Pergamon

Biochemical Pharmacology, Vol. 50, No. 10, pp. 1701–1709, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02073-X

INTERACTION OF DRUGS WITH P-GLYCOPROTEIN IN BRAIN CAPILLARIES

LUCIE JETTÉ,* GÉRARD F. MURPHY,† JEAN-MARIE LECLERC‡ and RICHARD BÉLIVEAU*§

*Laboratoire d'Oncologie Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, Montréal, Québec, Canada H3C 3P8; †Sandoz Canada, Dorval, Québec, Canada; and ‡Hôpital Ste-Justine, Montréal, Québec, Canada

(*Received* 21 *April* 1995; *accepted* 29 *June* 1995)

Abstract—P-glycoprotein (P-gp) is expressed at high levels in a variety of non-cancerous tissues such as the endothelial cells of the blood-brain barrier (BBB) capillaries. These thin capillaries tightly regulate the movement of substrates from the circulating blood into the brain. P-gp may be involved in the exclusion of various drugs from the capillary endothelial cells, blocking their entry into the brain. However, interactions of drugs with P-gp expressed in brain capillaries remain to be characterized. We have performed photoaffinity labeling studies using [^{125}I]arylazidoprazosin (IAAP) to evaluate the inhibitory efficiency of various compounds. Cyclosporin A (CsA) and its derivative PSC 833 (PSC) were the most effective inhibitors of IAAP binding among the drugs tested. The magnitude of inhibition was: PSC > CsA > quinidine > vinblastine > verapamil > actinomycin D > colchicine > reserpine > bilirubin > doxorubicin > progesterone. Cremophor El, the vehicle used to administer CsA and PSC intravenously, was also able to inhibit IAAP photolabeling of P-gp. Labeling experiments were also performed using a photoactivatable [^{3}H]CsA derivative. Photolabeling of P-gp with this compound was abolished almost completely by CsA and PSC. *In vivo* studies were also performed by treating rats with CsA [10 mg/(kg · day) for 10 days]. Following this treatment, no alteration in the level of P-gp expression in brain capillaries was observed. These results suggest that, at the proper dosage, administration of CsA to cancer patients could help to enhance the response of brain tumors to chemotherapeutic agents without modifying the intrinsic level of P-gp expression in this tissue.

Key words: P-glycoprotein; brain capillaries; cyclosporin A; PSC 833; photolabeling; cyclosporin treatment

Clinical resistance to chemotherapeutic agents is a major problem in the treatment of many cancers. One form of resistance, termed MDR[‖], is defined by the ability of cancer cells to become resistant to a variety of structurally unrelated cytostatic agents [1, 2]. This phenomenon is characterized by the overexpression of a glycoprotein, P-gp, at the surface of cancer cells [3]. P-gp is the product of the *mdr* genes and is believed to function as an energy-dependent drug efflux pump [4, 5].

P-gp is also expressed in non-cancerous tissues, such as the endothelial cells of the BBB capillaries [6–10]. The endothelial cells of the brain capillaries are responsible for the barrier properties of the BBB. These capillaries possess tight interendothelial cell junctions, sparse pinocytic vesicular transport and an absence of pores or fenestrations [11]. This barrier protects the brain from many exogenous toxins and sudden fluctuations in the levels of systemic substances. Hydrophobic antitumor agents, such as Vinca alkaloids and Adriamycin®, and lipid-soluble compounds, such as CsA, cannot accumulate in the brain [12, 13]. It has been proposed that P-gp expressed in the BBB capillaries is responsible for the extrusion of these compounds from the endothelial cells [7].

The class I isoform is the major form of P-gp expressed in brain capillaries [14, 15]. In immunohistochemical studies, P-gp has been shown to be expressed on the luminal membrane of those endothelial cells [7]. *In vitro* studies performed with cultured cells have demonstrated that P-gp of capillary endothelial cells transports vincristine and CsA [9, 16]. Recently, evidence of a direct implication of P-gp in the barrier functions of the BBB has been provided by generation of mice homozygous for a disruption of the class I isoform (mdr3) [14]. Injection of vinblastine to these mice lacking cellular expression of P-gp resulted in elevated drug levels in many tissues, especially in the brain. Thus, P-gp contributes to the barrier functions of the BBB by extruding compounds that are potentially toxic to the brain from the capillary endothelial cells.

The fact that P-gp plays such a role in the BBB capillaries also suggests that this protein is responsible for the lack of responsiveness of brain tumors to chemotherapy. Many studies have been performed in order to find compounds able to inhibit P-gp activity and restore sensitivity of cancerous cells to chemotherapeutic agents. The *in vitro* revertant activity of calcium-channel blockers, calmodulin inhibitors, antiarrhythmics, antimalarials, steroids, immunomodulators and antibiotics has been widely evaluated [17]. Among these compounds, CsA, an immunosuppressive cyclic undecapeptide of fungal origin, and its non-immunosuppressive analog, PSC, have been found to be very effective agents in reversing MDR [18, 19]. CrEl, which is the vehicle used

§ Corresponding author: Dr. Richard Béliveau, Laboratoire d'Oncologie Moléculaire, Département de Chimie, Université du Québec à Montréal, C.P. 8888, Succursale A, Montréal, Québec, Canada H3C 3P8. Tel. (514) 987-8551; FAX (514) 987-4054.

‖ Abbreviations: BBB, blood–brain barrier; CrEl, Cremophor El; CsA, cyclosporin A; IAAP, [^{125}I]iodoarylazidoprazosin; mAb, monoclonal antibody; MDR, multidrug resistance; PB, physiological buffer; P-gp, P-glycoprotein; PSC, PSC 833; and TBS, Tris-buffered saline.

to dissolve CsA and PSC, has also potential chemosensitizing properties [20, 21]. However, interaction of drugs with P-gp expressed in brain capillaries remains to be characterized.

In this study, we have performed photoaffinity labeling experiments using IAAP and a photoactivatable CsA derivative (SDZ-[^{3}H]-205-536) to characterize the interaction of various drugs with P-gp expressed in BBB capillaries. Among the drugs tested, CsA and PSC were the most effective inhibitors of IAAP and SDZ-[^{3}H]-205-536 photolabeling. We also treated rats with CsA [10 mg/(kg · day) for 10 days] to see if this compound could modulate the *in vivo* expression of P-gp in brain capillaries. Following this treatment, no alteration in the level of P-gp expression was observed. These results suggest that, at proper dosage, CsA and its non-immunosuppressive derivative, PSC, could be effective in reversing the chemoresistance of brain tumors, *in vivo*.

MATERIALS AND METHODS

Chemicals

Monoclonal antibody C219 (mAb C219) was purchased from ID Laboratories (London, Ontario). Horseradish peroxidase-conjugated rabbit anti-mouse antibody, enhanced chemiluminescence (ECL) reagents, and NCS tissue solubilizer were from Amersham (Oakville, Ontario). IAAP (2200 Ci/mmol) and En^{3}Hance were purchased from Dupont–New England Nuclear (Markham, Ontario). Cyclosporin A, PSC 833, and the photoactivatable CsA derivative SDZ-[^{3}H]-205-536 (19.6 μCi/μg) were provided by Sandoz Pharma Ltd. (Basel, Switzerland). All other reagents were from the Sigma Chemical Co. (St. Louis, MO).

Isolation of brain capillaries

Capillaries were isolated from Sprague–Dawley male rats. The brains were cleared of meninges, superficial large blood vessels and choroid plexus. The cerebral cortex was homogenized in 5 vol. of PB composed of 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, 15 mM HEPES–Tris, pH 7.4, with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario). The homogenates were mixed with an equal volume of 31% (w/v) Dextran T-70 in PB. Brain capillaries were then purified according to the procedure of Dallaire *et al.* [22]. The final pellets containing isolated microvessels were resuspended in PB and stored in liquid nitrogen until used. γ-Glutamyltranspeptidase activity was assayed using a slight modification of the method described by Orlowski and Meister [23] to evaluate enrichment factors of each preparation. Protein content was determined in all experiments using the method of Bradford [24].

Detection of P-gp

P-gp was detected by western blot analysis. SDS–PAGE was performed according to the method of Laemmli [25]. Brain capillaries were resuspended in sample buffer to a final protein concentration of 1 mg/mL and loaded on 6.0 or 7.5% acrylamide/bis-acrylamide (29.1: 0.9) gels, without prior heating. The proteins were transferred electrophoretically to 0.45-μm pore size Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked overnight at 4°, with 5% (w/v) nonfat powdered milk in TBS containing 50 mM Tris–HCl, 150 mM NaCl, 0.3% (w/v) Tween-20, pH 7.0. The membranes, washed three times with TBS, were incubated with the mAb C219 (200 ng/mL) for 2 hr at 37°. The antibody was diluted in TBS containing 1% (w/v) BSA. Horseradish peroxidase-conjugated rabbit anti-mouse antibody was used as secondary antibody. Detection was made with ECL reagents according to the manufacturer's instructions. The blots were exposed to preflashed Fuji films.

Photoaffinity labeling with IAAP

Capillary proteins (50 μg) were incubated with 20 nM IAAP in a reaction buffer containing 20 mM Tris–HCl, pH 8.0, and proteinase inhibitors (2 μg/mL aprotinin, 10 μg/mL pepstatin A, and 100 μg/mL bacitracin). The incubation was carried out for 1 hr at 25° in the dark and followed by cross-linking under a Spectroline UV lamp (Fisher Scientific, Montréal, Québec) at 254 nm for 5 min at 4°. The unincorporated radioactive prazosin analogue was removed by centrifugation (8000 *g*, 10 min). The labeled P-gp was recovered by immunoprecipitation with mAb C219. Immunoprecipitation was carried out for 16 hr at 4° in 0.25 mL of Buffer A (50 mM Tris–HCl, 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) Triton X-100, 0.5% (w/v) deoxycholate, pH 7.4). Immune complexes were isolated by incubation for 2 hr at 25° with protein A-Sepharose beads, followed by two washes in Buffer A, one wash in buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.01% (w/v) SDS, 0.1% (w/v) Triton X-100, 0.05% (w/v) deoxycholate, pH 7.4, and two final washes in buffer containing 50 mM Tris–HCl, 150 mM NaCl, pH 7.4. The beads were then resuspended in Laemmli sample buffer and electrophoresed on a 6.0% SDS–polyacrylamide gel. The gels were exposed to preflashed Kodak films with an intensifying screen (Picker, Montréal, Québec) at −80° for 2–4 weeks.

Photoaffinity labeling with a photoactivatable CsA derivative

Capillary proteins (100 μg) were incubated with the photoactivatable CsA derivative SDZ-[^{3}H]-205-536 (0.6 μCi) in a reaction buffer containing 20 mM Tris–HCl, pH 7.4. The incubation was carried out for 1 hr at 25° in the dark and followed by cross-linking under a UV lamp at 254 nm for 10 min at 4°. The unincorporated radioactive CsA analogue was removed by centrifugation (8000 *g*, 10 min). The pellets were resuspended in 20 mM Tris–HCl, pH 7.4. Laemmli sample buffer was then added, without boiling the samples. The proteins were separated using a 6.0 or 7.5% polyacrylamide gel. In some experiments, P-gp was immunoprecipitated with mAb C219 using the protocol described for the IAAP photolabeling experiments. The gels were stained with Coomassie Brilliant blue, sliced in 2-mm strips, and placed in individual vials. The strips were then treated with NCS tissue solubilizer, and the radioactivity was measured by liquid scintillation counting.

Cyclosporin treatments

Sprague–Dawley rats weighing 300–350 g were treated daily with s.c. injections of CsA. CsA was used in PB with 15% (w/v) CrEl and 3% (w/v) ethanol. Two different control groups were used in the experiments: the first group (Ctl) received only PB and the second group (CrEl) received the CsA vehicle (PB, CrEl and ethanol). Rats were injected with PB, CrEl or CsA [10

mg/(kg · day)] for 10 days. The rats were decapitated 24 hr after the last injection, and capillaries were purified from the brain cortex. Whole blood concentrations of CsA were determined using a fluorescence polarization monoclonal antibody assay (Abbott Laboratories, Abbott Park, IL).

Densitometric and statistical analyses

The absorbance of the bands obtained from western blot analysis and from photolabeling studies were evaluated with an LKB Ultrascan XL densitometer. Molecular mass determination was performed using the following standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Statistical analyses were made with a paired Student's *t*-test, using the StatView program and a Macintosh computer.

RESULTS

Inhibition of IAAP photolabeling with various drugs

Capillary proteins were incubated with increasing concentrations of IAAP and cross-linked under UV light. P-gp was immunoprecipitated with mAb C219. A protein with a molecular mass of 155 kDa corresponding to P-gp was photolabeled with IAAP (Fig. 1). The amount of photolabeled P-gp was directly proportional to the amount of IAAP used between 5 and 30 nM. According to these results, an IAAP concentration of 20 nM was chosen for drug inhibition studies since photolabeled P-gps could be well visualized when exposing the gels to preflashed Kodak films for 2–3 weeks.

Several agents were tested for their ability to inhibit IAAP photolabeling of P-gp (Fig. 2). Photolabeling was performed in the presence of a 100-fold molar excess of various compounds without preincubation of the brain capillaries with the drugs. At this concentration, progesterone, doxorubicin and bilirubin inhibited poorly the labeling of P-gp. Verapamil, vinblastine, quinidine, CsA and PSC were the most effective inhibitors of the photolabeling of P-gp. The efficiency of each agent is summarized in Table 1.

Since CsA and PSC were the most effective agents tested, the inhibition of IAAP labeling was further characterized by performing photoaffinity labeling experiments in the presence of increasing concentrations of these drugs (Fig. 3A and 4A). The concentrations of CsA and PSC that inhibited IAAP photolabeling of P-gp by 50% (IC_{50}) were 26.9- and 4.3-fold molar excess, respectively, as determined by densitometric analysis of the autoradiograms (Fig. 3B and 4B).

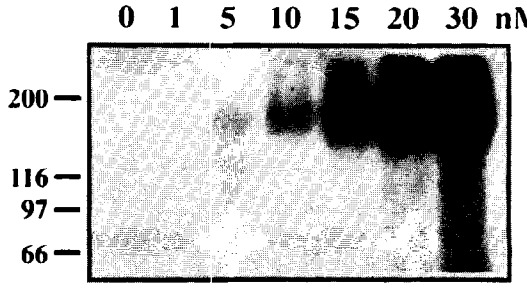

Fig. 1. Photoaffinity labeling of P-gp as a function of IAAP concentration. Protein samples (50 μg) from brain capillaries were incubated in the presence of 0, 1, 5, 10, 15, 20, and 30 nM IAAP for 60 min at 25° and irradiated at 254 nm for 5 min with a UV light. P-glycoprotein was immunoprecipitated with mAb C219, and the gels were exposed to preflashed Kodak films. (N = 2.)

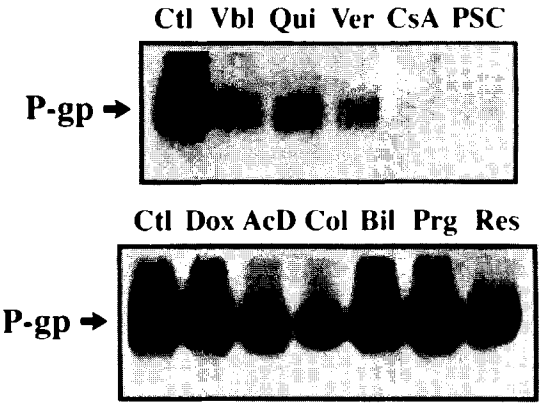

Fig. 2. Photoaffinity labeling of P-gp in the presence of various drugs. Protein samples (50 μg) from brain capillaries were incubated with IAAP (20 nM) and cross-linked with UV light. P-gp was immunoprecipitated with mAb C219, and the gels were exposed to Kodak films. Photolabeling was performed in the presence of 2% (w/v) ethanol (Ctl), which was the vehicle used to dissolve the drugs, or in the presence of a 100-fold molar excess of vinblastine (Vbl), quinidine (Qui), verapamil (Ver), cyclosporin A (CsA), PSC 833 (PSC), doxorubicin (Dox), actinomycin D (AcD), colchicine (Col), bilirubin (Bil), progesterone (Prg) and reserpine (Res). These inhibition studies were performed without preincubation of the brain capillaries with the drugs. (N = 2.)

Inhibition of IAAP photolabeling by CrEl

Inhibition studies were also performed with CrEl, the vehicle that is widely used clinically to administer CsA intravenously. A concentration of 0.001% (w/v) of CrEl

Table 1. Effects of various drugs on IAAP photoaffinity labeling of P-gp expressed in brain capillaries

Agent	IAAP photoaffinity labeling of P-gp (% of control)
Control	100
Progesterone	78.4 ± 3.1
Doxorubicin	75.8 ± 10.7
Bilirubin	69.6 ± 12.7
Reserpine	59.6 ± 5.3
Colchicine	56.5 ± 10.0
Actinomycin D	56.2 ± 1.5
Verapamil	32.7 ± 5.9
Vinblastine	32.0 ± 3.0
Quinidine	30.0 ± 2.8
Cyclosporin A	18.9 ± 5.5
PSC 833	14.3 ± 6.7

The photolabeling of P-gp with IAAP was performed in the presence of 100-fold molar excess of various drugs, as described in Materials and Methods. The absorbance of the photolabeled protein bands was estimated with an LKB Ultroscan XL densitometer. The relative area of the bands is expressed as a percentage of the total amount of the photolabeled protein detected in the absence of drugs. Values represent means ± SD for two experiments.

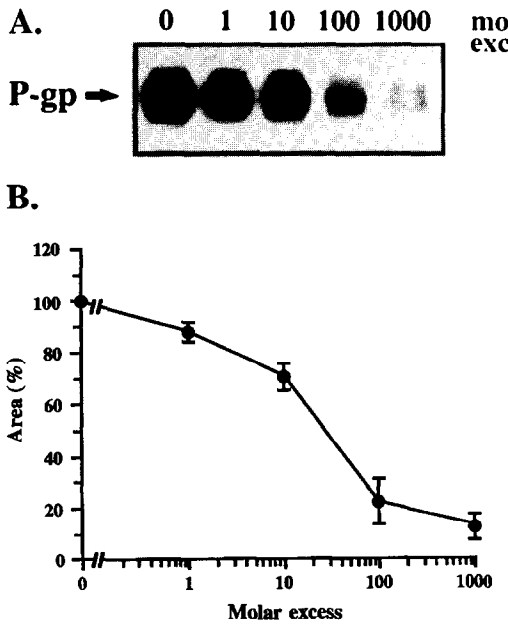

Fig. 3. Inhibition of IAAP labeling with CsA. (A) Protein samples (50 μg) from brain capillaries were incubated with IAAP (20 nM) and cross-linked with UV light. P-gp was immunoprecipitated with mAb C219, and the gels were exposed to Kodak films. Photolabeling was performed in the presence of 2% (w/v) ethanol (0), or 1-, 10-, 100-, and 1000-fold molar excess of CsA. These inhibition studies were performed without preincubation of the brain capillaries with the drugs. (B) The absorbance of the photolabeled protein bands was estimated with an LKB Ultroscan XL densitometer. The relative area of the bands is expressed as a percentage of the total amount of the photolabeled protein detected in the absence of drug. Values represent means ± SD for two experiments.

tively, as determined by densitometric analysis of the autoradiograms (Fig. 3B and 4B).

Inhibition of IAAP photolabeling by CrEl

Inhibition studies were also performed with CrEl, the vehicle that is widely used clinically to administer CsA intravenously. A concentration of 0.001% (w/v) of CrEl inhibited IAAP photolabeling of P-gp by more than 50% (Fig. 5A). This inhibition was not due to UV light absorption by CrEl since, at the lowest concentration where inhibition occurred, there was no significant absorbance by CrEl. Almost no UV light absorption could be measured at a CrEl concentration ranging from 0.0001 to 0.01% (w/v) (Fig. 5B).

Photolabeling of P-gp with the [^{3}H]CsA analog

Labeling experiments were also performed on brain capillaries using the photoactivatable CsA derivative SDZ-[^{3}H]-205-536. Capillary proteins were photolabeled with the [^{3}H]CsA analog and cross-linked under UV light, and P-gp was immunoprecipitated with mAb C219. The immune complexes were separated by gel electrophoresis, the gels were sliced, and the radioactivity was measured. This CsA derivative photolabeled a protein of 155 kDa from the brain capillaries, and this labeling was abolished almost totally in the presence of a 100-fold molar excess of vinblastine, quinidine, CsA or PSC (Fig. 6).

CsA chronic treatment

Interaction of CsA with P-gp expressed in brain capillaries was investigated further by treating rats with daily s.c. injections of CsA [10 mg/(kg · day)] for 10 days. Trough CsA blood concentration, measured 24 hr after the last injection, was 1927 ± 240 μg/L. Control rats were injected with PB or with the vehicle CrEl (ethanol 3% and CrEl 15% in PB) for the same period of time as the CsA-treated rats. Capillaries were isolated from cerebral cortex 24 hr after the last injection. γ-Glutamyltranspeptidase activity was measured in the homogenate and the isolated brain capillaries to evaluate the purity of the preparations. The enrichment of γ-glutamyltranspeptidase activity in the brain capillaries pre-

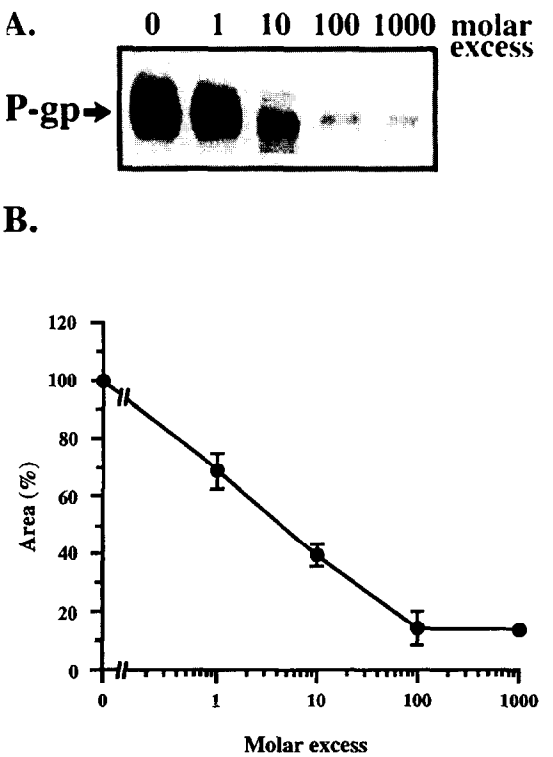

Fig. 4. Inhibition of IAAP labeling with PSC. (A) Protein samples (50 μg) from brain capillaries were incubated with IAAP (20 nM) and cross-linked with UV light. P-gp was immunoprecipitated with mAb C219, and the gels were exposed to Kodak films. Photolabeling was performed in the presence of 2% (w/v) ethanol (0), or 1-, 10-, 100-, and 1000-fold molar excess of PSC. These inhibition studies were performed without preincubation of the brain capillaries with the drug. (B) The absorbance of the photolabeled protein bands was estimated with an LKB Ultroscan XL densitometer. The relative area of the bands is expressed as a percentage of the total amount of the photolabeled protein detected in the absence of drug. Values represent means ± SD for two experiments.

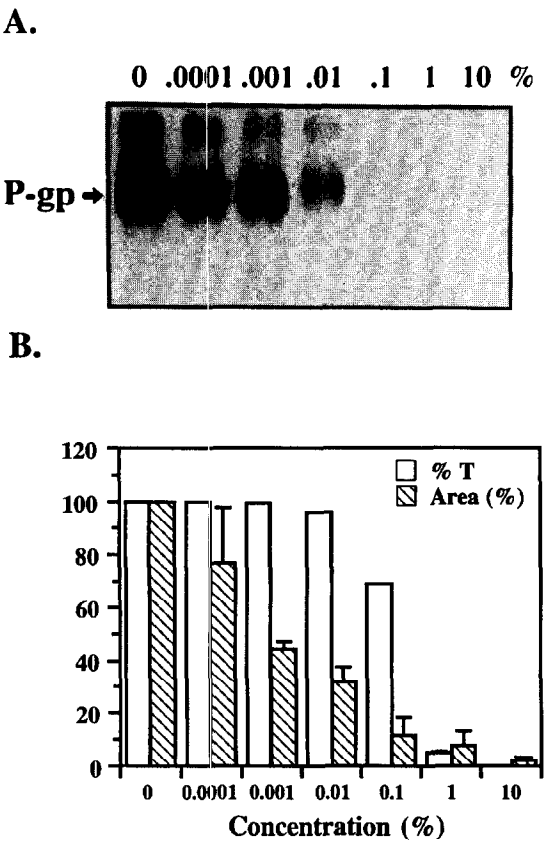

Fig. 5. Inhibition of IAAP labeling with CrEl. (A) Protein samples (50 μg) from brain capillaries were incubated with IAAP (20 nM) and cross-linked with UV light. P-gp was immunoprecipitated with mAb C219, and the gels were exposed to Kodak films. Photolabeling was performed in the presence of 0, 0.0001, 0.001, 0.01, 0.1, 1, and 10% (w/v) CrEl. These inhibition studies were performed without preincubation of the brain capillaries with the CrEl. (B) The absorbance of the photolabeled protein bands was estimated with an LKB Ultroscan XL densitometer. The relative area of the bands is expressed as a percentage of the total amount of the photolabeled protein detected in the absence of CrEl (▨). The absorbance of CrEl under UV light (254 nm) was measured with a spectrophotometer to verify if the inhibition of IAAP labeling observed was due to intrinsic UV light absorption of CrEl (□). Values represent means ± SD for two experiments.

pared from the PB-, CrEl- and CsA-treated rats was 34.2 ± 4.9, 28.5 ± 5.9 and 30.9 ± 8.6, respectively.

P-gp expression in brain capillaries isolated from the different groups was evaluated by western blot analysis. Capillary proteins were solubilized with 1% (w/v) Triton X-100 and 0.5% (w/v) deoxycholate and centrifuged, and the supernatant was used for immunoblot analysis. This solubilization step was performed because mAb C219 reacts with two proteins of the brain capillaries; P-gp and an unrelated 190-kDa protein [15, 26]. In contrast with P-gp, the 190-kDa protein is not solubilized with these detergents. Following this procedure, mAb C219 detected a protein of 155 kDa in capillaries isolated from all groups (Fig. 7A). The absorbances of the immunoreactive bands were evaluated with a laser densitometer. A correction was applied to the amount of P-gp detected in brain capillaries isolated from each group, in order to compare the levels of expression directly. This was done by dividing the scanned area of the autoradiograms by the enrichment factor of each preparation. The groups treated with PB were considered as the control groups, and levels of P-gp were expressed as a percentage of those obtained for this control. P-gp expression levels were not significantly different ($P > 0.14$) in capillaries isolated from groups treated with PB, CrEl or CsA (Fig. 7B).

Photoaffinity labeling studies using IAAP or the photoactivatable CsA derivative SDZ-[^{3}H]-205-536 were also performed on brain capillaries isolated from each group (Fig. 8). The amount of photolabeled P-gp in the CsA-treated groups was not significantly higher than the amount of photolabeled P-gp detected in the PB and the CrEl groups.

DISCUSSION

It has been shown that mice lacking cellular expression of P-gp accumulate elevated levels of vinblastine in many tissues, especially the brain, demonstrating the involvement of P-gp in the barrier functions of the BBB [14]. Finding compounds able to inhibit this drug efflux pump would help to establish new chemotherapeutic approaches for the treatment of brain tumors. We have used IAAP, a photoaffinity probe previously shown to label P-gp overexpressed in MDR cell lines [27], to study the interaction of drugs with P-gp expressed in BBB capillaries. Photolabeling of P-gp with IAAP was inhibited by various drugs. At a 100-fold molar excess, the efficiency of these compounds was in the order: PSC > CsA > quinidine > vinblastine > verapamil > actinomycin D > colchicine > reserpine > bilirubin > doxorubicin > progesterone.

The weak inhibition obtained with progesterone and doxorubicin, at the concentration tested, is consistent with results obtained in cancer cells where a 10,000-fold molar excess of progesterone and doxorubicin was needed to produce 67 and 69% inhibition of P-gp photolabeling with IAAP, respectively [27]. Progesterone has been shown to have a higher affinity for P-gp class II isoform than for the class I isoform [28]. Since we have shown that class I is the dominant isoform expressed in brain capillaries [15], this may explain the lack of inhibition by progesterone reported here. Doxorubicin has been reported to inhibit poorly photolabeling of P-gp by another probe, [^{3}H]azidopine [29], a finding that is consistent with our results.

Reserpine, colchicine and actinomycin D inhibited moderately the IAAP photolabeling of P-gp expressed in brain capillaries. These results are in agreement with IAAP photolabeling inhibition studies performed with vincristine-resistant human neuroblastoma cells [30]. Verapamil, vinblastine and quinidine inhibited photolabeling of P-gp with good efficiency. Vinblastine has been shown to be transported by P-gp and to inhibit with high efficiency the IAAP photolabeling of P-gp [27, 31]. Verapamil and quinidine are compounds known to interact with P-gp and reverse MDR [17]. Although these compounds are very potent inhibitors of IAAP photolabeling, CsA and PSC showed an even higher efficiency. PSC was at least 6 times more potent than CsA in inhibiting IAAP photolabeling of P-gp. PSC is a non-im-

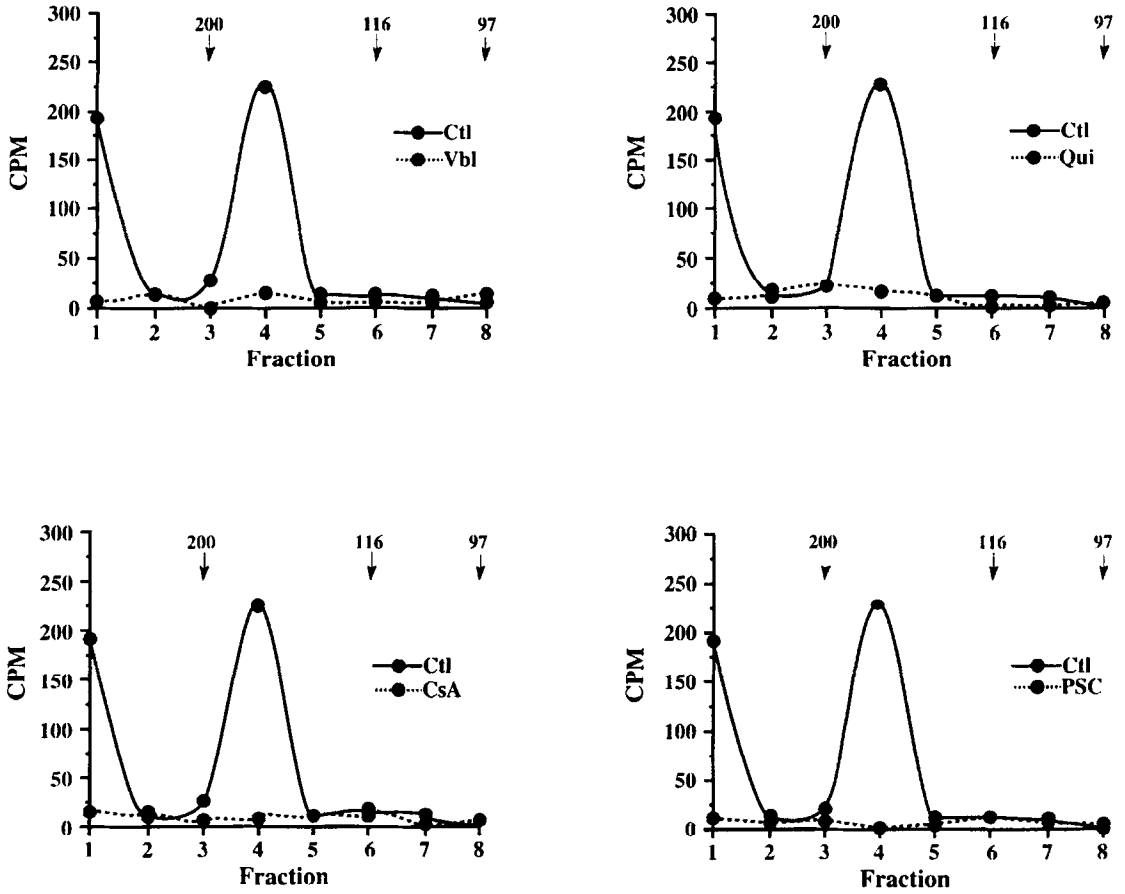

Fig. 6. Photolabeling of P-gp with the [^{3}H]CsA analog. Protein samples (100 μg) from brain capillaries were incubated in the presence of a 0.84 μM concentration of the photoactivatable CsA derivative SDZ-[^{3}H]-205-536 (0.6 μCi), and cross-linked with UV light. P-gp was immunoprecipitated using mAb C219. The immunoprecipitated complexes were separated by SDS–PAGE using a 7.5% polyacrylamide gel. The gels were sliced in 2-mm strips, and each fraction was placed in an individual vial. The strips were then treated with NCS tissue solubilizer, and the radioactivity was measured by liquid scintillation counting. Labeling was performed in the presence of 2% (w/v) ethanol (Ctl) or 100-fold molar excess of vinblastine (Vbl), quinidine (Qui), cyclosporin A (CsA) or PSC 833 (PSC). (N = 2.) The size of the molecular weight standards is indicated by arrows.

munosuppressive analog of CsA and has been reported to be up to 20-fold more potent than CsA in reversing MDR in cancer cells [32–34]. Our results support the assertion that PSC is one of the most potent agents tested so far to inhibit P-gp *in vitro*.

The potency of CrEl as an inhibitor of IAAP photolabeling of P-gp has also been tested since this compound has been shown to reverse the MDR phenotype in cancer cells [20, 21]. CrEl is a nonionic surfactant containing polyethoxylated castor oil. It is used to solubilize many hydrophobic drugs, including CsA and PSC. At low concentrations (0.001 to 0.1%), CrEl inhibited IAAP photolabeling of P-gp with very good efficiency. CrEl, at a similar concentration, has been reported to enhance the sensitivity of tumor bearing mice to Adriamycin [21]. These results suggest that CrEl used as a carrier for CsA or PSC in clinical trials has the potential to enhance the reversal of MDR mediated by these agents.

The interaction of CsA with P-gp expressed in brain capillaries was further investigated by treating rats with CsA for 10 days. The s.c. route was chosen since it provides reproducible and steady CsA blood levels with little variation over a 24-hr period in rats [35]. Following this treatment, neither CsA nor CrEl altered significantly the level of P-gp expression in brain capillaries. Although CsA is highly lipophilic, the accumulation of this compound into the cerebral compartment is extremely low [13, 36]. It has been suggested that the active efflux of CsA by P-gp present in the luminal membranes of brain capillaries is responsible for the low penetration of CsA into the brain [16]. We have show that CsA directly interacts with P-gp expressed in this tissue since this protein was photolabeled with the [^{3}H]CsA derivative. This labeling was inhibited by CsA, PSC, vinblastine, and quinidine. However, chronic administration of CsA to rats did not alter the intrinsic level of P-gp expressed in brain capillaries even though the CsA blood levels reached were adequate for MDR modulation [37].

In vitro studies on the modulation of P-gp expression by CsA have yielded contradictory results. An increase in P-gp expression in human colon carcinoma cell lines following treatment with verapamil, nifedipine, and CsA has been reported [38]. However, no modulation of P-gp expression was observed with sublines of the EMT6 mouse tumor cell line grown in the presence of various

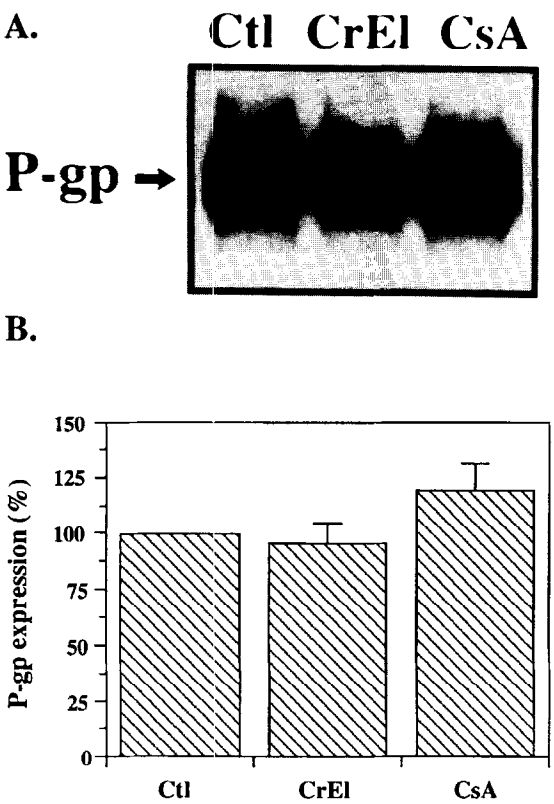

Fig. 7. Immunodetection of P-gp in brain capillaries following CsA chronic treatment. Rats were treated with daily s.c. injections of PB (Ctl), CrEl or 10 mg/(kg · day) of CsA for 10 days. Rats were killed 24 hr after the last injection, and brain capillaries were isolated from the three different rat groups. (A) Brain capillaries were solubilized with 1% (w/v) Triton X-100 and 0.5% deoxycholate for 30 min and centrifuged at 8000 *g* for 10 min. Protein samples (10 μg) from the supernatant were resolved by SDS–PAGE using a 6% polyacrylamide gel and immunoblots were performed using mAb C219, as described under Materials and Methods. (B) The immunoreactive bands were analyzed with a laser densitometer and the relative area is expressed as a percentage of the total amount of the immunoreactive protein present in the capillaries isolated from the PB-treated rats (Ctl). Values were corrected for the enrichment factor of each preparation and represent means ± SEM for four different treatments.

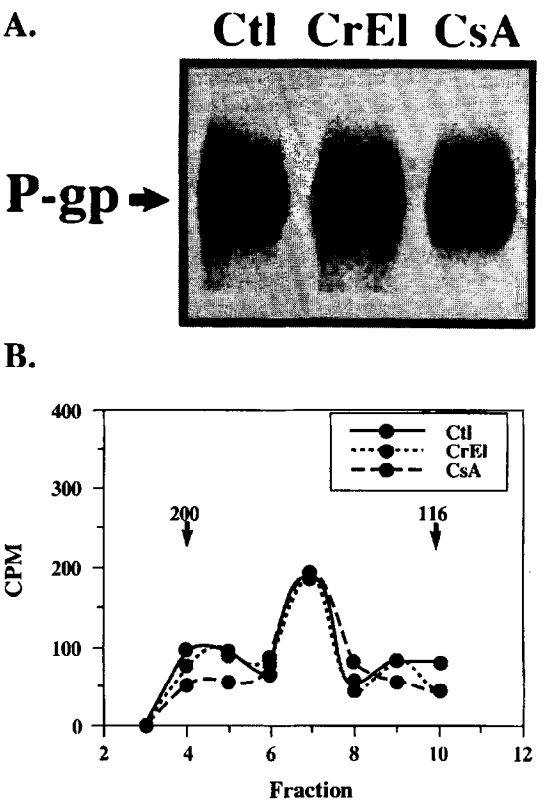

Fig. 8. Photolabeling of P-gp following CsA chronic treatment. Rats were treated with daily s.c. injections of PB (Ctl), CrEl or 10 mg/(kg · day) of CsA for 10 days. Rats were killed 24 hr after the last injection, and brain capillaries were isolated from the three different rat groups. (A) Protein samples (50 μg) from brain capillaries were incubated with IAAP (20 nM), and cross-linked with UV light. P-gp was immunoprecipitated with mAb C219, and the gels were exposed to Kodak films. Photolabeling was performed on brain capillaries isolated from 3 rat groups, for 4 different treatments. (B) Protein samples (30 μg) from brain capillaries were incubated with the photoactivatable CsA derivative SDZ-[^{3}H]-205-536 (0.6 μCi), cross-linked with UV light, and separated by SDS–PAGE using a 6.0% polyacrylamide gel. The gels were stained with Coomassie Brilliant blue and sliced in 2-mm strips. The strips were treated with NCS tissue solubilizer, and the radioactivity was measured by liquid scintillation counting. Data are means of a representative experiment performed in triplicate. The size of the molecular weight standards is indicated by arrows.

concentrations of CsA [39]. These different results may be explained by the CsA dosage used, the intrinsic level of P-gp expression in different cell lines, or physiological and morphological differences between tissues and cell lines. These factors have been shown to influence the relative sensitization of resistant cell lines *in vitro* [40]. This is an important issue since increased levels of P-gp expression following administration of an MDR reversing agent could alter the efficiency of therapeutic regimens. The lack of induction of P-gp expression in brain capillaries suggest that CsA has the potential to enhance the accumulation of chemotherapeutic agents into the cerebral compartment and may be useful in patients with brain tumors.

In conclusion, CsA and PSC were, among several agents tested, the most effective inhibitors of photolabeling of P-gp expressed in brain capillaries. Chronic treatment of rats with CsA did not alter the level of P-gp expression in BBB capillaries. These results suggest that, at the proper dosage, administration of CsA or PSC to patients with brain tumors could help to enhance the response of their tumors to chemotherapeutic agents.

Acknowledgements—The enthusiastic support of Prof. Ben Sulsky is gratefully acknowledged. We thank Dr. R. W. Yatscoff, Health Science Centre, University of Alberta, for measurement of CsA blood levels, and Dr. Michel Demeule and Dr. Vincent Vachon for their critical reading of the manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and by Sandoz Canada Inc. L. J. is a recipient of a fellowship from the Medical Research Council of Canada.

REFERENCES

1. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155–199, 1990.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
3. Kartner N, Riordan JR and Ling V, Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science* **221:** 1285–1288, 1983.
4. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J and Ling V, Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316:** 817–819, 1985.
5. Doige CA, Yu X and Sharom FJ, ATPase activity of partially purified P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. *Biochim Biophys Acta* **1109:** 149–160, 1992.
6. Jetté L, Têtu B and Béliveau R, High levels of P-glycoprotein detected in isolated brain capillaries. *Biochim Biophys Acta* **1150:** 147–154, 1993.
7. Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR, Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* **86:** 695–698, 1989.
8. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC, Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: Evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. *J Histochem Cytochem* **37:** 159–164, 1989.
9. Tatsuta T, Naito M, Oh-hara T, Sugawara I and Tsuruo T, Functional involvement of P-glycoprotein in blood-brain barrier. *J Biol Chem* **267:** 20383–20391, 1992.
10. Hegmann EJ, Bauer HC and Kerbel RS, Expression and functional activity of P-glycoprotein in cultured cerebral capillary endothelial cells. *Cancer Res* **52:** 6969–6975, 1992.
11. Goldstein GW and Betz AL, The blood-brain barrier. *Sci Am* **255:** 74–83, 1986.
12. Levin VA, Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *J Med Chem.* **23:** 682–684, 1980.
13. Cefalu WT and Pardridge WM, Restrictive transport of a lipid-soluble peptide (cyclosporin) through the blood-brain barrier. *J Neurochem* **45:** 1954–1956, 1985.
14. Schinkel AH, Smit JJM, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CAAM, van der Valk MA, Robanus-Maandag EC, te Riele HPJ, Berns AJM and Borst P, Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:** 491–502, 1994.
15. Jetté L, Pouliot J-F, Murphy GF and Béliveau R, Isoform I (mdr3) is the major form of P-glycoprotein expressed in mouse brain capillaries. Evidence for cross-reactivity of antibody C219 with an unrelated protein. *Biochem J* **305:** 761–766, 1995.
16. Tsuji A, Tamai I, Sakata A, Tenda Y and Terasaki T, Restricted transport of cyclosporin A across the blood–brain barrier by a multidrug transporter, P-glycoprotein. *Biochem Pharmacol* **46:** 1096–1099, 1993.
17. Lum BL, Gosland MP, Kaubisch S and Sikic BI, Molecular targets in oncology: Implications of the multidrug resistance gene. *Pharmacotherapy* **13:** 88–109, 1993.
18. Twentyman PR, Cyclosporins as drug resistance modifiers. *Biochem Pharmacol* **43:** 109–117, 1992.
19. Boesch D, Gavériaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P and Loor F, *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res* **51:** 4226–4233, 1991.
20. Schuurhuis GJ, Broxterman HJ, Pinedo HM, van Heijningen THM, van Kalken CK, Vermorken JB, Spoelstra EC and Lankelma J, The polyoxyethylene castor oil Cremophor EL modifies multidrug resistance. *Br J Cancer* **62:** 591–594, 1990.
21. Woodcock DM, Linsenmeyer ME, Chojnowski G, Kriegler AB, Nink V, Webster LK and Sawyer WH, Reversal of multidrug resistance by surfactants. *Br J Cancer* **66:** 62–68, 1992.
22. Dallaire L, Tremblay L and Béliveau R, Purification and characterization of metabolically active capillaries of the blood–brain barrier. *Biochem J* **276:** 745–752, 1991.
23. Orlowski M and Meister A, γ-Glutamyl-*p*-nitroanilide: A new convenient substrate for determination and study of L- and D-γ-glutamyltranspeptidase activities. *Biochim Biophys Acta* **73:** 679–681, 1963.
24. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
25. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
26. Beaulieu E, Demeule M, Pouliot J-F, Averill-Bates DA, Murphy GF and Béliveau R, P-glycoprotein of blood barrier: Cross-reactivity of MAb C219 with a 190 kDa protein in bovine and rat isolated brain capillaries. *Biochim Biophys Acta* **1233:** 27–32, 1995.
27. Greenberger LM, Yang C-PH, Gindin E and Horwitz SB, Photoaffinity probes for the α1-adrenergic receptor and the calcium channel bind to a common domain in P-glycoprotein. *J Biol Chem* **265:** 4394–4401, 1990.
28. Yang C-PH, Cohen D, Greenberger LM, Hsu SI-H and Horwitz SB, Differential transport properties of two *mdr* gene products are distinguished by progesterone. *J Biol Chem* **265:** 10282–10288, 1990.
29. Friche E, Demant EJF, Sehested M and Nissen NI, Effect of anthracycline analogs on photolabeling of P-glycoprotein by [^{125}I]iodomycin and [^{3}H]azidopine: Relation to lipophilicity and inhibition of daunorubicin transport in multidrug resistant cells. *Br J Cancer* **67:** 266–231, 1993.
30. Safa AR, Agresti M, Byrk D and Tamai I, *N*-(*p*-Azido-3-[^{125}I]iodophenethyl)spiperone binds to specific regions of P-glycoprotein and another multidrug binding protein, spiperophilin, in human neuroblastoma cells. *Biochemistry* **33:** 256–265, 1994.
31. Horio M, Gottesman MM and Pastan I, ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc Natl Acad Sci USA* **85:** 3580–3584, 1988.
32. Keller RP, Altermatt HJ, Nooter K, Poschmann G, Laissue JA, Bollinger P and Hiestand PC, SDZ PSC 833, a non-immunosuppressive cyclosporine: Its potency in overcoming P-glycoprotein-mediated multidrug resistance of murine leukemia. *Int J Cancer* **50:** 593–597, 1992.
33. Twentyman PR and Bleehen NM, Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin A. *Eur J Cancer* **27:** 1639–1642, 1991.
34. te Boekhorst PAW, van Kapel J, Schoester M and Sonneveld P, Reversal of typical multidrug resistance by cyclosporin and its non-immunosuppressive analogue SDZ PSC 833 in Chinese hamster ovary cells expressing the *mdr*1 phenotype. *Cancer Chemother Pharmacol* **30:** 238–242, 1992.
35. Wassef R, Cohen Z and Langer B, Pharmacokinetic profiles of cyclosporine in rats. *Transplantation* **40:** 489–493, 1985.
36. Begley DJ, Squires LK, Zlokovic BV, Mitrovic DM, Hughes CCW, Revest PA and Greenwood J, Permeability

of the blood–brain barrier to the immunosuppressive cyclic peptide cyclosporin A. *J Neurochem* **55:** 1222–1230, 1990.

37. Lum BL, Fisher GA, Brophy NA, Yahanda AM, Adler KM, Kaubisch S, Halsey J and Sikic BI, Clinical trials of modulation of multidrug resistance. *Cancer* **72:** 3502–3514, 1993.
38. Herzog CE, Tsokos M, Bates SE and Fojo AT, Increased *mdr*-1/P-glycoprotein expression after treatment of human colon carcinoma cells with P-glycoprotein antagonists. *J Biol Chem* **268:** 2946–2952, 1993.
39. Wright KA and Twentyman PR, Derivation and characterization of a mouse tumour cell line with acquired resistance to cyclosporin A. *Eur J Cancer* **29A:** 389–394, 1993.
40. Twentyman PR, Reeve JG, Koch G and Wright KA, Chemosensitisation by verapamil and cyclosporin A in mouse tumour cells expressing different levels of P-glycoprotein and CP22 (sorcin). *Br J Cancer* **62:** 89–95, 1990.