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Effects of γ- and Hydroxypropyl-γ-cyclodextrins on the Transport of Doxorubicin across an in Vitro Model of Blood-Brain Barrier

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ABSTRACT

Association between doxorubicin (DOX) and γ-cyclodextrin (γ-CD) or hydroxypropyl-γ-CD (HP-γ-CD) has been examined to increase the delivery of this antitumoral agent to the brain. The stoichiometry and the stability constant of γ-CD or HP-γ-CD and DOX complexes were determined in physiological medium by UV-visible spectroscopy. By using an in vitro model of the blood-brain barrier (BBB), endothelial permeability and toxicity toward the brain capillary endothelial cells of DOX, γ-CD, and HP-γ-CD were performed. For each CD, endothelial permeability was relatively low and a disruption of the BBB occurred at 20 μM, 20 mM, and 50 mM DOX, γ-CD, and HP-γ-CD, respectively. Increasing amounts of CDs were added to a fixed DOX concentration. Addition of γ-CD or HP-γ-CD, up to 15 and 35 mM, respectively, decreased the DOX delivery, probably due to the low complex penetration across the BBB and the decrease in free DOX concentration. Higher CD concentrations increased the DOX delivery to the brain, but this effect is due to a loss of BBB integrity. In contrast to what was observed on Caco-2 cell model with various drugs, CDs are not able to increase the delivery of DOX across our in vitro model of BBB.

Despite advances in brain research, central nervous system disorders remain very difficult to treat because the majority of drugs do not cross the blood-brain barrier (BBB) (Pardridge, 2003). Indeed, the BBB acts as an anatomical and transporter barrier notably due to the presence of tight junctions and the ATP-dependent efflux pump P-glycoprotein (P-gp), respectively. The BBB limits the ability of many drugs to penetrate brain tissue by restricting paracellular and transcellular transport. Ohnishi et al. (1995) have reported that the brain distribution of doxorubicin (DOX), an antitumoral agent widely used in the treatment of several cancers, is mainly restricted by P-glycoprotein on the BBB under normal physiological conditions. Consequently, DOX delivery to the brain is still a challenge for numerous research groups. Indeed, to circumvent the limited access of DOX into the brain, different approaches have been investigated, including drug delivery systems such as liposomes (Saito et al., 2004) or nanoparticles (Gulyaev et al., 1999), peptide-vector strategy using DOX linked to cationic peptides (Rousselle et al., 2001), P-gp modulator (Fenart et al., 1998), or osmotic pressure modification (Neuwelt et al., 1981). A bradykinin analog, RMP-7, can also increase the tight junction permeability of the endothelial cells and enhance delivery of therapeutic agents across the BBB (Bartus et al., 1996). Interestingly, cyclodextrins (CDs) could be envisaged as permeation enhancer on the BBB. Indeed, CDs are widely used to increase the delivery or bioavailability of drugs in nasal (Merkus et al., 1999), ocular (Loftsson and Järvinen, 1999), dermal (Matsuda and Arima, 1999), or oral field. For example, it was described on Caco-2 cell monolayer (as an intestinal model) that dimethyl-β-CD increased permeability of tacrolimus (an immunosuppressor) by its inhibitory effect on the P-gp-mediated efflux (Arima et al., 2001). Because Caco-2 cell monolayer is often used to predict toxicity or permeability for a drug toward the BBB (Lohmann et al., 2002), it was of great interest to evaluate the role of CDs on DOX transport across the BBB. Recently, we have described the behavior of various CDs on an in vitro model of BBB (Monnaert et al., 2004). The γ-CD and HP-γ-CD have been found to be the less toxic CDs

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ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; DOX, doxorubicin; CD, cyclodextrin; γ-CD, γ-cyclodextrin; HP-γ-CD, hydroxypropyl-γ-cyclodextrin; BCEC, brain capillary endothelial cell; RH, Ringer-HEPES; DMEM, Dulbecco's modified Eagle's medium; PBS-CMF, calcium- and magnesium-free phosphate-buffered saline; Pe, endothelial permeability; S9788, a triazino-aminopiperidine derivative; RMP-7, Cereport.

toward the brain capillary endothelial cell (BCEC) monolayer and were able to cross slightly the BBB. Because these two γ-CDs and DOX formed an inclusion complex, their association has been studied to modify the P-gp efflux and BBB permeability to increase DOX delivery.

Materials and Methods

Chemicals and Antibodies. [^{3}H]Inulin (0.54 mCi/mmol) and [^{14}C]doxorubicin hydrochloride (58 mCi/mmol) were obtained from Amersham Biosciences Inc. (Les Ulis, France). Doxorubicin was obtained from Sigma (Saint Quentin Fallavier, France). γ-CD and HP-γ-CD (with an average degree of substitution equal to 4.8) were purchased from Cyclolab (Budapest, Hungary) and Wacker Chemie GmbH (Lyon, France), respectively. CDs were used as received without further purification. S9788 was synthetized at the Servier Research Institute (Courbevoie, France). All other chemicals and solvents were of analytical reagent grade. Primary antibody was detected with appropriate combination of fluorescently labeled secondary antibodies: rabbit polyclonal anti-human occludin (Zymed Laboratories, South San Francisco, CA) and goat anti-rabbit Alexa Fluor 488 IgG (Molecular Probes, Eugene, OR), respectively.

Characterization of Cyclodextrin-Doxorubicin Complexes. The continuous variation method (Job's method) was used to determine the stoichiometry of the complex (Connors, 1987). For each CD, a series of samples containing variable ratios (r varying from 0 to 1) of CD and DOX were prepared keeping the total concentration of species constant (10 μM in this present case). The samples were prepared in physiological buffer (Ringer-HEPES; RH). The differences of absorption intensity at 479 nm in UV-visible spectroscopy (Lambda 19; PerkinElmer Life and Analytical Sciences, Courtaboeuf, France) were measured in function of molar ratio. The plot of Δabs.[DOX] versus the mole ratio of DOX (r) was traced. The UV spectra's derivatives (recorded at 479 nM) were chosen so that no effect from the refractive index relative to the CD was observed (Landy et al., 2000).

The titration method was used to determine association constants. This method was applied for a fixed concentration of DOX (10 μM) and varying concentrations of CD. The concentrations used for each CD have been chosen to obtain 0, 25, 50, 75, and 95% of complex form (an approximate knowledge of the constant is necessary). An algorithmic treatment was used to calculate the association constants. The algorithmic treatment was applied to UV-spectra derivatives (recorded at 479 nm). Assuming a 1:1 stoichiometry, the observed UV-visible absorption of DOX (A_{OBS}) and the complex concentration [COMP] are described as follows:

$$A_{OBS} = (A_{DOX} \times [DOX] + A_{COMP} \times [COMP])/[DOX]_T \quad (1)$$

$$[COMP] = -1/2\ \{(1/K_f + [CD]_T + [DOX]_T)^2 - 4\ [CD]_T[DOX]_T]^{1/2} + 1/2\ (1/K_f + [CD]_T + [DOX]_T) \quad (2)$$

where K_f and $[]_T$ stand for binding constant and total concentration, respectively. For a given value of K_f, [COMP] is known and A_{COMP} may be calculated from eq. 1 for each $[CD]_T$. Standard deviation over A_{COMP} is minimized relative to K_f to obtain the 1:1 association constant.

Brain Capillaries Endothelial Cells. BCECs were isolated and characterized as described by Méresse et al. (1989). The use of cloned endothelial cells allowed us to obtain a pure endothelial cell population without contamination by pericytes. The cells were cultured in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated calf serum and 10% (v/v) horse serum (Invitrogen, Auckland, New Zealand), 2 mM glutamine, 50 μg/ml gentamicin, and basic fibroblast growth factor (1 ng/ml, added every other day).

Rat Glial Cells. Primary cultures of mixed glial cells composed of 60% astrocytes, 20% oligodendrocytes, and 20% microglia were prepared from newborn rat cerebral cortex (Descamps et al., 2003). After removing the meninges, the brain tissue was forced gently through a nylon sieve, as described by Booher and Sensenbrenner (1972). Glial cells were plated on six multiwell dishes at a concentration of 1.2×10^5 cells/ml in 2 ml of DMEM supplemented with 10% (v/v) fetal calf serum (Invitrogen), and the medium was changed twice a week. Three weeks after seeding, cultures of glial cells were stabilized and used for coculture.

Preparation of Filters for Coculture. Culture plate inserts (Millicell PC 3 μm, 30 mm diameter; Millipore, SAS, Molsheim, France) were coated on the upper side with 150 μl of a 2-mg/ml solution of rat tail collagen containing 10-fold concentrated DMEM plus 0.3 M NaOH. The coated inserts were dried for 1 h at 37°C and were rinsed twice with water and once with calcium- and magnesium-free phosphate-buffered saline (PBS-CMF) before being placed in complete medium.

Experimental Method for Coculture. Cultures of glial cells were prepared as described above. After 3 weeks, coated filters were set in six multiwell dishes containing glial cells. 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 was the same as that for BCECs. Under these conditions, BCECs formed a confluent monolayer after 7 days. Experiments were performed 5 days after confluence.

Transport Experiments. During the permeability study, RH solution was added to the lower compartment (abluminal side) of a six-well plate (2.5 ml/well). Filters containing endothelial cells were transferred into six-well plate containing RH solution. RH solution (1.5 ml) containing CD and/or DOX (or not, control) was placed, at time 0, in the upper compartment (luminal side). Incubations were performed at 37°C. At 30, 60, and 120 min, inserts were transferred to other wells to minimize the possibility of passage from the lower compartment. For each condition, three inserts with BCEC monolayer and three without cells were assayed. Amounts of CD in the lower compartment were analyzed by liquid chromatography-mass spectrometry. These analyses were performed using a Finnigan P4000 pump (Courtaboeuf, France). The column was a Polymer Laboratories PLRPS-S column (250 × 4.6 mm; Marseille, France). The binary eluent system consisted of acetonitrile + 0.05% formic acid (eluent A) and water + 0.05% formic acid (eluent B). Gradient elution was performed using 2% eluent A at the beginning of the analysis and then a linear gradient from 2 to 20% eluent A for 20 min at the flow rate of 400 μl/min for γ-CD or a linear gradient from 2 to 60% eluent A for 20 min at the flow rate of 400 μl/min for HP-γ-CD. An injection volume of 20 μl was used for samples without pretreatment.

Mass spectrometry analysis was performed with a Finnigan LCQ-DUO mass spectrometer that was equipped with an electrospray interface operating in the positive ion mode. Selected ion monitoring was used for quantitative analysis monitoring the $(M+H)^+$ of m/z 1297.1 for γ-CD and 1587.2 for HP-γ-CD corresponding at a degree of substitution equal to 5.

Amounts of DOX in the lower compartment were measured in a liquid scintillation counter (Tri-Carb 2100TR; Packard, Warrenville, IL). The total DOX concentration (1 μM) was composed of radioactive DOX (431 nM) plus unlabeled DOX (569 nM).

Using the same methodology, the integrity of the BCEC monolayer was checked by adding [^{3}H]inulin in the upper compartment containing the tested solutions. Amounts of radiotracer in the lower compartment were measured in a liquid scintillation counter.

The amount of DOX, inulin, or CD crossing the BBB was expressed as endothelial permeability (Pe). It was calculated in centimeters per minute as described by Dehouck et al. (1992). In this calculation, both filter permeability (Pf = insert filter + collagen coating) and filter plus cell permeability (Pt = filter + collagen +

endothelial cells) were taken into account, according to the formula $1/Pe = 1/Pt - 1/Pf$.

Electrical Resistance Measurements. Electrical resistance of the cultures was measured using a Millicell-ERS apparatus (Millipore). In each experiment, the resistance of the collagen-coated filter was measured and subtracted from the resistance of the cells on the filter to yield the resistance due to the endothelial monolayer itself.

Fluorescence Microscopy. Tight junctions were visualized by occludin staining. BCECs were fixed with 1% paraformaldehyde in PBS-CMF at room temperature. After washing with PBS-CMF, cells were permeabilized with 0.1% (v/v) Triton X-100/PBS-CMF for 10 min and preincubated in 10% (v/v) normal goat serum/PBS-CMF for 30 min. Primary antibody against occludin was added (1/200 dilution in 2% (v/v) normal goat serum/PBS-CMF) for 60 min. Secondary antibody was added for 60 min. The filters and their attached monolayers were mounted on glass microscope slides with Mowiol mountant (Hoechst, Frankfurt, Germany), and the specimens were visualized and photographed with fluorescence microscope (Leica, Wetzlar, Germany).

Results

Characterization of γ-CD/DOX and HP-γ-CD/DOX Complexes. The γ- or HP-γ-CD has been used to modify the transport of DOX across the BBB. In aqueous medium, γ-CD forms an inclusion complex with DOX as proved by NMR (Djedaïni et al., 1990), fluorescence (Husain et al., 1992), and absorbance spectroscopy (Bekers et al., 1990). The stoichiometry has been determined to be 1:1, and the association constant value differed according to experimental technique varying between 200 and 1000 M^{-1}. Because our transport experiments across the BBB were undertaken in a physiological buffer (RH), the RH influence has also been investigated. Thus, the stoichiometry and the stability constant of γ-CD or HP-γ-CD/DOX complex were determined in RH.

The stoichiometry of these inclusion complexes was provided by the continuous variation technique. In presence of each CD, the absorption intensity of DOX exhibited variations. The Job's plots derived show a maximum for a ratio (r) equal to 0.5, indicating the formation of a 1:1 inclusion complex in RH. The association constants of each inclusion complex were evaluated by the way of UV-visible spectroscopy at 298 K. The values calculated by assuming a 1:1 inclusion complex are equal to $1360 \pm 70\ M^{-1}$ and $320 \pm 20\ M^{-1}$ for γ- and HP-γ-CD/DOX complexes, respectively.

Transport Studies of DOX Alone. Before studying the influence of CDs on the transport of DOX across the BBB, the transport of DOX alone was performed. All experiments were carried out on our in vitro model of BBB presenting most of the in vivo BBB characteristics (Cecchelli et al., 1999). DOX at varying concentrations was added on the luminal side of the monolayer of BCECs, and the DOX transport was observed after a 120-min incubation. The results were expressed by using the endothelial permeability coefficient for DOX [$Pe_{(DOX)}$]. Using the same procedure, the integrity of the BCEC monolayer was checked by adding radiolabeled inulin on the monolayer. Inulin was used as an indicator of the functional integrity of the tight junctions, and an endothelial permeability coefficient for inulin [$Pe_{(inulin)}$] higher than 0.4×10^{-3} cm/min was indicative of a leaky BBB. In our in vitro BBB model, the $Pe_{(inulin)}$ across the monolayer was less than 0.2×10^{-3} cm/min (mean value of $0.15 \pm 0.03 \times 10^{-3}$ cm/min) under control conditions. Moreover, the initial integrity of BCECs before each experiment was checked by measuring the electrical resistance; the mean value was equal to $660 \pm 50\ \Omega \cdot cm^2$. As shown in Fig. 1, transport of DOX across the BCEC monolayer was low and constant [$Pe_{(DOX)} = 0.4 \times 10^{-3}$ cm/min] for concentrations varying from 1 to 10 μM. However, fluorescent cell nuclei revealed an accumulation of DOX in the BCEC at concentrations as low as 1 μM (Fig. 5a). The $Pe_{(DOX)}$ increased sharply at 20 μM to reach a maximum at 50 μM ($Pe = 4.2 \times 10^{-3}$ cm/min). An increase in $Pe_{(inulin)}$ was equally observed at 20 μM DOX. This increase was directly related to the loss of BBB integrity as shown by the change in endothelial permeability of inulin (0.2×10^{-3} cm/min for control versus 0.75×10^{-3} cm/min for 20 μM). So, the BBB integrity is not modified up to 10 μM DOX.

Transport Studies of γ-CD and HP-γ-CD. Transport of γ-CD and HP-γ-CD alone across the BBB were equally performed and the integrity of the BCEC monolayer was checked by adding inulin. As shown in Fig. 2a, the endothelial permeability coefficient for γ-CD [$Pe_{(\gamma\text{-CD})}$] was about 0.3×10^{-3} cm/min for concentrations varying from 1 to 15 mM and increased at 20 mM (0.78×10^{-3} cm/min). In Fig. 2b, the same behavior was observed for HP-γ-CD: the $Pe_{(HP\text{-}\gamma\text{-}CD)}$ was approximately 0.3×10^{-3} cm/min up to 35 mM and reached the value of 1.96×10^{-3} cm/min at 50 mM. In each case, the rise of $Pe_{(CD)}$ is correlated with the loss of barrier properties as indicated by the higher Pe of inulin.

Effect of S9788 as a Reversing Agent of P-Glycoprotein on DOX, γ-CD, and HP-γ-CD Transport. S9788 is a triazino-aminopiperidine derivative and a P-gp reversing agent. To measure the reversal activity of S9788 on P-gp, BCECs were incubated with DOX, γ-CD, or HP-γ-CD either alone or with S9788; and in each case $Pe_{(DOX)}$ or $Pe_{(CD)}$ and $Pe_{(inulin)}$ are presented in Fig. 3. A concentration of 1 μM was chosen for S9788 because it has already been used for in vitro experiments in this BBB model (Fenart et al., 1998). As a control experiment, 1 μM S9788 was incubated on BCECs, and the BBB integrity was preserved as shown by inulin permeability (0.15×10^{-3} cm/min). In presence of S9788 and DOX (1 μM), $Pe_{(DOX)}$ and $Pe_{(inulin)}$ increased by a factor 2.2 and 4.5, respectively, in comparisons with DOX alone. This result shows clearly that DOX transport is increased in presence of a P-gp inhibitor but that this increase is due to a loss of BBB integrity. In this case, the threshold of DOX toxicity is decreased. No change in $Pe_{(CD)}$ and $Pe_{(inulin)}$ was observed for a coincubation of γ-CD or HP-γ-CD (1 mM) and S9788. Therefore, we can conclude that these two CDs are unlikely to be P-gp substrates.

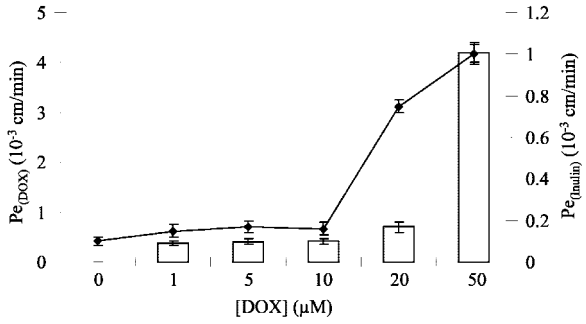

Fig. 1. Effect of DOX concentration on endothelial permeability coefficient for DOX (column) and inulin (curve) after 120 min of incubation. Transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments.

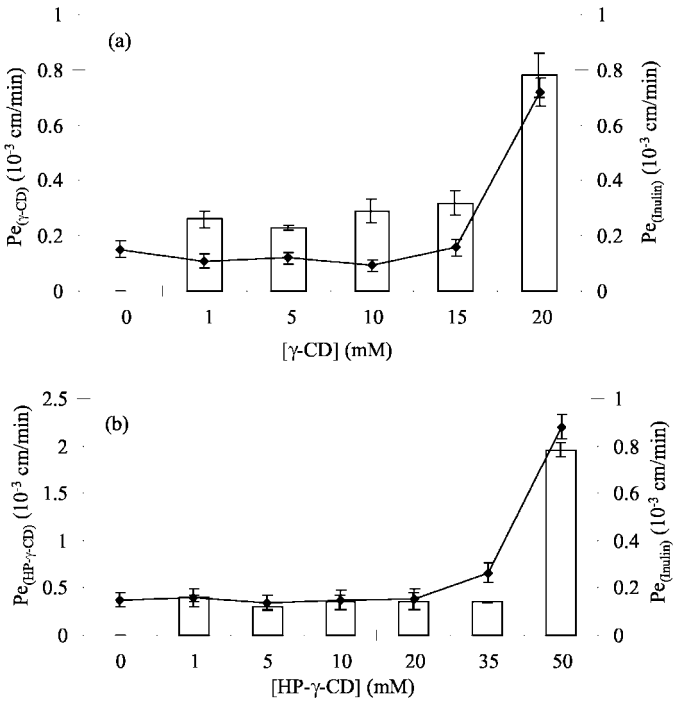

Fig. 2. Effect of CD concentration on endothelial permeability coefficient for γ-CD (a, columns) and HP-γ-CD (b, columns) and inulin (curve) after 120 min of incubation. Transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments.

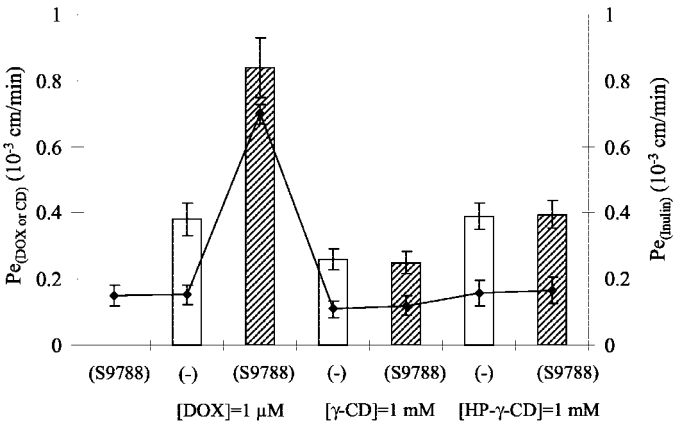

Fig. 3. Effect of S9788 on endothelial permeability for DOX, γ-CD, and HP-γ-CD (columns) and on endothelial permeability for inulin (curves) after 120 min of incubation. BCECs were incubated with 1 μM DOX or 1 mM γ-CD or 1 mM HP-γ-CD in absence (open columns) or in presence (hatched columns) of 1 μM S9788. Transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments.

Transendothelial Transport Studies of DOX in the Presence of γ-CD or HP-γ-CD. To evaluate the ability of CD/DOX to modify the brain delivery of DOX, the DOX concentration was fixed at 1 μM to avoid any toxic effect toward the integrity of the BBB. Amount of CD added has been chosen in function of percentage of free or complexed DOX. Table 1 summarizes the different percentages in CD or DOX used, according to the initial CD concentration and association constant. A high ratio CD/DOX led to a low free DOX concentration. To complex 90% of DOX, the CD concentration must be equal to 10 and 35 mM for γ-CD and HP-γ-CD, respectively. In these cases, the percentage of free CD is greater than 99.5%.

TABLE 1
Percentage of complexed DOX and CD as a function of CD concentration and association constant (in each case [DOX] = 1 μ M)
The percentage of complexed species was calculated from eq. 2 described under *Materials and Methods.*

	CD concentration	Complexed DOX	Complexed CD
	mM	*%*	
γ-CD	1	56	1.00
	5	87	0.20
	10	93	0.10
	15	95	0.07
	20	96	0.05
HP-γ-CD	1	23	1.00
	5	60	0.20
	10	75	0.10
	20	85	0.05
	35	91	0.03
	50	93	0.02

Figure 4a represented the evolution of DOX transport as a function of γ-CD concentration. DOX transport to the brain at 1 μM without CD corresponds to 100% and is used as a reference. Addition of γ-CD, up to 10 mM, decreased slightly the transport of DOX, whereas in presence of 20 mM γ-CD, an increase in DOX delivery was observed (up to 180%). $Pe_{(inulin)}$ decreased equally up to 10 mM and increased from 20 mM (0.09×10^{-3} cm/min at 10 mM versus 0.38×10^{-3} cm/min at 20 mM). The latter increase is directly related to the loss of cell monolayer integrity.

Interestingly, addition of HP-γ-CD, at 1 mM, led to a slight increase of 20% in DOX transport (Fig. 4b). However, this increase was nonsignificant as demonstrated by the mean scores obtained using one-way analysis of variance/Dunnett's

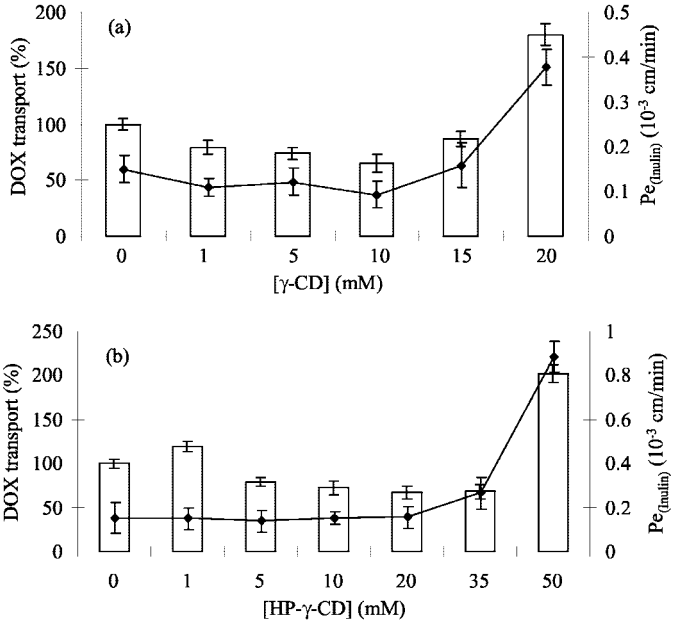

Fig. 4. Effect of γ-CD (a) and HP-γ-CD (b) concentration on DOX transport (columns) and on endothelial permeability for inulin (curves) after 120 min of incubation. The transport of DOX across BCECs was expressed according 100% to the reference (1 μM DOX without CD). Transport studies were conducted at 37°C in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments.

test ($p = 0.122$ versus control). Then, addition of HP-γ-CD, from 5 to 35 mM, decreased the DOX delivery, and at 50 mM, an increase up to 200% was observed. $Pe_{(inulin)}$ was not modified up to 20 mM, and the increase (up to 0.9×10^{-3} cm/min), at 50 mM, is directly related to the loss of cell monolayer integrity.

Immunofluorescence Stainings. The previous results were confirmed by immunofluorescence stainings of occludin (a tight junction protein) after the BCECs have been incubated with or without γ-CD in presence of 1 μM DOX. Occludin stainings of BCECs, without γ-CD, revealed a reticular pattern at intercellular regions between all cells, indicating that the cells were sealed together by highly differentiated tight junctions (Fig. 5a). Additional immunostainings were undertaken in the presence of increasing γ-CD concentrations. No difference between control and BCECs in presence of γ-CD at 5 mM was observed (Fig. 5b). When the cells were incubated with γ-CD at 20 mM, the occludin staining depicted disruptions and large gaps between cells, showing a loss of integrity (Fig. 5c). The results are in accordance with our inulin permeability studies. Immunofluorescence stainings in presence of HP-γ-CD were comparable with those of γ-CD (data not shown).

Discussion

In this work, γ-CD or HP-γ-CD has been used to increase DOX transport across the BBB. The transport of DOX alone was first examined. At low doses from 1 to 10 μM, DOX crosses slightly the BBB. These low permeabilities could be explained by the presence of P-gp. Indeed, DOX is a P-gp substrate and so actively pumped out the BCECs as shown by Fenart et al. (1998) and confirmed by the experiments with a P-gp inhibitor (Fig. 3). Nevertheless, our results cannot exclude that the breast cancer resistance protein (BCRP/ABCG2) may also play a role in the disposition of DOX in our cell system. Indeed, this protein has been recently detected at the BBB and acts as a drug efflux pump (Eisenblatter et al., 2003; Cisternino et al., 2004). Moreover, DOX has been recently described as a BCRP substrate (Han and Zhang, 2004). At higher doses (>20 μM), we observe that DOX reached the brain compartment due to the BBB breakdown as indicated by $Pe_{(inulin)}$. DOX accumulates in the BCEC nucleus as shown by our fluorescence study (data not shown), and this accumulation leads to a BBB disruption. Indeed, DOX is capable of generating a variety of free radical species that are toxic for the cells (Keizer et al., 1990; Leblanc et al., 1991).

The transport of γ- and HP-γ-CD alone was studied. The Pe of each CD is relatively similar, but the toxicity toward the BBB integrity is 2-fold lower for HP-γ-CD. The origin of toxicity is probably related to interactions between CDs and membrane lipids. Indeed, as shown on our in vitro BBB model, γ-CD is able to extract cholesterol (13% compared with control) and phospholipids (20% compared with control) from BCEC membranes (Monnaert et al., 2004). Furthermore, a marked reduced toxicity for HP-γ-CD in comparison with γ-CD has already been described toward P388 murine leukemia cells (Leroy-Lechat et al., 1994).

Then, the association between CD and DOX has been performed to increase the DOX transport to the brain. The abrupt increase in DOX delivery in presence of each CD is directly related to the opening of the BBB tight junctions as indicated by $Pe_{(inulin)}$ and immunostainings. This opening corresponds to concentrations where each CD alone leads to a loss of BBB integrity and no effect on P-gp inhibition by CD is observed. So, this increase in DOX concentration into the brain compartment is directly related to CD toxicity, and no supplementary toxic effect due to DOX was observed. As already described above, lipid effluxes by CD were probably responsible for this BBB breakdown. In nontoxic conditions, the behavior of γ- and HP-γ-CD is almost similar. The complexation of DOX decreases its transport to the brain and its toxicity toward BCECs. This phenomenon shows clearly that the inclusion complex does not improve the DOX transport and that only the free DOX is available. A similar behavior has been observed with the association of flutamide (used in prostate cancer chemotherapy) and HP-β-CD using the Caco-2 in vitro model (Zuo et al., 2000). The permeability coefficient for this drug across a Caco-2 cell monolayer increased when HP-β-CD concentrations were reduced, indicating that the passage was due to freely available flutamide.

So, the increase in DOX delivery in presence of CDs is due to the loss of BBB integrity. These results could be compared with studies performed on a Caco-2 cell monolayer, which is often used to predict toxicity or permeability for a drug toward the BBB. Indeed, CDs are able to increase the delivery or bioavailability of drugs in the Caco-2 cell system. Arima et al. (2001) reported the enhancing effect of dimethyl-β-CD on the oral bioavailability of tacrolimus in Caco-2 cells. In addition, dimethyl-β-CD and HP-β-CD exhibited 22- and 19-fold permeability coefficient enhancement of cosalane (a potent inhibitor of HIV), respectively (Udata et al., 2003). The authors concluded that CDs can promote transport by altering membrane fluidity with a minimal disruption of cellular bar-

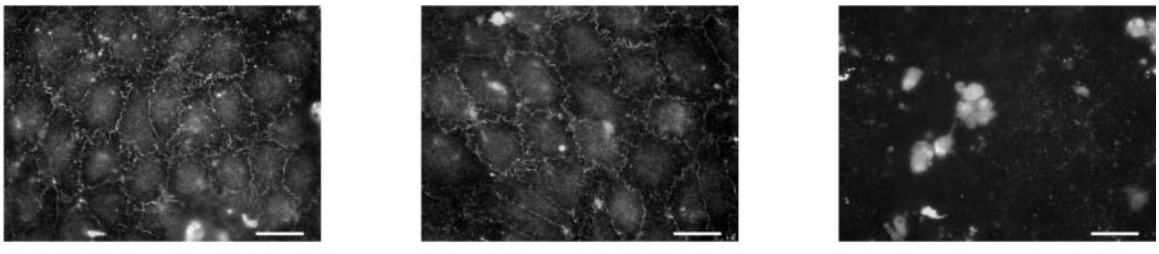

Fig. 5. Occludin immunofluorescent staining of BCECs after 120 min of incubation in the absence (a) or the presence of 5 mM (b) or 20 mM γ-CD (c). In each case, [DOX] = 1 μM. Scale bar, 25 μm.

rier. These selected results and others (Hovgaard and Brondsted, 1995; Ventura et al., 2003) show without ambiguity the beneficial effect of CD to increase drug delivery on in Caco-2 cell monolayers. So, as already described by Lundquist et al. (2002), our study reinforces the fact that correlation between Caco-2 and BBB model must be proscribed. BCECs are more sensitive and contrary to other cell systems only a slight extraction and/or reorganization of the membrane components is acceptable, otherwise a loss of BBB integrity occurs.

In conclusion, the use of γ-CD or HP-γ-CD at nontoxic concentrations does not increase the DOX delivery to the brain. Moreover, as DOX and the CDs used form inclusion complexes, DOX delivery seems to be decreased. So, CDs could slightly modify the BBB fluidity to increase the delivery of noncomplexed drug. Other experiments are running to study the coadministration of a CD and a drug that do not associate.

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