Acta Oncologica Vol. 34, No. 2, pp. 235–241, 1995

COMPARATIVE STUDY ON REVERSAL EFFICACY OF SDZ PSC 833, CYCLOSPORIN A AND VERAPAMIL ON MULTIDRUG RESISTANCE IN VITRO AND IN VIVO

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A non-immunosuppressive cyclosporin, SDZ PSC 833 (PSC833), shows a reversal effect on multidrug resistance (MDR) by functional modulation of MDR1 gene product, P-glycoprotein. The objective of the present study was to compare the reversal efficacy of three multidrug resistance modulators, PSC833, cyclosporin A (CsA) and verapamil (Vp). PSC833 has approximately 3–10-fold greater potency than CsA and Vp with respect to the restoring effect on reduced accumulation of doxorubicin (ADM) and vincristine (VCR) in ADM-resistant K562 myelogenous leukemia cells (K562/ADM) in vitro and also on the sensitivity of K562/ADM to ADM and VCR in in vitro growth inhibition. The in vivo efficacy of a combination of modifiers (PSC833 and CsA: 50 mg/kg, Vp 100 mg/kg administered p.o. 4 h before the administration of anticancer drugs) with anticancer drugs (ADM 2.5 mg/kg i.p., Q4D days 1, 5 and 9, VCR 0.05 mg/kg i.p., QD days 1–5) was tested in ADM-resistant P388-bearing mice. PSC833 significantly enhanced the increase in life span by more than 80%, whereas CsA and Vp enhanced by less than 50%. This reversal potency, which exceeded that of CsA and Vp, was confirmed by therapeutic experiments using colon adenocarcinoma 26-bearing mice. These results demonstrated that PSC833 has signficant potency to reverse MDR in vitro and in vivo, suggesting that PSC833 is a good candidate for reversing multidrug resistance in clinical situations.

Resistance to anticancer drugs is one of the major causes of failure in cancer chemotherapy, despite many advances in treatment. The development of acquired resistance during treatment, inherent resistance to anticancer drugs, and heterogeneous sensitivity in a population may contribute to the resistance in cancer chemotherapy. In the last decade, it has been clarified that a membrane glycoprotein termed P-glycoprotein plays an important role in the drug resistance to hydrophobic and structurally unrelated antitumor agents such as anthracyclines and vinca alkaloids (multidrug resistance (MDR)) (1–3). The P-glycoprotein level in plasma membrane often associates with the resistance index, reduced drug accumulation, and enhanced active efflux from cells (1, 2). It has been established that P-glycoprotein functions as an ATP-hydrolyzing drug efflux pump to transport the hydrophobic agents out of the cell (2, 4), thereby keeping the cytosolic concentration of the substrate low.

The discovery of the reversal effect of verapamil (Vp) on MDR (5) has led to the identification of many agents that have a reversing effect on MDR, e.g., several calcium antagonists (6–8), calmodulin antagonists (7, 8), cyclosporins (9, 10), FK506 (11), AHC 52 (12) and S9733 (13). Although the potential mechanism(s) involved in the

Received 29 September 1993.
Accepted 19 October 1993.
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Presented at the 18th International Congress of Chemotherapy, Stockholm, Sweden, June 27–July 2, 1993.

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modulation of P-glycoprotein-mediated resistance by the modulators have not yet been fully explored, the finding of direct binding of photoanalog of Vp (14) and cyclosporin (15) to P-glycoprotein suggests that MDR modifiers directly bind to P-glycoprotein and interfere with the binding of the MDR-related anticancer agents to this transporter.

Cyclosporin A (CsA) has clear reversing effects on multidrug resistance in vitro and in vivo (9, 10, 16). However, its immunosuppressive effects and nephrotoxicity limit its clinical usefulness for reversal of MDR (16). SDZ PSC833 (PSC833), which has no immunosuppressive or nephrotoxic effects, has been developed as a candidate for circumvention of MDR for clinical trials. Recent studies have demonstrated that PSC833 has greater efficacy than CsA in reversing MDR in vitro and in vivo (17–20). In the present study, we compared the reversing potency of PSC833 with two reference agents, CsA and Vp, in vitro and in vivo, in the following experimental items: effect on 1) reduced accumulation of doxorubicin (ADM) and vincristine (VCR) in ADM-resistant subline of K562 myelogenous leukemia (K562/ADM), 2) sensitivity of K562/ADM to ADM and VCR in in vitro growth inhibition and 3) antitumor activity of ADM and VCR in ADM-resistant P388 leukemia (P388/ADM)- and colon adenocarcinoma 26 (Colon26)-bearing mice models.

Material and Methods

Drugs. VCR and ADM formulated for clinical use were obtained from Shionogi Co., Ltd., Osaka, and Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan respectively. For oral application, PSC 833 (Sandoz, Basel) and CsA (Sandoz, Basel) were dissolved in ethanol, and then mixed with labrafil (Sandoz, Basel). These solutions were diluted with corn oil to the concentration of 10 mg/ml (standard solution: ethanol/labrafil/corn oil 2.1 : 4.2 : 3.7 v/v/v). The standard solution was further diluted with corn oil before application in a volume of 5 or 10 ml/kg body weight. Vp (Sigma Chemical Co. Ltd., Tokyo) was dissolved in distilled water for oral application in a volume of 0.01 ml/g body weight. $[^{3}H]$VCR (6.6 Ci/mmol) and $[^{14}C]$ADM (55 mCi/mmol) were obtained from Amersham Japan Ltd., Tokyo. All other chemicals were purchased commercially and were of analytical grade.

Cell culture and drug treatment. The human myelogenous leukemia K562 cell line (K562) was provided by Dr. Ezaki, and its subline resistant to ADM was established in our laboratory (21, 22). K562 and K562/ADM were maintained in culture dishes in RPMI1640 supplemented with 5% fetal calf bovine serum and 100 μg/ml kanamycin. The in vitro growth inhibition assay was carried out according to the method described previously (5–8, 11). In brief, K562 and K562/ADM cells (4×10^{4} cells/2 ml culture medium) in culture tubes were cultured at 37°C for 3 h in a humidified atmosphere comprising 5% CO_2/95% air, following by further incubation for 72 h in the presence or absence of the anticancer drug (ADM or VCR) and the MDR modulator (PSC833, CsA or Vp). The number of cells was counted in a model ZM Coulter Counter. The median concentration of drug necessary to inhibit the growth of the cells by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration against the growth rate (percent of control) of drug-treated cells.

Intracellular accumulation of $[^{14}C]$ADM and $[^{3}H]$VCR. K562 and K562/ADM at a cell density of 10^{6} cells/ml culture medium were incubated in Corning 60 mm tissue culture dishes in the presence or absence of the MDR modulators (PSC833 at 0.1–3 μM, CsA and Vp at 1–30 μM) at 37°C for 10 min, followed by further incubation for 2 h after the addition of the radiolabeled drug. The intracellular accumulation of the radiolabeled ligand was measured as described previously (5–8, 14).

Evaluation of antitumor activity. Colon26 and P388/ADM were kindly supplied by the National Cancer Institute, NIH, Bethesda, Md. Female 6-week-old BALB/c and female 6-week-old BALB/c × DBA/2CrF$_1$ (CD2F1) mice weighing 19–23 g each were purchased from Charles River Japan, Inc., Tokyo, Japan. A volume (0.1 ml) of cell suspension, 10^{6} of P388/ADM cells in 0.1 ml of Hanks' balanced salt solution (HBSS), was inoculated intraperitoneally (i.p.) into the CD2F1 mice (day 0). The in vivo efficacy of the combination of modifiers (PSC833 and CsA: 50 mg/kg, Vp 100 mg/kg administered p.o. 4 h before the application of anticancer drug) with anticancer drug (ADM 2.5 mg/kg i.p., Q4D days 1, 5 and 9, VCR 0.05 mg/kg i.p., QD days 1–5) was tested in CD2F1 mice inoculated with 10^{6} of P388/ADM. Each drug-treated and control group of mice consisted of five or six and ten mice respectively. Antitumor activity was evaluated by an increase in life span (ILS (%)), calculated by the equation:

$$ILS = (T/C - 1) \times 100$$

where T and C denote the mean survival time of the drug-treated and control groups, respectively. Antitumor activity using Colon26-bearing mice was evaluated as described previously (23, 24). Colon26 were prepared from surgically removed tumors which were inoculated s.c. bilaterally in BALB/c mice. The surgically removed tumor fragments were minced in HBSS to make the tumor cell suspension. Obtained tumor homogenate was passed through 40 mesh sieves. Thereafter 10^{5} of viable Colon26, as determined by trypan blue dye exclusion, were implanted s.c. bilaterally in CD2F1 mice (day 0). ADM (8 mg/kg i.v.) and VCR (0.3 mg/kg i.v.) in combination with the MDI modulators were administered on day 1 and days 1, 5 and 9 respectively. Mice of each group were weighed 2 or 3 times a week. Long and short diameters were measured using calipers on the same day of the weighing. The tumor volume (v) was calculated by the

following equations:

$$v = 1/2 \times a \times b^2$$

where a and b are the long and short diameters of the tumor mass in mm. The weight of mice and tumor volume were measured until the death of the mice. Antitumor activity was evaluated by the T/C (precentage ratio of the mean tumor volume of the drug-treated group to that of the control group) and ILS values.

Results

In vitro effect of MDR modulators on restoration of reduced accumulation of [^{14}C]ADM and [^{3}H]VCR in K562/ADM. The intracellular accumulation of [^{14}C]ADM and [^{3}H]VCR in K562 and K562/ADM was evaluated after incubation with the radiolabeled ligand in the presence or absence of various concentrations of the MDR modulator (PSC833 0.1–3 μM, CsA and Vp 1–30 μM), as shown in Fig. 1A and B. K562/ADM, a P-glycoprotein- and MDR1 expression-positive cell line (22), in the absence of the MDR modulators accumulated [^{14}C]ADM and [^{3}H]VCR at a lower level than K562 (K562; 5.6 pmol of [^{14}C]ADM and 0.65 pmol of [^{3}H]VCR per 10^6 cells, and K562/ADM 4.0 pmol of [^{14}C]ADM and 0.06 pmol of [^{3}H]VCR per 10^6 cells). The dose intensity of the restoration of reduced accumulation in K562/ADM by the three MDR modulators shows that PSC833 even at 1 μM in the medium was sufficient to restore the uptake of [^{14}C]ADM and [^{3}H]VCR by K562/ADM to a level comparable to that of K562, while 0.1 and 0.3 μM of PSC833 had partial restoration ability. PSC833 slightly increased the accumulation of [^{14}C]ADM and [^{3}H]VCR also in K562 (Fig. 1), although these levels were not statistically different from the level in the absence of PSC833 in the medium ($p > 0.05$). In the comparison of the dose intensity of the three MDR modulators, PSC 833, was much more effective than CsA and Vp in increasing the intracellular accumulation of ADM or VCR in K562/ADM. The concentration of PSC 833 required to increase the drug accumulation level to that of sensitive cells (K562) was 10 times lower than that of CsA and Vp. At 1 μM of the MDR modulators in the medium; PSC833 was the only modulator that restored accumulation levels of both [^{14}C]ADM and [^{3}H]VCR to levels that were statistically different from the levels in the absence of the MDR modulator ($p < 0.01$).

In vitro reversal effect of MDR modulators on sensitivity to ADM and VCR in K562/ADM. Examination of the growth inhibition of K562/ADM by either ADM or VCR alone showed that K562/ADM was resistant to ADM and VCR by 140 ± 32 and 220 ± 76-fold (mean $\pm$ standard deviation in triplicate determination) respectively. All of the MDR modulators (PSC833, CsA and Vp) enhanced the sensitivity of K562/ADM to ADM and VCR in vitro

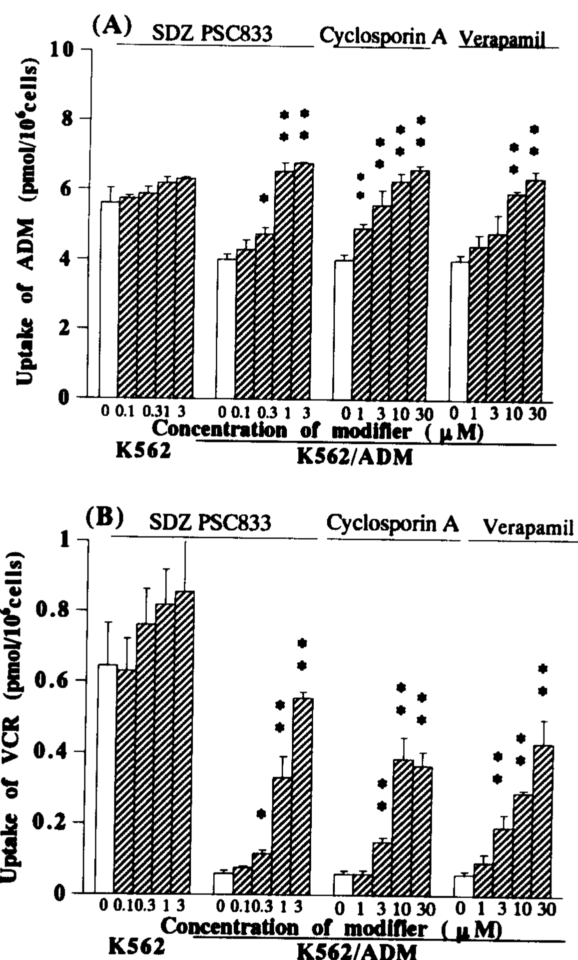

Fig. 1. Effects of MDR modulators on the intracellular accumulation of (A) [^{14}C]ADM and (B) [^{3}H]VCR. Cell suspensions of K562 and K562/ADM at a cell density of 10^6 cells/ml of culture medium were incubated at 37°C for 2 h with the radio-labeled ligand ([^{14}C]ADM at 170 nM or (B) [^{3}H]VCR at 23 nM) in the presence or absence of either PSC833, CsA or verapamil as indicated above the bars. The intracellular accumulation was estimated as described in Material and Methods. Data represent mean ± standard deviation of triplicate determinations. Error bar denotes the standard deviation. * and **: statistically different from intracellular accumulation in the absence of MDR modulator by Student's t-test ($p < 0.05$: * and $p < 0.01$: **).

(Fig. 2). This sensitizing ability of PSC833 was much more potent than that of CsA and Vp as the restoring ability of PSC833 in the intracellular accumulation of ADM and VCR. PSC833 (1.5 μM) enhanced the sensitivity of K562/ADM to ADM cytotoxicity by a factor of 55 and to VCR cytotoxicity by a factor of 99, whereas 3 μM CsA and Vp enhanced ADM cytotoxicity 5.3- and 9.3-fold and to VCR cytotoxicity 4.1- and 6.8-fold respectively. PSC833 was the only agent which completely restored the sensitivity of K562/ADM to ADM and VCR to the levels of K562 (IC_{50}: ADM17.7 nM, VCR 3.3 nM). In the sensitive cells, maximum sensitization ratio, fold decrease in IC_{50} by the

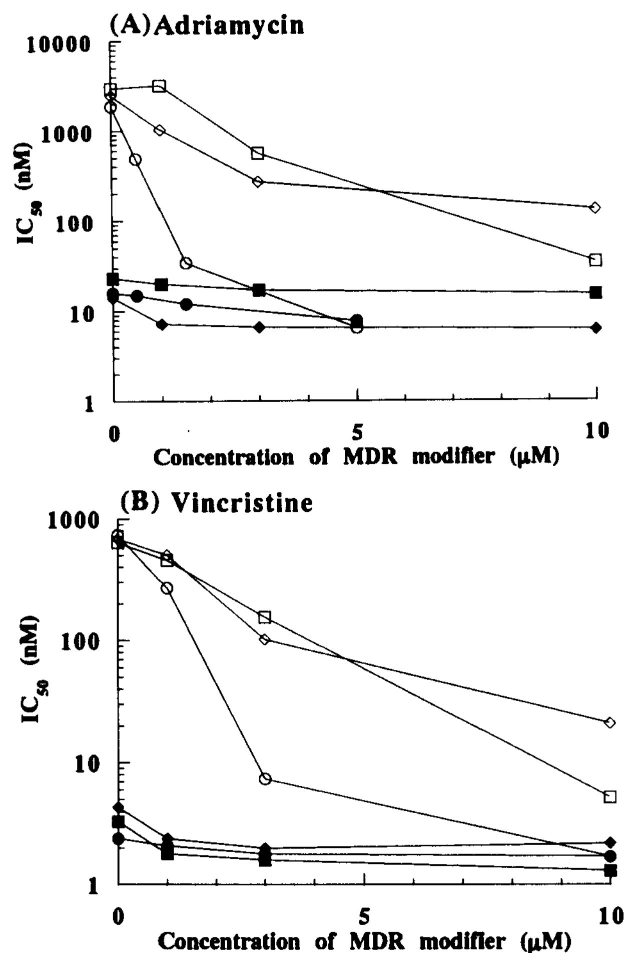

Fig. 2. In vitro sensitization of K562/ADM to (A) ADM and (B) VCR by the MDR modulators. Growth inhibitory effects of ADM or VCR in the presence or absence of MDR modulators (○●PSC833, ◇◆: Vp, □■: CsA) were determined after 72 h culture as described in Material and Methods. The IC_{50} values were plotted against the concentration of MDR modulator in the medium. Open and closed marks denote the IC_{50} values in K562/ADM and K562 respectively.

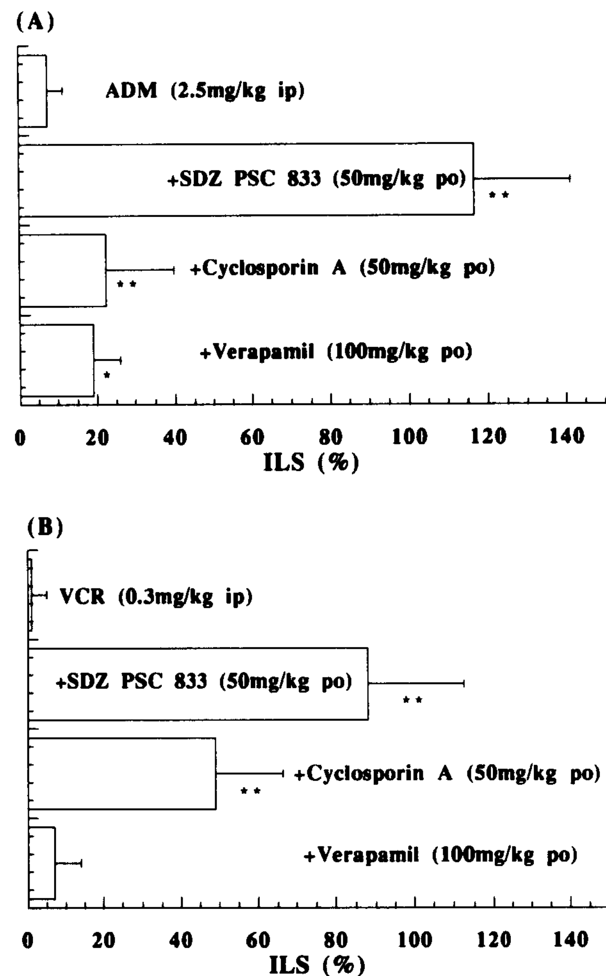

Fig. 3. In vivo therapeutic effect of MDR modulators on antitumor activity of (A) ADM and (B) VCR in P388/ADM-bearing mice. 10^6 cells of P388/ADM were inoculated i.p. in CD2F1 mice. MDR modulators (PSC833, CsA or Vp) were administered p.o. 4 h prior to iv administration of the anticancer drug. Antitumor activity of ADM or VCR in combination with the modulators was expressed as ILS (%) as described in Material and Methods. Each group consists of 5 (Fig. 3(B)) or 6 (Fig. 3(A)) mice and the control consists of 10 mice. * and **; statistically different from ILS, in the group of mice treated with ADM or VCR alone by Student's t-test ($p < 0.05$: * and $p < 0.01$: **).

presence of the modulator, being less than 3.0 indicated that the three MDR modulators poorly enhanced the sensitivity of K562 to ADM and VCR.

In vivo reversal of MDR by MDR modulators in P388/ADM and Colon26-bearing mice models. In vivo studies confirmed the in vitro findings. Colon26 and P388/ADM were used in the therapeutic model for inherent and acquired multidrug resistance respectively, since P388/ADM and Colon26 are MDR1 expression- and P-glycoprotein-positive cell lines (S. Sone, personal communication). In the therapy using the tumor-bearing mice models, an equal dose of the two cyclosporins (50 mg/kg p.o.) was set to compare the total potency of the cyclosporins to reverse MDR in tumor and to increase bioavailability of the anticancer drugs. The dose of Vp was set as 100 mg/kg p.o., which gave maximum solubility of Vp in the oral dosing solution. Mice inoculated with P388/ADM were treated with ADM or VCR in combination with the MDR modifiers. Application of the MDR modulator or the antitumor drug alone had negligible antitumor activity in P388/ADM-bearing mice (ILS < 10%). Oral administration of SDZ PSC 833 significantly potentiated the antitumor activity of ADM in P388/ADM-bearing mice, as shown in Fig. 3A. Although the application of CsA and Vp also potentiated the antitumor activity of ADM, the ILS values in the case of CsA and Vp were much less than that in the case of PSC833 (ILS of CsA and Vp were less than 25%). PSC833 was also the most potent agent

Table

Effect of SDZ PSC 833, cyclosporin A and verapamil on antitumor activity of doxorubicin (ADM) and vincristine (VCR) in colon adenocarcinoma 26-bearing mice

Drug and dosage	Schedule	MST[a] (days)	ILS[b] (%)	Tumor volume (mm^3 on day 18)	T/C[c] (%)	Body weight change[d] (g)
Control		27.6 ± 5.6[e]		791 ± 393[e]	100	−1.6
ADM (8 mg/kg i.v.)	day 1	27.7 ± 8.2	0.2	898 ± 568	114	−1.1
+SDZ PSC 833 (50 mg/kg po)	day 1	39.0 ± 6.4*1*2	41	112 ± 94*1*2	14.1	−2.3
+cyclosporin A (50 mg/kg po)	day 1	36.8 ± 7.3*3	34	502 ± 316	63.5	−1.4
+verapamil (100 mg/kg po)	day 1	35.6 ± 8.2	29	615 ± 454	60.3	−1.5
VCR(0.3 mg/kg iv)	days 1, 5 and 9	28.8 ± 8.7	4.5	837 ± 117	106	−1.1
+SDZ PSC 833 (50 mg/kg po)	days 1, 5 and 9	38.7 ± 9.7*3	40	375 ± 377	47.4	−1.0
+cyclosporin A (50 mg/kg po)	days 1, 5 and 9	31.8 ± 9.0	15	805 ± 377	102	−0.4
+verapamil (100 mg/kg po)	days 1, 5 and 9	30.0 ± 8.2	8.7	615 ± 454	77.8	−0.4

Colon26 cells were implanted s.c. in CDF1 mice. MDR modulator was administered po 4 hours prior to the anticancer drug administration. ADM and VCR were administered iv on day 1 and days 1, 5 and 9 respectively.
a) MST, mean survival time.
b) ILS, increase in life span, described in Material and Methods.
c) T/C (%) denotes tumor volume ratio of treated to control mice.
d) Difference in body weight (g) between days 9 and 1.
e) Values of MST and tumor volume are shown as mean ± standard deviation.
*1 Significant difference form control group by Student's t-test ($p < 0.01$).
*2 Significant difference from group treated wtih ADM or VCR alone by Student's t-test ($p < 0.05$).
*3 Significant difference from control group by Student's t-test ($p < 0.05$).

(ILS > 80%) in the potentiative ability of antitumor activity of VCR in P388/ADM-bearing mice (Fig. 2B). Application of CsA revealed moderate reversal (ILS approximately 50%) while application of Vp led to negligible activity (ISL < 10%). In the Colon26-bearing mice model, the anticancer drug alone or in combination with the MDR modulator was applied on day 1 (ADM) or days 1, 5 and 9 (VCR). On day 18, the tumor volume, being approximately 800 mm^3 in the control mice, was determined to evaluate the T/C value. Although the dosages of ADM (day 1, 8 mg/kg i.v.) and VCR (days 1, 5 and 9, 0.3 mg/kg i.v.) had negligible antitumor activity in this Colon26-bearing mice model, which presents the basis for in vivo inherent resistance in Colon26, the combination with the MDR modulators made enhancement of antitumor activity. Antitumor activity of ADM and VCR in combination with or without the MDR modulators was summarized in the Table. SDZ PSC 833 alone had negligible effect on the tumor growth and survival of mice (data not shown). PSC833 was the most potent of the MDR modulators in prolonging the mean survival time of the mice and inhibition of tumor growth in this model.

Discussion

PSC833 is a novel non-immunosuppressive and MDR-modulating cyclosporin (17–20). In the present study, PSC833 was the most potent of the three MDR modulators, CsA, Vp and PSC833, in reversing MDR in vitro (Fig. 1 and 2) and in vivo (Fig. 3 and Table). The approximately 3–10-fold greater potency of PSC833 than CsA and Vp in vitro as well as its dramatic reversing effect on MDR in vivo suggest that PSC833 is a good candidate for overcoming MDR in clinical trials. These results are consistent wtih comparative studies on the reversing activity of several MDR modulators in vitro and in vivo (17–20). Friche et al. (25) did not demonstrate greater potency of PSC833 than CsA in the restoring effect on reduced accumulation of daunorubicin in a daunorubicin-resistant Ehrlich ascites in vitro, which is not consistent with observations made in our laboratory and by other groups (17–20, 26). This may be attributed to differences in the applied cell line and ligands in the transport experiments.

Special attention was paid to the following characteristics in the reversal activity of PSC833 in vivo: 1) PSC833 had a significant reversal effect (ILS > 80%) in the P388/ADM-bearing mice model. There are few compounds having reversing activity in P388/ADM-bearing mice models, while many compounds were tested in our laboratory using mice-bearing vincristine-resistant (P388/VCR) and ADM-resistant P388 cells (supplied from National Cancer Institute, NIH, resistance index of P388/VCR was approximately one-order lower than P388/ADM (12)). Maximal ILS values in the case of AHC52 (12), calcium channel blockers (27) and MS-209 (28) were 41, 51 and 94% respectively. Although the dose schedule and experimental design were slightly varied in these evaluations, the present result of the maximal ILS (>100%) of PSC833 suggest a significant potential of PSC833 to reverse MDR in vivo. 2) PSC833 had a reversing effect in the Colon26-bearing mice model. Tsuruo et al. (27, 28) reported

that Vp reversed inherent resistance to vinca alkaloids using murine solid tumor-bearing mice models. In the present study, Vp also enhanced the antitumor activity of ADM and VCR in Colon26-bearing mice. PSC833 was more potent than Vp and CsA in this model, although we cannot directly compare the previous (29) and present results due to some difference in the therapeutic procedures.

The reversal efficacy of various compounds has been evaluated according to the following items as a standard (3): 1) Inhibitory effect on photolabeling of P-glycoprotein using photo-active compounds, e.g., [^{3}H]azidopine and analogs of anthracycline and vinca alkaloid (depending on the affinity of MDR modulator to this transporter), 2) effect on transport of MDR-related anticancer agents and 3) effect on in vitro growth inhibition by the MDR-related anticancer drugs (depending on the affinity to this transporter plus cellular pharmacokinetics and dynamics of the anticancer drug and MDR modulator), and 4) in vivo reversal effect in multidrug resistant tumor-bearing mice (depending on the factors described in 2) and 3) plus in vivo pharmacokinetics, pharmaco- and toxico-dynamics of MDR-related anticancer drugs and modulators). The present study indicated that PSC833 was the most potent of the three modulators with respect to in vitro and in vivo efficacy to reverse MDR, relating to the items described in 2)–4). The ability to inhibit the in vitro photolabeling of P-glycoprotein using plasma membrane fraction derived from P-glycoprotein-overexpressed cells should be elucidated for indication of the affinities of the modulating agents to P-glycoprotein.

In addition to the potency of PSC833 in in vitro and in vivo reversal of MDR, PSC833 enhanced the toxic effect of ADM and VCR in mice, when the effect of PSC833 on maximal tolerated doses of ADM and VCR was examined (data not shown). However, the dose intensities of PSC833 and CsA in this enhancement of the toxicity of ADM and VCR in CD2F1 mice were comparable, suggesting that PSC833 is a better candidate than CsA for clinical trials. Pharmacokinetic interaction in P-glycoprotein-rich organs would, at least in part, contribute to the toxic interaction. Keller et al. (20) reported that application of PSC833 increased the AUC values of etoposide in normal rats, suggesting that dose of etoposide in combination with PSC833 should be reduced in clinical situations. Since MDR1 gene product is located in the bile canalicular surface of hepatocytes and the brush-border membrane of the proximal tubles (2, 3), PSC833 possibly inhibits the biliary and urinary excretion of etoposide, which presents the basis for the potentiative toxicity induced by the combination of MDR modulators with MDR-related anticancer drugs. This possibility is supported by the finding that Vp inhibited the biliary excretion of VCR in perfused rat livers (30). MDR modulators potentially enhanced tissue residence time of MDR-related agents, so that MDR modulators could enhance cardiac toxicity of ADM. To consider the clinical application of the combination therapy of MDR-related anticancer drugs and MDR modulator, it is important to clarify the mechanisms contributing to the enhancement of toxicity of MDR-related anticancer drugs by the application of MDR modulator.

In conclusion, PSC833 is the most potent agent for reversing MDR among the three modulators studied and the combination of ADM or VCR with PSC833 reversed acquired and inherent resistance in rodents. Exerting much less immunosuppressive effect and renal toxicity than CsA, PSC833 is a good candidate for clinical application for reversal of MDR.

ACKNOWLEDGEMENTS

We wish to thank Drs. Dalia Cohen, Yohiro Itoh, Yuji Nakayama and Saburo Sone for critical reading of the manuscript and Miss Noriko Kokubu for secretarial help in preparation of the manuscript.

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