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Effect of p-glycoprotein inhibitor combinations on drug efflux from rat brain microvessel endothelial cells

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In an effort to develop a clinically useful approach to inhibit the drug efflux across the blood brain barrier (BBB) mediated by P-glycoprotein (P-gp), the combined inhibitory effect of four P-gp inhibitors: cyclosporin A (CsA), verapamil (Ver), tetrandrine (Tet) and doxorubicin (Dox), was evaluated by determining the intracellular concentration of rhodamine 123 in *in vitro* cultured rat brain microvessel endothelial cells (BMEC). The results showed that CsA combined with Ver or Tet synergistically inhibited P-gp mediated efflux of Rh123 from rat BMEC, suggesting that the combined application of P-gp inhibitors would possibly be a useful approach to increase drug concentration in brain tissues, enhance the therapeutic effect and reduce the toxicity of drugs.

1. Introduction

P-Glycoprotein (P-gp), a 170 kD plasma membrane glycoprotein and initially discovered in tumor tissues, has been proved to be one of the major factors responsible for the cellular extrusion and decreasing intracellular concentration of a number of anticancer drugs (Krishna et al. 2000) and inducing multidrug resistance (MDR) in a ATP-dependent manner. P-Gp was also found in many important non-tumor tissues, for instance, small and large intestine, liver, adrenal gland, blood brain barrier (BBB), and blood testis barrier (Cordon-Cardo et al. 1989; Thiebaut et al. 1987). It is apparent that except for leading to MDR, this protein also has multiple physiological functions (Johnstone et al. 2000). In brain capillaries constructing BBB, P-gp plays an important role in preventing hydrophobic molecules (drugs or other substrates) from crossing the BBB and reaching the central nerve system (CNS), resulting in a low accumulation of these compounds in the brain (Schinkel 1999). As we know, the limited penetration of drugs into the brain tissue is a major obstacle for drug therapy of CNS diseases, such as brain tumors, Parkinson's disease, and Alzeimer's disease. Therefore, elevating the concentration of therapeutic drugs in brain tissue by inhibiting the activity of P-gp is considered promising in the treatment of CNS diseases. A number of studies have been done and proved that this approach is feasible and effective (Tsiji et al. 1992).

Numerous drugs have been demonstrated to effectively inhibit the drug efflux activity of P-gp, thereby overcoming MDR or increasing the penetration of drugs into brain tissues. Unfortunately, the optimum concentration for overcoming the drug efflux driven by P-gp is usually far beyond the maximally tolerable plasma levels for many these inhibitors. This seriously limits the clinical use of P-gp inhibitors (Lampidis et al. 1986, 1990). Consequently, better approaches are needed to inhibit the activity of P-gp in drug therapy of CNS diseases, and to increase drug concentration in tumor tissues.

Some experiments have shown that the synergistic inhibition of P-gp activity by the combination of inhibitors is a potential method to decrease the effective concentration of a single inhibitor, thus avoiding their side effects and enhancing their reverse effect in some type of tumor cells (Lehnert et al. 1991; Hu et al. 1990). However, little has been reported on the effect of combination of P-gp inhibitors on drug efflux across BBB.

Based on the study on the interaction of several P-gp inhibitors on ATPase activity of P-gp in rat BMEC in our laboratory (He Ling 2002), we investigated the synergistic inhibitory effect of four classical inhibitors: Cyclosperin A (CSA), doxorubicin (Dox), verapamil (Ver) or Tetrandrine (Tet) on P-gp activity in rat BMEC, in order to explore whether this combined application of drugs would allow lower concentrations of single agents to modulate drug penetration into BBB, and to demonstrate a new valuable and feasible method to improve drug accumulation in brain tissues.

2. Investigations and results

2.1. Effects of individual P-gp inhibitors on the accumulation of Rh123 in rat BMEC

The determination of the intracellular fluorescence of Rh123 showed that Rh123 in rat BMEC incubated with CsA ($0.1–5\ \mu mol \cdot L^{-1}$), Ver ($0.5–10\ \mu mol \cdot L^{-1}$), Dox ($1–20\ \mu mol \cdot L^{-1}$) or Tet ($0.5–10\ \mu mol \cdot L^{-1}$.) was significantly increased in a dose-dependent manner, respectively. The Inhibitory effect was CsA > Tet > Ver > Dox.

Table 1: Effect of CsA alone and in combination with Ver, Tet or Dox on the accumulation of Rh123 in rat BMEC

Group	Concentration of inhibitor ($\mu mol \cdot L^{-1}$)	Content of Rh123 ($nmol \cdot mg^{-1}$protein)	Q	Type of combination
CsA	Control	4.09 ± 0.49		
	0.1	4.67 ± 0.26		
	0.5	$5.90 \pm 0.36^{**}$		
	1	$8.36 \pm 0.44^{**}$		
	2.5	$10.86 \pm 0.96^{**}$		
	5	$12.50 \pm 2.19^{**}$		
CsA/Ver	0/0.5	4.50 ± 0.27		
	0.1/0.5	$5.27 \pm 0.34^{**b}$	1.47	Synergistic
	0.5/0.5	$7.00 \pm 10.49^{**b}$	1.59	Synergistic
	1/0.5	$10.09 \pm 0.93^{**b}$	1.57	Synergistic
	2.5/0.5	$12.83 \pm 0.83^{**b}$	1.49	Synergistic
	5/0.5	$15.58 \pm 0.92^{**b}$	1.59	Synergistic
CsA/Tet	0/0.5	4.44 ± 0.36		
	0.1/0.5	$5.10 \pm 0.34^{**}$	1.00	Additive
	0.5/0.5	$6.87 \pm 0.63^{**b}$	1.34	Synergistic
	1/0.5	$9.64 \pm 0.62^{**b}$	1.32	Synergistic
	2.5/0.5	$13.55 \pm 0.70^{**c}$	1.49	Synergistic
	5/0.5	$18.05 \pm 1.73^{**c}$	1.80	Synergistic
CsA/Dox	0/1	4.55 ± 0.51		
	0.1/1	$5.07 \pm 0.45^{*}$	0.96	Additive
	0.5/1	$6.42 \pm 0.65^{**}$	1.14	Additive
	1/1	$8.25 \pm 1.10^{**}$	1.02	Additive
	2.5/1	$10.89 \pm 0.98^{**}$	1.11	Additive
	5/1	$12.58 \pm 0.61^{**}$	1.13	Additive

Intracellular concentration of Rh123 were determined in the presence of increasing concentration of CsA (0.1, 0.5, 1, 2.5 and 5 $\mu m \cdot L^{-1}$) alone or combined with fixed concentration of Ver (0.5 $\mu m \cdot L^{-1}$), Tet (0.5 $\mu m \cdot L^{-1}$) or Dox (1$\mu m \cdot L^{-1}$) respectively. Results were presented as mean $\pm$ SD (n = 5) and evaluated with Student's t test (* $P < 0.05$,** $P < 0.05$ vs control ;^{b} $P < 0.05$, ^{c} $P < 0.01$ vs CsA group) and Q criterion. The combination was judged as synergistic when $P < 0.05$ and $Q > 1.15$ at the same time.

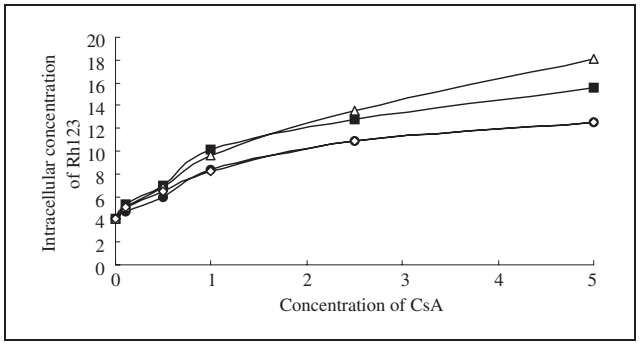

Fig. 1: Effect of CsA alone and in combination with Ver, Tet or Dox on the accumulation of Rh123 in rat BMEC. Intracellular concentration of Rh123 were determined in the presence of increasing concentration of CsA alone (●) or combined with fixed concentration of Ver (■), Tet (△) or Dox (◇) (n = 5)

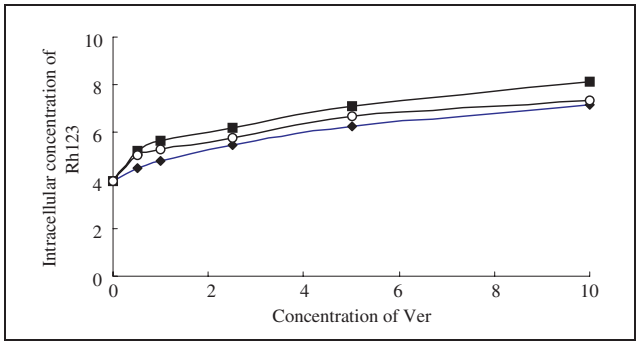

Fig. 2: Effect of Ver alone and in combination with CsA, or Dox on the accumulation of Rh123 in rat BMEC. Intracellular concentration of Rh123 were determined in the presence of increasing concentration of Ver alone (◆) or combined with fixed concentration of CsA (■), or Dox (○) (n = 5)

2.2. Effects of combined use of P-gp inhibitors on the accumulation of Rh123 in rat BMEC

2.2.1. Effect of CsA in combination with Ver, Tet or Dox on the accumulation of Rh123 in rat BMEC

After rat BMEC were treated with CsA (0.1, 0.5, 1, 2.5 and 5 $\mu m \cdot L^{-1}$) in combination with Ver (0.5 $\mu m \cdot L^{-1}$), intracellular accumulation of Rh123 was significantly increased as compared with that in rat BMEC treated with comparable concentration of CsA alone ($p < 0.05$, $Q > 1.15$). It suggested that the CsA and Ver have synergistic action. Similarly, synergism also was observed in CsA plus Tet (0.5 $\mu m \cdot L^{-1}$) group ($p < 0.05$ as compared with 0.5 or 1 $\mu m \cdot L^{-1}$ CsA, and $p < 0.01$ with 2.5 or 5 $\mu m \cdot L^{-1}$ CsA, $Q > 1.15$). CsA plus Dox (0.5 $\mu m \cdot L^{-1}$) did not significantly increase the intracellular concentration of Rh123 as compared to that treated with comparable concentration of CsA alone ($p > 0.05$, $0.85 < Q < 1.15$). It suggested the Dox only interacted additively with CsA (Table 1, Fig. 1).

2.2.2. Effect of Ver in combination with CsA or Dox on the accumulation of Rh123 in rat BMEC

As compared with Ver (0.5, 1, 2.5, 5 and 10 $\mu m \cdot L^{-1}$) alone, combination of Ver with CsA (0.1 $\mu m \cdot L^{-1}$) significantly enhanced intracellular concentration of Rh123 ($p < 0.05$, $Q > 1.15$), suggesting that a synergistic interaction existed between these two inhibitors. Combination of Ver with Dox (1.0 $\mu m \cdot L^{-1}$) only slightly increased intracellular concentration of Rh123 ($p > 0.05$, $0.85 < Q < 1.15$). Therefore, an additive action between them was identified (Table 2, Fig. 2).

2.2.3. Effect of Tet in combination with CsA or Dox on the accumulation of Rh123 in rat BMEC

The increasing of accumulation of Rh123 in rat BMEC treated with Tet (0.5, 1.0, 2.5, 5 and 10 $\mu ml \cdot L^{-1}$) alone, was significantly enhanced by CsA (0.1 $\mu m \cdot L^{-1}$) ($p < 0.05$, $Q > 1.15$ as compared with 5 and 10 $\mu ml \cdot L^{-1}$

Table 2: Effect of Ver alone and in combination with CsA or Dox on the accumulation of Rh123 in rat BMEC

Group	Concentration of Inhibitor ($\mu mol \cdot L^{-1}$)	Content of Rh123 ($nmol \cdot mg^{-1}$protein)	Q	Type of combination
Ver	Control	3.98 ± 0.46		
	0.5	4.50 ± 0.27		
	1	$4.79 \pm 0.41^{*}$		
	2.5	$5.50 \pm 0.22^{**}$		
	5	$6.29 \pm 0.62^{**}$		
	10	$7.14 \pm 0.42^{**}$		
Ver/CsA	0/0.1	4.66 ± 0.26		
	0.5/0.1[b]	$5.27 \pm 0.36^{**b}$	1.23	Synergistic
	1/0.1[b]	$5.66 \pm 0.54^{**b}$	1.30	Synergistic
	2.5/0.1[b]	$6.20 \pm 0.43^{**b}$	1.16	Synergistic
	5/0.1[b]	$7.11 \pm 0.23^{**b}$	1.29	Synergistic
	10/0.1[b]	$8.11 \pm 0.61^{**b}$	1.23	Synergistic
Ver/Dox	0/0.5	4.44 ± 0.36		
	0.5/1	$5.08 \pm 0.39^{**}$	1.06	Additive
	1/1	$5.28 \pm 0.35^{**}$	1.02	Additive
	2.5/1	$5.81 \pm 0.43^{**}$	0.98	Additive
	5/1	$6.66 \pm 0.38^{**}$	1.06	Additive
	10/1	$7.33 \pm 0.46^{**}$	1.03	Additive

Intracellular concentration of Rh123 were determined in the presence of increasing concentration of Ver (0.5, 1, 2.5, 5 and 10 $\mu m \cdot L^{-1}$) alone or combined with fixed concentration of CsA (0.1 $\mu m \cdot L^{-1}$) or Dox (1 $\mu m \cdot L^{-1}$) respectivel. Results were presented as mean ± SD (n = 5) and evaluated with Student's t test ($^{*}P < 0.05$, $^{**}P < 0.05$ vs control; $^{b}P < 0.05$, $^{c}P < 0.01$ vs Ver group) and Q criterion. The combination was judged as synergistic when $P < 0.05$ and $Q > 1.15$ at the same time.

Table 3: Effect of Tet alone and in combination with CsA or Dox on the accumulation of Rh123 in rat BMEC

Group	Concentration of inhibitor ($\mu mol \cdot L^{-1}$)	Content of h123 ($nmol \cdot mg^{-1}$protein)	Q	Type of combination
Tet	0	3.92 ± 0.24		
	0.5	4.44 ± 0.36		
	1	$5.29 \pm 0.33^{**}$		
	2.5	$5.57 \pm 0.52^{**}$		
	5	$6.48 \pm 0.55^{**}$		
	10	$8.93 \pm 0.67^{**}$		
Tet/CsA	0/0.1	4.66 ± 0.26		
	0.5/0.1	$4.98 \pm 0.35^{*}$	1.00	Additive
	1/0.1	$6.10 \pm 0.76^{**}$	0.97	Additive
	2.5/0.1	$6.28 \pm 0.68^{**}$	1.07	Additive
	5/0.1	$8.83 \pm 1.36^{**b}$	1.74	Synergistic
	10/0.1	$10.21 \pm 0.67^{**b}$	1.27	Synergistic
Tet/Dox	0/1	4.55 ± 0.51		
	0.5/1	$4.97 \pm 0.30^{**}$	0.93	Additive
	1/1	$5.69 \pm 0.41^{**}$	0.96	Additive
	2.5/1	$5.94 \pm 0.48^{**}$	0.96	Additive
	5/1	$6.86 \pm 0.65^{**}$	1.03	Additive
	10/1	$9.20 \pm 0.55^{**}$	1.06	Additive

Intracellular concentration of Rh123 were determined in the presence of increasing concentration of Tet (0.5, 1, 2.5, 5 and 10 $\mu m.L^{-1}$) alone or combined with fixed concentration of CsA (0.1 $\mu m \cdot L^{-1}$) or Dox (1 $\mu m \cdot L^{-1}$) respectivel. Results were presented as mean ± SD (n = 5) and evaluated with Student's t test ($^{*}P < 0.05$, $^{**}P < 0.05$ vs control; $^{b}P < 0.05$, $^{c}P < 0.01$ vs Tet group) and Q criterion. The combination was judged as synergistic when $P < 0.05$ and $Q > 1.15$ at the same time

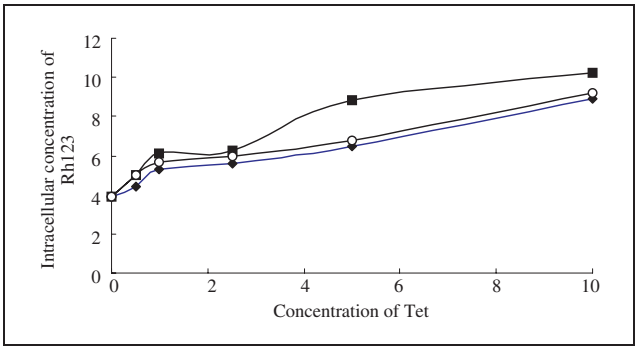

Fig. 3: Effect of Tet alone and in combination with CsA, or Dox on the accumulation of Rh123 in rat BMEC. Intracellular concentration of Rh123 were determined in the presence of increasing concentration of Tet alone (◆) or combined with fixed concentration of CsA (■), or Dox (○) (n = 5)

Tet), suggesting that CsA could synergize with Tet. No significant difference was observed when Tet was combined with Dox (1.0 $\mu m \cdot$ L-1) ($p > 0.05$, and $0.85 < Q < 1.15$). Therefore, interaction between them was identified as additive (Table 3, Fig. 3).

2.2.4. Effect of combination of Dox with CsA, Ver or Dox on the accumulation of Rh123 in rat BMEC

Dox (1, 2.5, 5, 10 and 20 $\mu ml \cdot L^{-1}$) in combination with CsA (0.1 $\mu ml \cdot L^{-1}$), Ver (0.5 $\mu ml \cdot L^{-1}$), or Tet (0.5 $\mu ml \cdot L^{-1}$) respectively only showed a slightly enhanced intracellular accumulation of Rh123 ($p > 0.05$, $0.85 < Q < 1.15$) as compared with corresponding concentration of Dox alone. Consequently, an additive action between Dox and CsA, Tet or Ver was identified in our study (Table 4).

Table 4: Effect of Dox alone and in combination with CsA, Tet or Ver on the accumulation of Rh123 in rat BMEC

Group	Concentration of inhibitor ($\mu mol \cdot L^{-1}$)	Content of Rh123 ($nmol \cdot mg^{-1}$protein)	Q	Type of combination
Dox	0	4.05 ± 0.27		
	1	4.70 ± 0.45		
	2.5	$5.26 \pm 0.47^{*}$		
	5	$5.76 \pm 1.10^{**}$		
	10	$6.48 \pm 0.95^{**}$		
	20	$7.12 \pm 0.65^{**}$		
Dox/CsA	0/0.1	4.66 ± 0.26		
	1/0.1	$5.01 \pm 0.37^{*}$	0.94	Additive
	2.5/0.1	$5.48 \pm 0.39^{**}$	0.95	Additive
	5/0.1	$6.04 \pm 0.39^{**}$	1.03	Additive
	10/0.1	$6.55 \pm 0.55^{**}$	0.99	Additive
	20/0.1	$7.51 \pm 0.57^{**}$	1.12	Additive
Dox/Tet	0.5	4.44 ± 0.36		
	1/0.5	$5.12 \pm 0.35^{**}$	0.97	Additive
	2.5/0.5	$5.61 \pm 0.36^{**}$	0.98	Additive
	5/0.5	$6.16 \pm 0.45^{**}$	1.04	Additive
	10/0.5	$6.86 \pm 0.75^{**}$	1.06	Additive
	20/0.5	$7.57 \pm 0.66^{**}$	1.10	Additive
Dox/Ver	0/0.5	4.50 ± 0.27		
	1/0.5	$5.16 \pm 0.39^{**}$	1.10	Additive
	2.5/0.5	$5.66 \pm 0.56^{**}$	1.08	Additive
	5/0.5	$6.13 \pm 0.49^{**}$	1.08	Additive
	10/0.5	$6.73 \pm 0.42^{**}$	1.06	Additive
	20/0.5	$7.49 \pm 0.67^{**}$	1.07	Additive

Intracellular concentration of Rh123 were determined in the presence of increasing concentration of Dox (1, 2.5, 5, 10 and 20 $\mu m \cdot L^{-1}$) alone or combined with fixed concentration of CsA (0.1 $\mu m \cdot L^{-1}$), Tet (0.5 $\mu m \cdot L^{-1}$) or Ver (0.5 $\mu m \cdot L^{-1}$) respectivel. Results were presented as mean ± SD (n = 5) and evaluated with Student's t test ($^{*}P < 0.05$, $^{**}P < 0.05$ vs control; $^{b}P < 0.05$, $^{c}P < 0.01$ vs Dox group) and Q criterion. The combination was judged as synergistic when $P < 0.05$ and $Q > 1.15$ at the same time.

3. Discussion

Along with the discovery of the important role of P-gp in pathology and physiology, the use of P-gp inhibitors was considered a promising way to overcome MDR and to increase the penetration of drugs through physiological barriers, including BBB (Tsuji and Tamai 1997). However, the results of early clinical studies have been inconclusive and rather disappointing. One of the major obstacles was that the tested P-gp inhibitors were intolerable in clinical practice, because these agents modulated MDR at very high concentrations at which unacceptable side effects was produced (Lum et al. 1993). One potential solution of this problem is to develop new inhibitors that are more effective and less toxic. Nevertheless, it is quite difficult to find a potent, specific and safe drug able to inhibit the efflux activity of P-gp. At present, such a substance is still lacking (van Zuylen 2000) although the first drug able to reverse MDR was discovered more than 20 years ago. Recently, as it has been confirmed that there are multiple binding sites for substrates and inhibitors on P-gp, another approach to inhibit P-gp has been proposed (Ayesh et al. 1996), which is the combination of P-gp inhibitors, each at a concentration below its toxic level. The use of combination of P-gp inhibitors with synergisic interaction is satisfactory if the toxicity of the combination is significantly lower than that of the individual drugs, at the dose required to inhibit P-gp activity. Obviously, it is necessary in the first place to identify a synergism of drugs to inhibit P-gp activity.

P-gp has been demonstrated to transport substrates in an ATP dependent manner, and hydrolysis of ATP is the energy source of substrate transporting (Sharom et al. 1995; DiDodato 1997). Previous research on ATPase activity of P-gp in our laboratory has shown that there was a non-competitive inhibitory interaction between three structurally unrelated P-gp inhibitors, CsA, Tet and Dox, suggesting that they bind at different sites of P-gp. This implies that a synergism among these inhibitors might exist (He Ling 2002).

On the base of this study, we tried to further characterize the synergism between these three inhibitors and Ver, on the Rh123 transport mediated by P-gp in rat BMEC. Because dose-limiting toxicity mechanisms of the single drugs must not overlap, the combination of Ver and Tet was not examined, as they both are Ca^{2+} antagonist. For the purpose of the reliability of the synergy, firstly, the fixed concentration of drug was chosen, at which there was no significant effect ($p > 0.05$) of drugs alone on the intracellular accumulation of Rh123. Secondly, t-test and Q criterion was simultaneously used to determine an interaction between the inhibitors. Using these methods, we found that mutual synergistic actions existed between CsA and Ver, or CsA and Tet. The interaction between CsA and Ver is interesting. Most studies on ATPase activity showed a competitive interaction between them (He Ling 202; Ayesh 1996). However, some authors have reported a synergistic interaction on substrate transport or cytotoxic activity in tumor cells (Xiu 1990; Osann et al. 1992). This discrepancy was presumed to be due to the difference of cell types. This discrepancy also is the reason why we examined the combination of CsA and Ver in our study. Our results demonstrated a synergistic action between these drugs, which is consistent with that in tumors. It demonstrates the complexity of action of P-gp in different assay systems. A further disclosure of the mechanism of P-gp will be helpful to explain this phenomenon.

In summary, our results suggest that the combined use of CsA and Ver, or CsA and Tet may be a valuable way to inhibit P-gp in BBB with lower doses of the single agents. More inhibitors should be examined in this respect; particularly an *in vivo* study is needed to further confirm the effect and feasibility of this approach.

4. Experimental

4.1. Drugs and reagents

Cyclosporin A (CsA), Verapamil (Ver) were generously provided by Liu Xiaodong (China pharmaceutical university, P.R China). Tetrandrine (Tet) was generously provided by Hua Weiyi (China pharmaceutical university, P.R China). Doxorubicin (Dox) and Rhodamine123 were purchased from Sigma Co. All other chemicals were of analytical and commercially available.

4.2. Primary culture of rat brain microvessel endothelial cells

Endothelial cells were isolated from rat brain tissues. Briefly, cortex was obtained from rat brain. After remove of surface vessels and meninges, gray matter was minced and incubated for 20 min at 37 °C in 0.05% trypsin. Then the samples were passed through a 150 μm nylon mesh. After that the filtrate was passed through a 72 μm nylon mesh and washed with PBS. The material retained on the nylon mesh was collected and centrifuged at 1500 × g for 5 min. Then the pellet was resuspended and incubated at 37 °C for 30 min in 0.1% collagenase II. After centrifugation at 1500 × g for 5 min, the deposit was resuspended and cultured in DMEM/F12 medium supplemented with 20% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2.

4.3. Effect of single P-gp inhibitors on the accumulation of Rh123 in rat BMEC

Rat BMEC were seeded at a density of $5 \times 10^5 mL^{-1}$ in 24-well plates. After reaching confluent, cell monolayers were preincubated with serum-free Dulbecco MEM(DMEM) medium for 30 min at 37 °C, then cells were exposed to 10 μmL^{-1} Rh123 in DMEM medium with CsA, Ver, Dox or Tet respectively for 90 min at 37 °C. Cell monolayers were washed with PBS and disrupted in 1% Triton X-100. Fluorescence of Rh123 was determined using a 1420 Victor^2 Multilabel Hits Counter with 492 nm excitation wavelength and 525 nm emission wavelength. The 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 standardized with the amount of protein in each sample as determined by the Blandford assay (Fontaine et al. 1996). Inhibition of Rh123 accumulation(%) was calculated from obtained fluorescence value (F) obtained according to the followed formula:

$$\text{Inhibition of Rh123 accumulation}(\%) = [F_{drug} - F_{control}]/F_{control} \times 100\%$$

4.4. Effect of combined use of P-gp inhibitors on the accumulation of Rh123 in rat BMEC

The effect of CsA, Ver, Dox and Tet in combinations was analyzed as above by determining the inhibitory effect of a fixed concentration of one inhibitor in the presence of increasing concentrations of the other one.

4.5. Statistical analysis

To evaluate whether the effects of combined inhibitors were synergistc, the "Student" t-test was used and statistical significance was defined as $P < 0.05$. The data was also assayed by the Q value criterion (Hu Xiangjie 1999) according to the following formula:

$$Q = \text{Actual combined effect/expected combined effect}$$
$$= \text{Actual combined effect}/(E_A + E_B - E_A \cdot E_B).$$

where E_A and E_B is the effect of the two inhibitors individually.
If $Q < 0.85$, effects were considered to be antagonistic, if $0.85 < Q < 1.15$ effects were considered to be additive, and if $Q > 1.15$ synergistic. The combined effects with $p < 0.05$ and $Q > 1.15$ were simultaneously was synergistic ultimately in this study.

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