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Role of P-glycoprotein in Colchicine and Vinblastine Cellular Kinetics in an Immortalized Rat Brain Microvessel Endothelial Cell Line

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ABSTRACT. Uptake and efflux of colchicine and vinblastine, whose effects are related to their high-affinity binding to tubulin, were studied in the immortalized rat brain microvessel endothelial cell line RBE4. At 10 nM extracellular drug concentration, uptake equilibrium was approached at 45 hr for colchicine, but at only 3.5 hr for vinblastine. After 1 hr preincubation with 200 nM colchicine or vinblastine, drug efflux fitted biexponential kinetics with an initial fast phase (half-life = 2.2 min and 9.6 min, respectively) and a later slow phase (half-life = 3.6 hr and 1.8 hr, respectively). After 6 hr preincubation with 200 nM colchicine, only the slow phase (half-life = 3.6 hr) could be observed. The colchicine and vinblastine uptake rate was increased by cyclosporin A, an inhibitor of the drug efflux pump P-glycoprotein, which is expressed at the blood-brain barrier. Whereas cyclosporin A decreased vinblastine efflux, its effect on colchicine efflux was apparent after only 13 hr washout and was associated with the re-uptake by cells of colchicine molecules. Differences in uptake kinetics of colchicine and vinblastine could be related to differences in their lipid solubility, and mainly in their binding affinities to tubulin. Differences in efflux kinetics could in addition be explained by the involvement of P-glycoprotein in the efflux of vinblastine, whereas efflux of colchicine was not influenced by this pump. Indeed, binding of colchicine to tubulin would imply that most intracellular colchicine may be inaccessible to P-glycoprotein. In the case of a cytotoxic drug such as colchicine, which is tightly bound to intracellular receptors, the role of P-glycoprotein within the blood-brain barrier would be more to protect the brain against entry of this drug than to detoxify the brain by its extraction. BIOCHEM PHARMACOL 53;11:1735–1742, 1997.

KEY WORDS. blood-brain barrier; colchicine; endothelial cells; P-glycoprotein; tubulin; vinblastine

Colchicine is a potent therapeutic drug that has been used in the short- and long-term treatment of acute gout [1] and is effective in a variety of inflammatory disorders, including familial Mediterranean fever [2]. Both its therapeutic effects and toxic effects induced by overdose are related to its interaction with tubulin [3]. Colchicine first binds to soluble tubulin and forms a poorly reversible final state tubulin-colchicine complex which can then bind to microtubule ends [4, 5]. In contrast to colchicine, vinblastine, another tubulin-binding compound, inhibits exchange of microtubules with tubulin by binding directly to microtubule ends [5, 6]. Since colchicine and vinblastine bind to different forms of tubulin, the soluble and polymerized forms respectively, association constants of colchicine-binding sites (1 to 10 μM^{-1}) and vinblastine-binding sites (0.5 to 1 μM^{-1}) are different [4, 5].

In the brain, colchicine and vinblastine are able to disrupt axoplasmic transport [7]. Their neurotoxicity appears to be related to their ability to bind to tubulin, resulting in inhibition of microtubule assembly. The blood-brain barrier (BBB) protects the brain from many exogenous toxins and sudden fluctuations in the levels of systemic substances. The brain microvessel endothelial cells have several transport systems that influence BBB permeability by regulating the passage of essential nutrients into the brain and restricting the entry of polar solutes. It has been reported that endothelial cells of the BBB, unlike those of most other tissues, strongly express the transmembranous P-glycoprotein (P-gp) [8, 9], the product of the multidrug resistance (MDR) gene. P-gp has been studied extensively because of its role in drug resistance in various tumors and neoplastic cell lines [10–12]. P-gp reduces intracellular anticancer drug accumulation by rapidly pumping such drugs out of these cells and would be responsible for extrusion back into the circulation of the

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Abbreviations: AUC, area under the curve; BBB, blood-brain barrier; P-gp, P-glycoprotein; CsA, cyclosporin A; MDR, multidrug resistance; GTP, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase.

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hydrophobic xenobiotics that diffuse into endothelial cells at the BBB [13].

Colchicine and vinblastine have been shown to be actively transported out of MDR cells by P-gp in order to maintain intracellular drug concentrations at subtoxic levels. These two substrates of P-gp possess different affinities for the pump, as shown by Liu and Sharom with purified P-glycoprotein [14]. The purpose of the present study was to examine whether P-gp influences the cellular kinetics of colchicine and vinblastine in cultured BBB endothelial cells and to investigate whether intracellular mechanisms other than passive diffusion through the plasma membrane and P-gp transport affect exchange of these drugs between extra- and intracellular compartments.

For this study, we used an immortalized rat brain microvessel endothelial cell line RBE4. The immortalized cellular clone RBE4 was isolated after transfection of primary rat brain microvessel endothelial cells with the plasmid pE1A-neo. This plasmid carries the entire E1A region of adenovirus 2 and the neogene for resistance to the aminoglycoside neomycin. RBE4 cells display a nontransformed endothelial phenotype, exhibit contact inhibition, growth factor and anchorage-dependent proliferation [15]. These immortalized cells remain sensitive to astroglial factors for the expression of gamma-glutamyl transpeptidase (GTP), alkaline phosphatase (ALP) [16] and P-gp [El Hafny B, Chappey O, Piciotti M, Boval B and Roux F; submitted for publication].

We show differences in the uptake characteristics of colchicine and vinblastine which could be related to differences in lipid solubility, and mainly in association constants of the two drugs to tubulin. The functional importance of P-gp in RBE4 cells is demonstrated with the P-gp inhibitor cyclosporin A, which increases intracellular uptake of both drugs. P-glycoprotein induces an efflux of vinblastine at high concentration, but not for colchicine under the same experimental conditions. The high-binding affinity of colchicine to tubulin would imply that most intracellular colchicine may be inaccessible to P-gp, whereas the affinity of vinblastine for P-gp, higher than its affinity for microtubules, would allow its extraction by P-gp. Our findings suggest that high-affinity binding of toxins to intracellular receptors may be a limiting factor for cell detoxication by P-gp.

MATERIALS AND METHODS

Reagents

Fetal calf serum and basic fibroblast growth factor were obtained from Boehringer (Mannheim, Germany) and media from Life Technologies (Cergy Pontoise, France). [^{3}H] colchicine (70 Ci/mmol) and [^{3}H] vinblastine sulphate (11 Ci/mmol) were purchased from Amersham France SA (Les Ulis, France). Colchicine, vinblastine sulphate, verapamil and vincristine sulphate were from Sigma (St Louis, MO, U.S.A.). Colchicine-specific Fab fragments were obtained from purified polyclonal goat immunoglobulin G specific to colchicine by papain treatment as described by Sabouraud *et al.* [17]. Polyclonal digoxin-specific Fab fragments were obtained from Boehringer (Mannheim GmbH, Germany). Cyclosporin A was a generous gift from Sandoz Pharma Ltd (Basel, Switzerland). Cyclosporin A was stored as a stock solution in ethanol at 8.3×10^{-2} M with dilutions then made in culture medium. Control cultures were treated with the same final concentration of 0.016% ethanol.

Cell Cultures

RBE4 immortalized rat brain microvessel endothelial cells were plated onto collagen I-coated 4- or 24-well multiplates (rat tail collagen) and maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 10 mM HEPES, 2mM glutamine, 10% heat-inactivated fetal calf serum, 300 μg/mL neomycin (G418) and 1 ng/mL basic fibroblast growth factor (bFGF) in humidified 5% CO_2/95% air at 37°C. The experiments were performed on 4-day confluent cultures.

Drug Uptake Studies

Cellular uptake of [^{3}H] vinblastine and [^{3}H] colchicine was measured according to Tatsuta *et al.* [18]. Cells were preincubated in culture medium with or without 10 μM cyclosporin A (CsA) for 30 min at 37°C. [^{3}H] colchicine or [^{3}H] vinblastine were then added and, after different incubation times at 37°C, cells were rapidly washed three times with ice-cold PBS to eliminate the extracellular drug. Two sets of experiments were carried out. Uptake of 10 nM colchicine or 10 nM vinblastine was measured over short periods of up to 3.5 hr, and uptake of colchicine concentrations ranging from 0.5 nM to 200 nM was measured over long periods of up to 75 hr. The amount of [^{3}H] colchicine or [^{3}H] vinblastine retained in the cells was counted in Pico-fluor 40 (Packard, Rungis, France) by β scintillation counting after the cells had been lysed with 0.1 N NaOH. An aliquot of cell lysate was used in parallel to determine cellular protein concentration by the Lowry assay [19]. Intracellular colchicine or vinblastine was expressed as pmol/mg protein.

Drug Efflux Studies

RBE4 cells grown on 4-well plates were washed three times with ice-cold PBS and preincubated for either 1 hr in culture medium containing 10 or 200 nM [^{3}H] vinblastine or [^{3}H] colchicine, or for 6 hr in medium containing 200 nM [^{3}H] colchicine. After washing with PBS at 4°C to eliminate the extracellular drugs, cells were incubated in culture medium with or without P-gp modulators (washout medium).

For measurement of colchicine long-term efflux, colchicine-specific Fab fragments were added to the washout medium in order to avoid re-uptake by the cells of the colchicine molecules released into the medium. The

amount of Fab fragments added to the 0.2 mL medium covering the cells in a multiplate well was equal to the amount of colchicine accumulated in the cells after 6 hr incubation. Control cells were incubated in the presence of the same amount of digoxin-specific Fab fragments [20].

The incubation medium was pipetted at the designated time points. The cells were then washed with ice-cold PBS and lysed by 0.1 N NaOH. The radioactivity associated with the incubation medium and the lysed cells was counted in Ready Solv HP (Beckman) by β scintillation counting. Intracellular colchicine is expressed as the percentage of total extra- and intracellular colchicine in each well.

Pharmacokinetic Analysis

The area under the curve (AUC) was calculated by the trapezoidal rule using the INPLOT computer program (GraphPAD software, ISI Sorrento Valley, CA, U.S.A.).

Estimates of slow and rapid elimination half-lives ($t_{1/2}$) were obtained by fitting the curve to the intracellular colchicine or vinblastine concentration-time data with a mono- or biexponential equation using the non-linear least squares SIPHAR program (SIMED, Créteil, France). To assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed. In addition to the likelihood test, Akaike and Schwarz criteria were tested to select the most appropriate model.

RESULTS

Vinblastine and Colchicine Uptake in RBE4 Cells

In order to compare the vinblastine and colchicine accumulation rate in RBE4 cells, uptake was measured at an extracellular drug concentration of 10 nM, i.e., the level attained in human serum during clinical treatment with colchicine [20].

The time-course of vinblastine uptake in confluent RBE4 cells is shown in Fig. 1A. Vinblastine accumulation rapidly approached equilibrium during the first 3.5 hr. In the presence of 10 μM cyclosporin A, uptake followed an identical pattern but the intracellular vinblastine concentration was increased at all experimental times, resulting in a 38.5% increase in the AUC of vinblastine accumulation vs time.

Uptake of colchicine with time is shown in Fig. 1B. After an initial rapid entry, colchicine accumulated at a constant rate over the 3 hr of the uptake evaluation. After addition of 10 μM cyclosporin A to the incubation medium, the cell content of colchicine was increased at all experimental times, resulting in a 30.2% increase in the AUC of colchicine accumulation vs time.

Fig. 2 shows that within a colchicine concentration range of 0.5 nM to 20 nM, equilibrium was approached at 45 hr incubation. However, with the toxic concentration of 200 nM, the intracellular colchicine level was maximum and stable after a 6-hr incubation. Intracellular content of

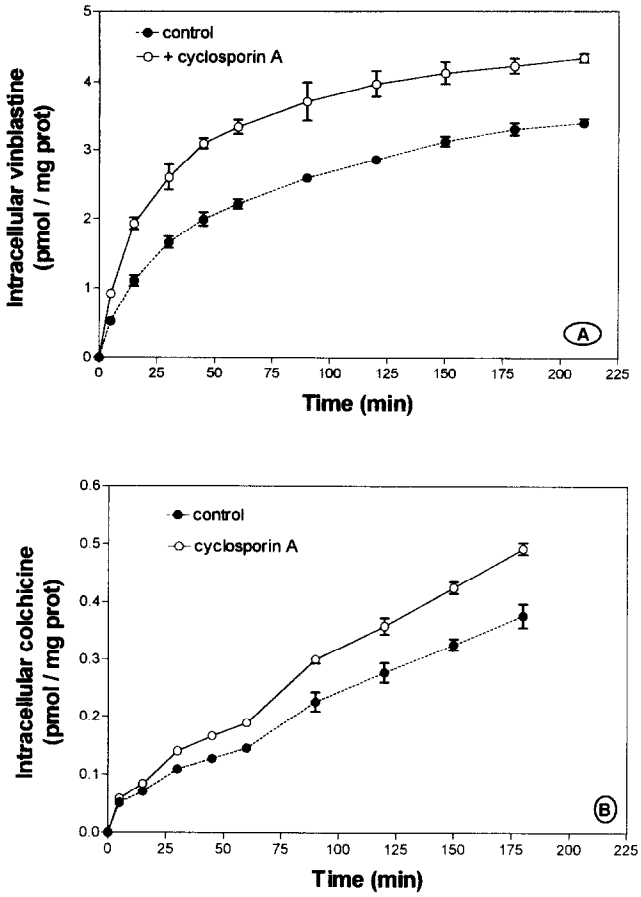

FIG. 1. Time-course for intracellular [^{3}H] vinblastine (A) and [^{3}H] colchicine (B) uptake by RBE4 cells. Effect of cyclosporin A. RBE4 cells were preincubated with growth medium alone (control) or with growth medium containing 10 μM CsA. After 30 min, 10 nM [^{3}H] colchicine or 10 nM [^{3}H] vinblastine were added, and the uptake was measured for the indicated times as described in Materials and Methods. Values are means ± SD of 4 separate wells of one representative experiment. $P < 0.01$ vs control in Student's *t*-test at all experimental times.

colchicine decreased after 45 hr incubation with the different tested colchicine concentrations.

Vinblastine and Colchicine Short-term Efflux from RBE4 Cells

Efflux of both antitubulin agents was measured at low (10 nM) and high (200 nM) drug concentrations.

Semilogarithmic plots of vinblastine efflux for 2.5 hr of washout are shown in Fig. 3A (10 nM vinblastine) and Fig. 3B (200 nM vinblastine), after preloading of RBE4 cells with vinblastine for 1 hr. The different curves of vinblastine efflux from RBE4 cells can be characterized by biexponential kinetics. After preloading with 10 nM vinblastine, the efflux pattern was not modified by the addition of cyclosporin A to the washout medium. In the control cells, the values for the half-life of the initial fast phase and later slow phase were 8.9 min and 2.5 hr respectively, while in the CsA-treated cells, they were 8.1 min and 2.6 hr. However, after preloading with 200 nM vinblastine, addi-

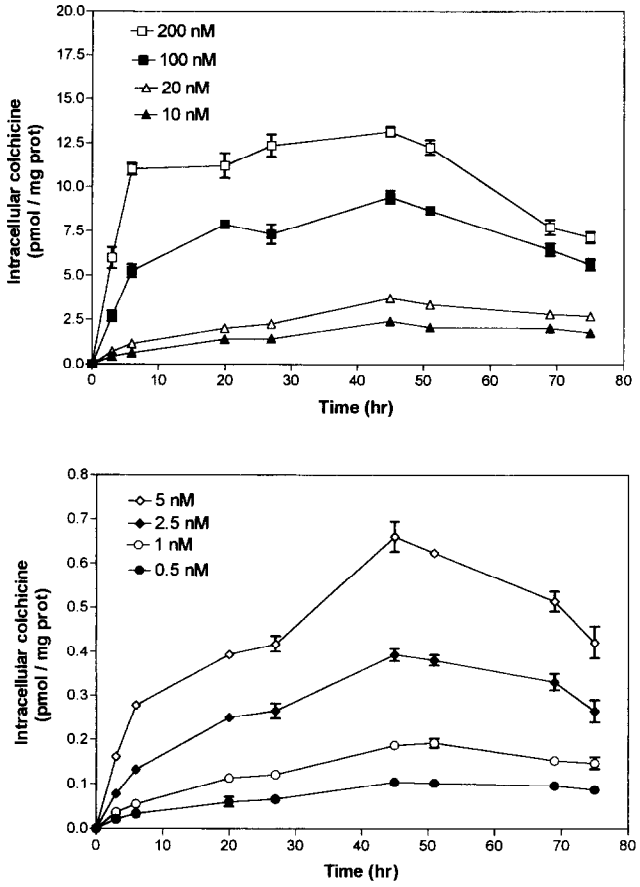

FIG. 2. Time-course for uptake by RBE4 cells of different extracellular colchicine concentrations. Cells were incubated in the presence of increasing concentrations of colchicine ranging from 0.5 nM to 200 nM and sampled at graded time intervals to measure the cell-associated radioactivity as described in Materials and Methods. Values are means ± SD of 4 separate wells of one representative experiment.

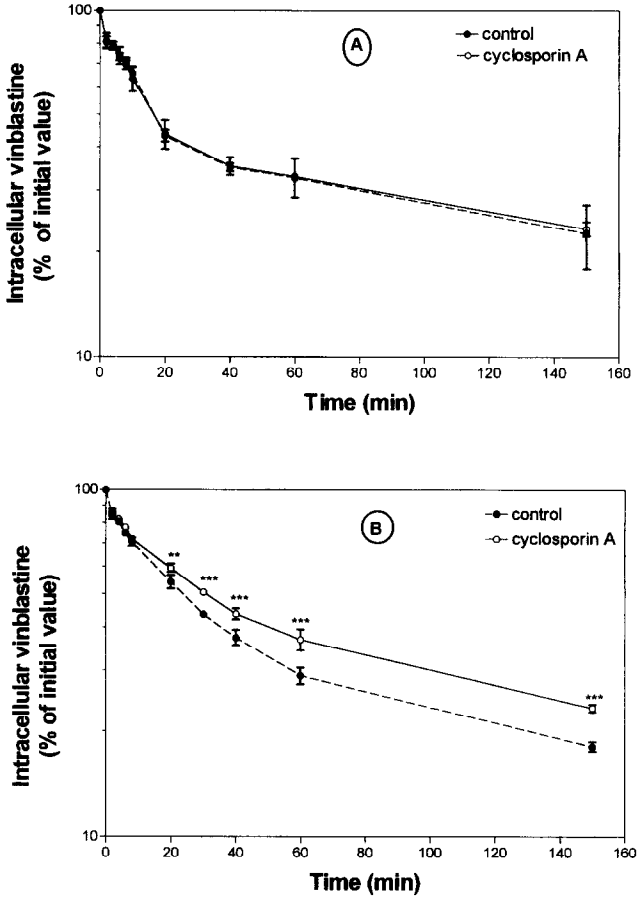

FIG. 3. Semilogarithmic time profiles of vinblastine efflux from RBE4 cells preloaded with different amounts of the drug. RBE4 cells were preincubated with 10 nM (A) or 200 nM (B) [^{3}H] vinblastine for 1 hr. The cells were then washed and incubated in drug-free medium (control) or in medium containing 10 μM CsA. At increasing time intervals, the amount of [^{3}H] vinblastine retained in the cells was determined and expressed as the percentage of total extra- and intracellular vinblastine. Values are means ± SD of 4 separate wells of one representative experiment. **P < 0.01, ***P < 0.001 vs control in Student's *t*-test.

tion of CsA increased the half-life values of both phases. Indeed, in control cells, the half-life values were 9.6 min for the fast phase and 1.8 hr for the slow phase, whereas in CsA-treated cells, the values were 11.6 min and 2.5 hr respectively. Such a CsA-induced increase in half-life values resulted in a 18% increase in the AUC of vinblastine efflux versus time. The slow phase half-lives obtained with the CsA-treated cells preloaded with 200 nM vinblastine and with the control cells preloaded with 10 nM vinblastine were not different.

Fig. 4A (10 nM colchicine) and Fig. 4B (200 nM colchicine) show semilogarithmic plots of colchicine efflux as a function of time, for 2.5 hr of washout, when RBE4 cells were preloaded with colchicine for one hr. Colchicine efflux from RBE4 cells can be described by biexponential kinetics with parameters that do not differ significantly whether cells are preloaded with 10 nM or 200 nM of colchicine. Addition of cyclosporin A to the washout medium did not change the kinetic parameters significantly. Thus, the different efflux curves can be analysed into an initial fast phase, defined by a half-life ranging from 2.2 to 2.6 min, and a slow phase, defined by a half-life ranging from 3.6 to 4.2 hr.

To further analyse colchicine efflux characteristics, experiments were performed at equilibrium (Fig. 4C). Fig. 2 shows that steady-state was reached after preloading the RBE4 cells for 6 hr with 200 nM colchicine. After a very short fast phase from time 0 to time 2 min, the half-life of which could not be evaluated, the efflux curves for the RBE4 cells washed out with or without cyclosporin A were described by a monoexponential kinetics with a half-life ranging from 3.6 to 3.8 hr. Again, addition of cyclosporin A did not induce any change in kinetic parameters.

Colchicine Long-term Efflux from RBE4 Cells

Colchicine efflux after 20 hr of washout was analysed after preloading the RBE4 cells for 6 hr with 200 nM colchicine (Fig. 5). In order to eliminate re-uptake of the colchicine

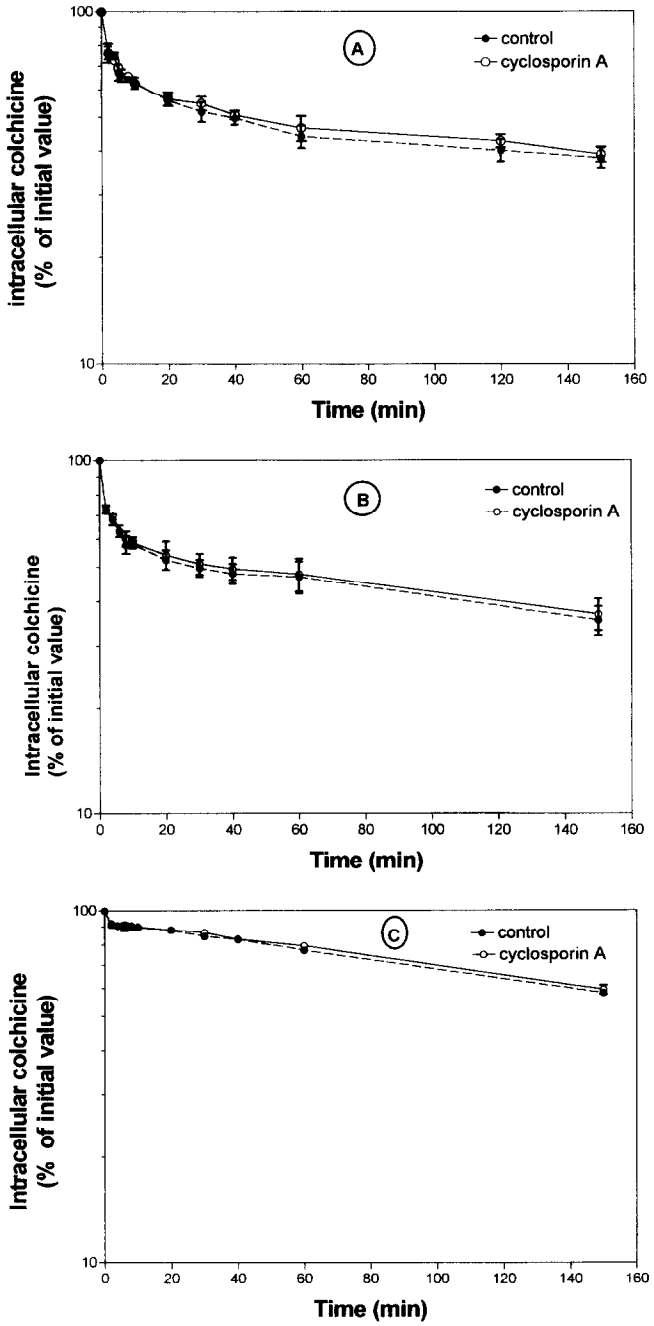

FIG. 4. Semilogarithmic time profiles of colchicine efflux from RBE4 cells preloaded with different amounts of drug. RBE4 cells were preincubated with 10 nM [^{3}H] colchicine for 1 hr (A) or with 200 nM [^{3}H] colchicine either for 1 hr (B) or 6 hr (C). The cells were then washed and incubated in drug-free medium (control) or in medium containing 10 μM CsA. At graded time intervals, the amount of [^{3}H] colchicine retained in the cells was determined and was expressed as a percentage of the sum of extra- and intracellular colchicine, as described in Materials and Methods. Values are means ± SD of 4 separate wells of one representative experiment.

molecules released into the incubation medium, colchicine-specific Fab fragments were added at the start of washout. Control cells were incubated with the same concentration of digoxin-specific Fab fragments to take into account the potential interactions of the Fab fragments with the cells. Semilogarithmic plots of colchicine cell content as a function of time show that long-term efflux in the presence of colchicine-specific Fab fragments followed monoexponential kinetics. The half-life value was 3.7 hr for the cells incubated with colchicine-specific Fab fragments. A decrease in colchicine content of cells incubated with colchicine-specific Fab fragments was significant after 13 hr of washout, while increases in colchicine content in cyclosporin A-treated cells were significant after 11 hr. The decreases induced by colchicine-specific Fab fragments were not modified by simultaneous addition of CsA to the washout medium (inset of Fig. 5).

Likewise, addition of 10 μM verapamil to the washout medium induced a significant increase in intracellular concentration of colchicine only after 12 hr of incubation. However, in the presence of 50 μM vincristine, the efflux rate of colchicine was dramatically reduced, since the half-life value calculated during the first 10 hr of washout was 16.5 hr, instead of 3.45 hr with control cells (Fig. 6).

DISCUSSION

In this study, uptake and efflux kinetics of colchicine and vinblastine have been analysed in the immortalized rat brain microvessel endothelial cell line RBE4. The uptake studies suggested that P-gp can limit entry of colchicine and vinblastine into the BBB endothelial cells and that differences in intracellular content of these drugs can mainly be related to differences in their binding affinities to tubulin. The efflux studies showed that P-gp cannot extract colchicine efficiently from the endothelial cells because this drug is tightly bound to intracellular receptors.

It has previously been demonstrated that P-gp is expressed in the RBE4 cell line and is active in expelling colchicine and vinblastine from these cells [21]. In the present study, CsA, an inhibitor of the drug efflux pump P-glycoprotein, was able to enhance the intracellular accumulation of the P-gp substrates colchicine and vinblastine. This result indicates that P-gp limits the passive intracellular diffusion of these drugs and is a factor decreasing their intracellular accumulation in RBE4 cells. CsA has also been shown to increase drug accumulation in MDR variants of the human lung tumor cell line COR-L23. These variants do not express P-gp, but overexpress MRP, the multidrug resistance-associated protein [22]. Thus, we cannot exclude that other efflux pumps may be implicated in the expulsion of colchicine and vinblastine from RBE4 cells. This possibility is currently under study.

We showed that after incubating RBE4 cells with 10 nM of labelled drug for 1 hr, vinblastine accumulated in the cells approximately 10 times faster than colchicine and that the uptake equilibrium was approached at 3.5 hr incubation for vinblastine and at 45 hr incubation for colchicine. These patterns of colchicne and vinblastine uptake are comparable to the accumulation kinetics of both drugs as described by Safa *et al.* in human KB cells [23]. Intracellular

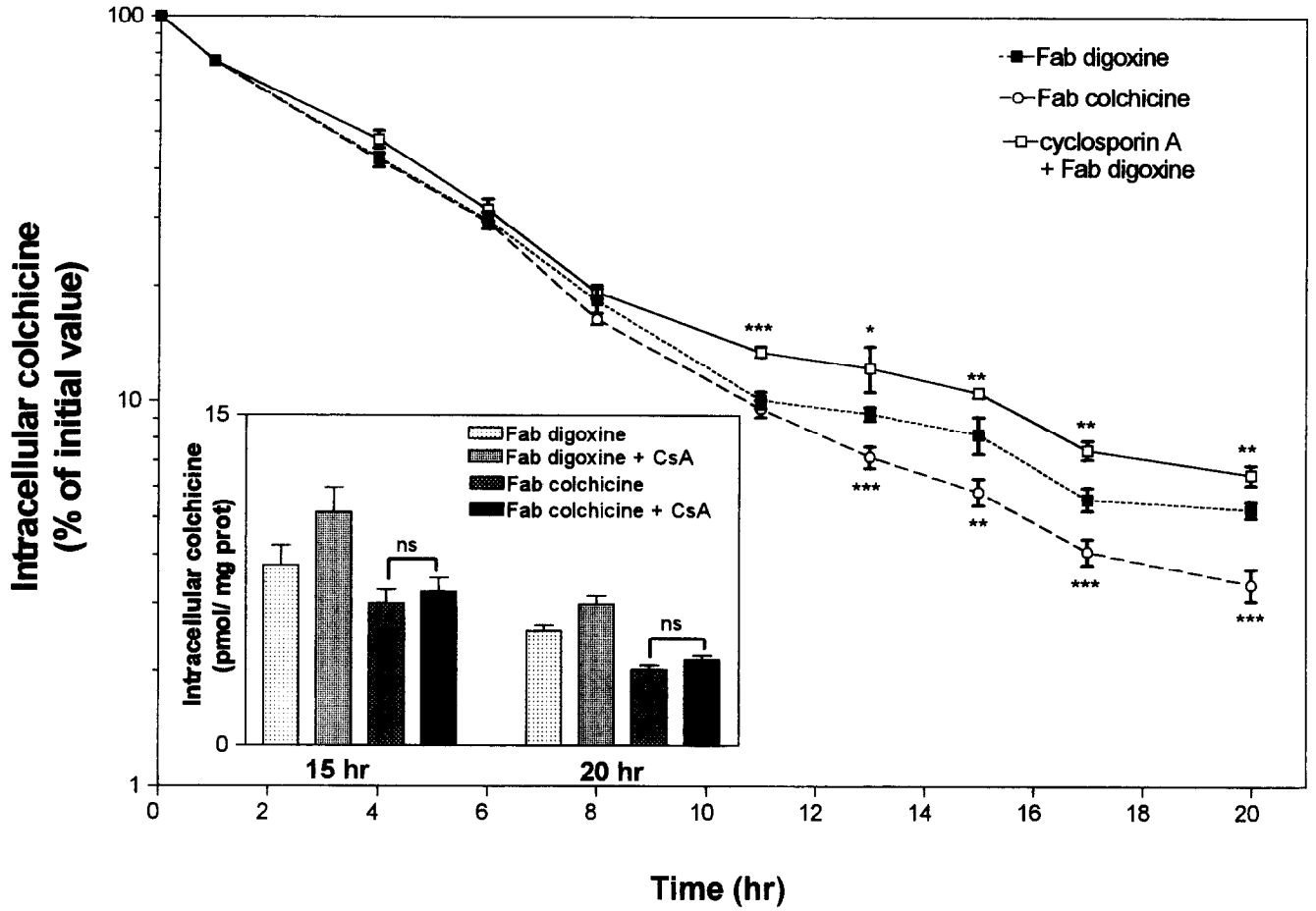

FIG. 5. Effects of cyclosporin A and colchicine-specific Fab fragments on the long-term colchicine efflux kinetics. After 6 hr colchicine preincubation with 200 nM [^{3}H] colchicine, the cells were washed and incubated in drug-free medium (control), in medium containing 10 μM CsA, or in drug-free medium containing colchicine-specific Fab fragments. Control and CsA-treated cells were incubated with digoxin-specific Fab fragments. The amount of extracellular Fab fragments in medium was equal to the amount of intracellular colchicine at time 0. At increasing time intervals, the amount of [^{3}H] colchicine retained in the cells was determined and expressed as the percentage of total colchicine. Inset: Comparison at 15 hr and 20 hr of cells incubated in medium containing 10 μM CsA and colchicine-specific Fab fragments. Values are means ± SD of 4 separate wells of one representative experiment. ns: not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control in Student's *t*-test.

content of colchicine declined after 45 hr incubation with higher drug concentrations. This evolution suggests that a steady-state cannot be reached with such high colchicine doses, which may induce progressively nonspecific cell death with denaturation of tubulin colchicine sites [24].

The cellular content of colchicine and vinblastine at different experimental uptake times depends on a balance between three factors: drug lipid solubility affecting passive distribution into the cell; drug expulsion out of the cell by P-glycoprotein; and drug retention in the cell due to different relative affinities to intracellular receptors. Partitioning into the plasma and vesicle membrane bilayers would be higher for vinblastine than for colchicine, since the octanol/water partition coefficient is logP = 1.68 for vinblastine [25] and logP = 1.28 for colchicine [26]. However, such differences are not large enough to explain the 10-fold differences in the uptake rates of both drugs. Under our experimental conditions, the P-gp-mediated efflux would not contribute to differences in cellular content of vinblastine and colchicine, since the relative increase in drug accumulation induced by CsA in RBE4 cells was approximately similar for both drugs. Colchicine has been shown to bind to tubulin with association constants ranging from 1 to 10 μM^{-1} [4], whereas vinblastine binds to microtubules with lower association constants ranging from 0.5 to 1 μM^{-1} [5]. Thus, in RBE4 cells, differences in vinblastine and colchicine uptake kinetics as well as in the time required to reach equilibrium could largely be explained by differences in drug-binding affinities to tubulin.

In order to analyse the relationship between the intracellular content and the rate of efflux of colchicine or vinblastine, cells were preloaded with either 10 nM or 200 nM of drug. When the efflux rates were measured after 1 hr preincubation with either concentration, colchicine and vinblastine efflux from RBE4 cells followed biexponential kinetics. There was a rapid exit of the drug during the first minutes followed by a slow exit requiring several hours. The half-life of the fast phase of vinblastine efflux was higher

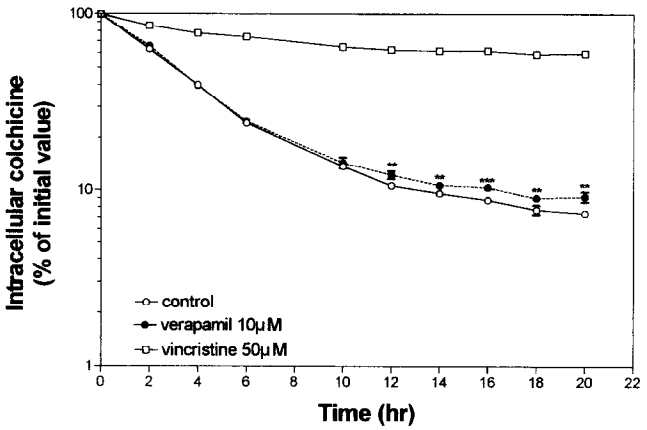

FIG. 6. Effects of verapamil and vincristine on the long-term colchicine efflux from RBE4 cells. RBE4 cells were preincubated with 200 nM [^{3}H] colchicine for 6 hr. The cells were then washed and incubated in drug-free medium (control) or in medium containing 10 μM verapamil or 50 μM vincristine. At indicated times, the [^{3}H] colchicine retained in the cells was determined and expressed as a percentage of extra- and intracellular colchicine. Values are means ± SD of 4 separate wells of one representative experiment. **$P < 0.01$, ***$P < 0.001$ vs control in Student's *t*-test.

than that of colchicine, whereas the half-life of the slow phase was lower.

The fast phase of drug efflux may represent the outflow of unbound molecules distributed in the intracellular fluid space and the higher value of the vinblastine efflux fast-phase half-life could partly be explained by the higher partitioning of vinblastine into the plasma membrane bilayer [12]. The slow phase may represent the outflow of molecules released from their intracellular receptors. Indeed, dissociation of the purified tubulin-colchicine complex has been described as a single exponential kinetics with an apparent dissociation rate constant of 38.5×10^{-6} s^{-1}, leading to a dissociation half-life of 5 hr at 37°C [24]. Thus, drug efflux for the slow phase would be rate-limited by the dissociation of colchicine from its tubulin binding sites. After 6 hr preincubation of 200 nM colchicine, i.e., when equilibrium between intracellular and extracellular compartments was reached, colchicine efflux from RBE4 cells followed only a monoexponential kinetics with the same cell elimination half-life as that measured for the slow phase after 1 hr colchicine preincubation. This result suggests that after 6 hr preincubation most intracellular colchicine molecules were bound to tubulin. Addition of vincristine to washout medium greatly increased intracellular colchicine in RBE4 cells compared to controls, with an elimination half-life of 16.5 hr instead of 3.45 hr. Using purified rat brain tubulin, McClure and Paulson [27] also observed that the dissociation of colchicine from tubulin was reduced after tubulin preincubation with vinblastine, since the time required to lose one half the colchicine binding activity increased from 7.5 hr in controls to 24.5 hr after preincubation with vinblastine. These different observations confirm that high intracellular colchicine binding affinity to tubulin limits the efflux of this drug from RBE4 cells. Differences between the association constants of vinblastine to microtubules (0.5 to 1 μM^{-1}) [5] and colchicine to tubulin (1 to 10 μM^{-1}) [4] suggest that vinblastine was released faster from microtubules into the intracellular fluid space, with a consequent shorter efflux slow-phase half-life.

Although RBE4 cells express functional P-gp as demonstrated by drug uptake kinetics, CsA had no effect on the colchicine efflux from RBE4 cells, since the efflux half-lives were similar in the presence or absence of the P-gp inhibitor. Contrary to colchicine efflux, vinblastine efflux was inhibited by CsA when RBE4 cells were preincubated for 1 hr with 200 nM vinblastine, since the efflux half-life increased in the presence of CsA for both the fast and slow phases. The association constant of vinblastine to P-gp (1.3 μM^{-1}) [14] is slightly higher than its association constant to microtubules (0.5 to 1 μM^{-1}). Thus, when vinblastine is released from microtubules into the cytosol, the molecules can bind to P-gp. In contrast, the association constant of colchicine to P-gp (6.3 nM^{-1}) [14] is much lower than its association constant to tubulin (1 to 10 μM^{-1}) and the molecules would preferentially bind to tubulin. Furthermore, because of the strong binding of colchicine to tubulin, the amount of exchangeable drug distributed in the intracellular fluid space would be very low. Since only the cytosolic free drug has access to P-gp, the concentration of P-gp substrate in the cytosol would thus be a limiting factor for evaluation of P-gp activity.

Inhibition of vinblastine efflux by CsA was dependent on the amount of intracellular drug, since the inhibitory effects of CsA were observed after 1 hr preincubation with a high (200 nM) but not with a low (10 nM) extracellular concentration. For 10 nM vinblastine, as for 10 and 200 nM colchicine, the cytosolic free drug concentration would be too low for P-gp activity to be detected. Similarly, it has been reported that the fraction of daunorubucin tightly bound to intracellular structures or confined to compartments other than the cytosol would be inaccessible for P-gp-mediated export functions [28].

When efflux rates were measured for a longer time (20 hr), we washed out the external colchicine and added colchicine-specific Fab fragments to bind the extracellular colchicine and to avoid re-uptake of the extracellular colchicine by the cells. When the efflux was measured after washing out the external colchicine for times longer than 13 hr, intracellular colchicine was significantly decreased in the presence of the colchicine-specific Fab fragments. Only then was CsA or verapamil able to inhibit colchicine efflux in this phase when extracellular drug is taken up again. P-gp was then able to export colchicine again because a sufficient amount of free substrate had access to P-gp immediately after crossing the plasma membrane. Absence of the CsA effect when colchicine-specific Fab fragments were added to the washout medium confirmed that P-gp activity was measurable only when enough colchicine molecules were taken up again by the cells. The *in vitro* efficiency of

the antibody has already been reported by Chappey *et al.* [20], who demonstrated that both periodically washing out external colchicine and adding colchicine-specific Fab fragments induce efflux of intracellular colchicine from lymphocytes with similar first-order kinetics.

Thus, differences in vinblastine and colchicine efflux kinetics in RBE4 cells may be explained, like differences in uptake kinetics, by differences in lipid solubility and drug binding-affinities to tubulin. However, P-glycoprotein could also be involved if the amount of exchangeable drug in the cytosol was high enough for P-glycoprotein activity to be evaluated. In conclusion, P-gp cannot actively expel and consequently reduce the intracellular concentration of drugs such as colchicine, whose efflux is rate-limited by their high-affinity binding to intracellular receptors. We suggest that the role of P-gp within the blood-brain barrier would be more to protect the brain against cytotoxic drug entry than to detoxify the brain by extraction of the drugs that are tightly bound to intracellular receptors.

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