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# Intracellular Levels of Two Cyclosporin Derivatives Valspodar (PSC 833) and Cyclosporin A Closely Associated with Multidrug Resistance-modulating Activity in Sublines of Human Colorectal Adenocarcinoma HCT-15

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**P-Glycoprotein, which mediates multidrug resistance (MDR) in cancer chemotherapy, is a principal target of cyclosporin A and [3′-keto-Bmt[1]]-[Val[2]]-cyclosporin (valspodar; PSC 833). To clarify mechanisms contributing to the different MDR-modulating activities of valspodar and cyclosporin A, we investigated the relation of the intracellular levels of the two cyclosporin derivatives to their modulating effect on MDR in different P-glycoprotein-expressing human colorectal carcinoma HCT-15 cells (parental HCT-15 and adriamycin-resistant sublines). In this study, valspodar was found to be much more potent than cyclosporin A in both sensitizing resistant cells to MDR-related anticancer drugs (e.g., adriamycin, vincristine and paclitaxel (taxol)) and increasing 2-[6-amino-3-imino-3H-xanthen-9-yl]benzoic acid methyl ester (rhodamine 123) retention and [G-$^3$H]vincristine sulfate ([$^3$H]vincristine) accumulation in these cells. Furthermore, a good correlation was detected between P-glycoprotein levels and the MDR-reversing effect of valspodar. In contrast, the effects of cyclosporin A could not be linked to P-glycoprotein levels in the MDR cells. In addition, the intracellular accumulation of valspodar was found to be 3–6 fold higher than that of cyclosporin A in four sublines and verapamil, an inhibitor of P-glycoprotein-mediated transport, enhanced the accumulation of cyclosporin A, but not valspodar. These results suggested that valspodar accumulation is not actively regulated by the P-glycoprotein-mediated efflux system.**

Key words: P-Glycoprotein — Valspodar — Cyclosporin A — Multidrug resistance — Correlation

Multidrug resistance (MDR) is one of the major obstacles to cancer chemotherapy. It has been well established that P-glycoprotein encoded by *MDR1* gene plays an important role in MDR.[1)] A variety of reversing agents has been investigated for the ability to reverse P-glycoprotein-mediated MDR. These include calcium channel antagonists (verapamil[2)]), calmodulin antagonists (trifluoperazines[3, 4)]), immunosuppressants (cyclosporin A[5, 6)], FK506[7)]) and antimalarial drugs (quinidine[8)]). These agents have been called first-generation MDR modulators. Unfortunately, clinical studies of many of these first-generation MDR modulators revealed severe toxic effects such as cardiotoxicity and nephrotoxicity through modulation of physiological pathways.[9)] These toxic effects make it difficult to achieve therapeutically effective blood levels of the agents.

To overcome these problems, less toxic and more specific second-generation inhibitors of P-glycoprotein have been investigated by several investigators. Nifedipine derivatives (AHC52[10)]), quinoline derivatives (MS209[11)]) and cyclosporin derivatives ([3′-keto-Bmt[1]]-[Val[2]]-cyclosporin (valspodar; PSC 833[12–14)])) have been reported to be potent in reversing MDR with less toxicity. Valspodar and cyclosporin A differ from each other in only two chemical modifications. In valspodar, the β-hydroxy amide in cyclosporin A is replaced by a β-keto amide, and an ethyl group is replaced by an isopropyl group.[14)] In spite of the minor nature of the structural modifications, valspodar, in contrast to cyclosporin A, is non-immunosuppressive and non-nephrotoxic, and was found to be a more potent P-glycoprotein inhibitor than cyclosporin A in terms of both restoring the sensitivity of MDR cells and prolonging the survival time of tumor-bearing mice.[12–14)] Furthermore, it was reported that photoactive analogues of cyclosporin bind directly to P-glycoprotein and this binding was inhibited by valspodar and cyclosporin A.[15)] These results indicate that the molecular target of two cyclosporin derivatives is P-glycoprotein. However, it has been unclear what mechanisms contribute to the differential potency of valspodar and cyclosporin A in overcoming MDR. This prompted us to study the possibility that the level of P-glycoprotein in resistant cells could be correlated to the difference in activity found between valspodar and cyclosporin A.

Due to the diversity of the species and origin of MDR cell lines, little is known about the difference in potency between valspodar and cyclosporin A in an isogenic phe-

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notype background. Thus, we established a series of adriamycin-resistant sublines of a human colorectal carcinoma HCT-15 by continuous exposure to adriamycin and confirmed that they express different amounts of P-glycoprotein. In this study, we demonstrated that intracellular accumulation of valspodar, unlike cyclosporin A, is not influenced by verapamil, a competitive inhibitor of P-glycoprotein. This suggests that P-glycoprotein regulates the intracellular level of cyclosporin A, but not valspodar, and this might be the basis for the differential potency between valspodar and cyclosporin A in reversing MDR.

## MATERIALS AND METHODS

**Materials** Valspodar and cyclosporin A were prepared by Novartis Pharma, Ltd. (Basle, Switzerland). Adriamycin, epirubicin and mytomycin C were purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo), and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), 2-[6-amino-3-imino-3H-xanthen-9-yl]-benzoic acid methyl ester (rhodamine 123), paclitaxel (taxol) and verapamil were from Sigma (St. Louis, MO). Vincristine was purchased from Shionogi Co., Ltd. (Osaka), and mitoxantrone from Lederle Japan, Ltd. (Tokyo). Etoposide was purchased from Nihon Kayaku, Co., Ltd. (Tokyo).

[$^3$H]Cyclosporin A (specific activity, 8.7 Ci/mmol) and [$^{14}$C]valspodar (specific activity, 54.5 mCi/mmol) were prepared by Novartis Pharma, Ltd. [G-$^3$H]Vincristine sulfate ([$^3$H]vincristine, specific activity, 6.9 Ci/mmol) was purchased from Amersham (Tokyo). The purity of the ligand was confirmed by using HPLC or TLC, and [$^3$H]-cyclosporin A, [$^{14}$C]valspodar, and [$^3$H]vincristine were 98.9, 98 and 97.6% pure, respectively. Anti-P-glycoprotein monoclonal antibody JSB-1 was obtained from Nichirei (Tokyo) and MRK-16 was from Kyowa Medex, Co., Ltd. (Tokyo). All other chemicals were of analytical grade.

**Tumor cells** Human colorectal carcinoma HCT-15 (CCL 225) cells were obtained from American Type Culture Collection (Rockville, MD) through Dainippon Pharmaceutical, Co. (Osaka). Adriamycin-resistant sublines of HCT-15, HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2, were established by continuous exposure of the parental cells to adriamycin at concentrations of 25, 50 and 200 ng/ml. Parental and resistant sublines of HCT-15 were maintained in RPMI-1640 medium (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 m*M* glutamine, and 100 $\mu$g/ml kanamycin in an atmosphere containing 5% $CO_2$ at 37°C. HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 cells were maintained in the medium containing 25, 50 and 200 ng/ml adriamycin.

**Immunoblotting for P-glycoprotein** Plasma membrane-enriched fractions from parental and resistant sublines of HCT-15 were prepared by differential centrifugation as described previously.[16] The immunoblotting of P-glycoprotein was performed as previously described[17, 18] with minor modifications. In brief, the samples were dissolved in the Laemmli sample buffer[19] without boiling prior to loading. Protein (20 $\mu$g) was separated by 4–20% sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to Imobilon sheets (Millipore, Tokyo) for immunoblot analysis as described previously.[20] The blots were probed with 1 $\mu$g/ml of JSB-1, followed by horseradish peroxidase-conjugated anti-mouse IgG (1/300 dilution). The expression of P-glycoprotein was detected on X-ray film (Kodak, Tokyo) using an enhanced chemiluminescence system (Amersham, Rochester, NY). The density of bands derived from sublines of HCT-15 was quantitated using scanning densitometry (EPScan 1.20, Image 1.43 for Macintosh). Relative expression levels of P-glycoprotein in resistant sublines were calculated as ratios to that of parental HCT-15.

**Detection of P-glycoprotein expression by flow cytometry** For determination of P-glycoprotein expression,[21] $3\times10^6$ cells in staining buffer (Dulbecco's phosphate-buffered saline supplemented with 5% FBS and 0.2% sodium azide) were incubated with 10 $\mu$g/ml of either MRK-16 or isotype-matched mouse IgG2a for 1 h at 4°C. The cells were washed twice with staining buffer, and then incubated with 0.175 mg/ml of FITC-labeled goat anti-mouse IgG for 1 h at 4°C. After several washes with staining buffer, the cells were passed through a 200 $\mu$m nylon mesh and resuspended in staining buffer. Fluorescence intensity (excitation wavelength=488 nm) was determined by means of an Epics flow cytometer (Coulter Electronics, Hialeah, FL).

The specific binding of primary antibody was estimated by subtracting the fluorescence intensity of samples incubated with isotype-matched IgG from the fluorescence of samples incubated with primary antibody. The relative expression level was expressed as a ratio of the specific fluorescence intensity of resistant sublines to that of parental cells.

***In vitro* growth inhibition assay** XTT assay was carried out as described previously.[22] In brief, the cells were seeded in 96-well tissue culture plates at a density of $5\times10^3$ cells/well. The cells were incubated for 24 h, then drug treatments were initiated. The cells were exposed to the drugs for 72 h, and relative cell growth was assessed by means of staining with XTT. After a 4 h incubation with the tetrazolium dye, the absorbance at 450 nm was measured by use of a microplate reader (Molecular Devices Corp., Menlo Park, CA). The $IC_{50}$ value was defined as the concentration inhibiting the tumor cell growth by 50%. The increase of drug-sensitivity by cyclosporin derivatives was expressed as a sensitization factor.

The sensitization factor was determined by dividing the $IC_{50}$ value without modulator by the $IC_{50}$ value with modulator. Relative resistance to anticancer drugs was determined by dividing the $IC_{50}$ value of a resistant subline by that of the parental cells.

**P-Glycoprotein function assay** P-Glycoprotein function was investigated by rhodamine 123 assay. Rhodamine 123[23] is a fluorescent substrate for P-glycoprotein. The four sublines of HCT-15 in growth medium were seeded in 24-well tissue culture plates ($10^6$ cells/well) and incubated for 24 h. The growth medium was removed and replaced by incubation medium (Hanks' balanced salt solution supplemented with 10 m*M* Hepes buffer and 10% FBS) with or without cyclosporin derivatives, and then cells were incubated for 15 min at 37°C. Rhodamine 123 was added to reach a final 10 $\mu$g/ml and the cells were further incubated for 30 min. After several washes with ice-cold phosphate-buffered saline (PBS), the cells were trypsinized and suspended in PBS. Intracellular fluorescence intensity (cumulative fluorescence, emission wavelength=530 nm, excitation wavelength=485 nm) was determined by means of a fluorescence plate reader (CytoFluor 2350, Millipore, Corp., Bedford, MA).

To estimate rhodamine 123 retention, the cells were further incubated with or without cyclosporin derivatives in rhodamine 123-free incubation medium for 30 min at 37°C. They were washed, and the fluorescence intensity (retention fluorescence) was determined by a fluorescence plate reader. Cell surface-associated fluorescence was also determined after adding ice-cold rhodamine 123 to the cells followed by immediate washing. Rhodamine 123 retention was determined by dividing the retention fluorescence by the cumulative fluorescence of the cells.

$$\text{Rhodamine 123 retention} = \text{retention fluorescence}/\text{cumulative fluorescence}$$

Rhodamine 123 retention ratio was expressed as a percentage ratio and calculated as follows:

$$\text{Rhodamine 123 retention ratio (\%)} = \frac{\text{rhodamine 123 retention with modulator}}{\text{rhodamine 123 retention without modulator}} \times 100$$

**Drug accumulation studies** Accumulation studies were carried out as described previously.[7] Four sublines of HCT-15 in growth medium were seeded in 24-well tissue culture plates ($10^6$ cells/well) and incubated for 24 h at 37°C. Cells were incubated with 0.3 $\mu M$ [$^3$H]cyclosporin A, 0.3 $\mu M$ [$^{14}$C]valspodar or 7.7 n*M* [$^3$H]vincristine with and without 1 $\mu$g/ml valspodar, 1 $\mu$g/ml cyclosporin A or 6.8 $\mu$g/ml verapamil at 37°C for 2 h. Following the incubation, the cells were washed with ice-cold PBS and then lysed with 1 *N* NaOH at room temperature overnight. Aliquots were then assayed for radioactivity with a liquid scintillation counter (LS-6000TA, Beckman, Palo Alto, CA) and protein amount was determined by using a BCA kit (Pierce, Rockford, IL). Cellular drug levels of [$^3$H]vincristine were expressed as a percentage ratio calculated as follows:

$$\text{Percent ratio of intracellular level} = \frac{[^3\text{H}]\text{vincristine accumulation with modulator}}{[^3\text{H}]\text{vincristine accumulation without modulator}} \times 100$$

**Statistical analysis** In the transport experiments, significant differences of intracellular level between cyclosporin derivatives were identified by repeated measures analysis of variance (ANOVA) (Bonferroni/Dunn). Pearson's correlation test was used for correlation analyses of MDR-

Table I. Resistance Profile to MDR-related Drugs and Relative P-Glycoprotein Expression Level of Parental and Adriamycin-resistant Sublines of HCT-15

| Drug | HCT-15 | HCT-15/ADM1 | HCT-15/ADM2 | HCT-15/ADM2-2 |
|---|---|---|---|---|
| Adriamycin | 252±115[a] (1)[b] | 562±219 (2.23) | 1110±295*** (4.40) | 1960±810*** (7.77) |
| Epirubicin | 1980±384 (1) | 3130±935 (1.58) | 6600±1490*** (3.34) | 8550±2340*** (4.33) |
| Mitoxantrone | 87±16 (1) | 124±32 (1.42) | 470±54*** (5.40) | 272±133*** (3.12) |
| Vincristine | 921±146 (1) | 1870±415** (2.03) | 2020±393** (2.19) | 2730±883** (2.96) |
| Taxol | 326±37 (1) | 916±500** (2.81) | 859±162** (2.63) | 1570±426*** (4.81) |
| Etoposide | 1740±110 (1) | 3940±544*** (2.27) | 4420±1250*** (2.55) | 5480±1270*** (3.16) |
| Mitomycin C | 3530±1710 (1) | 2840±544 (0.80) | 6230±109** (1.77) | 901±538 (0.26) |
| Relative P-glycoprotein level | | | | |
| | 1±0.06[c] | 1.69±0.24 | 2.33±0.63 | 4.73±0.68 |

*a*) The $IC_{50}$ values (ng/ml) were defined as the concentration giving a cell growth of 50%. Significant differences from $IC_{50}$ values of HCT-15 cells are indicated (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ by Bonferroni/Dunn (two tail)). The values are mean±SD of triplicate experiments.

*b*) Relative resistance was determined by dividing the $IC_{50}$ values of resistant sublines by that of parental cells.

*c*) P-Glycoprotein expression level in intact cells was determined by a flow-cytometric assay using a monoclonal antibody MRK-16, recognizing an external domain of P-glycoprotein. Relative P-glycoprotein level of resistant sublines was expressed as a ratio to that of parental HCT-15. The values are mean±SD of three experiments.

reversing efficacy of cyclosporin derivatives. These analyses were done by Stat View II software (version 4.02 for Macintosh, Abacus Concepts, Inc., Berkeley, CA) and GraphPad PRISM software (version 2.0 for Windows, GraphPad Software, Inc., San Diego, CA).

## RESULTS

**Relative resistance to P-glycoprotein-transported anticancer drugs** We first evaluated relative resistance of the parental and three adriamycin-resistant sublines to anticancer drugs. The three resistant sublines, HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 exhibited 2.23, 4.20 and 7.77 fold resistance to adriamycin, the selection agent, compared to the parental cells. The resistant sublines were also cross-resistant to other anticancer drugs known to be substrates of P-glycoprotein. The cells demonstrated relative high resistance to epirubicin and taxol, relative low resistance to mitoxantrone, vincristine, etoposide, and no resistance to non-MDR-related anticancer drug, mitomycin C (Table I).

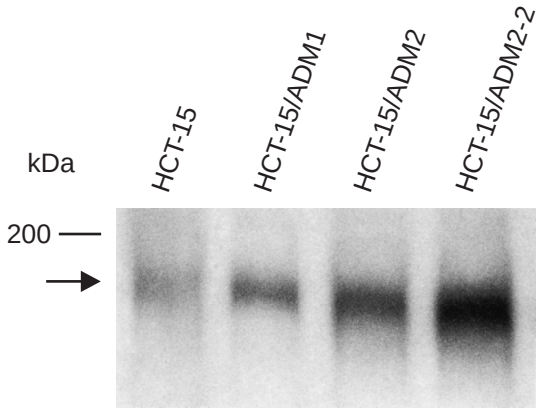


Fig. 1. P-Glycoprotein expression in membrane fraction from four HCT-15 human colorectal carcinoma sublines. Membrane fractions derived from tumor cells were subjected to immunoblot analysis with anti-P-glycoprotein antibody JSB-1. Lanes were loaded with 20 $\mu$g of each cell lysate. The arrow indicates the position of P-glycoprotein (170–180 kDa). Density of the sample was quantitated in each blotting sheet. This figure presents the results of a typical experiment.

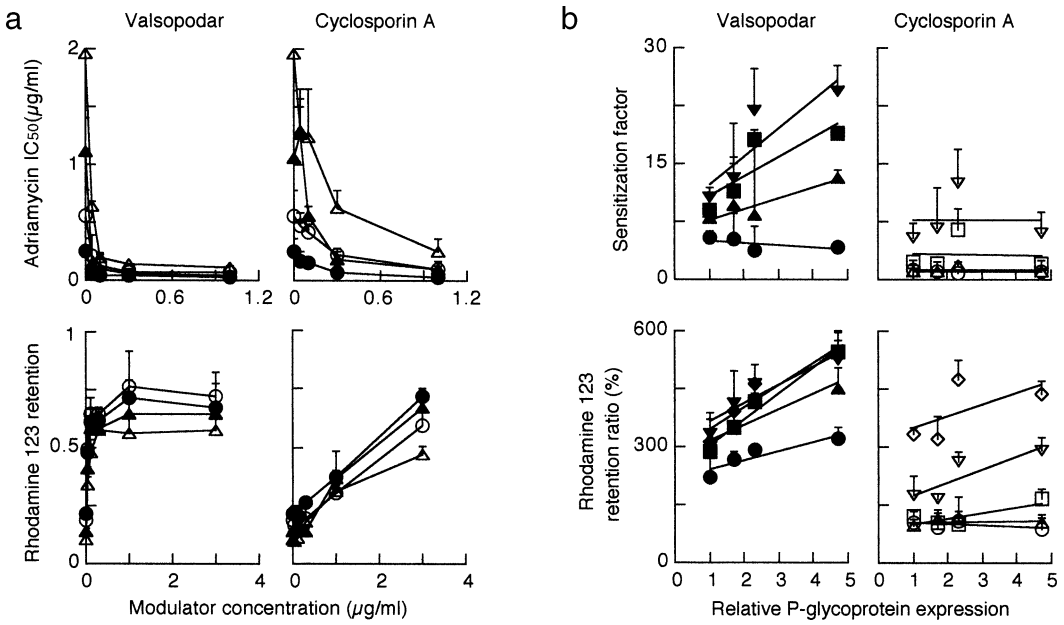


Fig. 2. a. Concentration-dependent enhancement of adriamycin anti-growth activity and rhodamine 123 retention by cyclosporin derivatives. The $IC_{50}$ values of adriamycin to inhibit the cell growth and rhodamine 123 retention of the four sublines of HCT-15 were determined by the XTT and rhodamine 123 assays. The $IC_{50}$ values and rhodamine 123 retention data were plotted against cyclosporine derivative concentrations to evaluate MDR reversal activity. Each point and vertical line represents the mean±SD of triplicate experiments, respectively. ● HCT-15, ○ HCT-15/ADM1, ▲ HCT-15/ADM2, △ HCT-15/ADM2-2. b. Relation of adriamycin sensitization factor and rhodamine 123 retention ratio to P-glycoprotein expression level. The adriamycin sensitization factor and the ratios of rhodamine 123 retention with MDR modulator to that without MDR modulator were plotted against the relative P-glycoprotein level of four sublines of HCT-15, which were separately determined by flow-cytometric assay. A positive correlation is seen between P-glycoprotein level, adriamycin sensitization factor and the ratio of rhodamine 123 retention with valspodar at least above 0.3 $\mu$g/ml, while no correlation was seen in the case of cyclosporin A (Pearson's correlation test). Each point and vertical line represents the mean±SD of triplicate experiments, respectively. Concentration ($\mu$g/ml): ● ○ 0.05, ▲ △ 0.1, ■ □ 0.3, ▼ ▽ 1, ◆ ◇ 3.

**Immunoblot analysis** Parental HCT-15 cells intrinsically express a moderate level of P-glycoprotein, as was reported previously.[24, 25] To determine whether acquisition of adriamycin resistance is due to altered expression of P-glycoprotein, the plasma membrane fractions prepared from resistant cells were subjected to immunoblot analysis with JSB-1, a P-glycoprotein antibody. HCT-15 resistant sublines demonstrated an increase in P-glycoprotein levels by approximately 2–5 fold (Fig. 1). The expression levels of HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 were increased by 1.72±0.73, 2.80±1.70 and 4.83±0.96 fold, respectively, compared to the parental cells (mean± SD of three experiments).

The results were confirmed by flow-cytometric analysis with MRK-16 antibody. The relative P-glycoprotein expression ratios of HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ADM2-2 to the parental HCT-15 were 1.69± 0.24, 2.33±0.63 and 4.73±0.68 fold, respectively (mean± SD of three experiments, Table I). We also determined expression of multidrug resistance protein (MRP), since MRP is often overexpressed when cells are selected for resistance to adriamycin. Flow-cytometric analysis with an MRP-specific monoclonal antibody demonstrated MRP overexpression in these resistant sublines. The expression levels of HCT-15/ADM1, HCT-15/ADM2 and HCT-15/ ADM2-2 were increased 1.13±0.08, 1.37±0.15 and 1.43±

Table II. $IC_{50}$ Values Modulated by Cyclosporin Derivatives and Sensitizing Factor of Parental and Adriamycin-resistant Sublines of HCT-15

| Drug | HCT-15 | HCT-15/ADM1 | HCT-15/ADM2 | HCT-15/ADM2-2 |
|---|---|---|---|---|
| Valspodar 0.3 $\mu$g/ml | | | | |
| Adriamycin | 37.2±6.4$^{**, a)}$ (8.98)$^{b)}$ | 64.6± 24.2$^{**}$ (11.5) | 56.3±4.1$^{**}$ (18.2) | 140±5$^{**}$ (18.9) |
| Epirubicin | 67.0±16.4$^{**}$ (30.8) | 78.3±16.5$^{**}$ (41.1) | 124±17$^{**}$ (54.0) | 145±49$^{**}$ (63.5) |
| Mitoxantrone | 31.8±8.5$^{**}$ (2.91) | 39.3±7.3$^{**}$ (3.22) | 63.3±7.7$^{**}$ (7.50) | 38.5±17.8$^{**}$ (8.60) |
| Vincristine | 17.7±6.3$^{**}$ (56.0) | 26.9±2.5$^{**}$ (69.9) | 21.0±4.4$^{**}$ (99.2) | 33.5±6.4$^{**}$ (83.4) |
| Taxol | 6.91±0.64$^{**}$ (47.5) | 8.69±0.90$^{**}$ (106) | 6.63±8.31$^{**}$ (131) | 9.94±3.88$^{**}$ (172) |
| Etoposide | 1110±252$^{**}$ (1.61) | 1960±295$^{**}$ (2.04) | 1830±15$^{**}$ (2.41) | 3310±2560 (2.30) |
| Mitomycin C | 1360±753 (3.08) | 2380±270 (1.20) | 3340±748$^{*}$ (1.94) | 495±36 (1.83) |
| Valspodar 1 $\mu$g/ml | | | | |
| Adriamycin | 31.1±3.4$^{**}$ (10.6) | 69.7±50.9$^{**}$ (13.2) | 48.5±12.4$^{**}$ (21.9) | 11.0±14.0$^{**}$ (24.4) |
| Epirubicin | 62.7±9.2$^{**}$ (32.0) | 59.4±13.6$^{**}$ (54.9) | 88.7±7.4$^{**}$ (74.7) | 128±29$^{**}$ (69.0) |
| Mitoxantrone | 30.0±12.2$^{**}$ (3.41) | 31.4±3.1$^{**}$ (3.97) | 66.9±3.6$^{**}$ (7.03) | 32.9±3.3$^{**}$ (8.32) |
| Vincristine | 16.1±1.9$^{**}$ (57.7) | 19.7±1.9$^{**}$ (95.6) | 19.0±4.8$^{**}$ (111) | 21.7±1.0$^{**}$ (126) |
| Taxol | 5.25±0.48$^{**}$ (62.6) | 6.25±1.51$^{**}$ (153) | 4.57±1.06$^{**}$ (194) | 6.46±2.24$^{**}$ (270) |
| Etoposide | 635±407$^{**}$ (3.78) | 1540±286$^{**}$ (2.62) | 1720±400$^{**}$ (2.66) | 1810±188$^{**}$ (3.04) |
| Mitomycin C | 2000±1450 (3.35) | 2440±599 (1.22) | 3590±1360$^{*}$ (1.89) | 203±9 (4.44) |
| Cyclosporin A 0.3 $\mu$g/ml | | | | |
| Adriamycin | 75.7±7.3$^{*}$ (2.23) | 229±46$^{*}$ (2.04) | 187±67$^{*}$ (6.49) | 626±155$^{*}$ (2.11) |
| Epirubicin | 362±146$^{**}$ (6.22) | 629±330$^{**}$ (6.74) | 1610±449$^{**}$ (4.31) | 2960±1210$^{**}$ (3.17) |
| Mitoxantrone | 57.5±16.6$^{*}$ (1.59) | 59.6±10.8$^{**}$ (2.12) | 191±19$^{**}$ (2.47) | 168±50 (1.72) |
| Vincristine | 251±76$^{**}$ (3.91) | 410±145$^{**}$ (4.94) | 720±46$^{**}$ (3.55) | 1050±139$^{**}$ (2.62) |
| Taxol | 35.3±2.5$^{**}$ (9.28) | 87.5±48.2$^{**}$ (14.7) | 132±21$^{**}$ (6.62) | 242±121$^{**}$ (7.57) |
| Etoposide | 1170±408$^{*}$ (1.59) | 1820±116$^{**}$ (2.17) | 2800±273 (1.59) | 2990±1020$^{*}$ (1.96) |
| Mitomycin C | ND$^{c)}$ | ND | ND | ND |
| Cyclosporin A 1 $\mu$g/ml | | | | |
| Adriamycin | 34.3±12.7$^{**}$ (5.35) | 99.9±68.0$^{**}$ (6.75) | 94.5±28.6$^{*}$ (12.4) | 244±121$^{**}$ (6.10) |
| Epirubicin | 73.3±21.7$^{**}$ (28.4) | 68.0±10.6$^{**}$ (46.7) | 205±45$^{**}$ (33.1) | 461±165$^{**}$ (20.2) |
| Mitoxantrone | 39.9±2.7$^{**}$ (2.19) | 30.6±2.6$^{**}$ (4.06) | 71.8±3.7$^{**}$ (6.55) | 40.4±21.1$^{**}$ (8.52) |
| Vincristine | 31.3±19.7$^{**}$ (36.8) | 65.4±9.5$^{**}$ (29.0) | 50.0±28.4$^{**}$ (49.3) | 228±93$^{**}$ (13.3) |
| Taxol | 14.0±1.6$^{**}$ (23.5) | 19.0±5.7$^{**}$ (51.6) | 15.7±2.2$^{**}$ (55.5) | 29.4±4.1$^{**}$ (54.1) |
| Etoposide | 830±328$^{**}$ (2.31) | 1740±274$^{**}$ (2.30) | 1720±298$^{**}$ (2.63) | 2280±324$^{**}$ (2.43) |
| Mitomycin C | ND | ND | ND | ND |

*a*) The $IC_{50}$ values (ng/ml) were defined as the concentration giving a cell growth of 50%. For all cell lines for each anticancer drug, significant differences from $IC_{50}$ values without modulator as shown in Table I are indicated (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ by Bonferroni/Dunn (two tail)). The values are mean±SD of triplicate experiments.
*b*) The sensitization factor was determined by dividing the $IC_{50}$ values without modulator by the $IC_{50}$ values with modulator.
*c*) ND, not done.

0.16 fold, respectively, compared to the parental cells (mean±SD of three experiments). However, the absolute MRP mRNA amount in HCT-15 cells was very small (data not shown), suggesting that MDR in the resistant HCT-15 sublines is mediated mainly by P-glycoprotein.

**Effect of cyclosporin derivatives on drug sensitivity and rhodamine 123 retention** To determine the effect of valspodar and cyclosporin A on adriamycin sensitivity in the four sublines, cell viability was assessed by XTT assay. A dose-dependent decrease in adriamycin $IC_{50}$ values was observed in the presence of valspodar or cyclosporin A (Fig. 2a). Valspodar at 0.3 $\mu$g/ml restored the adriamycin sensitivity of resistant cells to a level comparable to that of parental cells. Cyclosporin A also restored adriamycin sensitivity, but 1 $\mu$g/ml cyclosporin A was required to achieve the same reversing effect as that of 0.3 $\mu$g/ml valspodar. Valspodar also enhanced the cytotoxicity of other P-glycoprotein-transported anticancer drugs (e.g., epirubicin, mitoxantrone, vincristine, taxol and etoposide, Table II) much more efficiently than cyclosporin A.

Similarly, cyclosporin A up to 3 $\mu$g/ml did not significantly increase rhodamine 123 retention, but valspodar at 0.1 $\mu$g/ml restored full rhodamine 123 retention in the resistant cells (Fig. 2a).

**Correlation between P-glycoprotein expression and activity of the cyclosporin derivatives** The correlation between P-glycoprotein levels and the effects of cyclosporin derivatives were analyzed in the HCT-15 sublines. As shown in Fig. 2b and Table III, there was a positive correlation between P-glycoprotein levels and adriamycin sensitization factor at 0.3 or 1 $\mu$g/ml valspodar. No positive correlation (Pearson's correlation coefficients; $r \leq 0.350$, $P \leq 0.500$) was detected at valspodar

Table III. Correlation Coefficient between Modulating Effects by Cyclosporin Derivatives and P-Glycoprotein Expression Level

| | Valspodar | | Cyclosporin A | |
|---|---|---|---|---|
| | 0.3 $\mu$g/ml | 1 $\mu$g/ml | 0.3 $\mu$g/ml | 1 $\mu$g/ml |
| Adriamycin | 0.770[a), **] | 0.728[**] | −0.0455 | −0.00356 |
| Vincristine | 0.416 | 0.763[**] | −0.469 | −0.481 |
| Taxol | 0.809[**] | 0.763[**] | −0.234 | 0.519 |
| Rhodamine 123 retention ratio (%) | 0.944[***] | 0.731[**] | 0.659[**] | 0.767[***] |
| Percent ratio of intracellular [$^3$H]vincristine level | ND[b)] | 0.834[***] | ND | 0.221 |

Correlation coefficients were calculated from the data in Figs. 2b and 3.
*a*) Pearson's correlation coefficient (significance of positive correlation, * $P<0.05$, ** $P<0.01$, *** $P<0.001$).
*b*) ND, not done.

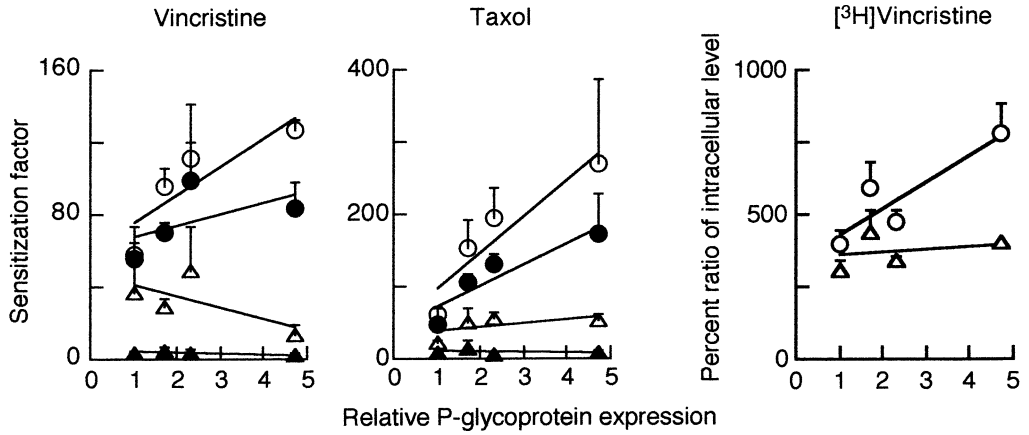


Fig. 3. Relation of sensitization factors of vincristine, taxol and [$^3$H]vincristine accumulation to P-glycoprotein expression level. P-Glycoprotein expression level-dependent valspodar effects on the sensitization factors of vincristine and taxol and percent ratio of intracellular [$^3$H]vincristine level are shown. A positive correlation ($P>0.416$) was found by Pearson's correlation coefficient analysis in all the valspodar combination studies. Each point and vertical line represents the mean±SD of triplicate experiments, respectively. ● 0.3 $\mu$g/ml valspodar, ○ 1 $\mu$g/ml valspodar, ▲ 0.3 $\mu$g/ml cyclosporin A, △ 1 $\mu$g/ml cyclosporin A.

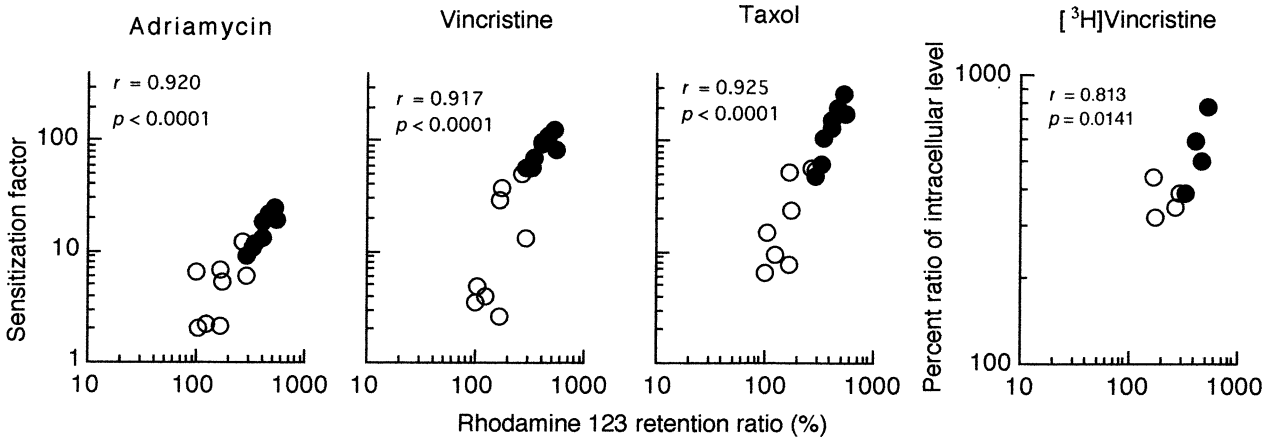


Fig. 4. Correlation between sensitization factors and inhibitory activity of P-glycoprotein function by cyclosporin derivatives. The sensitization factors and percent ratio of intracellular [$^3$H]vincristine level were plotted as a function of rhodamine 123 retention ratio (%). The positive correlation of MDR-inhibitory activity of cyclosporine derivatives was statistically evident (Pearson's correlation test). ● 0.3, 1 $\mu$g/ml valspodar, ○ 0.3, 1 $\mu$g/ml cyclosporin A.

concentrations of less than 0.3 $\mu$g/ml. This lack of correlation with P-glycoprotein at low concentrations suggested that valspodar did not completely inhibit P-glycoprotein function. For cyclosporin A, no positive correlation between P-glycoprotein levels and adriamycin sensitization factor was observed at any concentration ($r \leq -0.129$, $P \leq 0.690$; Fig. 2b and Table III). Similar results were obtained with other MDR-related anticancer drugs, and Fig. 3 presents the results for vincristine and taxol.

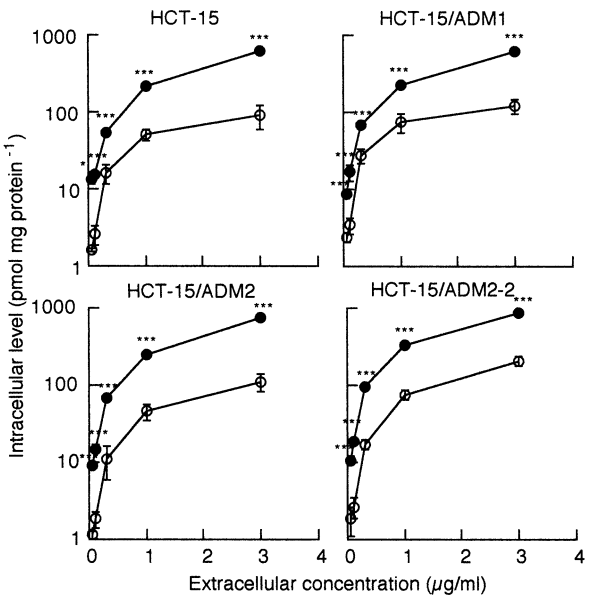


Fig. 5. Intracellular accumulation of [$^{14}$C]valspodar and [$^3$H]cyclosporin A in four sublines of HCT-15. Intracellular accumulation of cyclosporin derivatives was determined by means of a transport study. The amount of intracellular cyclosporin derivatives was expressed as pmol mg protein$^{-1}$ and as the mean±SD of triplicate experiments, respectively. *** Significant difference from cyclosporin A concentration externally added in the transport study ($P$<0.001 by Bonferroni/Dunn test (two tail)). ● [$^{14}$C]valspodar, ○ [$^3$H]cyclosporin A.

The relation between P-glycoprotein levels and the inhibitory effects of cyclosporin derivatives on the transport function of P-glycoprotein was also studied in the HCT-15 sublines. At 0.05 to 3 $\mu$g/ml valspodar, the increase in rhodamine 123 retention showed a good correlation with P-glycoprotein expression level ($r \geq 0.712$, $P \leq 0.0020$; Fig. 2b and Table III). In the presence of cyclosporin A, the ratio of rhodamine 123 retention at concentrations above 0.3 $\mu$g/ml also showed a positive correlation with P-glycoprotein levels (Fig. 2b and Table III). These results indicated that the reversal of MDR by modulation of P-glycoprotein function was dose-dependent. In addition, we determined the intracellular accumulation of [$^3$H]vincristine in the resistant sublines and found that valspodar and cyclosporin A significantly enhanced intracellular accumulation levels of [$^3$H]vincristine. We found a good correlation between P-glycoprotein levels and the effect of valspodar on the accumulation of [$^3$H]vincristine (Fig. 3). Such a correlation was not found for cyclosporin A.

Next, the correlations between the MDR-reversing activities of 0.3 and 1 $\mu$g/ml valspodar and cyclosporin A were analyzed in the four sublines of HCT-15. Positive correlations were observed between sensitization factors to adriamycin, vincristine and taxol, increase in rhodamine 123 retention and vincristine accumulation (Fig. 4, $r \leq 0.925$, $P \leq 0.0141$).

**Intracellular accumulation of the two cyclosporin derivatives** The intracellular accumulations of two cyclosporin derivatives were determined in transport studies. We found that the intracellular accumulation of valspodar was 3–6 fold higher than that of cyclosporin A in the four resistant sublines (Fig. 5). To verify the relation of valspodar and cyclosporin A intracellular accumulation levels to the modulating effects on MDR, the sensitizing effects to anticancer drugs, increase in rhodamine 123 retention and vincristine accumulation were plotted against intracellular modulator level (Fig. 6). The comparisons indicated that the intracellular levels of valspodar and cyclosporin A showed good correlations with the sensitization factors to adriamycin, vincristine, taxol ($r \geq 0.743$, $P \leq 0.0010$), increase in rhodamine 123 retention and vincristine accumulation ($r \geq 0.730$, $P \leq 0.0086$).

**Verapamil effect on accumulation of the two cyclosporin derivatives** To clarify the mechanism contributing to the difference in intracellular levels of the two cyclosporin derivatives, we investigated the effects of 6.8 $\mu M$ verapamil on the intracellular accumulation of valspodar and cyclosporin A. Verapamil was reported to be a competitive inhibitor of P-glycoprotein.[26] Vincristine was used as a reference compound in these experiments. Accumulations of vincristine and cyclosporin A were significantly enhanced by verapamil (Fig. 7), but no effect on valspodar accumulation was observed.

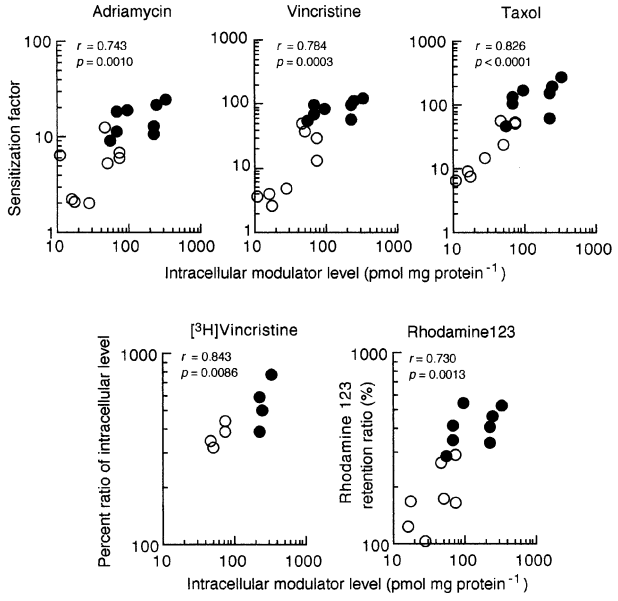


Fig. 6. Correlation between sensitization factors, inhibitory activity of P-glycoprotein function and intracellular level of cyclosporin derivatives. The sensitization factors, intracellular [$^3$H]vincristine level and rhodamine 123 retention ratio (%) were plotted against intracellular modulator level. A positive correlation between the activity of cyclosporin derivatives and intracellular modulator level was found (Pearson's correlation test). ● valspodar, ○ cyclosporin A.

## DISCUSSION

Overexpression of drug efflux transporters by cancer cells is an important factor in some of the clinical failures of cancer chemotherapy. In recent years, efforts have been made to identify agents that reverse the activity of P-glycoprotein, the most widely characterized drug efflux pump, and several compounds have been found. These

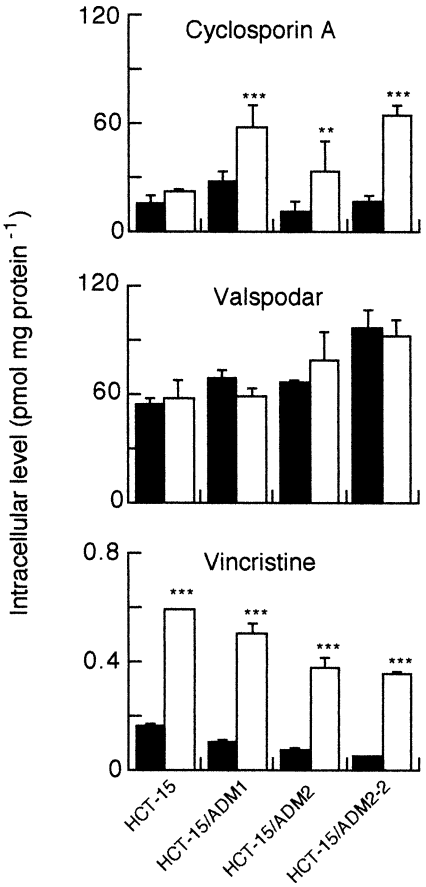


Fig. 7. Effects of verapamil on intracellular drug accumulation and its relation to P-glycoprotein expression level. Drug accumulation experiments were carried out with [$^3$H]cyclosporin A, [$^{14}$C]valspodar and [$^3$H]vincristine in parental and adriamycin-resistant sublines of HCT-15. The amount of intracellular drug was expressed as pmol mg protein$^{-1}$ and as the mean±SD of triplicate experiments, respectively. The open and closed columns denote the intracellular accumulation with or without verapamil. Verapamil significantly increased the accumulation of [$^3$H]cyclosporin A and [$^3$H]vincristine, but not [$^{14}$C]valspodar (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, Bonferroni/Dunn test).

include two cyclosporin derivatives, valspodar and cyclosporin A, that were identified as potent reversing agents. Although only minimal structural differences exist between the two drugs, valspodar was found to be a more potent MDR-reversing agent than cyclosporin A.[12–14] However, the basis for this difference in the potencies of valspodar and cyclosporin A is still unknown. In this study, we investigated the possibility that the level of P-glycoprotein in resistant cells could be correlated to the difference of potency between the two cyclosporin derivatives. HCT-15 sublines expressing relatively high levels of P-glycoprotein were more resistant to adriamycin, and other P-glycoprotein-transported anticancer drugs than the parental cells (Table I). As previously described,[12–14] we observed that valspodar showed a 3 fold higher potency than cyclosporin A in restoring the sensitivity of the resistant cells to adriamycin (Fig. 2a). In addition, the potency of valspodar, but not cyclosporin A, in sensitizing resistant cells to adriamycin, vincristine and taxol was correlated to the P-glycoprotein levels of the cells (Figs. 2b and 3).

Similarly, differences in the potencies of the two cyclosporin derivatives were found in the function assay using rhodamine 123, a substrate of P-glycoprotein (Fig. 2a). As observed in previous studies,[27] valspodar was also found to be 30 fold more potent than cyclosporin A in restoring rhodamine 123 retention (Fig. 2a). Changes in the adriamycin, vincristine and taxol resistance-modulating patterns of the two cyclosporin derivatives also were correlated with the increasing rhodamine 123 retention (Fig. 4). Therefore, the MDR-reversing activity of the two cyclosporin derivatives was dependent on the different restoring ability for P-glycoprotein-transport of the substrate in the resistant sublines.

In this study, we also evaluated intracellular accumulation of valspodar and cyclosporin A in the cells. The concentrations of valspodar in the cells were 3–6 fold higher than that of cyclosporin A (Fig. 5). A good correlation was found between the intracellular concentrations of the two cyclosporin derivatives and their reversing effects on adriamycin, vincristine and taxol resistance or ability to restore [$^3$H]vincristine accumulation and rhodamine 123 retention (Fig. 6). This indicated that the higher intracellular level of valspodar in the cells resulted in the different potencies of the two cyclosporin derivatives.

To clarify the mechanisms contributing to the difference in intracellular levels of valspodar and cyclosporin A, we next determined the intracellular accumulations of two drugs with or without verapamil, a P-glycoprotein inhibitor that competes for vinblastine binding sites on P-glycoprotein.[26] Verapamil appeared to have different effects on the transport of the two cyclosporin derivatives in the cells. The accumulation of cyclosporin A in resistant sublines was enhanced by verapamil, while no increase in the accumulation of valspodar was induced by verapamil in the same sublines (Fig. 7). Therefore, in spite of the minimal nature of the structural difference between valspodar and cyclosporin A, the cyclosporin derivatives appear to have different transport characteristics in P-glycoprotein-expressing cells. As previously reported, since cyclosporin A can be effluxed by P-glycoprotein,[15] the lower intracellular accumulation of cyclosporin A than valspodar in the resistant cells may be a result of the ability of P-glycoprotein to pump cyclosporin A. Valspodar, on the other hand, cannot be pumped by P-glycoprotein. Such changes in the intracellular accumulation of cyclosporin A, but not valspodar, could result in the apparently greater MDR-reversing effect of valspodar than cyclosporin A in the resistant cells. Furthermore, in the absence of verapamil, the accumulation of cyclosporin A in the P-glycoprotein-overexpressing resistant cells seems not to be lower than that in the parental HCT-15 (Fig. 7). In contrast to cyclosporin A, the accumulation of vincristine, a reference P-glycoprotein substrate, is clearly decreased in resistant cells. This discrepancy might be explained by the difference in electric charge between cyclosporin A and vincristine, vincristine being cationic whereas cyclosporin A is electronically neutral. A recent study demonstrated that cations are better substrates for P-glycoprotein than neutral agents.[28] Therefore, cyclosporin A may be transported much less efficiently than vincristine by P-glycoprotein, and this could result in the indistinct decrease of intracellular accumulation in resistant cells.

In another study, Smith *et al.*[29] demonstrated a low rate of efflux of valspodar in MDR1-transfected LLC-PK1 pig kidney cells. In their study, the Michaelis constant ($K_m$) of valspodar for transport was 4 fold lower than that of cyclosporin A and the maximal transport rate ($V_{max}$) of valspodar was 20 fold lower, demonstrating that the transport ($k_{cat}$) of valspodar is much slower than that of cyclosporin A. It is still not completely clear whether valspodar is transported by P-glycoprotein or not. However, we previously found[30] that the apparent $K_i$ value of P-glycoprotein for valspodar is 0.134 $\mu M$, by kinetic analyses of the inhibition by valspodar of verapamil-stimulated P-glycoprotein ATPase. Although direct comparison of the valspodar concentration is not meaningful because these values are dependent on the assay system used, the concentration of valspodar in the transport study was much higher than the above $K_i$ value. Smith *et al.* performed their transport study using 0.1 to 2 $\mu M$ valspodar, and 0.3 $\mu M$ was used in our study. The higher concentration seems to completely inhibit P-glycoprotein transport activity by itself. In addition, $^{14}$C-labeled valspodar could not be used appropriately at lower concentration due to its low specific activity. Lower concentrations of $^3$H-labeled valspodar might be more suitable for a transport study to evaluate whether valspodar is transported or is a poor substrate for P-glycoprotein.

The mechanism by which ATP hydrolysis causes a structural change of the protein to transport substrates is not completely understood. However, an alternative explanation for differential potency of valspodar and cyclosporin A might be different conformational changes in P-glycoprotein, which might influence ATP hydrolysis. This idea is supported by our previous results, showing that combined therapy with valspodar/cyclosporin A and antibody MRK-16 resulted in a synergistic inhibition of P-glycoprotein function. Pretreatment of HCT-15/ADM2-2 cells with valspodar modified the binding of antibody MRK-16 differently compared with the case of pretreatment with cyclosporin A.[30] From these findings it was suggested that alterations of P-glycoprotein structure by the two cyclosporin derivatives might account for the difference in their MDR-reversing potency. In addition, although both cyclosporin derivatives inhibited basal P-glycoprotein-ATPase with the same affinity, valspodar was more potent than cyclosporin A in inhibiting the substrate-stimulated activity.[31]

Our study suggested that one of the clinical benefits of valspodar is that the extent of modulation in tumors seems to be greater than that in normal tissues. However, in recent reports of randomized trials, valspodar failed to improve complete remission rates or survival when used in combination chemotherapy against leukemia.[32, 33] Since valspodar changes not only intracellular pharmacokinetics in tumors, but also systemic pharmacokinetics of drugs in normal tissues, the administration dose had to be reduced to avoid increased toxicity by combined use of anticancer drugs and valspodar. Altered adriamycin pharmacokinetics, increased central nervous system toxicities, and augmented emetic reaction indicated that systemic pharmacokinetics was changed by valspodar in normal tissues of patients.

In conclusion, our results demonstrated that valspodar was much more effective than cyclosporin A in four HCT-15 sublines with a range of P-glycoprotein levels. Intracellular accumulation of valspodar, unlike cyclosporin A, is not regulated by P-glycoprotein-mediated efflux in spite of the structural similarity of the two agents. These differences in transport characteristics between the two cyclosporin derivatives should provide a better insight into the basis of the different potencies of valspodar and cyclosporin A to reverse MDR.

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