Inhibition of P-Glycoprotein: Rapid Assessment of Its Implication in Blood–Brain Barrier Integrity and Drug Transport to the Brain by an *In Vitro* Model of the Blood–Brain Barrier

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Purpose. The objective of this work was to assess, *in vitro*, the passage of P-glycoprotein dependent drugs across brain capillary endothelial cells, when these drugs are associated with a reversing agent.

Methods. An *in vitro* model of the blood–brain barrier consisting of a coculture of brain capillary endothelial cells and astrocytes was used.

Results. We demonstrate that P-glycoprotein expression is upregulated by the presence of astrocytes. Uptake in the cells and transport across endothelial cell monolayers of vincristine, cyclosporin A and doxorubicin were studied. Using S9788 or verapamil as reversing agents, we found an increase in vincristine transport across the endothelial cell monolayers. On the other hand, the association of S9788 or verapamil with cyclosporin A failed to increase the transport of this drug. An increase in the transport of doxorubicin from luminal to abluminal compartment was also observed, due to endothelial cell monolayer breakdown.

Conclusions. Using this model, it is possible to predict the passage of a P-glycoprotein dependent drug to the brain or its sequestration in brain capillary endothelial cells when this drug is associated with a reversing agent, or its toxicity on the blood–brain barrier integrity.

KEY WORDS: brain capillary; endothelial cells; coculture; P-glycoprotein reversing agents; anticancer drugs; cyclosporin A.

INTRODUCTION

The blood–brain barrier is one of the main factors of chemotherapy failure in central nervous system tumors (1). Indeed the movement of compounds from the circulating blood to the extracellular space of the brain is strictly regulated by the brain capillary endothelial cells which are connected to each other by continuous tight junctions and constitute this blood-brain barrier (BBB). Nutrients needed for brain cells are selectively transported from the blood to the brain by means of specific receptors or transporters. Transcellular passage of drugs across the BBB is usually believed to be dependent on lipid solubility and molecular size. The immunomodulator cyclosporin A was expected to cross the BBB freely because of its cyclic structure with an internal hydrogen-bond that confers a highly lipid-soluble character, but cyclosporin A cannot enter the brain (2,3). In the same way, the passage across the BBB of anticancer drugs such as vincristine (*Vinca* alkaloid) and doxorubicin (Anthracyclin) which are highly lipophilic is unexpectedly low (4).

Cordon-Cardo *et al.* (5) showed that brain capillary endothelial cells (ECs) express P-glycoprotein (P-gp). These low permeabilities could then be explained by the occurrence of this specific transmembrane protein at the luminal face of the BBB. It has been verified that cyclosporin A (6), vincristine (7,8) and doxorubicin (9) are actively pumped out of brain capillary ECs by P-gp which is localized at the luminal side of the BBB. This results in a decreased accumulation within the ECs, explaining their low permeability through the BBB.

By searching for potential substrates and reversal agents of P-gp activity, we may be able to improve drug delivery to the brain, which could be important for the treatment of brain cancer. Indeed in the brain, contrary to the neovasculature of other primary tumors, P-gp is expressed in the ECs of the newly formed capillaries of low grade gliomas (10), impeding anticancer drug access to tumor cells that are located around the capillaries and do not express P-gp (11). *In vivo* studies have shown that when rats were pretreated with verapamil, colchicine and vinblastine uptake in the rat brain was substantially enhanced (12). In the same way, PSC 833, a P-gp inhibitor has been shown to increase cyclosporin A and vincristine penetration in rat brain (13). *In vitro*, verapamil is able to inhibit efflux of vincristine (7,8) and cyclosporin A (6) from brain capillary ECs. An uptake of the drugs by brain capillary ECs was demonstrated in these *in vitro* experiments, but no information about the potential transport of these drugs to the brain parenchyma was given.

To investigate various aspects of BBB functions, including transport of compounds, we have developed an *in vitro* BBB model consisting of a coculture of brain capillary ECs and astrocytes (14,15). As a strong correlation was demonstrated for a large number of molecules between the permeability coefficient of ECs obtained *in vitro* and the brain uptake index obtained *in vivo* (16,17), this model could be considered as an useful system to predict the transport of drugs to the brain.

In this study, we compare P-gp expression in brain capillary ECs in solo culture or in coculture with astrocytes. Vincristine, doxorubicin and cyclosporin A show, *in vitro* as *in vivo*, a very low BBB permeability. To investigate whether P-gp modulator agents may increase the transport of P-gp dependent drugs, a multidrug resistance reversal agent S9788, a triazino-amino piperidine derivative, was used in this study. The effects of this compound that has never been tested at the BBB level, were compared with those of verapamil a widely used inhibitor of vincristine and cyclosporin A efflux, *in vitro*.

Using these two P-gp reversing agents, we have shown that the *in vitro* model can be used to predict the passage of a

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ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; EC(s), endothelial cell(s); Pe, permeability coefficient of endothelial cells; PeS, endothelial cell permeability surface area product; PfS, filter permeability surface area product; PtS, total permeability (filter and endothelial cells) surface area product.

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P-gp dependent drug to the brain, or its sequestration in brain capillary ECs.

MATERIALS AND METHODS

Chemicals and Antibodies

[U-^{14}C]-sucrose (677 mCi/mmol), [^{3}H]-inuline (1,02 Ci/mmol), [G-^{3}H]-vincristine (6 Ci/mmol), [^{14}C]-doxorubicin hydrochloride (60 mCi/mmol) and enhanced chemiluminescence reagents were obtained from Amersham Laboratories (Les Ulis, France). [^{3}H]-cyclosporin (15,5 mCi/mg) was a gift from Novartis (Basel, Switzerland). S9788 was synthetized at the Servier Research Institute (Courbevoie, France). Verapamil hydrochloride was obtained from Sigma (St. Quentin Fallavier, France). The C219 monoclonal antibody was purchased from Cis bio Industries (Gif/Yvette, France).

Cell Culture

Bovine Brain Capillary Endothelial Cells

Endothelial cells were isolated and characterized as described by Méresse *et al.* (18). The use of cloned ECs allowed us to obtain a pure EC population without contamination by pericytes. The cells were cultured in the presence of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated calf serum and 10% (v/v) horse serum (Hyclone Laboratories, Logan), 2 mM glutamine, 50 μg/ml gentamycin and basic fibroblast growth factor (bFGF, 1 ng/ml, added every other day).

Rat Astrocytes

Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex. After removing the meninges, the brain tissue was forced gently through a nylon sieve. Astrocytes were plated on 6 multiwell dishes (Nunclon) at a concentration of 1.2×10^5 cells/ml in 2 ml of DMEM supplemented with 10% (v/v) fetal calf serum (Hyclone Laboratories, Logan) and the medium was changed twice a week. Three weeks after seeding, cultures of astrocytes were stabilized and used for coculture. Astrocytes were characterized with glial fibrillary acidic protein (GFAP), more than 95% of the astrocyte population was GFAP positive (14).

Preparation of Filters for Coculture

Culture plate inserts (Falcon, 3 μm, 30 mm diameter, from Falcon) were coated on the upper side with 150 μl of a 1.5 mg/ml rat tail collagen solution containing 10 fold concentrated DMEM plus NaOH 0.3 M. The coating dried for one hour at 37°C and is rinced twice with water and once with PBS-CMF, before putting it in complete medium.

Experimental Method for Coculture

Cultures of astrocytes were prepared as described above. After three weeks, coated filters were set in 6 multiwell dishes containing astrocytes. Endothelial cells were plated on their upper side in 1.5 ml of medium at a concentration of 4×10^5 cells/ml. The coculture medium is the same as for the brain capillary EC medium. Under these conditions, ECs formed a confluent monolayer after 7 days. Experiments were performed 5 days after confluence.

Detection of P-Glycoprotein by Western Blot Analysis

Brain capillaries were isolated as described by Méresse *et al.* (18). The freshly isolated capillaries suspended in phosphate buffered saline (PBS) were homogenized 1 min with a polytron and stored at −180°C. Brain capillary ECs on filters cultured with or without astrocytes for 12 days, were washed twice with PBS and recovered by gentle scraping and resuspended in PBS. They were then, homogenized 1 min with a polytron and stored at −80°C.

On the day of the experiment, brain capillaries and cells were suspended in Laemmli sample buffer containing 5% β-mercaptoethanol. The samples were agitated 30 min at 25°C. Proteins (25 μg in both cases) were then resolved on polyacrylamide gel electrophoresis (3–19% gradient), electrotransferred to nitrocellulose and incubated with anti P-gp (C219) at a concentration of 200 ng/ml. Immunoreactivity was detected by indirect immunoperoxidase method and enhanced chemiluminescence detection (Amersham Laboratories).

Transendothelial Transport Studies

On the day of the experiment, buffered Ringer solution was added to the lower compartments of a six well plate (2 ml per well). One insert containing a confluent monolayer of ECs was transferred to the first well of the six-well plate containing buffered Ringer solution. Indeed, the arrangement of the coculture readily permits the use of different cell types, which were separated easily after coculture by removing the insert. Then, 2 ml of buffered Ringer solution containing [^{14}C]-labeled or [^{3}H]-labeled test compound, were placed, at time zero, in the upper compartment. The incubations were performed at 37°C on a rocking platform. At time 10, 15, 20, 30, and 45 min after time 0, the insert was transferred to another well to minimize the possible passage from the lower to the upper compartment. Triplicate inserts coated with collagen, seeded or not with ECs, were incubated for 12 days with astrocytes and used for each drug. At the end of the experiments, the monolayers were washed twice with 2 ml ice-cold buffered Ringer solution and the radioactivity in the cells was counted, after scraping the cells. Then, amounts of the radiotracers in the lower compartment and in the cells were measured in a liquid scintillation counter (Wallac 14110, Pharmacia). The endothelial permeability coefficient (Pe in cm/min) was calculated as previously described (16). During all experiments, the integrity of the brain capillary EC monolayers was checked using [^{14}C]-sucrose or [^{3}H]-inulin as a tracer. In the case of coincubation experiments, the ratio of [^{14}C]/[^{3}H] was 1:10.

Effects of S9788 and Verapamil as Reversing Agents

Transendothelial transport studies were performed as previously described. First, the integrity of EC monolayers incubated with or without S9788 was checked using [^{14}C]-sucrose as a tracer. Then, transendothelial transport of radiolabeled drug in the absence or presence of reversing agents was carried out. Cells were incubated with [^{3}H]-cyclosporin A or [^{3}H]-vincristine (50 nM) for 45 min at 37°C with or without

1 μM S9788 or 25 μM verapamil. [^{14}C]-doxorubicin (420 nM) was associated with or without 1 μM S9788. Transendothelial transport was measured by counting the radioactivity in the lower compartments. Results were expressed as a percentage compared to the control (which was not incubated with the reversing agent). After 45 min transport experiment, cells were washed twice with ice-cold buffered Ringer solution and scraped gently to measure cell uptake. The radioactivity in the cells was determined as described above. These steps were performed quickly in order to prevent any drug efflux. The amount of intracellular drugs was expressed as a percentage compared to the control. Triplicate cocultures were assayed for each compound. During all experiments, the integrity of the EC monolayers was checked using [^{14}C]-sucrose or [^{3}H]-inulin as a tracer.

RESULTS

P-glycoprotein Detection in Cultured Brain Capillary ECs

The expression of P-gp in bovine brain capillary ECs cultured alone or cocultured with rat astrocytes was examined using Western blotting. Since C219 monoclonal antibody cross-reacts with human, rat and bovine isolated capillaries (19), an enriched preparation of capillaries isolated from bovine brain was used as a positive control. A protein with a molecular mass of 175 kDa was detected in isolated brain capillaries and in brain capillary ECs cultured alone or with astrocytes (Fig. 1). In each case (ECs alone or cultured with astrocytes), the amount of P-gp was maintained through several passages. No P-gp expression was detected in aortic ECs, which were used as a negative control (data not shown). As 25 μg of proteins were loaded for both ECs cultured alone or in the presence of astrocytes, a large increase in P-gp expression was apparent in ECs cultured with astrocytes (Fig. 1B).

These results indicate that cultured brain capillary ECs express P-gp, as *in vivo*. Coculture with astrocytes increases the expression of P-gp in ECs.

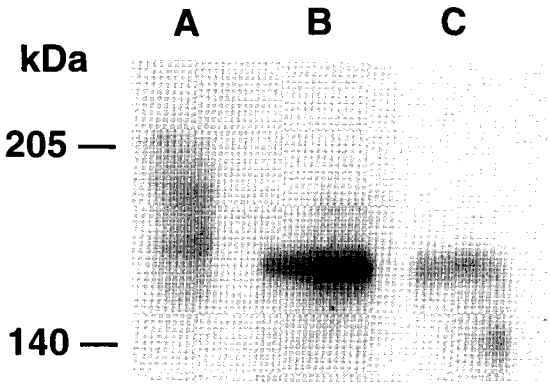

Fig. 1. Immunodetection of P-glycoprotein on Western blots. 25 μg of solubilized proteins from isolated brain capillaries (A), brain capillary ECs cocultured with astrocytes (B), brain capillary ECs in solo culture onto filters coated with collagen (C), were loaded per well.

Transendothelial Transport of Vincristine, Cyclosporin A and Doxorubicin Across Brain Capillary EC Monolayers

Apical-to-basal transport of vincristine, cyclosporin A and doxorubicin was measured across EC monolayers. A typical transport assay using triplicate inserts with brain capillary ECs is shown for doxorubicin in Fig. 2. The permeability coefficients of ECs were for doxorubicin, $Pe_{doxorubicin} = 0.72 \times 10^{-3}$ cm/min; vincristine, $Pe_{vincristine} = 2.41 \times 10^{-3}$ cm/min; and cyclosporin A, $Pe_{cyclosporin\ A} = 3.70 \times 10^{-3}$ cm/min.

After each 45 min transport experiment, intracellular accumulations of drugs were measured and expressed as a percentage of the drug quantity placed at the luminal face: 0.15% of vincristine, 0.87% of doxorubicin, and 0.60% of cyclosporin A were found in the cells.

Since doxorubicin, vincristine and cyclosporin A are very lipophilic and use the transcellular pathway, their action on the paracellular pathway (possible opening of the tight junctions) was checked. Coincubation of these drugs with [^{14}C]-sucrose or with [^{3}H]-inulin were performed. Indeed, sucrose and inulin diffuse very slowly across the BBB in physiological conditions *in vitro* as well as *in vivo* (16,17), and are used as indicators of the functional integrity of tight junctions. The permeability coefficient of ECs for sucrose or inulin when associated with the different drugs was not significantly different from the one obtained for sucrose or inulin alone (Table I). These results show that the integrity of EC monolayers is maintained during the transport experiment and that drug concentrations used for these experiments are not toxic for the cells.

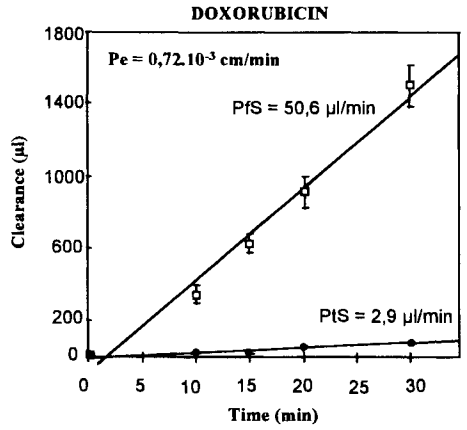

Fig. 2. Apical-to-basal transport of doxorubicin across brain capillary EC monolayers. Confluent monolayers of ECs and filters coated with collagen were incubated with 420 nM doxorubicin for 30 min at 37°C. Transports were measured by counting the amount of doxorubicin in the basal compartments. To obtain a concentration-independent transport parameter the clearance principle was used. Clearance for inserts covered with collagen and EC monolayers (●) and clearance for inserts coated with collagen (□) were plotted versus time. The slopes of the clearance curves for the cultured inserts were PtS (where PS is permeability × surface area product), the slopes of the clearance curves for the control filter were PfS. Permeability of EC monolayers (PeS) for each drug was calculated from: $1/PeS = 1/PtS - 1/PfS$ PeS values were divided by the surface area of the insert to generate the permeability coefficient of ECs (Pe). Each point is a mean of three different filters, and the curves are representative of 4 series of independent experiments.

Table I. Integrity Assessment of Endothelial Cell Monolayers in Contact with Drugs

[a]Pe for [^{14}C] sucrose ($\times 10^{-3}$ cm/min)	
	With [^{3}H]-vincristine 0.31 ± 0.06
Without drug 0.29 ± 0.03	
	With [^{3}H]-cyclosporin A 0.30 ± 0.02
[a]Pe for [^{3}H] inulin ($\times 10^{-3}$ cm/min)	
Without drug 0.11 ± 0.01	With [^{14}C]-doxorubicin 0.12 ± 0.02

[a] Pe: Permeability coefficient of EC monolayers.

Effect of S9788 as a Reversing Agent of P-glycoprotein: Integrity of EC Monolayers Incubated with S9788

To measure the reversal activity of S9788, cells were incubated with vincristine or cyclosporin A or doxorubicin either alone or with 1 μM S9788. The concentration of 1 μM was chosen as experimental data showed that this concentration is achievable in clinical situations. This concentration has already been used for *in vitro* experiments (20).

1 μM S9788 was not cytotoxic for cultured brain capillary ECs. Indeed, permeability coefficients for [^{14}C]-sucrose measured on ECs incubated in HEPES-buffered Ringer solution containing or not 1 μM S9788 were not significantly different ($Pe_{sucrose} = 0.51 \pm 0.09 \times 10^{-3}$ cm/min without S9788; $Pe_{sucrose} = 0.52 \pm 0.08 \times 10^{-3}$ cm/min with S9788), showing that the integrity of the monolayers was preserved when cells were in contact with 1 μM S9788.

Cellular Accumulation of Vincristine, Cyclosporin A and Doxorubicin

Cellular accumulation of vincristine was carried out with ECs in HEPES-buffered Ringer solution containing vincristine alone or vincristine plus 1 μM S9788. As shown in Fig. 3, a 45 min incubation of ECs with vincristine in association with S9788, resulted in a four-fold increase in vincristine uptake by the cells compared to incubation with vincristine alone. The same four-fold increase was observed with cyclosporin A associated with S9788. However, when the same experiment was performed with doxorubicin, a decrease in the uptake of doxorubicin was observed when doxorubicin was associated with S9788.

Transendothelial Transport of Vincristine, Cyclosporin A and Doxorubicin Across Brain Capillary EC Monolayers

When 1 μM S9788 was added to vincristine, transendo thelial transport of vincristine increased 3-fold (Fig. 4), but there was no increase in the transport of cyclosporin A across the EC monolayers when cells were incubated with both cyclosporin A and 1 μM S9788, compared to cyclosporin A alone. Transport of doxorubicin increased 2-fold in presence of S9788.

As S9788 failed to increase the cellular accumulation of doxorubicin after 45 min experiment, but allowed an increase in the transport of doxorubicin, a toxic effect was suspected. Since endothelial permeability coefficients for sucrose and inulin were not affected in the presence of doxorubicin alone or in the presence of S9788 alone, we wondered whether the association of these two drugs could disturb the monolayer integrity.

Transport assays using brain capillary EC monolayers were compared for [^{14}C]-doxorubicin+[^{3}H]-inulin and for [^{14}C]-doxorubicin+unlabeled 1 μM S9788 + [^{3}H]-inulin. As shown in Fig. 5, when clearance was plotted versus time, the slope of the clearance curve was linear up to 45 min for doxorubicin and inulin in the absence of S9788. But, when S9788 was associated to doxorubicin and inulin, a dramatic increase in the permeability of the monolayers was observed after 30 min for both doxorubicin and inulin, corresponding to a 2-fold increase in doxorubicin transport (Fig. 5) This increase was similar to the one observed when doxorubicin and S9788 were associated for a 45 min experiment (Fig. 4). The increase for inulin showed that after a 30 min experiment, a disruption of the monolayer integrity occurred.

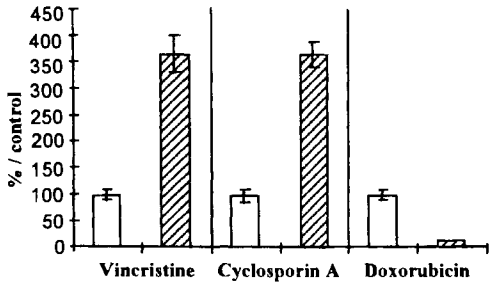

Fig. 3. Effect of S9788 on intracellular accumulation of vincristine, cyclosporin A and doxorubicin. EC monolayers were incubated with 50 nM vincristine or 50 nM cyclosporin A or 420 nM doxorubicin for 45 min at 37°C in absence (open column) or in presence (hatched column) of 1 μM S9788. After washing with ice-cold HEPES-buffered Ringer solution, the amount of vincristine, cyclosporin A and doxorubicin incorporated into the cells was determined. Results were expressed as a percentage compared to the control (in absence of S9788). Each point is a mean of three different filters, and the histograms are representative of 4 series of independent experiments.

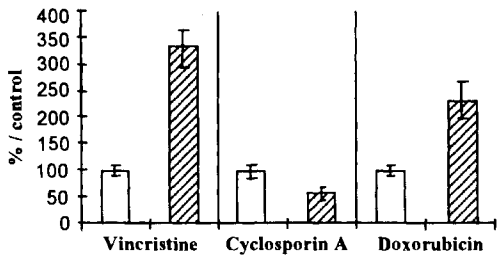

Fig. 4. Effect of S9788 on apical-to-basal transport of vincristine, cyclosporin A and doxorubicin. Confluent EC monolayers were incubated with 50 nM vincristine or 50 nM cyclosporin A or 420 nM doxorubicin for 45 min at 37°C in absence (open column) or in presence (hatched column) of 1 μM S9788. Transendothelial transports were measured by counting the amount of vincristine, cyclosporin A and doxorubicin in the basal compartments. Results were expressed as a percentage compared to the control (in absence of S9788). Each point is a mean of three different filters, and the histograms are representative of 4 series of independent experiments.

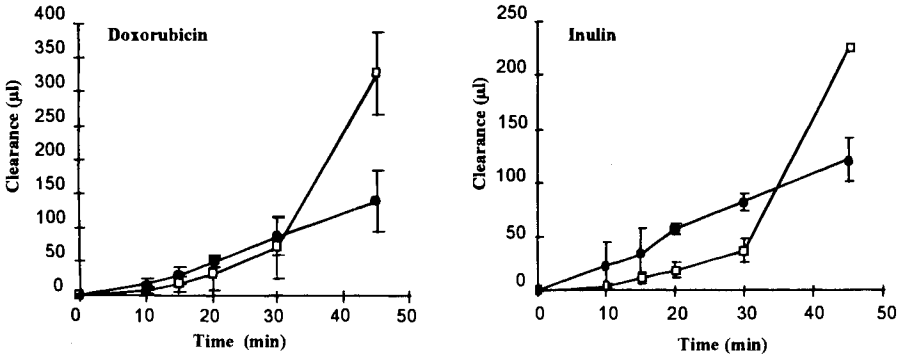

Fig. 5. Effect of association of doxorubicin+S9788 on the integrity of the EC monolayer. Transport assays using brain capillary EC monolayers were compared for doxorubicin + inulin (•) chosen as indicator of the functional integrity of tight junctions and for doxorubicin + S9788 + inulin (□). Clearances of doxorubicin and inulin were plotted versus time in the two conditions. Each point is a mean of three different filters, and the curves are representative of 4 series of independent experiments.

When clearance versus time was plotted for [^{3}H]-vincristine+unlabeled 1 µM S9788+[^{14}C]-sucrose, the slope was linear up to 45 min, allowing the calculation of the permeability coefficient of ECs for sucrose in presence of vincristine and S9788. The values ($Pe_{sucrose}$ = 0.39 ± 0.05 × 10^{-3} cm/min with vincristine+S9788) were in the same range as those obtained for sucrose associated with vincristine alone ($Pe_{sucrose}$ = 0.31 ± 0.06 × 10^{-3} cm/min). These results suggest that in this case, the 3-fold increase in vincristine transport in the presence of S9788 is not due to a breakdown of the EC monolayer.

Comparison with Verapamil

As verapamil is one of the most frequently used P-gp inhibitors, the effects of S9788 and verapamil on vincristine or cyclosporin A accumulation and transport were compared. As previously observed, a 4-fold increase in vincristine uptake and transport occurred when ECs were incubated with vincristine+S9788 (Fig. 6). The efficiency of 25 µM verapamil was not significantly different from the efficiency of 1 µM S9788 for vincristine accumulation and transport. Indeed a 3.5-fold increase in vincristine uptake and a 4.5-fold increase in vincristine transport were observed with verapamil instead of S9788. Lower concentrations than 25 µM verapamil were not as efficient as 1 µM S9788 (data not shown).

A 2.5-fold increase in cyclosporin A uptake was observed with 25 µM verapamil compared to a 3.5-fold increase with 1 µM S9788. However, a slight increase in the transport of cyclosporin A occurred with 25 µM verapamil (1.3-fold) whereas, as previously shown, S9788 did not improve the passage of cyclosporin A to the brain compartment (Fig. 7).

DISCUSSION

In previous investigations using the coculture system, it has been shown that, as *in vivo*, cyclosporin A cannot cross the *in vitro* BBB (17). It was suggested that this low permeability in the brain, despite its lipophilicity, was caused by the presence of P-gp that actively transports cyclosporin A to the outside of the ECs. It has already been shown that P-gp is expressed in

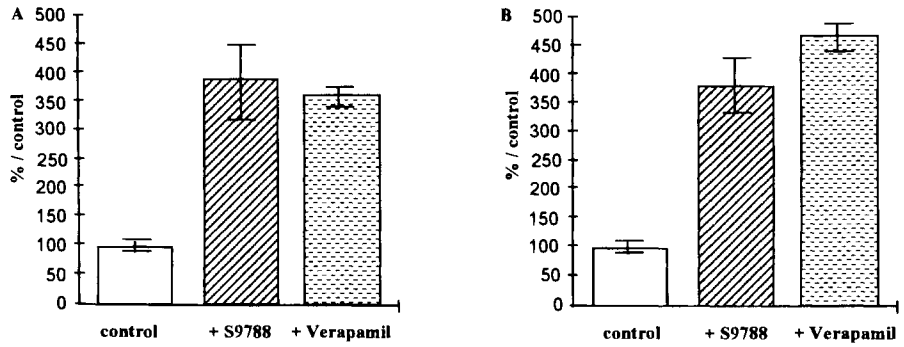

Fig. 6. Comparison of effects of verapamil and S9788 on intracellular accumulation (A) and transport (B) of vincristine. Confluent monolayers of ECs were incubated for 45 min at 37°C with 50 nM vincristine in absence (control) or in presence of 1 µM S9788 (+ S9788) or 25 µM verapamil (+ verapamil). Transendothelial transports were measured by counting the amount of vincristine in the basal compartment. After washing with ice-cold HEPES-buffered Ringer solution, the amount of vincristine incorporated into the cells was determined. Results were expressed as a percentage compared to the control. Each point is a mean of three different filters, and the histograms are representative of 3 series of independent experiments.

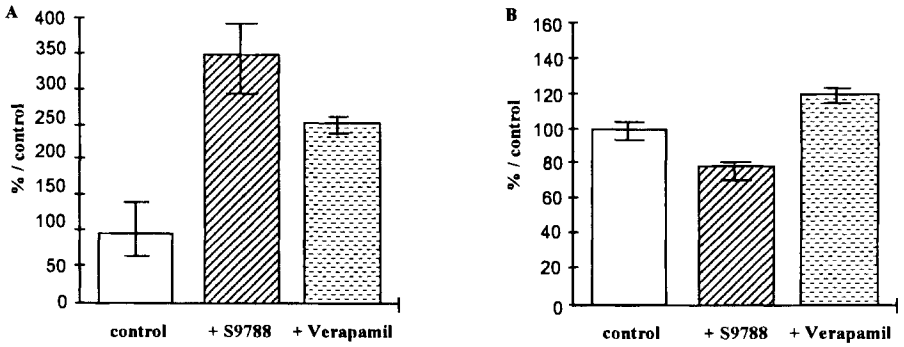

Fig. 7. Comparison of the effects of verapamil and S9788 on intracellular accumulation (A) and transport (B) of cyclosporin A. Confluent EC monolayers were incubated for 45 min at 37°C with 50 nM cyclosporin A in absence (control) or in presence of 1 μM S9788 (+ S9788) or 25 μM verapamil (+ verapamil). Transendothelial transports were measured by counting the amount of cyclosporin A in the basal compartment. After washing with ice-cold HEPES-buffered Ringer solution, the amount of cyclosporin A incorporation into the cells was determined. Results were expressed as a percentage compared to the control. Each point is a mean of three different filters, and the histograms are representative of 3 series of independent experiments.

solo culture of brain capillary ECs from bovine (7) and in the coculture system (21). In our experiments, low levels are present in ECs cultured in solo compared to the cocultured ECs. It is well known that BBB environment, such as the presence of glial cells, can modify BBB protein content in brain capillary ECs (14). The Western blotting experiment clearly shows that the coculture of brain capillary ECs with astrocytes upregulates EC P-gp expression. As Tatsuta *et al.* (22) have shown that P-gp expression level in brain capillary endothelium is regulated by a tissue specific factor in the brain matrix, we can suggest that this factor could be secreted by the astrocytes and trapped within the brain matrix, explaining why other tissue matrices did not have significant effects, and why this factor is specific to brain capillary ECs.

Apical-to-basal transport of vincristine, cyclosporin A and doxorubicin was measured across the EC monolayers. *In vitro* results are in agreement with *in vivo* data confirming the extremely low transport of cyclosporin A, vincristine and doxorubicin despite their high lipophilicity. These results confirm previous investigations showing a good correlation between *in vivo* brain uptake index and *in vitro* permeability coefficients (16,17).

In the brain, P-gp expressed on the luminal surface of capillary ECs, acts as an efflux pump from the endothelial cytoplasm to the blood resulting in a barrier against selected drugs. By administration of P-gp reversing agents, it could be possible to improve the P-gp mediated drug delivery to the brain. This study was designed to assess, *in vitro*, the effect of a potent P-gp inhibitor on transport across the brain capillary ECs of doxorubicin, vincristine and cyclosporin A. S9788 has previously been demonstrated to be a potent reversal of the multidrug resistance phenotype both *in vitro* and *in vivo* (20,23). In this study, the results show that S9788 is a potent inhibitor of P-gp at the BBB level by increasing the uptake of vincristine and cyclosporin A within the brain capillary ECs. Such an *in vitro* demonstration has already been made using verapamil, and has shown an increase in the uptake of cyclosporin A (6) or vincristine (7,8). In this study, 1 μM S9788 was as efficient as 25 μM verapamil in increasing cellular accumulation of vincristine and cyclosporin A within the cells. However, the decrease in cellular accumulation of doxorubicin after 45 min in the presence of S9788 was surprising.

For the first time *in vitro*, it was possible to show an increase in the transport of vincristine from apical-to-basal side, using 1 μM S9788 or 25 μM verapamil. Indeed, although it has already been shown that it is possible to increase, *in vitro*, the uptake of vincristine by brain capillary ECs, it has never been shown that vincristine is therefore able to cross the BBB. These results are in agreement with the *in vivo* results of Lemaire *et al.* (13) showing a significant increase in BBB transport of vincristine using PSC 833. In our experiment, the inhibition of P-gp efflux of vincristine by 1 μM S9788 or 25 μM verapamil allows a passive diffusion of vincristine across the EC monolayer without any disruption.

Although it was possible to increase the uptake of cyclosporin A within the cells using S9788, it was impossible to increase its apical-to-basal transport across brain capillary EC monolayers, cyclosporin A remaining blocked within the cells. Two reasons could explain the exceptionally low distribution of cyclosporin A into the brain and our failure to increase the transport of cyclosporin A across the brain capillary ECs *in vitro*. Even if P-gp mediated efflux from brain capillary ECs has been well documented for cyclosporin A (6), sequestration of cyclosporin A in brain capillary ECs has also been described (24). Indeed brain capillary ECs possess numerous mitochondria with intracellular binding sites for cyclosporin A such as calmodulin (25) and cyclophilin (26). These results disagree with those of Lemaire *et al.* (13). Coadministrating PSC 833 as a reversing agent and cyclosporin A to rats, they showed a significant enhancement of cyclosporin A BBB transport.

In the same way, in this study, by using 25 μM verapamil, we were not able to increase significantly cyclosporin A transport across brain capillary ECs. These results are in agreement with those of Shirai *et al.* (6) who mentionned that distribution of cyclosporin A in mice brains did not increase significantly when inhibitors of P-gp were administered before cyclosporin A. *In vitro*, similar results were reported by Saeki *et al.* (27) using porcine kidney epithelial cells. As cyclosporin A has been described to be neurotoxic when it penetrates the brain parenchyma (28), the low penetration of cyclosporin A into the

brain, even in the presence of P-gp inhibitors, could be considered as an advantageous effect.

The rupture of the EC monolayers incubated in the presence of S9788 and doxorubicin was shown using inulin. This breakdown may explain the increase in doxorubicin transport to the abluminal side. These results give support to the observation that a patient who was undergoing chronic cyclosporin A therapy, presented with neurological symptoms and coma, after doxorubicin administration (29). Like S9788, cyclosporin A is known to be a potent inhibitor of P-gp (30). The authors concluded that extreme caution should be given when doxorubicin is administered to a patient presenting with a cancer and undergoing cyclosporin A therapy. Indeed doxorubicin concentration in brain tissue of 4-month cyclosporin A treated rats increased after a single injection of doxorubicin (29). Rats presented neurological disorders. Ohnishi *et al.* (9) increased the uptake of doxorubicin, by decreasing the ATP content of cultured ECs. In that case no cytotoxic effect was mentioned. Indeed by recovering intracellular ATP content, uptake was rapidly reversed. In our study, it is the association of the two components S9788 and doxorubicin, which causes a cytotoxic effect, and consequently a disruption of the *in vitro* BBB.

To summarize, we have shown that, P-gp is expressed in cultured ECs and moreover upregulated in presence of astrocytes. As *in vivo*, despite their high lipophilicity, vincristine, cyclosporin A and doxorubicin cannot cross brain capillary EC monolayers. The main subject of this study was to check whether, using reversing agents (S9788 and verapamil), we could study the transport of P-gp dependent drugs across the BBB and predict the possibility for these drugs to reach the brain parenchyma. We have shown that the incubation of ECs with vincristine in association with S9788 resulted in a four fold-increase in vincristine uptake by the cells. Moreover, an increase in the BBB permeability for vincristine was observed. The same results were obtained with 25 μM verapamil. Such observations are of importance since it has been shown that in the brain, P-gp is expressed in the ECs of the tumor capillaries excluding chemotherapeutic agents from tumor cells that are located around the capillaries. On the other hand, the association of S9788 with cyclosporin A increased its uptake four-fold but failed to increase its BBB permeability.

The incubation of ECs with doxorubicin, in association with S9788, caused a disruption of EC monolayer that could trigger off neurological disorders if it occurred *in vivo*. Thus, the technique of opening the BBB using a reversing agent has been carefully studied with the *in vitro* BBB. Opening by inhibition of P-gp or the breakdown of the BBB were distinguished.

By administrating a P-gp reversing agent, it may be possible to improve the P-gp mediated drug delivery to brain tumors. On the other hand, by attempting to restore the sensitivity of a resistant peripheral tumor by the administration of a P-gp reversing agent, an increase in the neurotoxicity of the chemotherapeutic agent could occur. As the modulating agent and the cytotoxic must be chosen carefully, the *in vitro* system of the BBB could be useful for easy and rapid test of newly developed reversing agents. Indeed the search for novel and more potent modulators of P-gp is of major importance, because most of the P-gp inhibitors currently known cause side effects *in vivo*.

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