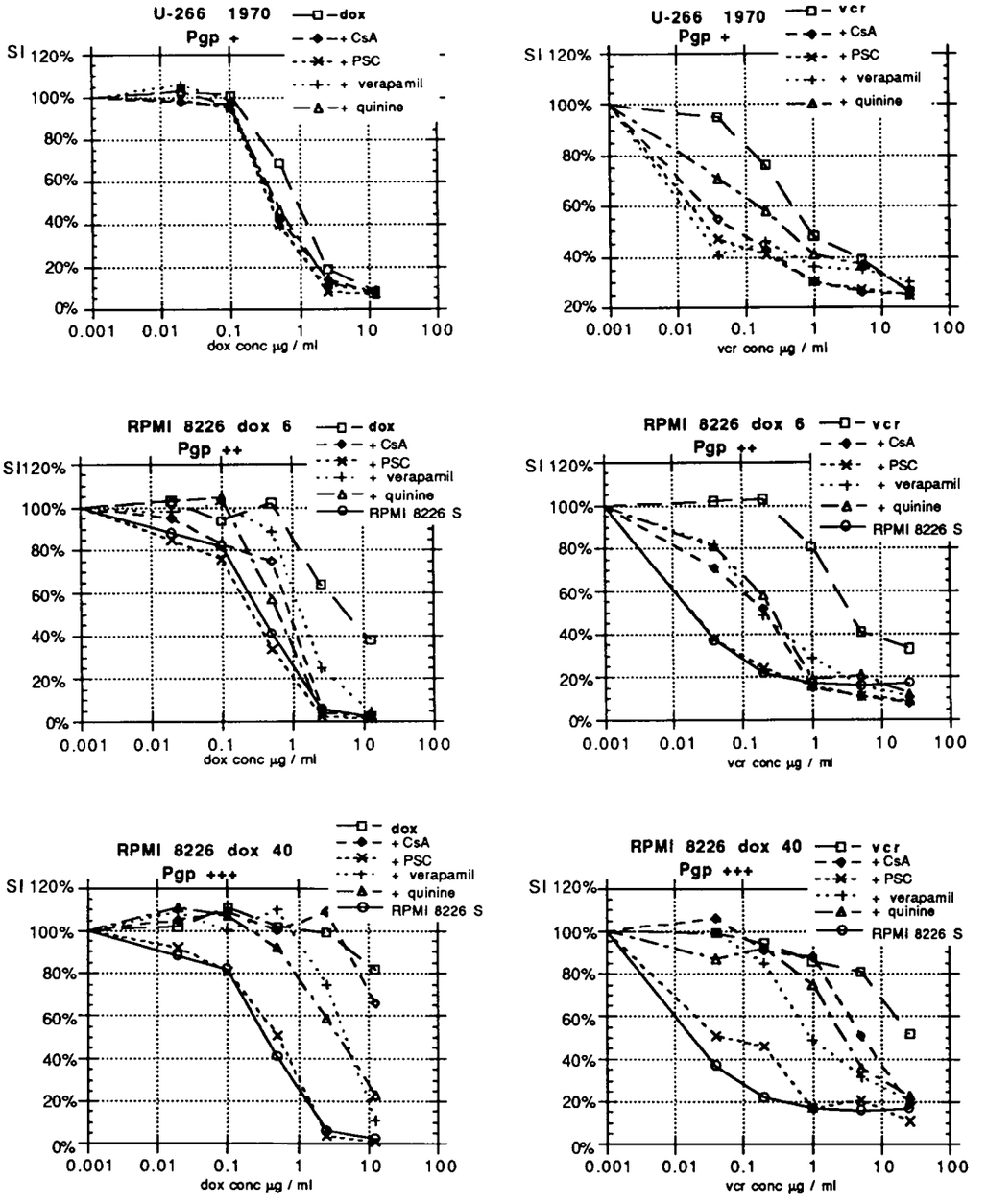

Figure 1. Effect of chemosensitizers on the cytotoxicity of dox and vcr in myeloma cell lines expressing Pgp. Pgp + to + + + denotes staining reaction to the antibody C219. CsA (1 µg/ml), PSC (1 µg/ml), verapamil (2.5 µg/ml) and quinine (10 µg/ml).

in 30% of the tumor cells. The FMCA indicated a borderline effect of the chemosensitizer (Table 2), compatible with increased cytotoxicity in the low percentage of Pgp positive tumor cells. However, the dye exclusion test did not support resistance modulation on morphologically identifiable myeloma cells. Furthermore, surviving cells after a single dox incubation were not enriched with Pgp positive cells.

Discussion

If not present at diagnosis, drug resistance seems to develop, inevitably, as a consequence of treatment

Table 2. SI, Pgp 170 expression and chemosensitizing in patient samples[a]

Patient	Pgp	SI (%) dox	SI (%) dox + CsA	SI (%) dox + PSC
1	+	97	51	50
2	–	78	33	38
3	+	44	30	31

[a] SI in patient samples in response to dox at EDCC (0.5 μg/ml) ± CsA (1 μg/ml) or PSC (1 μg/ml).

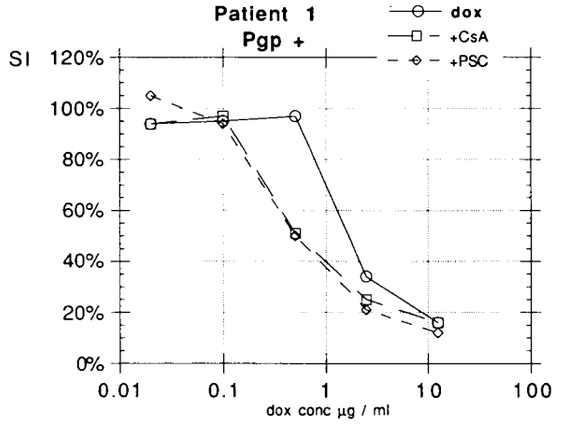

Figure 2. Effect of CsA (1 μg/ml) or PSC (1 μg/ml) on the effect of dox in patient 1.

in patients with multiple myeloma. Non-cross-resistant multidrug regimens have been tried for long periods in the treatment of patients with myeloma, without proven superiority to sequential introduction of new drugs when resistance supervenes.[14] With this background there is a need for new ways to detect, describe and circumvent drug resistance in myeloma patients. Pgp mediated MDR is a well characterized drug resistance mechanism which it is possible to circumvent pharmacologically and of relevance in patients with myeloma.

From a mechanistic point of view, where Pgp is seen as a molecular drug efflux pump, it is tempting to expect a correlation between the degree of resistance and the amount of Pgp. In heterogenous, usually small, patient samples with an admixture of normal cells, it is difficult to quantify Pgp with molecular techniques. If there is a correlation between the level of Pgp expression and clinical outcome, detection of the subpopulations of tumor cells with the highest expression of Pgp might also be most relevant. For these reasons we have used immunocytochemistry. This simple, semiquantitative technique gave a good correlation between Pgp expression and the degree of resistance in the cell lines.

There was also an inverse relationship between the degree of Pgp expression and the degree of resistance modulation by chemosensitizers, except for the novel CsA analog PSC. These results are in agreement with a recent report demonstrating reversing activity of very low concentrations of CsA in tumors with low expression of Pgp.[15]

PSC was a highly effective resistance modulator, almost completely reversing drug resistance in the most resistant cell line tested, RPMI 8226 dox 40; CsA, verapamil and quinine had more modest effects. If failure of chemosensitizers in the clinic is caused by too high expression of the Pgp drug efflux pump, PSC would be a good candidate for clinical trials.

In this paper we have described the use of a combination of two methods for the measurement of cytotoxicity. The FMCA method is well suited for use in samples with a low admixture of non-tumor cells, because of speed and reproducibility. This makes the assay suitable in the, albeit few, myeloma patients with more than approximately 70% tumor cells in the bone marrow, as in the cases presented. Also, in samples with a high percentage of tumor cells, we believe it is mandatory to verify effects by morphological examination, as differences in cell survival between tumor and non-tumor cells might invalidate FMCA results (patient 3). For most myeloma cases the dye exclusion test alone is more appropriate, due to the high non-tumor cell admixture.

In patient samples, there are well recognized problems associated with the use of immunocytochemical techniques for the detection of Pgp, e.g. epitope masking by sialinic acid.[16] Apart from method dependent sensitivity and specificity problems, there might also be a parallel expression of other mechanisms of resistance, e.g. rendering Pgp modifying drugs less active. The data on selected patients are included in this paper to illustrate a combined approach to detect reversible drug resistance.

In the first patient, with therapy induced resistance, addition of a resistance modifier according to the *in vitro* test was accompanied by a

clinical response. In this case Pgp determination and the cytotoxicity assays gave the same prediction. The myeloma cells in the second patient, with primary resistant disease, stained Pgp negative, but still the *in vitro* resistance pattern was influenced by chemosensitizers to the same degree as in the first case. This could indicate epitope masking or non-Pgp mediated MDR-type resistance. In the third patient no effect of chemosensitizers was detected in the Pgp positive subpopulation of tumor cells, and sensitivity to dox and vcr was higher than expected for Pgp positive myeloma cells. This could indicate detection of an antigen not associated with a functional Pgp drug efflux pump.

We could not detect any *in vitro* difference between PSC 833 and CsA in the patient samples, which is compatible with the degree of Pgp expression measured. Actually we have not found any patient among approximately 200 with hematological malignancies with a tumor as intensely Pgp positive as the RPMI 8226 dox 40 subline. Obviously this does not exclude the existence of small, undetected subpopulations of highly Pgp positive cells that could be enriched during treatment and become clinically important. In the patient treated with CsA, declining *in vitro* response at retesting does not seem to be due to *in vivo* selection of highly Pgp positive tumor cells and could reflect selection for other mechanisms of resistance.

In conclusion we have demonstrated an increased resistance modulating efficacy of the novel non-immunosuppressive CsA analog PSC compared with CsA, verapamil and quinine in highly Pgp expressing myeloma cells. The advantage of PSC over the other sensitizers, however, is lost in low grade resistant cells with low expression of Pgp, as commonly seen in the clinic. The feasibility of combining immunostaining with antibodies against Pgp and a cytotoxicity assay for resistance detection and modulation is shown. The presented data indicate the importance of including a functional assay to measure resistance modulation in clinical trials of chemosensitizers.

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