

Molecular Interactions of Cyclosporin A with P-glycoprotein

PHOTOLABELING WITH CYCLOSPORIN DERIVATIVES*

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The interaction between P-glycoprotein (140–180 kDa) from the multidrug-resistant Chinese hamster ovary cell line CH^RC5 and cyclosporin A was characterized using three different photoactivable cyclosporin A analogs. Two monoclonal antibodies, which are able to discriminate between two major domains of cyclosporin A (the cyclophilin and calcineurin binding domains), were used to detect the photolabeled proteins. A protein of 155 kDa corresponding to P-glycoprotein was much more strongly photolabeled in membranes of CH^RC5 cells than in membranes of their drug-sensitive parent cell line AuxB1. The antitumor drug vinblastine and the reversal agents verapamil and cyclosporin A inhibited the photolabeling, and the nonimmunosuppressive derivative PSC-833 caused a stronger inhibition than cyclosporin A. P-glycoprotein photolabeled with cyclosporin A analogs was only detected with the monoclonal antibody that recognizes cyclosporin A and its metabolites, indicating that the calcineurin binding domain recognized specifically by the other antibody is not exposed. These results suggest that the portion of cyclosporin A that binds to calcineurin plays a role in the interaction of cyclosporin A with P-glycoprotein.

Overexpression of P-glycoprotein (P-gp)¹ (1, 2), a plasma membrane protein that actively transports antitumor agents out of the cell and reduces their cytosolic concentration, is associated with multidrug resistance (3–5). P-gp was detected in several normal tissues and several kinds of tumors, some of which present higher expression after chemotherapy (6, 7).

A model based on the deduced amino acid sequence of this glycosylated protein of 150–180 kDa was proposed, consisting of 12 membrane-spanning segments and two intracytoplasmic ATP binding domains (5, 8). The uptake of colchicine and vinblastine into inside-out membrane vesicles derived from drug-resistant cells is stimulated by ATP and inhibited by different agents, including a wide variety of anticancer drugs and a large number of hydrophobic substances such as calcium channel blockers (verapamil and azidopine) and immunosuppressant drugs (cyclosporin A and FK506) (3, 9, 10). Chemo-

sensitizing agents (cyclosporin A and verapamil) are believed to reverse the multidrug resistance phenotype by inhibiting the energy-dependent efflux of the cytotoxic agents (2, 5). Furthermore, cyclosporin A (CsA) was previously reported to be a substrate for P-gp in renal cell lines (11). Understanding of the molecular mechanism involved in the interactions between P-gp and such a diversity of compounds appears essential for elucidating its mode of action and may contribute to the development of more effective anticancer chemotherapy.

Administration of CsA alone was also shown to cause an increase in P-gp expression in normal rat tissues (12). Many CsA metabolites and analogs inhibit P-gp *in vitro*, and some analogs such as PSC-833 possess stronger reversing properties than the parent compound (10, 13). Single serine residues within transmembrane domain 11 of P-gps encoded by mouse *mdr1* (Ser⁹⁴¹) and *mdr3* (Ser⁹³⁹) were shown to be critical for substrate specificity and P-gp interaction with CsA (14).

For its immunosuppressive activity, half of the CsA surface that includes amino acids 1–3 and 9–11 interacts with cyclophilin (15, 16). The exposed portion of CsA in the CsA-cyclophilin complex binds to calcineurin to form an immunosuppressant sandwich that blocks T-cell proliferation (17). However, for the reversal activity of CsA in multidrug resistance the exact molecular mechanism remains unknown, and the molecular events implicated in the interaction of CsA and its analogs with P-gp remain to be established.

In the present article photolabeling of P-gp by photoactivable CsA analogs (18) was characterized in the presence of different P-gp substrates and chemosensitizing agents. CsA bound to P-gp was detected by Western blots using monoclonal antibodies (mAbs) directed against different portions of CsA (19). One recognizes the calcineurin binding domain (anti-CsA_{CAL}) and the other recognizes the cyclophilin binding domain of CsA (anti-CsA_{CYP}). The use of these mAbs allowed us to bring new information on the molecular mechanism implicated in the interaction of CsA with P-gp.

EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium α and fetal bovine serum were purchased from Life Technologies, Inc. Penicillin and streptomycin were from Flow Laboratories (Mississauga, Ontario, Canada). The Mini-Protein II apparatus and electrophoresis reagents were from Bio-Rad. Polyvinylidene difluoride membranes and Milliblot-Graphite electroblotter I were from Millipore (Mississauga, Ontario, Canada). Anti-mouse IgG horseradish peroxidase-linked whole antibody and ECL reagents were purchased from Amersham Corp. The photoactivable CsA analogs (SDZ 212–122, SDZ 212–904, and SDZ 211–845), the parent drug, CsA analogs, and mAbs against CsA were kindly provided by Dr. R. M. Wenger (Sandoz Pharma Ltd.). [¹²⁵I]iodoarylazido prazosin (IAAP) was from DuPont NEN. The polyclonal antibody *mdr* (Ab-1) was from Oncogene Science (Uniondale, NY).

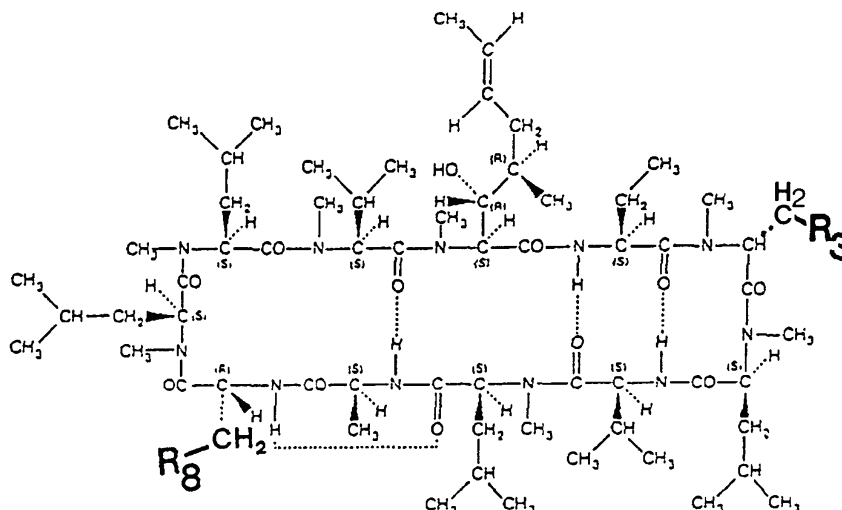
Cell Cultures and Crude Membrane Preparations—Cells of the pleiotropic drug-resistant CH^RC5 cell line, selected for resistance to colchicine (20), and of its drug-sensitive parent cell line AuxB1 were grown in monolayers in 175-cm² plastic tissue culture flasks at 37 °C under 5%

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¹ The abbreviations used are: P-gp, P-glycoprotein; CsA, cyclosporin A; mAb, monoclonal antibody; IAAP, [¹²⁵I]iodoarylazido prazosin; TBS-T, Tris buffered saline-Tween 20; CsD, cyclosporin D; SDZ, Sandoz; CyP, cyclophilin; CAL, calcineurin.

FIG. 1. Molecular structure of CsA and diazirine-CsA analogs. Cyclosporin A, R_3 and R_8 = H; SDZ 212-122, R_3 = H, and R_8 = $\text{OCOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}-\text{C}_6\text{H}_4-(4)\text{-CN}_2\text{-CF}_3$; SDZ 212-904, R_3 = H, and R_8 = $\text{OCOCH}_2\text{NHCO}-\text{C}_6\text{H}_4-(4)\text{-CN}_2\text{-CF}_3$; SDZ 211-845, R_3 = $\text{OCO}-\text{C}_6\text{H}_4-(4)\text{-CN}_2\text{-CF}_3$, and R_8 = H.



CO_2 in minimum essential medium α supplemented with 10% fetal bovine serum and 1% penicillin (50 units/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$). Cells were grown to confluence and incubated for 24 h at 37 °C with fresh culture medium. They were harvested with 15 mM sodium citrate in phosphate-buffered saline, which contained 138 mM NaCl, 5.4 mM KCl, and 5 mM $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7.4, washed by centrifugation, and resuspended in phosphate-buffered saline. Cells were homogenized in buffer (10 ml/g wet weight of cells) composed of 50 mM mannitol and 5 mM Hepes/Tris, pH 7.5, with a Polytron tissue homogenizer (Brinkmann Instruments) at position 5.5 for 1 min and centrifuged at $33,000 \times g$ for 30 min at 4 °C. The pellets were resuspended in the same buffer, stored at -80 °C, and used within 1 month.

Photolabeling of P-gp with Diazirine-CsA Analogs— $\text{CH}^{\text{RC}}5$ membranes (40 μg of protein) were incubated with diazirine-CsA analogs in 10 mM Tris/HCl, pH 7.0, and protease inhibitors (2 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 100 $\mu\text{g}/\text{ml}$ bacitracin) for 60 min at 25 °C and irradiated under a Spectrolene UV lamp (Fisher Scientific) at 254 nm on ice. Sample buffer was added to the suspensions, and proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gels according to the method of Laemmli (21) with a Mini-Protean II apparatus. The proteins were transferred electrophoretically onto a 0.45- μm pore size polyvinylidene difluoride membrane with a semidry electroblotter apparatus. The transfer was carried out at 1 mA/cm² for 90 min in the presence of 96 mM glycine, 10 mM Tris, and 10% methanol. Polyvinylidene difluoride membranes were incubated in Tris-buffered saline (50 mM Tris and 150 mM NaCl, pH 7.0) containing 0.2% Tween-20 (TBS-T) and 0.5 $\mu\text{g}/\text{ml}$ of the anti-CsA_{CYP} or anti-CsA_{CAL} mAb in TBS-T for 120 min at 25 °C. The membranes were washed three times for 15 min in TBS-T and incubated 60 min with horseradish peroxidase-conjugated anti-mouse IgG diluted 1:1000 in TBS-T containing 5% powdered milk. The membranes were finally washed three times in TBS-T for 15 min, and the immune complex was revealed with ECL reagents. Protein concentrations were estimated with the Bradford assay (22).

Photolabeling of P-gp with Iodoarylazido Prazosin— $\text{CH}^{\text{RC}}5$ membranes (50 μg of protein) were incubated with 20 nM IAAP in 10 mM Tris/HCl, pH 7.0, and protease inhibitors for 60 min at 25 °C and irradiated at 254 nm on ice for 5 min. P-gp was solubilized and incubated overnight at 4 °C with the polyclonal antibody *mdr* (Ab-1) in TBS containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS. The immune complex was precipitated with 40 μl of protein A-Sepharose (50%, v/v). Sample buffer was added to the suspension, and proteins were resolved by electrophoresis as described above. The gels were dried and exposed to preflashed Fuji films for 1 week at -80 °C.

RESULTS

Photolabeling of $\text{CH}^{\text{RC}}5$ Proteins with Diazirine-CsA—In the present study, a rapid method for the detection of CsA bound to P-gp from the multidrug-resistant Chinese hamster ovary cell line $\text{CH}^{\text{RC}}5$ was developed. Three different photoactivable CsA analogs, modified at position 8 (SDZ 212-122 and SDZ 212-904) or position 3 (SDZ 211-845) as described in Fig. 1, were assayed. Two anti-CsA mAbs directed against CsA, which are

able to discriminate between two portions of CsA, were used (Fig. 2). First, $\text{CH}^{\text{RC}}5$ proteins were incubated with 20 nM diazirine-CsA analog SDZ 212-122 and cross-linked under UV light for various lengths of time. Covalently bound CsA was revealed by Western blot analysis using the mAb against CsA and its metabolites (Fig. 3). Photolabeling increased with irradiation time, whereas, in the absence of UV light, no protein labeled with diazirine-CsA could be detected (Fig. 3A). As another control, a $\text{CH}^{\text{RC}}5$ protein gel stained by Coomassie Blue is also presented to demonstrate that photolabeling is specific for certain proteins, since the major proteins are not labeled by the CsA analog (Fig. 3A). This eliminates the concern that photolabeling may be artifactually related to free radicals generated on proteins during photolysis and scavenged by the CsA analog. The region corresponding to P-gp (150–180 kDa) was scanned with a laser densitometer, and the relative density of this region was plotted as a function of irradiation time (Fig. 3B). Since a high background level was observed with a long irradiation period (30 min), shorter irradiation times (10–15 min) were used in subsequent experiments.

Photolabeling of P-gp by Different Diazirine-CsA Analogs—Three CsA analogs modified at different positions were tested to evaluate their capacity to label P-gp (Fig. 4). Two of them, SDZ 212-122 and SDZ 212-904, have a diazirine group on position 8, and the third, SDZ 211-845, has a diazirine group on position 3. The amount of photolabeled P-gp increased as a function of the concentration of SDZ 212-122 or SDZ 212-904 (Fig. 4, A and B). CsA bound to P-gp could only be detected with the anti-CsA_{CYP} mAb (Fig. 4, A and B). The analog modified on position 3 was undetected with either antibody (Fig. 4C). In addition, two controls were performed for each analog. $\text{CH}^{\text{RC}}5$ proteins were incubated with 20 nM diazirine-CsA analog in the presence of 200 μM PSC-833 or without irradiation (-UV). In both controls, no labeled protein was detected when blots were probed with either mAb.

The region corresponding to P-gp (150–180 kDa) was scanned with a laser densitometer, and the relative density was plotted as a function of photoactivable CsA analog concentration (Fig. 5). Saturation has occurred for two photoactivable CsA analogs (SDZ 212-122 and SDZ 211-904). The concentrations needed to obtain 50% of the maximum density were 136 and 373 μM for SDZ 212-122 and SDZ 211-904, respectively. These results demonstrate site selectivity of the photolabeling and suggest that SDZ 212-122 has a higher affinity for P-gp than SDZ 211-904.

Inhibition of P-gp Photolabeling—To verify the specificity of

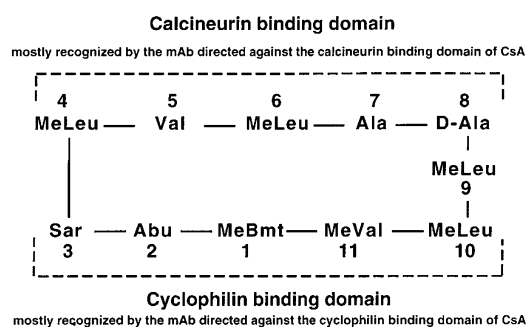


FIG. 2. **Identification of 11 amino acid residues of CsA.** The cyclophilin binding domain of the molecule includes residues 1–3 and 9–11, and the calcineurin binding domain corresponds to residues 4–9. *MeBmt*, 4-[(*E*)-2-butenyl]-4,4,4-trimethyl-L-threonine; *Abu*, L-aminobutyric acid; *Sar*, sarcosine; *MeLeu*, *N*-methylleucine; *MeVal*, *N*-methylvaline. The anti-CsA_{CYP} mAb recognizes CsA and its metabolites (16), and the anti-CsA_{CAL} mAb recognizes strongly the residues of the calcineurin binding domain. (Note that residue 1 may also be a part of the calcineurin binding domain; Ref. 30). These antibodies, anti-CsA_{CYP} and anti-CsA_{CAL}, correspond, respectively, to specific and nonspecific antibodies developed by Sandoz for the determination of CsA and its metabolites.

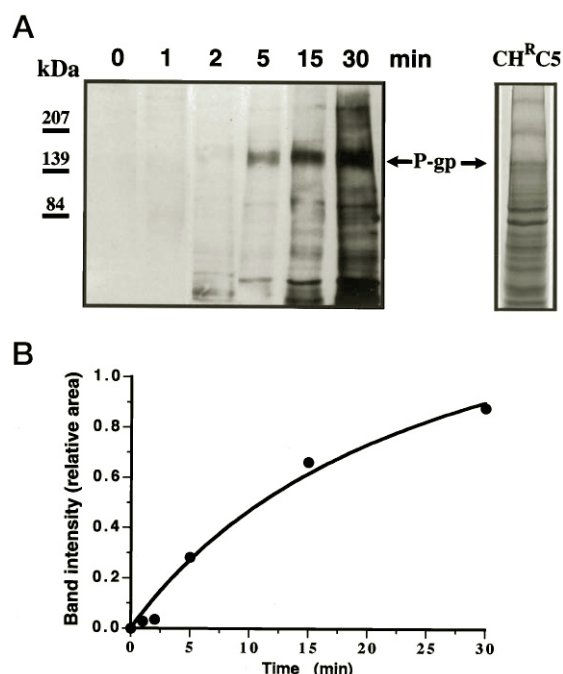


FIG. 3. **Photoaffinity labeling of CH^RC5 membrane proteins by diazirine-CsA as a function of irradiation time.** Membrane proteins (20 μ g) from CH^RC5 cells were incubated for 60 min at 25 $^{\circ}$ C with 20 nM diazirine-CsA SDZ 212–122 in 10 mM Tris/HCl, pH 7, and irradiated on ice. A, the CsA bound to CH^RC5 proteins was detected as described under “Experimental Procedures” using the mAb that recognizes the Cyp domain of CsA. B, the intensity of the band corresponding to P-gp (150–180 kDa) was measured with a laser densitometer. A representative experiment is shown ($n = 3$).

the photolabeling with diazirine-CsA analogs, photolabeling of CH^RC5 membranes was conducted in the presence of drugs that are known to interact with P-gp (Table I). A 50-fold molar excess of CsA, verapamil, and vinblastine reduced the photolabeling of the 155-kDa protein by 76, 50, and 64%, respectively. When proteins from the drug-sensitive parent cell line AuxB1 proteins were photolabeled with diazirine-CsA, only a small amount of the 155-kDa protein was detected compared with CH^RC5 proteins (Table I). CsA and PSC-833 inhibited the photolabeling of P-gp as a function of drug concentration (Fig. 6). PSC-833 was more efficient, since a 33 nM concentration of

the drug reduced the photolabeling by 50% compared with 82 nM for CsA, as estimated by densitometric analysis. These concentrations of PSC-833 and CsA correspond to 1.6- and 4.1-fold molar excesses over the diazirine-CsA. The stronger detection of the 155-kDa protein in membranes from CH^RC5 cells, compared with AuxB1 cells, and the inhibition of its photolabeling by these different drugs indicate that this protein is P-gp.

Competitive studies using different CsA analogs were also performed to determine whether photolabeling could be displaced by related molecules (Table II). At a 10-fold molar excess, PSC-833, a CsA derivative, inhibited the photolabeling of P-gp more strongly than CsA and cyclosporin D (CsD), which is the parent drug (not oxidized in position 1) of PSC-833. IAAP, another photoaffinity probe for P-gp (23–25), was also tested in the presence of the same analogs (Table II). Similar inhibition for CsA analogs was obtained when photolabeling was performed with IAAP or SDZ 212–122.

Inhibition of IAAP Photolabeling of P-gp by CsA Analogs—IAAP was used to further examine whether the CsA analogs SDZ 212–122 and SDZ 211–845 interact with P-gp. As shown in Fig. 7, A and B, CsA analogs caused a concentration-dependent inhibition of P-gp photolabeling by IAAP. However, a stronger inhibition was observed with SDZ 212–122. The concentrations required to cause a 50% inhibition of the IAAP photolabeling by these analogs was compared with that observed with CsA and PSC-833 at a 10-fold molar excess (Table II). SDZ 212–122, like PSC-833, caused a 90% inhibition, and SDZ 211–845 was even more effective than CsA in inhibiting the photolabeling of P-gp by IAAP.

DISCUSSION

Photoaffinity labeling is a useful approach for the characterization of P-gp in multidrug resistance. Photoaffinity probes such as IAAP and azidopine have allowed a partial topographical analysis of the protein binding sites for drugs (23–25). Analogs of vinblastine, colchicine, and verapamil were also used to photolabel P-gp and explore its function (for review, see Ref. 26). [³H]Cyclosporin diazirine (27) was used to show that CsA binds directly to P-gp, and uptake studies with [³H]CsA indicate that CsA is a substrate for P-gp (11).

In the present study, CH^RC5 proteins photolabeled with diazirine-CsA were immunodetected with a mAb that recognizes CsA and its metabolites (Fig. 2). A protein of 155 kDa was detected in CH^RC5 membranes but was virtually absent from AuxB1 membranes. The photolabeling of this protein was inhibited by verapamil and vinblastine, indicating that this protein is P-gp. In addition, this photolabeling was specific and did not result from a nonspecific binding of diazirine-CsA or from nonspecific interaction of the monoclonal antibody with CH^RC5 proteins, since there was no detection in the absence of UV light and diazirine-CsA.

We observed that CsA inhibited the photolabeling more efficiently than verapamil or vinblastine. This is in agreement with previous study on P-gp in brain capillaries, in which cyclosporin A was a better inhibitor than verapamil and vinblastine for P-gp photolabeling by IAAP (28). PSC-833 caused a stronger inhibition of the P-gp photolabeling by diazirine-CsA analogs than CsA and CsD, indicating that oxidation of position 1 of CsD increased its interaction with P-gp. These results are in accordance with previous reports showing that PSC-833 is 10 times more active than CsA, the latter being more active than verapamil as a reversal agent in cancer cells (10, 29). In the present study, photolabeling of P-gp by diazirine-CsA indi-

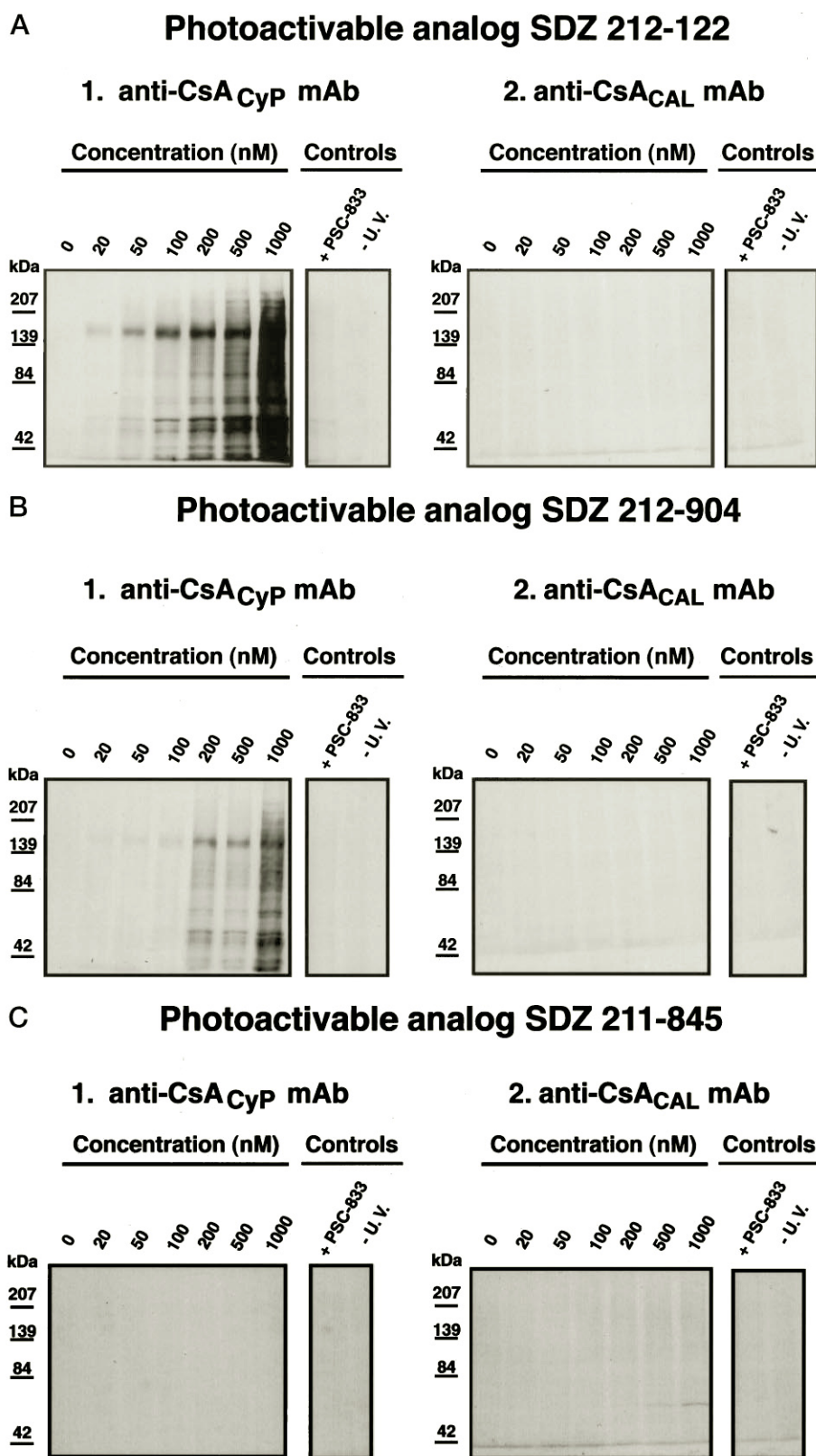


FIG. 4. Photoaffinity labeling of P-gp with CsA analogs. Membrane proteins (40 μ g) from CH^RC5 cells were incubated for 60 min at 25 °C with 20–1000 nM of the CsA analogs SDZ 212–122 (A), SDZ 212–904 (B), and SDZ 211–845 (C) and irradiated on ice for 10 min in 10 mM Tris/HCl, pH 7, and protease inhibitors. Western blots were then performed as described under “Experimental Procedures.” The two following controls were also performed. First, incubation with a 20 nM concentration of each photoactivable CsA analog was performed in the presence of 200 nM PSC-833. Second, the samples were not irradiated (–U.V.) after the incubation with 50 nM diazirine-CsA. The CsA covalently bound to P-gp was detected as described under “Experimental Procedures” with the anti-CsA_{CYP} mAb or the anti-CsA_{CAL} mAb directed against the calcineurin binding domain. ($n = 3$).

cated that PSC-833 is approximately 2.5 times more effective than CsA in displacing the photoactivable analog, confirming that this drug is one of the most potent chemosensitizers *in vitro*.

As indicated in Fig. 2, the two mAbs directed against CsA that we used could discriminate between the two major CsA domains, the calcineurin and the cyclophilin binding domains (19). Biospecific analysis with the BIAcore apparatus showed

that the diazirine-CsA analogs modified at position 8 (edge of both domains) are recognized by both antibodies, whereas the diazirine-CsA analog modified at position 3 (cyclophilin binding domain) is recognized only by the mAb that recognizes the calcineurin binding domain (data not shown). The mAb directed against the calcineurin binding domain does not react with the CsA-P-gp complex, suggesting that this domain is not available. The cyclophilin binding domain, on the other hand,

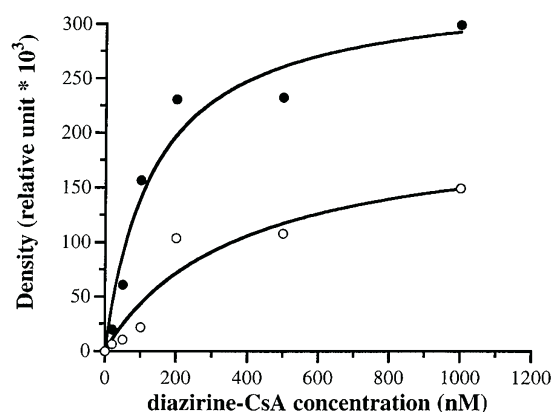


FIG. 5. Saturation of photoaffinity labeling of P-gp by CsA analogs. Autoradiograms from Fig. 4 were scanned. The density corresponding to P-gp was expressed as a function of photoactivable CsA analog SDZ 212-122 (●) and SDZ 212-904 (○) concentrations.

TABLE I
Diazirine-CsA photoaffinity labeling of P-gp

Membrane proteins (40 μ g) from CH^RC5 and AuxB1 cells were incubated for 60 min at 25 °C with 20 nM diazirine-CsA analog SDZ 212-122 in the presence of 1 μ M CsA, vinblastine, or verapamil and irradiated at 254 nm for 10 min at 4 °C. The covalently bound CsA analog was detected in Western blots with the mAb anti-CsA_{CYP}, and the autoradiograms were scanned with a laser densitometer. Values are expressed as the percentages of photolabeling and represent means \pm S.D. for four experiments.

Conditions	Photolabeling
	%
CH ^R C5	
Control	100 \pm 14
CsA	24 \pm 9
Verapamil	50 \pm 12
Vinblastine	36 \pm 18
AuxB1	5 \pm 5

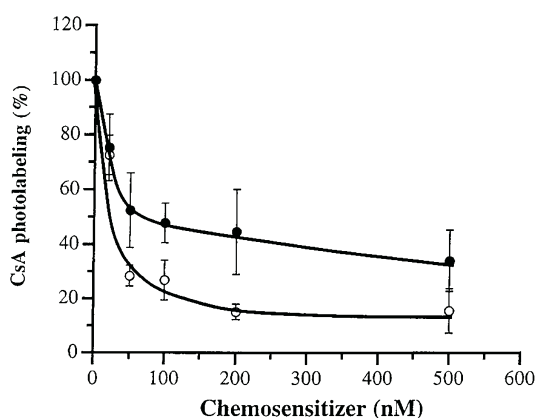


FIG. 6. Competition of diazirine-CsA photoaffinity labeling of P-gp with CsA and PSC-833. Membrane proteins (40 μ g) from CH^RC5 cells were incubated for 60 min at 25 °C with 20 nM diazirine-CsA analog SDZ 212-122 in the presence of 0–500 nM CsA (●) or PSC-833 (○) and irradiated at 254 nm for 10 min. Western blots were performed as described under “Experimental Procedures,” and the CsA bound to P-gp was detected with the anti-CsA_{CYP} mAb. The autoradiograms were scanned with a laser densitometer, and the band intensity obtained for the P-gp labeled was measured relative to control samples incubated without the competing compounds. The data represent the means \pm S.D. (bars) of three experiments.

appears to be exposed and detected by the other antibody. In addition, when the photolabeling was performed with the diazirine-CsA analog modified at position 3, located in the cyclophilin binding domain, the CsA-P-gp complex could not be

TABLE II
Diazirine-CsA photoaffinity labeling of P-gp in the presence of CsA analogs

Membrane proteins (40 μ g) from CH^RC5 cells were incubated for 60 min at 25 °C with 20 nM CsA analog SDZ 212-122 or 20 nM IAAP in the presence of 200 nM CsA analogs and irradiated as described under “Experimental Procedures.” The autoradiograms were scanned with a laser densitometer. Values were expressed as the percentages of photolabeling and represent means \pm S.D. for three experiments.

Analog	Photolabeling	
	diazirine-CsA	IAAP
	%	
CsA	53 \pm 15	56 \pm 2
PSC-833	27 \pm 12	12 \pm 2
CsD	72 \pm 18	80 \pm 3
SDZ 212-122	NA ^a	9 \pm 9
SDZ 211-845	NA	38 \pm 13

^a NA, not available.

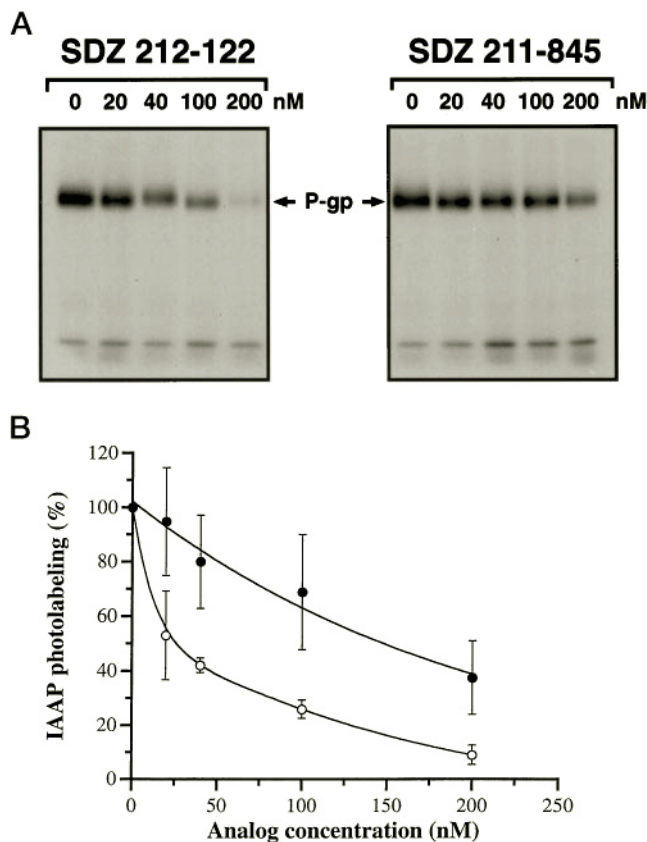


FIG. 7. Inhibition of IAAP photolabeling of P-gp by diazirine-CsA analogs. Membrane proteins (50 μ g) from CH^RC5 cells were incubated for 60 min at 25 °C with 20 nM IAAP in 10 mM Tris/HCl, pH 7, in the presence of protease inhibitors and increasing concentrations of the CsA analogs SDZ 212-122 or SDZ 211-845 and irradiated on ice for 5 min. A, P-gp was immunoprecipitated with the polyclonal antibody mdr (Ab-1), and the P-gp labeled by IAAP was detected as described under “Experimental Procedures.” B, the percentage of IAAP photolabeling of P-gp as a function of the SDZ 212-122 (○) and SDZ 211-845 (●) concentrations was measured by laser densitometry. The data represent the means \pm S.D. (bars) of three experiments.

detected with either antibody. However, both diazirine-analogs inhibited P-gp photolabeling by IAAP. The concentration of SDZ 212-122 needed to obtain a 50% inhibition of the IAAP photolabeling was 6-fold lower than that of SDZ 211-845. The efficiency of SDZ 212-122 and SDZ 211-845 was comparable to that of PSC-833 and CsA, respectively. Thus, the diazirine-CsA derivative modified at position 3 interacts with P-gp but could not be detected following photolabeling, indicating that the

domain detected by the anti-CsA_{CAL} antibody is not exposed or that this compound could not be cross-linked to P-gp after irradiation.

In the present work, we were able to detect P-gp labeled with CsA by Western blot analysis using a mAb directed against CsA. The NMR structure of CsA bound to cyclophilin showed that the side chains of residues 1–3 and 9–11 are in contact with cyclophilin (30). The results indicate that the portion of the CsA molecule that is important for binding to calcineurin is not accessible to the mAb following binding of the drug to P-gp. This suggests that the accessibility of the epitope to the antibody is limiting, because the calcineurin binding domain of CsA cross-links with P-gp after irradiation. In addition, the fact that the diazirine-CsA analogs modified in position 8 are covalently linked to P-gp after UV irradiation indicates that this position is also in close contact with P-gp. This position is a part of the other side of the CsA molecule available for calcineurin, which includes the surface formed by residues 4–8 and 9 and the tail of residue 1 (30, 31). However, PSC-833, which is modified at positions 1 and 2, showed a stronger inhibition of the photoaffinity labeling of P-gp than CsA, indicating that these two positions may be crucial for the interaction of CsA molecules with P-gp. The reason why PSC-833 is a better inhibitor than CsA could thus be due to its improved binding to P-gp caused by its modification in position 1, which modifies the orientation of this amino acid residue in the vicinity of the molecule, and its modification in position 2, which would allow a tighter interaction at its binding site. Our results suggest that a portion of the calcineurin may play a role in the interaction of CsA with P-gp, and that mAbs against different portions of the CsA peptide may be used to determine the exact amino acids implicated in the CsA interaction with P-gp.

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