Effect of E6, a novel calmodulin inhibotor, on activity of P-glycoprotein in purified primary cultured rat brain microvessel endothelial cells[1]

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KEY WORDS E6; P-glycoprotein; blood-brain barrier

ABSTRACT

AIM: To study the effect of E6, a novel calmodulin inhibitor, on the activity of P-glycorprotein (P-gp) in purified primary cultured rat brain microvessel endothelial cells (RBMEC). **METHODS:** Flow cytometry was used to purify RBMEC from other cell types. Fluorescence of P-gp substrate rhodamine123 (Rh123) in RBMEC was measured to evaluate the effect of E6 on the function of P-gp. **RESULTS:** Purified RBMEC was obtained after being sorted from other cell types by flow cytometry. Intracellular fluorescence of Rh123 in RBMEC increased after incubation with E6 3, 10 μmol/L or verapamil (VER) 50 μmol/L ($P<0.01$), respectively. After the treatment of E6 10 μmol/L, intracellular Rh123 accumulation was increased nearly to 3-fold. E6 and VER have no effect on accumulation of Rh123 in human umbilical vein endothelial cells (HUVEC), which do not express P-gp. **CONCLUSION:** E6 exhibited a strong inhibitory effect on the activity of P-gp in RBMEC.

INTRODUCTION

P-glycoprotein (P-gp) is an ATP-dependent drug efflux pump associated with multidrug resistance (MDR) in cancer chemotherapy. P-gp consists of a glycosylated 140-170 kDa protein with 12 transmembrane domains and two cytoplasmically located ATP-binding sites. It has been shown that P-gp could actively transport a wide variety of agents, including anticancer drugs, peptides, steroids, calcium channel blockers and antihistamines out of cells, thus reducing their intracellular concentrations[1].

In addition to its expression in some MDR tumors, P-gp is also found in a large number of normal tissues including adrenal medulla, liver, kidney, colon, and endothelial cells of brain microvessel[2,3]. Brain microvessel constitute a selective barrier between blood and brain-blood brain barrier (BBB). Although the function of P-gp in BBB remains under active investigation, the protein appears to protect the brain from many exogenous toxins and abrupt change in the levels of cerebral transmitters. Hydrophobic anticancer drugs, such as vincristine, and lipid-soluble agents, such as cyclosporin A (CsA), cannot accumulate in the brain. It has been proposed that P-gp expressed in the capillaries endothelial cells of BBB is responsible for the extrusion of these agents[3].

Some P-gp inhibitors, such as CsA, PSC833, verapamil (VER) have been shown to restore the intracellular accumulation of P-gp substrate drugs in MDR

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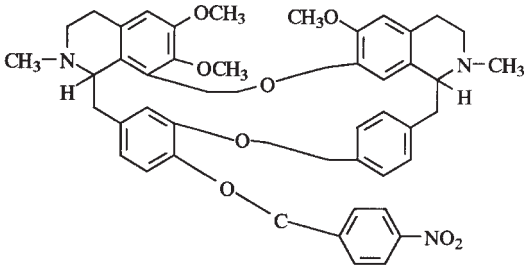
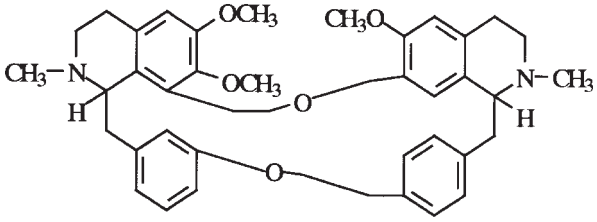
tumor cells[4]. They also inhibit the activity of the P-gp expressed in normal tissues including BBB[5]. Administration of P-gp inhibitors may result in an improvement of drug delivery into the brain to treat diseases of CNS, such as psychiatric disorders and primary malignant brain tumors[6].

E6, a derivative of berbamine which belongs to bi-benzylisoquinolines, is a potent calmodulin antagonist[7]. It is reported that E6 has the protective effects on cultured PC12 cells and cerebral ischemia in rats and mice. Some calmodulin inhibitors were found to have the inhibitory effect on the activity of P-gp in MDR tumor cells to increase the intracellular accumulation of P-gp substrates[8-10]. In this experiment, we investigated the effect of E6 on the accumulation of the fluorescent P-gp substrate, rhodamine123 (Rh123), in purified primary cultured RBMEC to evaluate the potential effect on the function of BBB using fluorescence spectrophotometry and flow cytometry.



E6



Berbamine

Chemical structures of E6 and berbamine

MATERIALS AND METHODS

Materials The cell line of human umbilical vein endothelial cells (HUVEC) was a gift from Dr HE Ling (China Pharmaceutical University). E6 was kindly provided by Prof HUANG Wen-Long (China Pharmaceutical University). Rh123 labeled acetylated-low density lipoprotein (Rh123-Ac-LDL) was kindly donated by Dr TANG Zhi-Hua (China Pharmaceutical University). P-gp monoclonal antibody (C219) was a product of Calbiochem-Novabiochem Corporation. FITC labeled sheep anti-mouse IgG, was purchased from Sino-American Biotechnology Company. Verapamil was a product of Sigma Co. All other chemicals were of analytical grade and commercially available.

Isolation of rat brain microvessel endothelial cells Endothelial cells were isolated from rat brain according to a modified method of Abbott *et al*[11]. Briefly, cortex was obtained from ten rats' brain and placed in ice cold phosphate-buffered saline (PBS). After remove of surface vessels and meninges, cortex gray matter was minced and incubated at 37 ºC for 20 min in D'hanks containing 0.05 % trypsine. Then the samples were passed through a 150-μm nylon mesh. After centrifugation at 800×*g* for 5 min, the pellet was resuspended in PBS containing 25 % bovine serum albumin (BSA) and centrifuged at 2000×*g* at 4 ºC for 10 min. Fat, cell debris, and myelin floating on BSA were discarded and the pellet containing microvessels was resuspended and incubated at 37 ºC for 30 min in Dulbecco's modified Eagle medium (DMEM) containing 0.1 % collagenase II. The microvessels were finally collected by centrifugation at 800×*g* for 5 min; then the pellet was washed twice with PBS and cultured in culture medium consisting of DMEM/F12 (1:1) supplemented with 20 % heated-inactivated fetal bovine serum, penicillin 100 kU/L and streptomycin 100 mg/L in culture dishes precoated with gelatin at 37 ºC in a 5 % CO_2 humidified atmosphere.

Purification of rat brain microvessel endothelial cells by flow cytometry Purification of RBMEC is based on Rh123-Ac-LDL being uptaken by microvessels endothelial cells via the "scavenger cell pathway" of LDL metabolism[12]. Primary cultured RBMEC were labeled with 10 mg/L Rh123-Ac-LDL at 37 ºC for 4 h. The labeled cells were washed twice with PBS and digested to single cell suspensions by 0.25 % trypsine. The samples were centrifuged at 800×*g* for 5 min and resuspended in serum-free DMEM. PC12 cells were treated in the same way as above for a negative control. Flow cytometry was used to sort Rh123-Ac-LDL labeled RBMEC from other cell types. Cells were analyzed with flow cytometry (Becton Dickinson, FACS Calibur) using excitation and emission wavelengths of 488 and 530 nm respectively. The cells of high fluorescent group were collected. The samples were gated on forward scatter versus side scatter to exclude cell debris and clumps. After the sort was completed, the cells were pelleted, washed once, and plated in gelatin-coated culture plates for routine culture.

Detection of P-gp by immunocytochemistry Expression of P-gp on confluent monolayers of cultured RBMEC was detected by immunocytochemistry. Briefly, monolayers of cells were washed, fixed with cold acetone for 10 min. After preincubation of PBS containing 1 % BSA at room temperature for 30 min to block sites of unspecific binding, cells were incubated with PBS containing 10 mg/L P-gp monoclonal antibody-C219 and 1 % BSA for 60 min. Then after two rinses in PBS, cells were incubated with 0.6 g/L sheep-anti-mouse-IgG-FITC at room temperature for 60 min. Fluorescence of FITC was examined with Fluorescence Microscope (Leica). Human umbilical vein endothelial cells (HUVEC), which do not express P-gp, were used as a negative control.

Rh123 accumulation study by fluorescence spectrophotometry Rh123 is a fluorescent dye that could be used as a function assay for P-gp[13,14]. RBMEC were seeded at a density of 5×10^7 cells/L in 24-well plates. After reaching confluence, cell monolayers were preincubated with serum-free DMEM at 37 ºC for 30 min, then the culture media were removed, and cell monolayers were exposed to 5 μmol/L Rh123 in DMEM containing E6 0.3, 1, 3, 10 μmol/L or VER 50 μmol/L as a positive control at 37 ºC for 90 min. After incubation, the dye solutions were removed, and cell monolayers were washed three times with ice-cold PBS and then solubilized in 1 % Triton X-100. Fluorescence of Rh123 was determined using fluorescence spectrophotometer and concentration of Rh123 was measured from the fluorescence value by the construction of a Rh123 standard curve. The amount of Rh123 in cell samples was normalized with the amount of protein in each sample as determined by the Blandford assay[15,16].

Flow cytometric analysis of Rh123 accumulation in RBMEC Single cell suspensions obtained by trypsinization from confluent monolayers of RBMEC, were incubated at 37 ºC for 90 min in the presence of E6 0.1, 1, 10 μmol/L or VER 50 μmol/L in serum-free DMEM containing Rh123 2 μmol/L. After incubation, the cells were collected by centrifugation at 800×*g* for 5 min, resuspended in ice-cold PBS containing 1 % fetal bovine serum and kept on ice until analysis by flow cytometry. Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 530 nm pass filter. As a negative control, human umbilical vein endothelial cells (HUVEC) were treated in the same way as above[17,18]. Data analysis was performed using Cell Quest software with cells gated on a dot plot of FSC versus SSC.

Statistical methods Data were represented as mean±SD. Statistical analysis was made on comparison of sample means using paired *t*-test.

RESULTS

Cell culture and purification After 1-3 d of primary culture, spindle-shaped endothelial cells were seen growing out of capillary fragments and became confluent at 8-10 d. In the initial stages of the culture, some non-spindle-shaped cells were found in the culture plates. The results of flow cytometric analysis demonstrated that the whole primary cultured cells could be divided into two groups depending on the difference of Rh123-Ac-LDL metabolism: high fluorescent group and low fluorescent group, in comparison with that, negative control-PC12 has just one group (Fig 1).

The cells of high fluorescent group were sorted by flow cytometry and cultured under normal condition. The monolayers of endothelial cell could be formed about 10 d later, and other type cells could hardly be found in the monolayers (Fig 2).

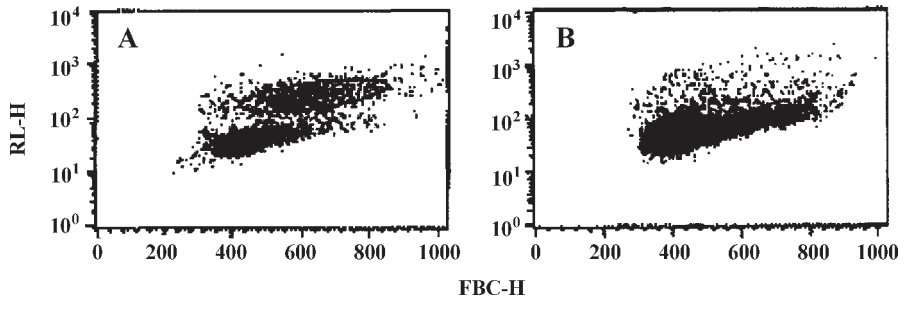


Fig 1. Primary cultured cells have two groups: high fluorescent group and low fluorescent group (A), and negative control-PC12 has just one group as a comparison (B).

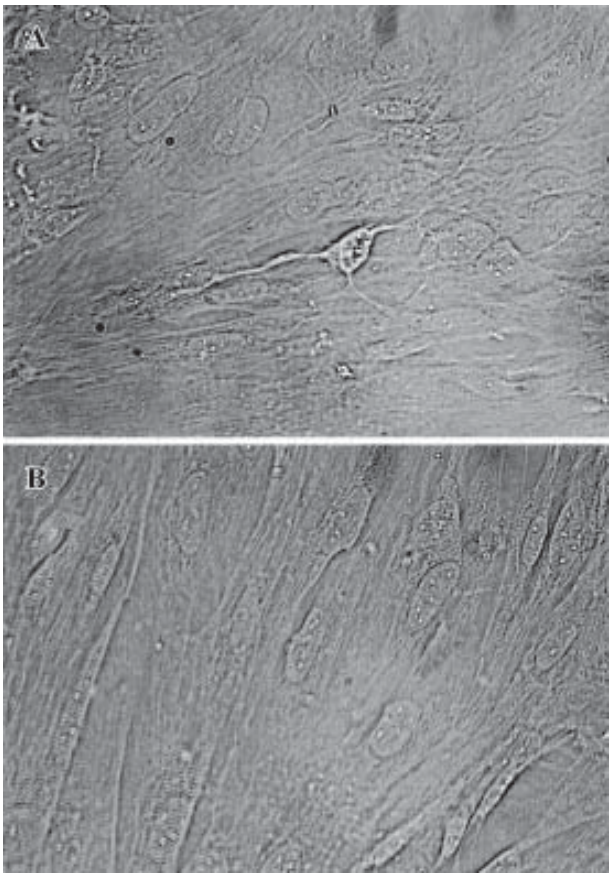


Fig 2. The primary cultured monolayer of endothelial cells contained some non-endothelial cells before purification (A). Other types of cells could hardly be found in the monolayer of endothelial cells after being sorted by flow cytometry (B). ×400.

Expression of P-gp in purified RBMEC P-gp monoclonal antibody C219 has been widely used to detect P-gp in RBMEC[19]. A strong fluorescence intensity of FITC which indicated the expression of P-gp was observed in RBMEC. The result showed that high levels of P-gp were expressed in purified RBMEC on our experimental conditions. No detectable expressions of P-gp were observed in HUVEC (Fig 3).

Effect of E6 on the accumulation of Rh123 in RBMEC determined by fluorescence spectrophotometry Obvious increases of Rh123 accumulation in RBMEC were observed following the treatment of P-gp inhibitor VER 50 μmol/L. In the E6 treated group, the amount of intracellular Rh123 increased after a 90 min-incubation period in the presence of E6 1, 3, 10 μmol/L. After the treatment of E6 10 μmol/L, intracellular Rh123 accumulation was increased nearly to 3-fold (Tab 1).

Flow cytometry Flow cytometric analysis was performed to evaluate the ability of P-gp to efflux its substrate Rh123, which could be excited effectively at a single wavelength (488 nm) and emission spectra taken at near 530 nm and could act as a target to monitor the pumping effect of P-gp. After a incubation at 37 ºC for 90 min in the presence of E6 0.1, 1, 10 μmol/L or VER 50 μmol/L, the amount of intracellular fluorescence of Rh123 was identified by the channel number of the fluorescent peak in fluorescence histograms. The results showed that P-gp inhibitor VER 50 μmol/L increased fluorescent intensity of Rh123 in RBMEC and move

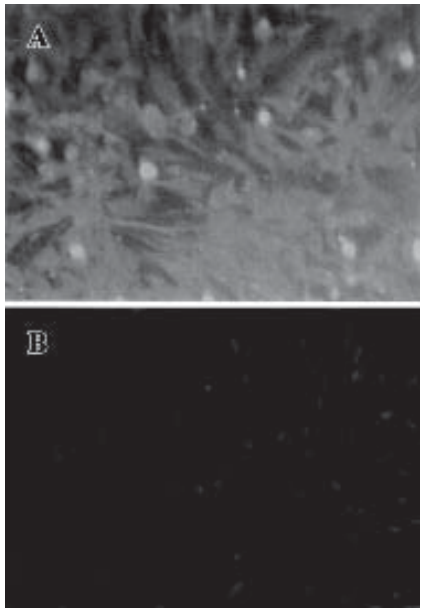


Fig 3. High levels of P-glycoprotein were expressed in purified RBMEC (A). No detectable expressions of P-glycoprotein were observed in HUVEC (B). ×200.

Tab 1. Effect of E6 on intracellular Rh123 accumulation in RBMEC. The intracellular accumulation of Rh123 was measured after incubation with Rh123 5 μmol/L at 37 ºC for 90 min in the presence of E6 or VER. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group.

Group	Drug concentration/ $\mu mol \cdot L^{-1}$	Rh123/ $nmol \cdot g^{-1}$ (protein)	Increase of cellular Rh123/%
Control	-	7.9±0.2	-
VER	50	22.8±2.1^{c}	189
E6	0.3	7.9±0.3	0
	1	11.4±1.2^{b}	44
	3	15.8±0.4^{c}	100
	10	20.4±1.0^{c}	158

the fluorescent peak to higher FL1- height, but had no effect on the fluorescence in HUMEC, which do not express P-gp. Rh123 fluorescence in RBMEC increased after incubation with E6 1, 10 μmol/L, but no change occurred in HUVEC under the same condition (Fig 4).

DISCUSSION

Primary cultured BMEC is a useful tool to study the function of BBB *in vitro*. But routine methods of isolation could not completely discard other cells such

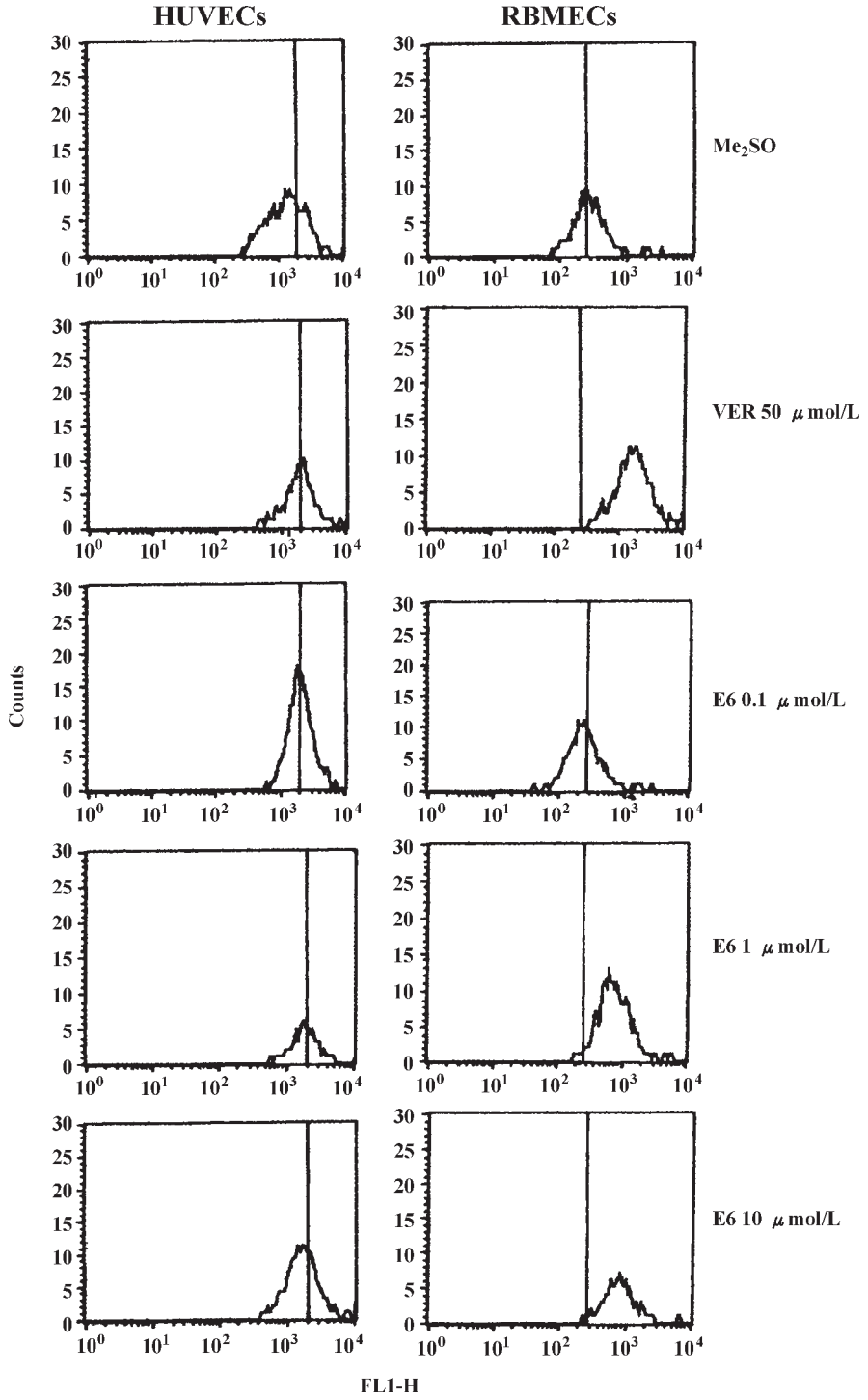


Fig 4. Intracellular Rh123 accumulation was determined by analysis of fluorescence histograms using flow cytometry. Rh123 fluorescence in RBMEC increased after the incubation with Rh123 2 μmol/L at 37 ºC for 90 min in the presence of VER 50 μmol/L or E6 1, 10 μmol/L. No difference was observed under the same condition in HUVEC which do not express P-gp.

as astrocytes and neurons which will influence the formation of tight junction. The uptake and degradation of LDL by endothelial cells is well documented and has provided a means for identification and isolation of endothelial cells[12]. In the present study, we used FCM to sort RBMEC from other cells based on the difference of LDL metabolism and obtained purified RBMEC. These cells displayed a typical capillary cell morphology and expressed a high level of P-gp.

BBB plays a major role in the maintenance of cerebral microenvironment. P-gp is expressed at high level in brain capillaries which consist of BBB[20]. P-gp in BBB is considered to regulate the access of certain molecules to the central nervous system, or in the secretory functions of the BBB. P-gp inhibitors could block the activity of P-gp located in MDR tumor cells and lead to recover the sensitivity of tumor cells to anticancer drugs. P-gp inhibitors also inhibited the activity of P-gp in BBB and increased the concentration of P-gp substrate drugs in brain. For example, Rh123 concentration in brain measured by microdialysis could be increased 3-4 fold by intravenous infusion of P-gp inhibitor-CsA[21].

Most of the central nervous system (CNS) drugs for the treatment of brain tumors, disfunction of brain neuronal cells, bacterial and viral infections in brain need to penetrate BBB and take pharmacological effect in brain. Optimal treatments of these diseases were severely limited by the difficulty to obtain sufficient concentrations of the therapeutic drug in brain. When potentially effective drugs are known to be good P-gp subatrates, it may be useful to improve the brain penetration of these drugs by co-administration of effective P-gp inhibitors[22]. Experiments in mice demonstrated that it may be feasible and acceptable to obtain an obvious increase in brain penetration by this procedure.

E6, a synthetic derivative of berbamine, has a potent calmodulin inhibitory effect. Some calmodulin inhibitors, such as trifluoperazine and nifedepine, have effects on reversion of MDR due to the inhibition of P-gp activity[23,24]. Our experiments demonstrated for the first time that E6 exhibited a strong inhibitory effect on the activity of P-gp in RBMEC and remarkably increased the intracellular accumulation of P-gp substrate Rh123. The inhibitory potency of E6 10 μmol/L was comparable to that of VER 50 μmol/L. The intracellular fluorescence of Rh123 in HUVEC, which has no P-gp expression, was not influenced by E6. Because of the particular role of P-gp in the function of BBB, these results indicated that E6 had a potential influence on the permeability of the BBB.

However, co-administration of P-gp inhibitors with other CNS therapeutic drugs has two effects: (1) P-gp inhibitors could be applied to improving drug delivery into CNS resulting in enhanced drug efficiency; (2) Inhibition of P-gp activity in BBB could increase the risk of central neurotoxicity.

In addition, according to inhibitory effect on the activity of P-gp in RBMEC, E6 may be a potential MDR reversal agent. Therefore, further study may be carried out to investigate the effect of E6 on the function of P-gp on MDR tumor cells.

In summary, our results indicated that flow cytometry could be used to purify RBMEC based on the difference of LDL metabolism between endothelium and other cells. We demonstrated for the first time that E6 exhibited a strong inhibitory effect on the activity of P-gp in RBMEC and had a potential influence on the permeability of the BBB. Although the mechanism of E6 inhibiting P-gp was not clear, our present study revealed that it is possible to improve therapies of some drugs on CNS disorders by co-administration of E6.

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