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Interaction of cyclosporin derivatives with the ATPase activity of human P-glycoprotein

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1 P-glycoprotein, a 170–180 kDa membrane glycoprotein that mediates multidrug resistance, hydrolyses ATP to efflux a broad spectrum of hydrophobic agents. In this study, we analysed the effects of three MDR reversing agents, verapamil, cyclosporin A and [3′-keto-Bmt[1]]-[Val[2]]-cyclosporin (PSC 833), on the adenosine triphosphatase (ATPase) activity of human P-glycoprotein.

2 P-glycoprotein was immunoprecipitated with a monoclonal antibody (MRK-16) and the P-glycoprotein-MRK-16-Protein A-Sepharose complexes obtained were subjected to a coupled enzyme ATPase assay.

3 While verapamil activated the ATPase, the cyclosporin derivatives inhibited both the substrate-stimulated and the basal P-glycoprotein ATPase. No significant difference was observed between PSC 833 and cyclosporin A on the inhibition of basal P-glycoprotein ATPase activity. PSC 833 was more potent than cyclosporin A for the substrate-stimulated activity.

4 Kinetic analysis indicated a competitive inhibition of verapamil-stimulated ATPase by PSC 833.

5 The binding of 8-azido-[α-^{32}P]-ATP to P-glycoprotein was not altered by the cyclosporin derivatives, verapamil, vinblastine and doxorubicin, suggesting that the modulation by these agents of P-glycoprotein ATPase cannot be attributed to an effect on ATP binding to P-glycoprotein.

6 The interaction of the cyclosporin derivatives with ATPase of P-glycoprotein might present an alternative and/or additional mechanism of action for the modulation of P-glycoprotein function.

Keywords: P-glycoprotein; ATPase; PSC 833; cyclosporin A; multidrug resistance

Introduction

P-glycoprotein, a 170–180 kDa membrane glycoprotein, is a member of the adenosine 5′-triphosphate (ATP)-binding cassette transporter family. It has been well documented that P-glycoprotein mediates one type of multidrug resistance (MDR) in tumour cells (for reviews, see Tsuruo, 1988; Gottesman & Pastan, 1993). P-glycoprotein has 12 transmembrane domains contained in two homologous halves and there are two ATP-binding cassette domains in each of the halves that catalyse ATP hydrolysis (Hamada & Tsuruo, 1988a,b; Sarkadi *et al*., 1992; Ambudkar *et al*., 1992; Sharom *et al*., 1993). Utilizing the energy produced by the hydrolysis of ATP, P-glycoprotein actively pumps out a broad spectrum of agents, such as, anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes and MDR reversing agents, including verapamil and cyclosporin A (CsA). Over-expression of P-glycoprotein results in resistance to multiple anticancer agents by maintaining the intracellular levels of the cytotoxic agents below their effective concentration.

A large number of compounds that interact with P-glycoprotein have been identified (for a review, see Ford & Hait, 1990). Some agents stimulate P-glycoprotein adenosine triphosphatase (ATPase) activity and others inhibit the substrate-stimulated ATPase activity (Hamada & Tsuruo, 1988b; Rao & Scarborough, 1994; Sarkadi *et al*., 1994; Sharom *et al*., 1995). Modulators such as verapamil, trifluoperazine and FK506 stimulate the ATPase of P-glycoprotein (Sharom *et al*., 1993; Rao & Scarborough, 1994). CsA was demonstrated to be transported by P-glycoprotein and to be a competitive inhibitor of the pump (Tamai & Safa, 1990; Saeki *et al*., 1993, Shirai *et al*., 1994). However, CsA does not stimulate P-glycoprotein ATPase and inhibits the verapamil-stimulated activity with high affinity (Rao & Scarborough, 1994; Sarkadi *et al*., 1994; Sharom *et al*., 1995; Rao, 1995). These findings suggest that CsA inhibits P-glycoprotein function by different modes of action compared with other MDR modulators.

[3′-keto-Bmt[1]]-[Val[2]]-cyclosporin (PSC 833), a non-immunosuppressive cyclosporin D derivative, is a much more potent MDR modulator than CsA (Boesch *et al*., 1991a,b; Archinal-Mattheis *et al*., 1995; Watanable *et al*., 1996b). Previously, we demonstrated that PSC 833 inhibits the binding of a photoactive analogue of cyclosporin to P-glycoprotein and selectively bound to P-glycoprotein-positive membrane vesicles (Archinal-Mattheis *et al*., 1995). From these results it was inferred that the molecular target of PSC 833 is P-glycoprotein. However, it remains unclear whether PSC 833 modulates P-glycoprotein ATPase activity.

In this study, we investigated the effects of PSC 833 as well as CsA and other modulators on the ATPase activity of human P-glycoprotein. These studies provide insight into the mechanism of action of the two cyclosporin derivatives in the modulation of P-glycoprotein function.

Methods

Tumour cells

The human myelogenous leukemia K562 cell line was provided by Dr Ezaki (Cancer Chemotherapy Center, Tokyo, Japan) and adriamycin-resistant subline of K562 mylogenous leukemia (K562/ADM) was established as previously described (Tsuruo *et al*., 1986). The cells were maintained in RPMI 1640

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supplemented with 10% heat-inactivated foetal bovine serum in a 5% CO_2/95% air atmosphere at 37°C. The K562/ADM cells were maintained in the presence of 0.5 μg ml^{-1} doxorubicin.

Preparation of plasma membrane-enriched fraction

A plasma membrane-enriched fraction of the cells was prepared as described previously (Georges *et al*., 1991; Archinal-Mattheis *et al*., 1995). Briefly, cell suspensions of K562 cells were centrifuged and resuspended in homogenate buffer (10 mM Tris-HCl, pH 8.0, 75 mM sucrose, 25 mM $MgCl_2$, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 150 mM KCl, and 5 mM dithiothreitol (DTT)). The pellets were disrupted by sonication for 10 s (Branson Sonifier model 450), and unbroken cells and nuclei were removed by centrifugation. The supernatants were laid onto a discontinuous sucrose gradient consisting of 16%, 31% and 45% sucrose. Centrifugation was carried out with an SW41Ti rotor (Beckman) for 18 h at $76900 \times g$ at 4°C. The opaque band at the 16/31% interface was collected, and diluted with STM buffer (Tris-HCl 10 mM, pH 7.5, 250 mM sucrose, 1.5 mM $MgCl_2$, 20 μg ml^{-1} aprotinin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)). The fraction was sedimented and the pellets were resuspended in STM buffer. The protein concentration was determined by the Lowry method (Lowry *et al*., 1951) with bovine serum albumin as standard.

Preparation of P-glycoprotein-MRK-16-Protein A complexes

P-glycoprotein was immunoprecipitated with anti-P-glycoprotein monoclonal antibody MRK-16 as described previously (Hamada & Tsuruo, 1988b). The plasma membrane-enriched fractions from the K562/ADM cells were solubilized in solubilizing buffer (Tris-HCl 50 mM, pH 7.5, NH_4Cl 150 mM, $MgCl_2$ 2 mM, and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphate (CHAPS) 1%) and immunoprecipitation was carried out by incubation of the membrane lysates (0.75 mg protein) with 20 μg of MRK-16 for 2 h at 4°C. Following the incubation, 100 μl of Protein A-Sepharose CL4B suspension (20% by volume in solubilizing buffer) was added to 1 ml of membrane lysates for 1 h. The precipitates were washed five times with the reaction buffer (Tris-HCl 50 mM, pH 7.5, NH_4Cl 150 mM, $MgCl_2$ 2 mM and CHAPS 0.1%). For the estimation of P-glycoprotein amount in the complexes, the suspension was dissolved in Laemmli sample buffer (0.13M Tris-HCl, pH 6.8, 6% sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol and 0.001% bromophenol blue (Laemmli, 1970)) and 10 μl of the resulting solution was analysed by 7.5% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). The amount of P-glycoprotein was estimated by Coomassie Blue staining (Daiichi, Tokyo, Japan) with bovine serum albumin as standard. For immunoblotting of P-glycoprotein, the protein separated by 4–20% SDS-PAGE was transferred electrophoretically to Imobilon sheets (Millipore, Tokyo, Japan) and the immunoblot analysis was carried out as described previously (Dong *et al*., 1995). The blots were probed with 1 μg ml^{-1} 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, Japan) by use of an enhanced chemiluminescence system (Amersham, Tokyo, Japan).

P-glycoprotein ATPase assay

The P-glycoprotein-MRK-16-Protein A complexes were subjected to an ATPase assay. The ATPase activity was estimated by a coupled enzyme assay as described previously (Garrigos *et al*., 1993). The immunocomplexes were dissolved in reaction buffer supplemented with 5 mM Mg^{2+}ATP, 0.1 mg ml^{-1} pyruvate kinase, 1 mM phosphoenolpyruvate, 0.1 mg ml^{-1} lactate dehydrogenase and 1 mM β-nicotinamide adenine dinucleotide (NADH). The absorbance at 340 nm was followed and the rate of NADH degradation was determined by linear regression. Statistical difference between IC_{50} values was analysed by ANOVA (Bonferroni/Dunn test; StatView ver 4.02).

Photoaffinity labelling and immunoprecipitation

The plasma membrane-enriched fractions from K562 and K562/ADM cells were incubated in distilled water for 30 min on ice, and then resuspended in reaction buffer supplemented with 2 mM PMSF, 2 μM leupeptin, 3 μM pepstatin and 50 mM DTT and preincubated for 1 h at 4°C in the presence or absence of either cyclosporin analogues, verapamil, doxorubicin, vinblastine or cold ATP. Then 8-azido-[α-^{32}P]-ATP (Amersham, 370 GBq mmol^{-1}) was added to a final concentration of 20 μM and the suspension incubated for 5 min. The mixtures were irradiated for 10 min at 4°C with a u.v. lamp (254 nm, R-52G, UVP). The photolabelled membranes were immunoprecipitated with MRK-16, and analysed by use of 7.5% SDS-PAGE and detected by autoradiography.

Kinetic analysis of PSC 833 inhibition of verapamil-stimulated ATPase

The P-glycoprotein ATPase inhibition constant for PSC 833 was determined as described previously (Rao & Scarborough, 1994; Rao, 1995). P-glycoprotein ATPase activity stimulated by verapamil at either 7.5, 10, 15 or 30 μM in the presence and absence of PSC 833 was determined. The ATPase activities were expressed as a percentage of the 30 μM verapamil-induced activity and then plotted in a double reciprocal plot.

To evaluate the inhibition constant of PSC 833, the following Michealis-Menten type equation for the competitive inhibition was used to model the experimental data:

$$V = \frac{V_{max} \times C}{K_m\left(1 + \frac{I}{K_i}\right) + C} \qquad \text{(Eq. 1)}$$

where V and V_{max} are the ATPase activity as percentage of the 30 μM verapamil-induced activity and the maximum velocity, respectively; K_m and K_i are the Michaelis constant of verapamil and inhibition constant of PSC 833, respectively; and I and C are the concentrations of PSC 833 and verapamil, respectively. K_i was determined by a nonlinear least-squares regression method (Yamaoka *et al*., 1981).

Materials

Anti-P-glycoprotein monoclonal antibody, JSB-1, was obtained from Nichirei, Tokyo, Japan. MRK-16, another anti-P-glycoprotein monoclonal antibody, was prepared as described previously (Hamada & Tsuruo, 1986). Doxorubicin was purchased from Kyowa Hakkou Kogyo, Co., Ltd. (Tokyo, Japan). Verapamil and vinblastine were purchased from Sigma (St. Louis, MO). CsA and PSC 833 were prepared at Sandoz Pharma., Ltd. (Basel, Switzerland). All other chemicals were of analytical grade.

Results

ATPase activity of the P-glycoprotein-MRK-16-Protein A-Sepharose complex

Immunoblotting of the human MDR1 gene product demonstrated the over-expression of P-glycoprotein in the plasma membrane fractions prepared from K562/ADM but not from K562 cells (Figure 1a). With the K562/ADM cell membranes, P-glycoprotein was immunoprepicitated with MRK-16 (Figure

1b) and the P-glycoprotein-MRK-16-Protein A-Sepharose complexes obtained were subjected to a coupled enzyme ATPase assay. The immunocomplexes demonstrated an ATPase activity of 102 ± 11 nmol min^{-1} mg^{-1} P-glycoprotein (mean ± s.e.mean of three experiments). This specific activity of P-glycoprotein ATPase was comparable with the previously obtained activity of immunoprecipitated P-glycoprotein (Hamada & Tsuruo, 1988b; Shimabuku *et al*., 1992). In addition, to validate the ATPase assay for P-glycoprotein used in this study, we examined the effects of the presence of verapamil, vinblastine and doxorubicin that are known P-glycoprotein ATPase stimulators (Ambudkar *et al*., 1992). It was found that the presence of 60 μM verapamil, 40 μM vinblastine or 90 μM doxorubicin stimulated ATPase of the immunocomplexes by 233, 154, and 142%, respectively (Figure 2).

The basal activity of the P-glycoprotein-MRK-16-Protein A-Sepharose complex of 102 nmol min^{-1} mg^{-1} P-glycoprotein was two orders of magnitude higher than that of purified P-glycoprotein (1.7 nmol min^{-1} mg^{-1} P-glycoprotein) (Hamada & Tsuruo 1988a). However, the activity of the P-glycoprotein-MRK-16-Protein A-Sepharose complex was about 30–50 times less than the activity of P-glycoprotein ATPase prepared from MDR1-infected Sf9 cells or P-glycoprotein reconstituted in liposomes (3–5 μmol min^{-1} mg^{-1} P-glycoprotein) (Sarkadi *et al*., 1992; Ambudkar *et al*., 1992). These differences might represent the distinct method used in this study for P-glycoprotein ATPase preparation.

Inhibition of P-glycoprotein ATPase activity by two cyclosporin analogues

The effects of PSC 833 and CsA treatments on the basal ATPase activity of P-glycoprotein were studied. As demonstrated in Figure 3, both cyclosporins inhibited the ATPase activity in a concentration-dependent manner, while verapamil caused activation to half maximum level (i.e., SC_{50}) at a concentration of 16.8 ± 11.9 μM (mean ± s.e.mean of three experiments). The IC_{50} of PSC 833 and CsA were 8.05 ± 2.96 and 12.3 ± 7.9 nM, respectively (mean ± s.e.mean of three experiments, Table 1). An almost complete inhibition (<20% of the control activity) of the basal ATPase activity was observed with 3 μM PSC 833 and CsA.

Subsequently, the effects of the cyclosporin derivatives on the ATPase activity stimulated by either 60 μM verapamil, 90 μM doxorubicin or 40 μM vinblastine were studied. The concentrations of verapamil, vinblastine and doxorubicin that achieved the maximum stimulation of P-glycoprotein ATPase activity were selected to study the effect of these stimulators (data not shown). As shown in Figure 4 and Table 1, PSC 833 and CsA inhibited the substrate-stimulated ATPase. The IC_{50} values of CsA for doxorubicin- and vinblastine-stimulated ATPase activity were more than 1 μM, since even at 1 μM CsA the relative ATPase activity was reduced by less than 50% (Figure 4). However, PSC 833 inhibited the doxorubicin- and vinblastine-stimulated activity with an IC_{50} of 37.9 ± 17.2 and 82.2 ± 51.4 nM (mean ± s.e.mean of three experiments), respectively. In addition, when the IC_{50} values of PSC 833 and CsA for the verapamil-stimulated activity were compared, PSC 833 was also found to be more potent than CsA in inhibiting verapamil-stimulated activity ($P < 0.05$).

Kinetic analyses of the inhibition by PSC 833 of verapamil-stimulated P-glycoprotein ATPase

Cyclosporin A and dihydrocyclosporin C were shown to inhibit competitively the verapamil-activation of P-glycoprotein ATPase (Rao & Scarborough, 1994; Rao, 1995). However, the mode of inhibition of verapamil stimulation by PSC 833 remains unclear. To characterize further the interaction of PSC 833 with the verapamil-stimulated P-glycoprotein ATPase, a kinetic analysis of this inhibition was performed. P-glycoprotein ATPase activity stimulated by 7.5, 10, 15 or 30 μM verapamil was measured in the presence and absence of PSC 833. The PSC 833 concentrations that were selected in this analysis resulted in partial inhibition of verapamil-stimulated ATPase (data not shown). The ATPase activities were expressed as percentages of the 30 μM verapamil-activated activity and then plotted in a double reciprocal plot (Figure 5). The presence of PSC 833 at 0.05 and 0.1 μM did not markedly change the in-

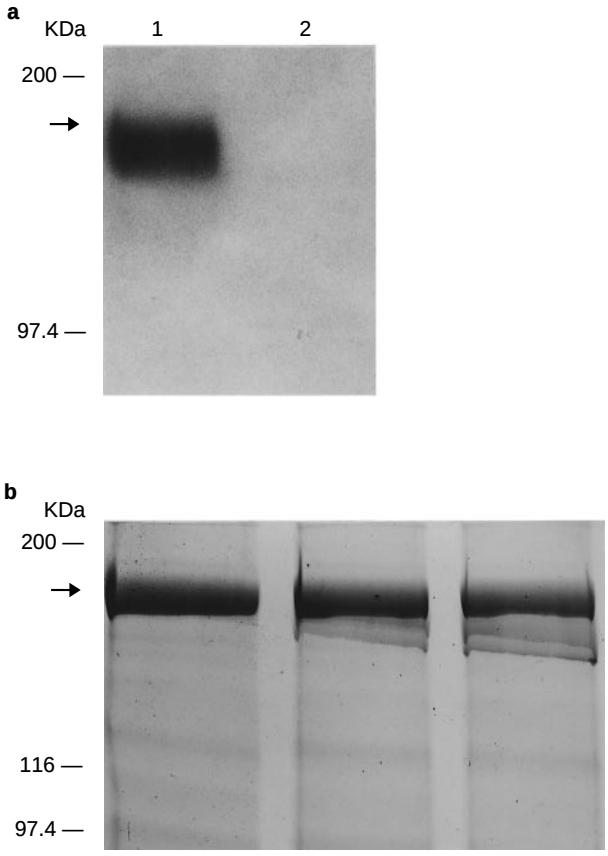

Figure 1 P-glycoprotein expression in membrane fraction from K562 and K562/ADM. (a) Membrane fractions derived from either K562 or K562/ADM cells were subjected to immunoblot analysis with anti-P-glycoprotein antibody JSB-1. Lane 1: K562/ADM 5 μg protein, Lane 2: K562 20 μg protein. The arrow indicates the position of P-glycoprotein. (b) P-glycoprotein-MRK-16-Protein A-Sepharose complexes were analysed by 7.5% SDS-PAGE and the gel was stained with Coomassie Blue. The positions of the molecular weight standards are indicated on the left and the corresponding position of P-glycoprotein is indicated with an arrow (170–180 kDa).

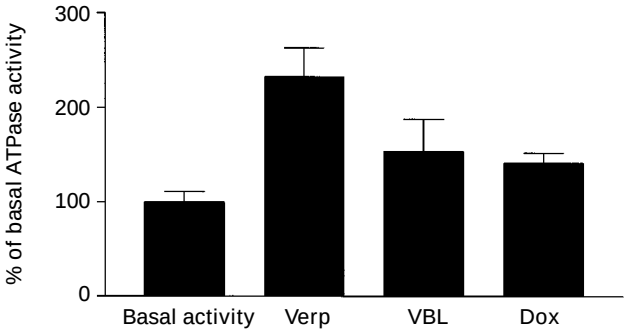

Figure 2 Stimulation of ATPase activity by verapamil, vinblastine and doxorubicin. The ATPase activity of P-glycoprotein-MRK-16-Protein A-Sepharose complexes were evaluated in the presence of stimulators. The concentrations of the stimulators used were 60 μM for verapamil (Verp), 40 μM for vinblastine (VBL) and 90 μM for doxorubicin (Dox). The ATPase activity is shown as a percentage of the basal ATPase activity of three independent membrane preparations (100% = 102 ± 11 nmol $min^{-1}mg^{-1}$ P-glycoprotein).

tercept on the ordinate scale in the double-reciprocal plot, indicating that PSC 833 competitively inhibited the verapamil activation. The K_i of PSC 833 was found to be 134 ± 30 nM (mean $\pm$ s.d. of three experiments).

Effect of 8-azido-[α-^{32}P]-ATP binding to P-glycoprotein

The binding of ATP to P-glycoprotein is believed to be the first step in ATP hydrolysis. A possible mechanism of the modulation of P-glycoprotein ATPase by cyclosporin derivatives and stimulators is thought to be the modification of ATP binding. To examine this, the effect of the agents on the photolabelling of P-glycoprotein with azido-[α-^{32}P]-ATP was investigated. Membrane fractions from K562/ADM cells were incubated in the presence of either PSC 833, CsA, verapamil, vinblastine or doxorubicin. After U.V. cross-linking, the photolabelled membranes were immunoprecipitated with MRK-16. The immunoprepicitated fraction was analysed by use of 7.5% SDS-PAGE and the photolabelled P-glycoprotein was detected by autoradiography (Figure 6). Neither of the two cyclosporin derivatives markedly changed the binding of the photoactive compound to P-glycoprotein. In addition, no marked increase in the binding of 8-azido-[α-^{32}P]-ATP to P-glycoprotein was observed in the presence of verapamil, vinblastine or doxorubicin.

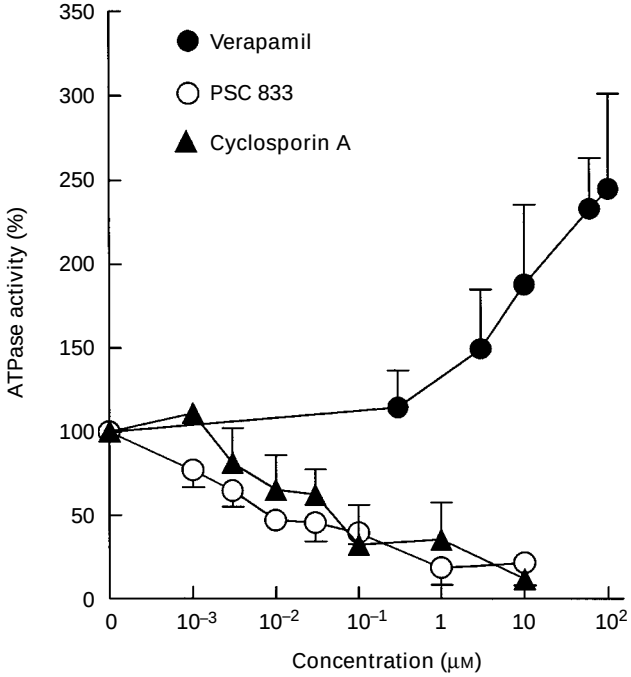

Figure 3 Effects of modulators on the basal ATPase activity of P-glycoprotein. P-glycoprotein in the membrane fraction from K562/ADM was immunoprecipitated with MRK-16. The P-glycoprotein-MRK-16-Protein A-Sepharose complexes were subjected to the ATPase assay. The ATPase activity with or without modulators is expressed as a percentage of the activity without the modulators. Each point and vertical line represent the mean value and s.e.mean of triplicate experiments, respectively.

Discussion

The results of this study extend our understanding of P-glycoprotein inhibition by PSC 833 and CsA by examining the effects of these MDR reversing agents on P-glycoprotein ATPase activity. Both cyclosporin analogues were found to inhibit the drug-activated and the basal ATPase activity of P-glycoprotein with high affinity. However, while PSC 833 and CsA inhibited the basal ATPase of P-glycoprotein with comparable affinity, PSC 833 was found to be more potent than CsA at inhibiting stimulated P-glycoprotein ATPase. A kinetic ana-

Table 1 Inhibition of P-glycoprotein ATPase activity by cyclosporin derivatives

	IC_{50}[a] (nM)	
Stimulator	*PSC 833*	*Cyclosporin A* (nM)
None	8.05 ± 2.96	12.3 ± 7.9
Verapamil (60 μM)	121 ± 117	759 ± 295*
Vinblastine (40 μM)	82.2 ± 51.4	> 1000
Doxorubicin (90 μM)	37.9 ± 17.2	> 1000

[a]Concentration producing half-maximal activity calculated from the data in Figures 3 and 4 (mean ± s.e.mean, $n = 3$). *Significant difference from PSC 833 ($P < 0.05$ by Bonferroni/Dunn test (two tail)).

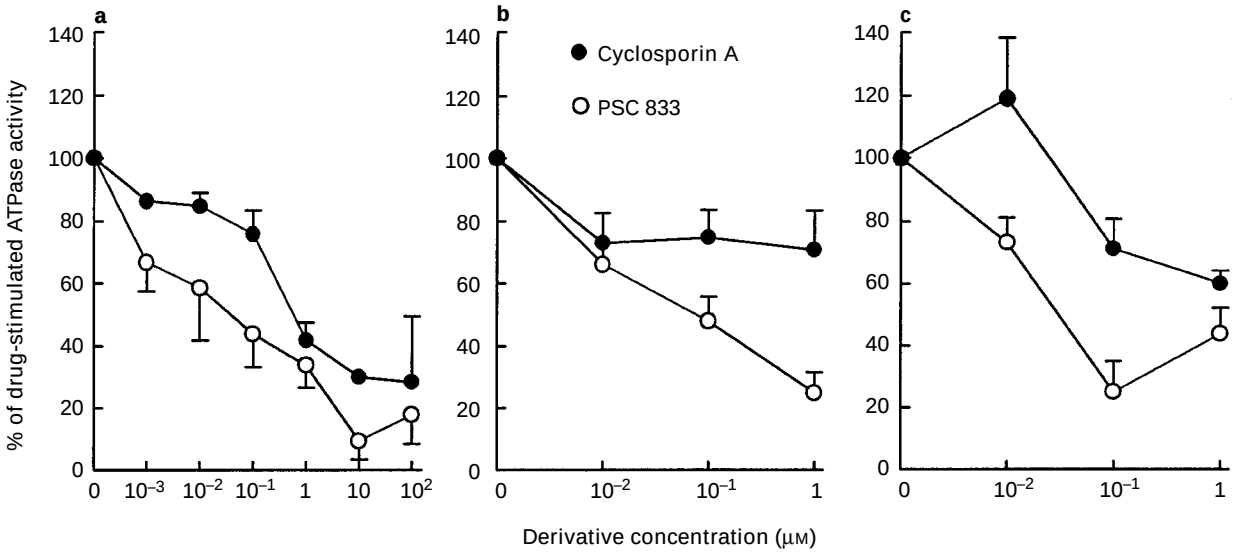

Figure 4 Effect of cyclosporin derivatives on the drug-stimulated ATPase activity of P-glycoprotein. P-glycoprotein ATPase was stimulated by either (a) 60 μM verapamil, (b) 40 μM vinblastine or (c) 90 μM doxorubicin. The drug-stimulated ATPase activity in the presence of the indicated concentrations of the cyclosporin derivatives was determined. The ATPase activity was expressed as a percentage of the activity without the cyclosporin derivatives. Each point and vertical line represent the mean value and s.e.mean of triplicate experiments, respectively.

lysis revealed a competitive inhibition of verapamil stimulation by PSC 833. Finally, the modulation of ATPase activity by the cyclosporin derivatives, verapamil, vinblastine and doxorubicin was not accompanied by changes in the binding of photoactive ATP analogue to P-glycoprotein. Delineation of the effects of PSC 833 and CsA on P-glycoprotein ATPase activity might give an insight into the mechanism of action of these cyclosporin derivatives in modulating P-glycoprotein function.

Several studies, in which the P-glycoprotein ATPase activity were measured, employed the vanadate-induced trap method with P-glycoprotein-positive membrane vesicles (Sarkadi *et al*., 1992; Rao & Scarborough, 1994; Sharom *et al*., 1995). In these studies, the difference between the ATPase activities with or without 100 μM vanadate in the presence of O, O'-bis(2-aminoethyl) ethyleneglycol-N,N,N′, N'-tetraacetic acid (EGTA), NaN_3 and ouabain were attributed to P-glycoprotein (c.a., vanadate method). Sarkadi *et al*., (1992) showed that 100 μM vanadate inhibited the ATPase activity by approximately 30% in P-glycoprotein-negative membrane vesicles even in the presence of EGTA, NaN_3 and ouabain. This indicated that the inhibitory effect of vanadate is not specific to P-glycoprotein. The less-specific effect of vanadate may impair the detection of the complete inhibition of basal P-glycoprotein ATPase by CsA (Sarkadi *et al*., 1994; Sharom *et al*., 1995). In contrast, the P-glycoprotein-MRK-16-Protein A-Sepharose complexes might represent a better alternative for evaluating the specific P-glycoprotein ATPase activity. However, with this method, the basal and stimulated activities of P-glycoprotein ATPase were found to be lower than those measured in membrane fractions by the vanadate method (Sarkadi *et al*., 1992; Sharom *et al*., 1995).

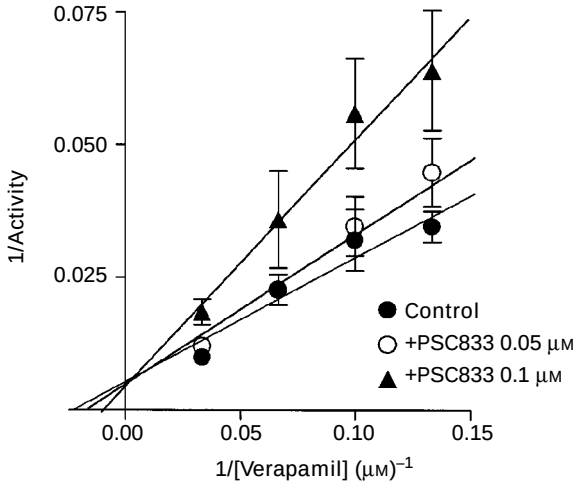

Figure 5 Double reciprocal plot of the inhibition of the verapamil-stimulated P-glycoprotein ATPase activity by PSC 833. P-glycoprotein ATPase was stimulated by verapamil at concentrations of 7.5, 10, 15 or 30 μM. The verapamil-stimulated activity in the presence of either 0, 0.05 or 0.1 μM PSC 833 was determined. Reciprocal values of the activity (as a percentage of the ATPase activity induced by 30 μM verapamil) are plotted against 1/verapamil concentrations.

With the P-glycoprotein-MRK-16-Protein A-Sepharose immunocomplexes, it was found that PSC 833 and CsA inhibited the basal ATPase activity of P-glycoprotein in a concentration-dependent manner (Figure 3). Although several studies have demonstrated inhibition of drug-stimulated P-glycoprotein ATPase by CsA, little is known about the effects of cyclosporin analogues on basal P-glycoprotein ATPase activity. Recently, it was shown in studies utilizing the vanadate-induced trap method to evaluate P-glycoprotein ATPase activity, that CsA did not markedly reduce the basal P-glycoprotein ATPase activity (Sarkadi *et al*., 1994; Sharom *et al*., 1995). However, in another study (Ramachandra *et al*., 1996) CsA was found to inhibit partially the basal activity. This controversy and the limited inhibition might be explained by the less-specific effect of vanadate. To our knowledge, this is the first study to show complete inhibition of basal P-glycoprotein ATPase by PSC 833 as well as CsA. The observation that the inhibition of basal P-glycoprotein ATPase activity by the two cyclosporin analogues was similar (Table 1), suggested a comparable affinity of these derivatives for P-glycoprotein. The different IC_{50} values for drug-activated ATPase indicate that PSC 833 is more potent than CsA in competing with the P-glycoprotein stimulators. Interestingly, both cyclosporin derivatives inhibited P-glycoprotein ATPase, but while CsA is transported by P-glycoprotein, PSC 833 is either not transported or is a poor substrate for P-glycoprotein (Archinal-Mattheis *et al*., 1995). Therefore, it was suggested that the ATPase inhibitory effects of the cyclosporin derivatives were independent of their being transported by P-glycoprotein.

It is possible that the modulation of P-glycoprotein ATPase by cyclosporin derivatives and the stimulators can be attributed to the modulation of ATP binding to P-glycoprotein. Recent studies (Georges *et al*., 1991; Kokubu *et al*., 1997) demonstrated that inhibition of P-glycoprotein ATPase by C219, a monoclonal antibody recognizing domains near the ATP-binding cassettes of P-glycoprotein, is associated with inhibition of the binding of azido-[α-^{32}P]-ATP, an ATP-like substrate (Al-shawi *et al*., 1994), to P-glycoprotein. However, none of the cyclosporin derivatives, verapamil, vinblastine and doxorubicin modulated the binding of azido-[α-^{32}P]-ATP to P-glycoprotein (Figure 6). The negligible effect of the cyclosporin derivatives and stimulators in these experiments suggests that these agents modulate ATP hydrolysis without modulating binding of ATP.

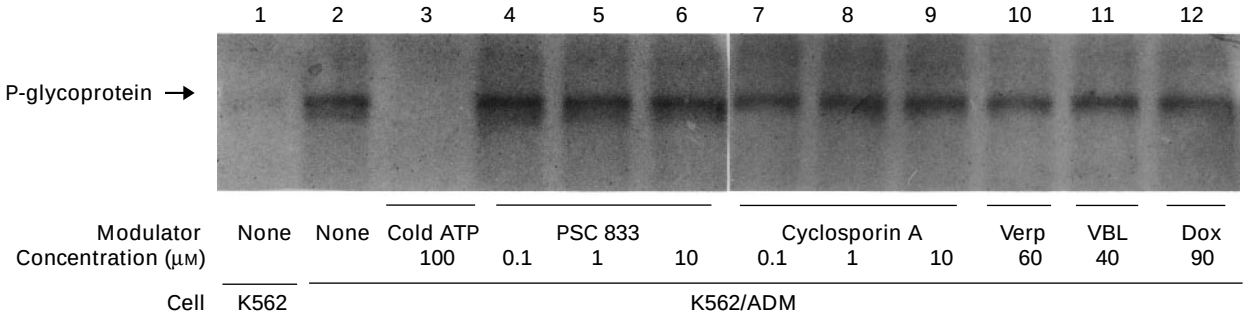

Figure 6 Effect of drugs on binding of 8-azido-[α-^{32}P]-ATP to P-glycoprotein. K562/ADM plasma membrane fractions were incubated with 8-azido-[α-^{32}P]-ATP in the presence or absence of the indicated concentrations of the modulators. P-glycoprotein was photolabelled with 20 μM 8-azido-[α-^{32}P]-ATP and immunoprecipitated with MRK-16. After the fractionation by SDS-PAGE, the labellings were detected by autoradiography. Lane 1: K562, lane 2: K562/ADM without agents, lane 3: K562/ADM with 100 μM of unlabelled ATP, lanes 4–6: K562/ADM with PSC 833, lanes 7–10: K562/ADM with CsA, lanes 10–12: K562/ADM with the indicated stimulator (Verp: verapamil, VBL: vinblastine, Dox: doxorubicin).

Similar to previously described activity of CsA (Rao & Scarborough, 1994; Rao, 1995), PSC 833 was found to inhibit competitively the stimulation of P-glycoprotein ATPase activity by verapamil (Figure 5). CsA was also demonstrated to be a competitive inhibitor of the stimulation of P-glycoprotein ATPase by vinblastine (Rao, 1995). These findings suggest that both cyclosporin derivatives compete with the stimulators for P-glycoprotein ATPase by interacting with the same or overlapping binding site(s). A previous finding (Tamai & Safa, 1990) of competitive inhibition by CsA of vinblastine binding to P-glycoprotein supports this hypothesis.

Studies in which photolabelling of P-glycoprotein with azidopine, prazosin and forskolin was investigated, followed by proteinase digestion, identified the drug binding sites, which are located in predicted transmembrane (TM) portions 6 and 12 and in close proximity to the ATP binding regions (Bruggeman *et al*., 1989; 1992; Greenberger, 1991; Greenberger *et al*., 1993). Similar to the photoactive agents, if the binding sites of the cyclosporin derivatives and stimulators are located near TM6 and/or TM12 close to the ATP-binding cassette, it is possible that ATPase hydrolysis by P-glycoprotein is modulated via allosteric effects produced by the binding of the modulators. The significance of the predicted TM11–12 of P-glycoprotein in the interaction of verapamil and CsA with the multidrug transporter was shown in recent mutagenesis studies of P-glycoprotein (Kajiji *et al*., 1993; 1994; Loo & Clarke, 1994; 1996). The capacity of verapamil and CsA to modulate P-glycoprotein transporter activity are both sensitive to substitution of Ser to Phe at position 941 in the mouse *mdr1* or at position 939 in the mouse *mdr3* gene product within TM11 (Kajiji *et al*., 1993; 1994). The sensitivity of both modulators to a point mutation within TM11 suggests a degree of similarity in the mode of binding.

It is not clear why agents like verapamil, an MDR-reversing agent, and anticancer drugs like vinblastine and doxorubicin have a stimulating effect on P-glycoprotein ATPase while cyclosporin analogues inhibit ATPase. Recently, it was suggested that P-glycoprotein ATPase has catalytic and inhibitory sites and that the affinities of various modulators for these two sites will determine whether P-glycoprotein ATPase is stimulated or inhibited (Ambudkar *et al*., 1996). Further, it was speculated that verapamil interacted with the catalytic and inhibitory sites while CsA has affinity for the inhibitory site alone. However, it remains unclear what domains of P-glycoprotein are responsible for the catalytic and inhibitory sites. An alternative explanation for the differing effect is that verapamil and the cyclosporin derivatives induced different conformational changes in P-glycoprotein that result in allosteric changes that influence ATP hydrolysis. Previously, PSC 833 and CsA, but not verapamil, were found to increase the binding of the monoclonal antibody MC57 to P-glycoprotein (Jachez *et al*., 1994). In addition, it was shown that a combined therapy of CsA/PSC 833 and MRK-16 resulted in a synergistic inhibition of P-glycoprotein functions, while that with verapamil/FK-506 and MRK-16 resulted in an additive effect (Naito *et al*., 1993; 1996). From these findings it was suggested that different conformational changes in P-glycoprotein are caused by these MDR reversing agents.

Studies with a mutant P-glycoprotein bearing a substitution of Gly to Val at position 185 have indicated that the cytoplasmic loop between predicted TM2 and TM3 is responsible for the functional roles of P-glycoprotein (Choi *et al*., 1988; Safa *et al*., 1990). This mutation has been shown to modulate substrate specificity as well as the activity of MDR-reversing agents. While the reversing activity of CsA and PSC 833 is more potent against the mutant P-glycoprotein bearing Val at position 185 than the wild-type P-glycoprotein, verapamil is not a potent modulator for the mutant (Cardarelli *et al*., 1995; Ramachandra *et al*., 1996; Watanabe *et al*., 1996a). These different effects of the Gly185 substitution on the reversing potency of the verapamil and cyclosporin derivatives lead us to speculate that the two modulators induce different conformational changes in the P-glycoprotein, even though these may share the same or overlapping binding site(s) (Figure 5; Tamai & Safa, 1990; Rao & Scarborough, 1994; Rao, 1995). In addition, it was found that verapamil inhibits the P-glycoprotein-associated chloride channel, while CsA has no effect (Mintenig *et al*., 1993). It is suggested that the different activity of the channel associated with P-glycoprotein between verapamil and CsA is correlated with the different activity of the P-glycoprotein ATPase, although both ATPase and transport activities of the multidrug transporter appear to be distinct and separable from the chloride channel activity (Gill *et al*., 1992).

Recent studies (Lampidis *et al*., 1997), with a selected series of anthracycline analogues with different positive charges, demonstrated that positively-charged analogues are better recognized by P-glycoprotein than their neutral counterparts. The study also showed that analogues with increasing lipophilicity, regardless of charge, are able to inhibit drug-binding to P-glycoprotein with more efficiency. These findings clarified that electronic charge is an important factor for P-glycoprotein substrate transport and that lipophilicity is important for MDR-reversing activity. One difference in chemical structure between the cyclosporin derivatives and the stimulator used in this study is that the stimulators are all cations whereas the cyclosporin derivatives are electronically neutral. Taken together, it is possible that electronic charge and lipophilicity are important factors for determining the mode of modulation (i.e., stimulation or inhibition) of P-glycoprotein ATPase. In addition, partition coefficient determinations revealed that PSC 833 is more lipophilic than CsA (Archinal-Mattheis *et al*., 1995). It is therefore suggested that the greater potency of PSC 833, compared to CsA, in inhibiting drug-stimulated P-glycoprotein-ATPase (Table 1) could be attributed to the different lipophilicity between PSC 833 and CsA.

The ATPase of P-glycoprotein might be an important target for MDR modulation, since the hydrolysis of ATP by P-glycoprotein is believed to produce the energy required for the active transport of anticancer drugs. It is possible that the inhibition by the cyclosporin derivatives of P-glycoprotein ATPase represents an additional and/or alternative mechanism of action for these MDR modulators.

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