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Preclinical report

A bioassay for the activity of PSC 833 in human serum for modulation of P-glycoprotein-mediated multidrug resistance

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We established a rapid and sensitive *ex vivo* bioassay to detect the multidrug resistance (MDR)-inhibitory activity of SDZ PSC 833 ([3′-keto-Bmt[1]]-[Val[2]]-cyclosporin (PSC 833)) in two RPMI 8226 human myeloma sublines (parent 8226 and doxorubicin-resistant subline Dox6) in 75% human serum. *In vitro* sensitivity of the tumor to doxorubicin was determined by 3-h drug exposure growth inhibition assay (MTT assay). PSC 833 in serum restored the IC_{50} of doxorubicin in the P-glycoprotein (P-gp)-positive resistant subline to the same level as in the sensitive cells at 1 μg/ml, which has been shown to be an achievable concentration in clinical trials. In addition, the cytotoxic effect of doxorubicin was enhanced by PSC 833 in the sera of the patient in whom the blood level was 705.7 ng/ml. However, 10 μg/ml PSC 833 in serum does not cause a complete recovery in the IC_{90} of doxorubicin in the resistant sublines. This MDR-inhibitory activity was supported by the finding that PSC 833 in serum does not increase accumulation of rhodamine 123 in doxorubicin-resistant cells in an *in vitro* functional assay. The present study provides evidence that PSC 833 in human serum is effective to modulate P-gp-mediated MDR but insufficient for the reversal of MDR from the clinicopharmacological point of view. [© 2000 Lippincott Williams & Wilkins.]

Key words: Multidrug resistance, P-glycoprotein, SDZ PSC 833, serum.

Introduction

Multidrug resistance (MDR) in cancer chemotherapy has thus far been a major obstacle. P-glycoprotein (P-gp), a member of the family of ATP-binding cassette transporters, was the first identified example of an anticancer drug efflux pump that confers one type of multidrug resistance.[1–3] In recent years, a number of competitive inhibitors for P-gp-mediated drug efflux, so-called MDR modulators such as cyclosporin A and verapamil, have been identified.[2,3]

A number of clinical trials of MDR modulators have been carried out to evaluate their clinical activity in malignant diseases such as myeloid leukemia, multiple myeloma or solid tumor neoplasias.[4] However, in such trials, especially for solid tumors, combination therapy using an MDR modulator has failed to re-sensitize resistant tumors. One explanation given for such an outcome is that effective plasma concentrations required to reverse MDR cannot be maintained without the induction of toxic effects in patients. For instance, the cardiovascular side effects of verapamil hamper maintenance of an effective plasma concentration.[2] The increase in cytotoxicity of anticancer drugs by a MDR modulator is due to the inhibition of P-gp activity in normal organs, such as intestine, liver and kidneys, causing increased tissue distribution and/or decreased excretion of the drugs.[5] Other possible mechanisms for the pharmacokinetic interaction that have been reported are the displacement of protein binding and decreased distribution of anticancer drugs to blood cells by the modulators.[6–8]

Frequently, a target concentration of MDR modulators has been set based on *in vitro* data from growth inhibition and P-gp transport assays. However, when high fractions of compounds exist as drug bound to plasma protein and/or distributed to blood cells, *in vitro* effective concentrations of MDR modulators may not be relevant to the effective concentration in blood in patients. Thus, setting target plasma concentrations for these MDR modulators has been one of the most critical issues in clinical trial design. To assess the ability of MDR modulators to reverse MDR in patients, *ex vivo* bioassays for analysis of patient serum containing MDR modulators might be helpful in predicting clinical response.[9] In some *in vitro* studies,

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accumulation assays of fluorescent P-gp substrates have been reported to be useful for analysis of P-gp function. However, the functional assay *in vitro* is of little relevance to response in patients, because alteration of pharmacokinetics of the anticancer drugs may be responsible for the clinical activity of MDR modulators.[10] Thus, to evaluate the efficacy of MDR modulators, growth inhibition assays have been thought to be more relevant than functional assays.

SDZ PSC 833 ([3′-keto-Bmt^1]-[Val^2]-cyclosporin (PSC 833)) is a P-gp-mediated MDR modulator currently undergoing clinical trials.[11–14] Several studies reported that PSC 833 is a potent MDR-reversing agent in drug accumulation assays in serum.[10,11,15] However, it remains unclear whether PSC 833 in human serum modulate P-gp-mediated MDR in growth inhibition assays. In this study, we determined the ability of PSC 833 in human serum to re-sensitize Dox6 cells, a doxorubicin-resistant subline of RPMI 8226, by using our *ex vivo* bioassay. The data from the *ex vivo* bioassay was evaluated in terms of degree and/or activity of the modulators to re-sensitize MDR tumor cells, since this will be the most clinically relevant endpoint.

Materials and methods

Patients

Thirty-one patients with advanced solid tumors were participating in a PSC 833 phase I study in combination with doxorubicin, given i.v. One of these patients with relapsed ovarian cancer was selected for the present investigation. The patient was a 66-year-old female who had received prior chemotherapy including anthracyclines, radiotherapy and surgery prior to entry into the trial.

PSC 833 in a Cremophor EL-based solution was given by i.v. infusion from a non-polyvinylchloride i.v. delivery system through an infusion pump. PSC 833 was administered as a 2 h loading dose and as a 24 h continuous infusion. Doses were 1 mg/kg (loading dose) and 1 mg/kg (continuous infusion). Doxorubicin was administered after the loading infusion of PSC 833 as a 5 min bolus i.v. at a dose of 30 mg/m^2. The patient served as her own control by being studied during a course without PSC 833 and doxorubicin.

Sampling

Whole-blood samples were obtained from the patient at the end of the loading infusion of PSC 833 (−1 h sampling time), and at 0 h during a course without PSC 833 and doxorubicin. Serum was separated from the blood samples by centrifugation and immediately frozen at −20°C in polypropylene tubes until the time of analysis. The concentration of PSC 833 in blood was determined by radioimmunoassay according to the method the Kornblau.[16]

Materials

PSC 833 was supplied by Novartis Pharma AG (Basle, Switzerland). Doxorubicin was purchased from Kyowa Hakkou Kogyo (Tokyo, Japan) and 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), 2-[6-amino-3-imino-3H-xanthen-9-yl]-benzoic acid methyl ester (rhodamine 123) and human serum (lot 125H9308, 87H85061) were purchased from Sigma (St Louis, MO). Sodium dodecyl sulfate (SDS) and *N*,*N*-dimethyl formamide (DMF) were purchased from Wako Pure Chemicals Industry (Osaka, Japan). The Gene Amp RNA PCR kit was purchased from PE Applied Biosystems (Foster City, CA) and PCR MIMIC™ from Clontech (Palo Alto, CA). Isogen was purchased from Nippon Gene (Tokyo, Japan). All other chemicals were of analytical grade.

Tumor cells

Human myeloma cell line RPMI 8226 (8226) and its doxorubicin-resistant subline (Dox6)[17] were kindly supplied by Dr Dalton (University of Arizona Cancer Center, Tucson, AZ). 8226 and Dox6 cells were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine and 100 μg/ml kanamycin in an atmosphere containing 5% CO_2 at 37°C. Dox6 cells were maintained in the same medium containing 60 nM doxorubicin.[17]

Ex vivo bioassay

Human serum and patient serum samples were filtered using a 0.45 μm membrane filter (Millipore, Bedford, MA). MTT assay was carried out as described previously.[18] In brief, 20 000 cells in 10 μl were suspended in phenol red-free RPMI medium (Sanko, Tokyo, Japan) and seeded into 96-well tissue culture plates. Then 60 μl of serum or phenol red-free RPMI medium containing no serum but with 10 μl of different concentrations of doxorubicin (final concentration: 0, 0.3, 1, 3, 10 and 30 μl/ml) were added to each well. The final serum concentration of each well was 75%. After a 3-h incubation at 37°C, cells were washed with phosphate-buffered saline [PBS(−)] and resuspended in 150 μl of phenol red-free RPMI medium supplemented with 10% FCS. Following

further incubation at 37°C for 69 h, 37.5 μl of MTT (final concentration: 1 mg/ml) was added to each well. Following a 4-h incubation at 37°C, 100 μl of 20% (w/v) SDS/DMF solution was added to dissolve the MTT formazan products. After a 12 h incubation, the absorbance was determined at 570 nm using a 96-well plate reader (Molecular Devices). The absorbance obtained from cells grown in the serum without doxorubicin or PSC 833 (or control serum) was considered to be the control (100%). The IC_{50} and IC_{90} values were defined as the concentration inhibiting the cell growth by 50 and 90%.

P-gp function assay

P-gp function was analyzed by the rhodamine 123[19] assay. Cells in phenol red-free RPMI medium or human serum were seeded in 96-well tissue culture plates (5×10^5 cells/well) and incubated with or without PSC 833 for 15 min at 37°C. Rhodamine 123 was added to a final concentration of 10 μg/ml and the cells were further incubated for 30 min. The microplates were centrifuged for 5 min at 200 *g* and the cells were washed and resuspended in cold PBS(−). Intracellular fluorescence intensity (accumulation fluorescence, emission wavelength 530 nm and excitation wavelength 485 nm) was determined with a fluorescence plate reader (Ascent version 2.2; Labsystems., Helsinki, Finland).

To estimate rhodamine 123 retention, the cells were further incubated with or without PSC 833 in rhodamine 123-free medium or human serum for 30 min at 37°C. After washing, fluorescence intensity (retention fluorescence) was determined. Cell surface-associated fluorescence was determined after adding ice-cold rhodamine 123 to the cells and then washed immediately. The retention (%) is the percentage ratio of the retention fluorescence divided by the accumulation fluorescence of the cells.

Detection of MDR1 mRNA by reverse transcription polymerase chain reaction (RT-PCR)

Expression of MDR1 in 8226 and Dox6 cells was analyzed by competitive RT-PCR using PCR MIMIC, a heterologous internal standard. The PCR MIMICs were 580 bp *Bam*HI–*Eco*RI fragments of v-*erbB1* conjugated with gene-specific composite primers of the target gene. The PCR MIMICs for MDR1 (designated as MDR1 MIMIC™, respectively) were prepared using the PCR MIMIC™ construction kit (Clontech, Palo Alto, CA). The primers for MDR1 MIMIC consist of primer residues of the *Bam*HI–*Eco*RI fragment of v-*erbB* conjugated with additional gene-specific primer residues from MDR1 (20 residues):[20] 5′-CCCATCATTGCAATAGCAGGCGCAAGTGAAATCTCCTCCG-3′ (MDR1 MIMIC sense strand); 5′-GTTCAAACTTCTGCTCCTGATTGAGTCCATGGGGAGCTTT-3′ (MDR1 MIMIC antisense strand). The MDR1 MIMIC was amplified for 16 cycles in the presence of the composite primers for MDR1 MIMIC, followed by secondary reaction for 18 cycles in the presence of the target gene-specific primers, (MDR1: sense 5′-CCCATCATTGCAATAGCAGG-3′ and antisense 5′-GTTCAAACTTCTGCTCCTGA-3′). The PCR cycle included heat denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and polymerization at 72°C for 90 s.

Total RNA was prepared from the tumor cells using Isogen (Nippon Gene, Tokyo, Japan). cDNA was prepared from 2 μg of total RNA using the Gene Amp RNA PCR kit (PE Applied Biosystems, Foster City, CA). The reaction mixture was incubated at 42°C for 30 min, and then heated for 5 min at 99°C to inactivate MuLV reverse transcriptase. The cDNA derived from 0.5 μg of total RNA was mixed with 20 μl PCR reaction mixture [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 188 nM gene-specific primers and 0.625 u AmpliTaq DNA polymerase] containing 0.312 amol of the MDR1 MIMIC. PCR reactions consisted of 30 cycles, and included 45 s of heat denaturation at 94°C, annealing at 55°C for 1 min and polymerization at 72°C for 2 min. The PCR products were analyzed by electrophoresis through 1.5% agarose gel and stained in 0.2 μl/ml ethidium bromide. The products were visualized by UV transillumination and photographed.

Statistical analysis

Statistical analysis of the dose–response curves for IC_{50} values and rhodamine 123 retention (%) was performed by two-way ANOVA. These analyses were done by Stat View II software (version 4.02 for Macintosh; Abacus Concepts, Berkeley, CA) and GraphPad PRISM software (version 2.0 for Windows; GraphPad, San Diego, CA).

Results

Expression of MDR1 mRNA

Expression of MDR1 mRNA in multidrug-resistant Dox6 and -sensitive 8226 cells was determined by a RT-PCR method using PCR MIMIC™ as a heterologous internal standard. As demonstrated in Figure 1, MDR1 mRNA encoding P-gp was overexpressed in Dox6, but little amplified product was detected in parental 8226.

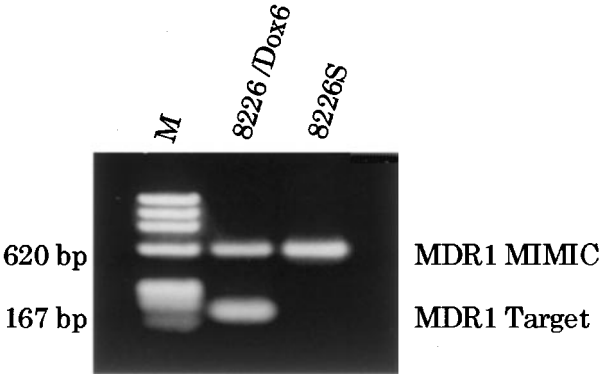

Figure 1. Target and MIMIC PCR products of MDR1. Total RNA (0.5 μg) extracted from 8226 and Dox6 cells, and MDR1 MIMIC (0.312 amol) were used in the PCR reactions. After 30 amplification cycles, the products were resolved on a 1.5% agarose gel and stained with ethidium bromide. Lane M represents ϕX174/*Hae*III DNA as size markers.

Comparable band intensity was seen for the MIMIC cDNA in the two samples, suggesting that the MDR in Dox6 can be attributed at least in part to MDR1 overexpression.

Cytotoxic effects of doxorubicin in combination with PSC 833 on multidrug-resistant and -sensitive cells

PSC 833, a potent P-gp-mediated MDR modulator, has shown potential in *in vitro* experiments and in clinical trials. In an initial experiment, PSC 833 concentrations to be used in the study were evaluated to determine whether PSC 833 by itself reduced cell growth rates. Cells were incubated in the absence or the presence of PSC 833 at concentrations from 0.3 to 10 μg/ml and the viability of the cells determined by a MTT assay. As shown in Figure 2, PSC 833 was found not to affect the cell viability of the two sublines in either human serum or serum-free media.

Next, we examined the effect of PSC 833 on the cytotoxic activity of doxorubicin, a substrate of P-gp, in the multidrug-resistant and -sensitive cells. The growth inhibition curves from doxorubicin on the two sublines demonstrated resistance to doxorubicin in Dox6 cells (Figure 3). The doxorubicin IC_{50} values verified a 2- to 3-fold increased resistance to doxorubicin in Dox6 as compared with the parental 8226 (human serum: Dox6, 4.61 ± 2.43 μg/ml, 8226, 2.80 ± 1.42 μg/ml, serum-free medium: Dox6, 4.71 ± 1.59 μg/ml, 8226, 1.21 ± 0.20 μg/ml). The results suggest that intracellular concentrations of doxorubicin in Dox6 might be kept low in relation to the transporter-negative 8226 cells. In addition, PSC

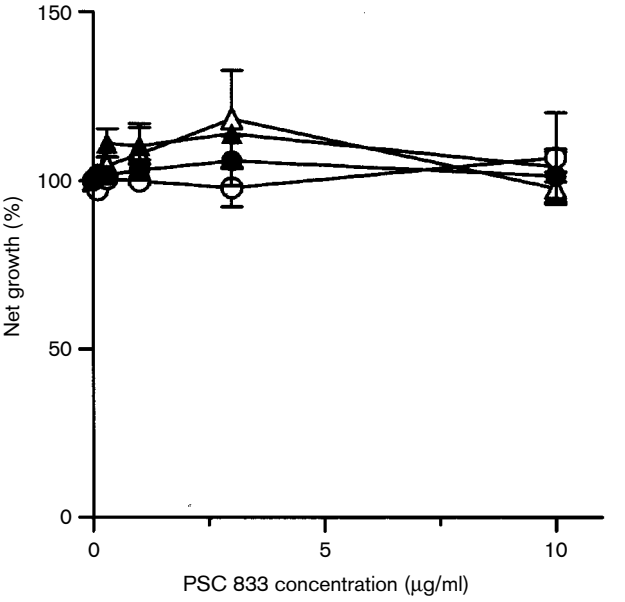

Figure 2. Cytotoxic effects of PSC 833 on two RPMI 8226 human myeloma sublines. Cytotoxic effects of PSC 833 on tumor cells were determined by the MTT assay in 75% serum or in serum-free medium. Net growth (%) was calculated relative to cells grown without PSC 833 as control. Each value represents the mean $\pm$ SD of three determinations. (●) Dox6 in RPMI medium. (○) Dox6 in human serum. (▲) 8226 in RPMI medium. (△) 8226 in human serum.

833 at non-cytotoxic concentrations (0.3–10 μg/ml) enhanced the doxorubicin cytotoxicity in Dox6 in both serum and serum-free medium. In contrast, 10 μg/ml PSC 833 did not markedly enhanced doxorubicin cytotoxicity in 8226 cells (data not shown).

The target concentration of PSC 833, which effectively reverses the sensitivity of the multidrug-resistant subline to the same level as that of the sensitive cells, was evaluated from concentration dependent sensitization curves. The curves for Dox6 and 8226 cells demonstrated that comparable doxorubicin IC_{50} values of the two sublines was obtained by PSC 833 at 0.3 μg/ml in serum-free medium (Figure 4). When serum was present, a much higher concentration of PSC 833, 1 μg/ml, was required for a reduction in the IC_{50} of doxorubicin in Dox6 cells. The statistical analysis indicates that the sensitivity to doxorubicin in serum was significantly less than in serum-free medium in both Dox6 and 8226 cells (two-way ANOVA; $p < 0.0001$). In clinical studies, a mean PSC 833 blood level of approximately 1–2 μg/ml can be achieved by an oral or i.v. administration regimen;[11–14] therefore, equivalent concentrations effectively modulate MDR in serum in terms of doxorubicin sensitivity. On the other hand, the data from IC_{90} values showed that

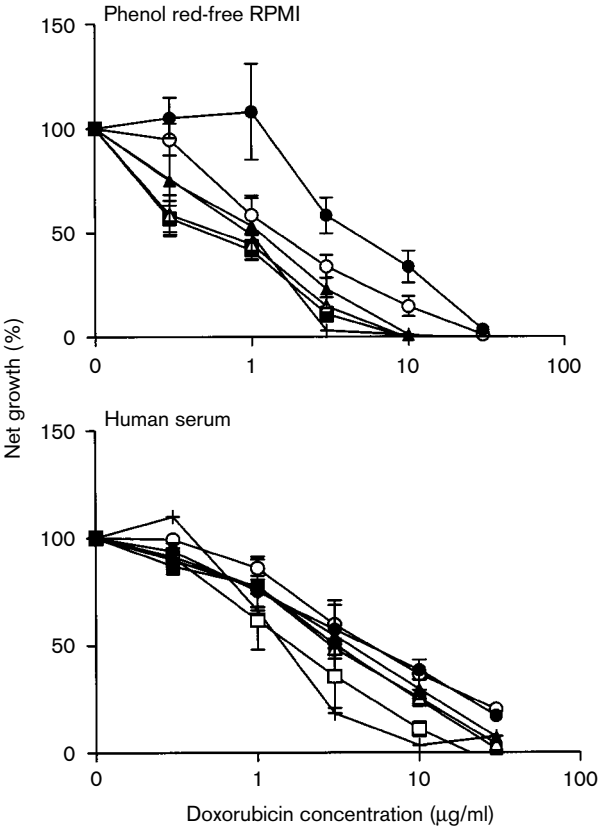

Figure 3. Enhancement of doxorubicin anti-growth activity by PSC 833. Growth inhibition of the drug-resistant Dox6 cells and parental 8226 cells by doxorubicin was determined by the MTT assay. Net growth (%) is plotted against doxorubicin concentration. Each value represents the mean ± SD of three determinations. (●) Doxorubicin alone in Dox6 cells. (○) + PSC 833 0.1 μg/ml. (▲) + PSC 833 0.3 μg/ml. (△) + PSC 833 1 μg/ml. (■) + PSC 833 3 μg/ml. (□) + PSC 833 10 μg/ml. (+) Doxorubicin alone in 8226 cells.

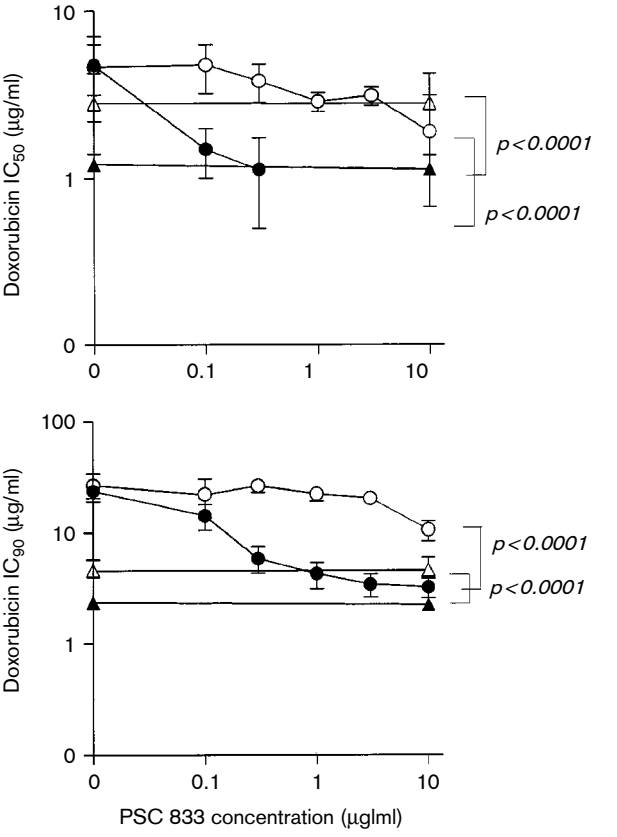

Figure 4. Concentration-dependent sensitization of Dox6 cells by PSC 833. The IC_{50} and IC_{90} values of doxorubicin on inhibition of the growth of tumor cells were plotted against the PSC 833 concentration. Each value represents the mean ± SD of three determinations. The *p* values (two-sided) shown were determined by two-way ANOVA for the comparison of entire curves. (●) Dox6 in RPMI medium. (○) Dox6 in human serum. (▲) 8226 in RPMI medium. (△) 8226 in human serum.

comparable doxorubicin sensitivity of the two sublines was obtained by 3 μg/ml PSC 833 in serum-free medium. In the presence of serum, however, even at a concentration of 10 μg/ml PSC 833 did not completely recover doxorubicin sensitivity of Dox6 cells comparable to those of non-resistant cells.

Effect of PSC 833 on the function of the multidrug transporter

Although PSC 833 sensitized Dox6 cells to doxorubicin, it is unclear whether PSC 833 can inhibit the function of P-gp. To examine whether PSC 833 inhibits transport functions of P-gp, we analyzed the intracellular accumulation of rhodamine 123, which is a substrate of the transporter. Figure 5 shows that PSC 833 enhanced intracellular fluorescence of rhodamine 123 in transporter-positive Dox6 cells in either serum or serum-free media. The concentrations that enhance accumulation in Dox6 as compared to 8226 cells were approximately 1 μg/ml in serum-free medium and 10 μg/ml in serum-containing medium, respectively. The entire dose–response curves in the presence or absence of serum were compared and a statistically relevant difference was evident between the two sublines (two-way ANOVA: 8226, p=0.001; Dox6, p=0.0002). The above *in vitro* function assays demonstrated that PSC 833 sensitized the multidrug-resistant Dox6 by inhibiting the multidrug transporter P-gp.

Sensitization of Dox6 cells to doxorubicin in the sera of a patient

To further explain the clinical relevance, patient sera were subjected to the *ex vivo* bioassay using MTT

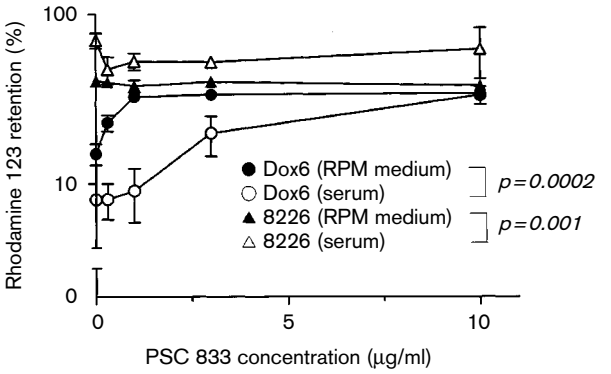

Figure 5. Effects of PSC 833 on rhodamine 123 retention (%) in tumor cells. Inhibition of P-gp function by PSC 833 was analyzed by using a fluorescent P-gp substrate, rhodamine 123. Rhodamine 123 retention (%) was determined by dividing the retention fluorescence by the accumulation fluorescence in the cells. In the absence of PSC 833, the accumulation fluorescence of rhodamine 123 in 8226 cells was 5.88 (arbitary units) in serum and 18.7 in serum-free medium. Each value represents the mean ± SD of three determinations. The *p* values (two-sided) shown were determined by two-way ANOVA for the comparison of entire curves.

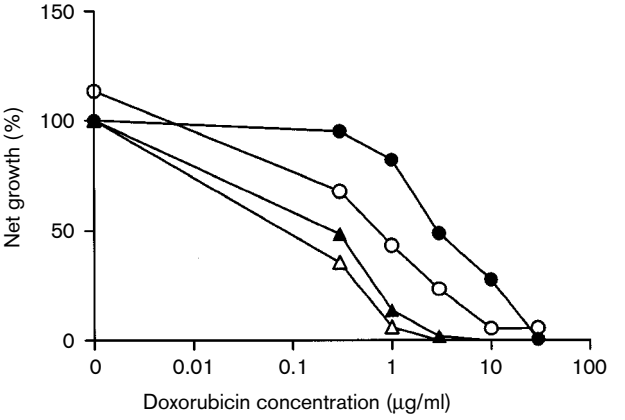

Figure 6. Enhancement of doxorubicin anti-growth activity by PSC 833 in the serum of a patient. The patient was given 30 mg/m^2 doxorubicin by short infusion and 1 mg/kg PSC 833 by continuous infusion. The serum samples before the doxorubicin infusion [−1 h sampling time, PSC 833 (+) and doxorubicin (−)] and the control serum [PSC 833 (−) and doxorubicin (−)] were subjected to the *ex vivo* bioassay to evaluate the sensitivity reversal activity of the serum. The growth inhibition of the drug-resistant Dox6 cells and parental 8226 cells by this serum supplemented with a series of dilutions of doxorubicin was determined by the MTT assay. Net growth (%) was plotted against the externally added doxorubicin concentration in the MTT assay. (○) Dox6 in serum containing PSC 833. (●) Dox6 in control serum. (△) 8226 in serum containing PSC 833. (▲) 8226 in control serum.

assay to evaluate the resistance reversal activity of PSC 833 in sera. One patient received 1 mg/kg PSC 833 in combination with 30 mg/m^2 doxorubicin, and serum samples before the doxorubicin infusion [−1 h sampling time, PSC 833 (+) and doxorubicin (−)] and before the PSC 833 infusion [control serum, PSC 833 (−) and doxorubicin (−)] were collected. Serum samples were supplemented with doxorubicin and added to 8226 or Dox6 cells in culture. In the control serum of the patient, the doxorubicin IC_{50} value of the resistant subline was approximately 10-fold higher than the value for parental cells (8226, 0.291 μg/ml, Dox6, 2.90 μg/ml). In the serum containing PSC 833, the concentration of PSC 833 measured in blood was 705.7 ng/ml by radioimmunoassay. This concentration of PSC 833 appears to be sufficient to partially reverse doxorubicin resistance in Dox6 cells (Figure 6, IC_{50}=0.730 μg/ml). In contrast, the serum containing PSC 833 had no effect on the doxorubicin sensitivity of 8226 cells (IC_{50}=0.164 μg/ml).

Discussion

Several studies utilizing *in vitro* cytotoxicity and drug accumulation assays have investigated the effect of human serum on the activity of MDR modulators.[21,22] In these studies, physiological serum protein concentrations could reduce the bioavailability of MDR-reversing activity by high plasma protein binding. Lehnert *et al.*[21] showed that amiodarone and trifluoperazine are almost completely inactivated by serum when used at a wide range of concentrations from 0.5 to 20 μM. Other agents, such as verapamil and cyclosporin A, are essentially unaffected at concentrations of more than 10 μM, but are markedly inhibited by serum at concentrations achievable in humans. This indicated that *in vitro* studies of MDR modulators in culture medium containing low serum protein concentrations can result in misleading conclusions regarding the potential clinical activity of such agents. Therefore, it is important to study the bioavailability of MDR modulators in human serum *in vitro*. A number of papers have described the bioassay in serum with PSC 833, functional assays with rhodamine 123 using $CD56^+$ cells[15] or $CD8^+$ T lymphocytes,[10] PSC 833 in plasma with H69/LX4 cell lines using radio-labeled daunorbicin accumulation.[11] In addition to the fact that radiolabeled drug is inconvenient to use, the functional assay is of little relevance to clinical response, despite measurement of the inhibition of P-gp-mediated drug efflux essential to overcome MDR. In this study, we examined the efficacy of PSC 833 in human serum in our growth inhibition bioassay for predicting the clinical bioavailability of PSC 833.

Determination of the MDR-modulating potency of PSC 833 was determined by 3 h drug exposure growth inhibition assays. A 3 h drug exposure did not reduce the bioavailability of Dox6 cells or the parental cells (data not shown). The decreased activity of PSC 833 at concentrations achievable in human serum was demonstrated when compared to serum-free conditions. In our experiments, although PSC 833 at approximately 1 μg/ml cause a marked reduction in the IC_{50} of doxorubicin in multidrug-resistant Dox6 cells in both normal adult and patient sera following administration of PSC 833, these conditions only provide about a 25% reduction in Dox6 cell growth. From the data on the IC_{90} value of doxorubicin, 10 μg/ml PSC 833 in serum showed almost complete attenuation of Dox6 cell growth; however, this concentration proved ineffective to modulate doxorubicin resistance and rhodamine 123 uptake to the same level as in the sensitive cells. Thus, the present results lead to the important conclusion that steady-state blood PSC 833 levels of more than 1–2 μg/ml may cause a marked but an insufficient attenuation of P-gp-positive cell growth in patients. Serum PSC 833 levels more than 1–2 μg/ml were not achieved in clinical trials, since PSC 833 that can be administrated to patients is limited by severe neurotoxicity, ataxia.[11] Our present bioassay results are consistent with the conclusions reported by Smith *et al.*[22] who found that PSC 833 could not completely reverse drug resistance at high concentrations in the presence of serum. Furthermore, Tunggal *et al.*[23] demonstrated that increasing cell density caused a decrease in doxorubicin uptake and MDR modulator efficacy in typical cell cultures at 10^5–10^6 cells/ml, which is much less than that found *in vivo* (around 10^9 cells/ml).

In addition to the activity of MDR modulators in serum, alteration of the pharmacokinetics of anticancer drugs may also contribute to their clinical activity. PSC 833 as well as other reversing agents modulate the pharmacokinetics of multidrug-resistant-related drugs.[11,24,25] When the unbound fraction of an anticancer drug in blood is increased by protein binding displacement and/or reduction in the distribution to blood cells by PSC 833, there is a transient rise in the unbound concentration of the anticancer drug in plasma. The increase in unbound concentration in plasma could increase the tissue distribution of the anticancer drugs.[6,7] Furthermore, enhanced tissue distribution of such drugs was indicated as being responsible for the increase in toxicity of the antitumor drugs in combination with PSC 833 in animals.[24,25] Previously, we reported that PSC 833 concentrations of at least 3 μg/ml did not markedly influence either serum protein binding or distribution to blood cells of doxorubicin, vincristine or etoposide in human serum.[26] The absence of an interaction between PSC 833 and the anticancer drugs in protein binding and distribution to blood cells suggested the existence of other mechanisms contributing to the increase in cytotoxicity of anticancer drugs after co-administration of PSC 833. Possible interactions are speculated to cause inhibition of the endogenous function of P-gp, such as efflux pump in tissues and excretion into bile and urine, and inhibition of drug metabolism mediated by cytochrome P450 3A. Ongoing clinical studies of PSC 833 should therefore consider appropriate dose modification of anticancer drugs since the effect of this modulator is not simply on pharmacokinetics.

In summary, we conclude that our established *ex vivo* bioassay system using growth inhibition assay can be used to evaluate the activity of PSC 833 in serum of patients receiving clinical treatment. We think the reversal activity of PSC 833 in our system will probably explain the most clinically relevant end-point in solid tumors.

Acknowledgments

Our colleague and friend Dr Toru Watanabe died on 14 December 1998. This paper is dedicated to his memory. The National Cancer Center Hospital East supported this study. We would also like to thank all of the clinical staff, particularly Dr Tomoko Ohtsu and Dr Yasutsuna Sasaki.

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