Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary information

Novel Ginkgolide B derivative attenuated the function and expression of P-glycoprotein at blood-brain barrier: presenting brain-targeting ability

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1. Preparation of ginkgolide B derivatives in Figure 1

General procedure for the preparation of ginkgolide B derivative

The GB derivatives were synthesized according to the procedure reported by Nakanishi et al. with minor modification. [1]

1 mmol GB (1.0 mmol, M = 424) and 1.3 mmol carboxylic acid were dissolved in 15-20 mL acetonitrile, and the mixture was stirred at 0°C ice bath. Subsequently, 25 mg DMAP (4-dimethylamino pyridine, 0.2 mmol, M = 122) and 268 mg EDC·HCl (1-(3-dimethylamino propyl)-3-ethyl carbodiimide hydro-chloride, 1.4 mmol, M = 192) were added. After being stirred at 0°C for 1 h, the mixture was stirred overnight at room temperature. All the reactions were monitored using TLC. The reaction solution was evaporated to dryness, and the crude product was dissolved in ethyl acetate (about 40 mL) and washed with 5% $NaHCO_3$ twice and saturated salt solution once. Then collected organic phase was dried, filtered and concentrated. The crude product was purified by column chromatography using silica gel (200-300 mesh) and eluting with petroleum ether/acetone (2:1-1:1, V/V) to obtain the desired solid product. All yields of the products refer to isolated yield.

The characterization data and spectra of some ginkgolide B derivatives

IR spectra were obtained on Thermo Nicolet 5700 system. NMR spectra were performed on Agilent VNMRS 600 or Bruker Avance 300 with TMS as internal standard, and the chemical shifts were recorded in ppm along with coupling constants (*J* values) in Hz. Multiplicities were designated as single (s), broad (br), double (d), triple (t), quadruple (q) and multiple (m). Mass spectra were recorded by electrospray ionization (ESI) at 70 eV in a Thermo LCQ Advantage MAX spectrometry with direct insertion probe. HRMS was recorded in a Waters Xevo G2 Q-TOF spectrometry with direct insertion probe.

10-O- nicotinic Ginkgolide B (**D4 in Fig. 1**)

Yield: 45%. The characterization data and spectra were reported by Wu et al. [2] ^{1}H NMR (300 MHz, d-DMSO) δ 9.13 (1H, d, *J* = 1.8 Hz Py), 8.85-8.87 (1H, dd, *J* = 6.6, 1.8 Hz,Py), 8.33-8.36 (1H, d, *J* = 10.4 Hz ,Py), 7.60-7.64 (1H, dd, *J* = 10.4, 6.6 Hz, Py), 6.73(1H, d, *J* = 4.8 Hz, 1-OH), 6.57 (1H, s, 3-OH), 6.08 (1H, s, 12-H), 5.78 (1H, d, *J* = 2.7 Hz, 10α-H), 5.55 (1H,d, *J* = 6.6 Hz, 6-H), 5.00 (1H, d, *J* = 4.8 Hz, 2-H), 4.93(1H, d, *J* = 4.8 Hz, 1β-H), 3.00 (1H, q, *J* = 6.9 Hz,14-H), 2.20-2.27 (1H, dd, *J* =

13.6, 6.0 Hz, 7β-H), 2.01-2.08 (1H, m, 7α-H), 1.75-1.80 (1H, m, 8-H), 1.16 (3H, d, *J* = 6.9 Hz, Me), 1.01 (9H, s, t-Bu). ^{13}C NMR (75 MHz, d-DMSO) δ 7.95, 28.77, 31.87, 36.56, 41.17, 48.29, 67.71, 68.59, 70.34, 74.83, 79.31, 83.22, 91.02, 98.6, 109.09, 123.93, 125.1, 137.19, 150.19, 153.83, 162.74, 169.78, 173.19, 175.86.

10-O-(5-methyl)-pyrazine-2-carboxylic Ginkgolide B (D6 in Fig. 1, namely PGB)

Colorless solid, Yield: 65%; FT-IR (KBr) υ_{max}/cm^{-1} 3462.0 (OH), 2966.0 (CH), 1789.5 (C=O); ^{1}H NMR (300 MHz, d-DMSO): δ 9.32 (1H, s, Py), 8.75 (1H, s, Py), 6.75 (1H, d, *J* = 4.8 Hz, 1-OH), 6.53 (2H, s, 12-H and 3-OH), 6.32 (1H, s, 10α-H), 5.48 (1H, d, *J* = 3.6 Hz, 6-H), 4.73 (1H, d, *J* = 7.2 Hz, 2-H), 4.17 (1H, dd, *J* = 7.2, 4.8 Hz, 1β-H), 2.90 (1H, q, *J* = 7.0 Hz, 14-H), 2.66 (3H, s, Me), 2.18-2.21 (1H, m, 7β-H), 1.80-1.90 (2H, m, 7α-H, 8-H), 1.14 (3H, d, *J* = 7.0 Hz, Me), 1.02 (9H, s, t-Bu); ^{13}C NMR (150 MHz, $CDCl_3$) δ 7.463, 22.429, 29.044, 32.348, 36.878, 41.929, 49.360, 67.272, 70.389, 73.171, 74.295, 80.072, 83.094, 92.215, 99.301, 111.094, 136.959, 143.505, 146.745, 161.028, 161.129, 167.526, 171.266, 175.556; ESI-MS (neg. ion mode) m/z 543.06 [M-H]$^-$, 1086.88 [2M-H]$^-$. HRMS (ESI, neg. ion mode) m/z calcd. For [M-H]$^-$ $C_{26}H_{27}N_2O_{11}$ 543.1615, found 543.1614.

The spectra of PGB was shown in Fig **1S-5S**.

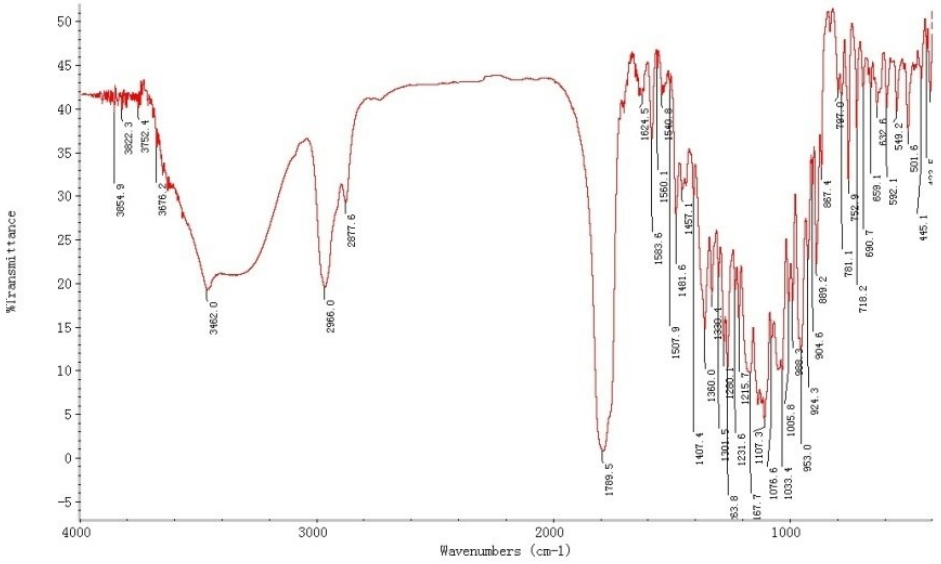


Fig. 1S FT-IR of PGB

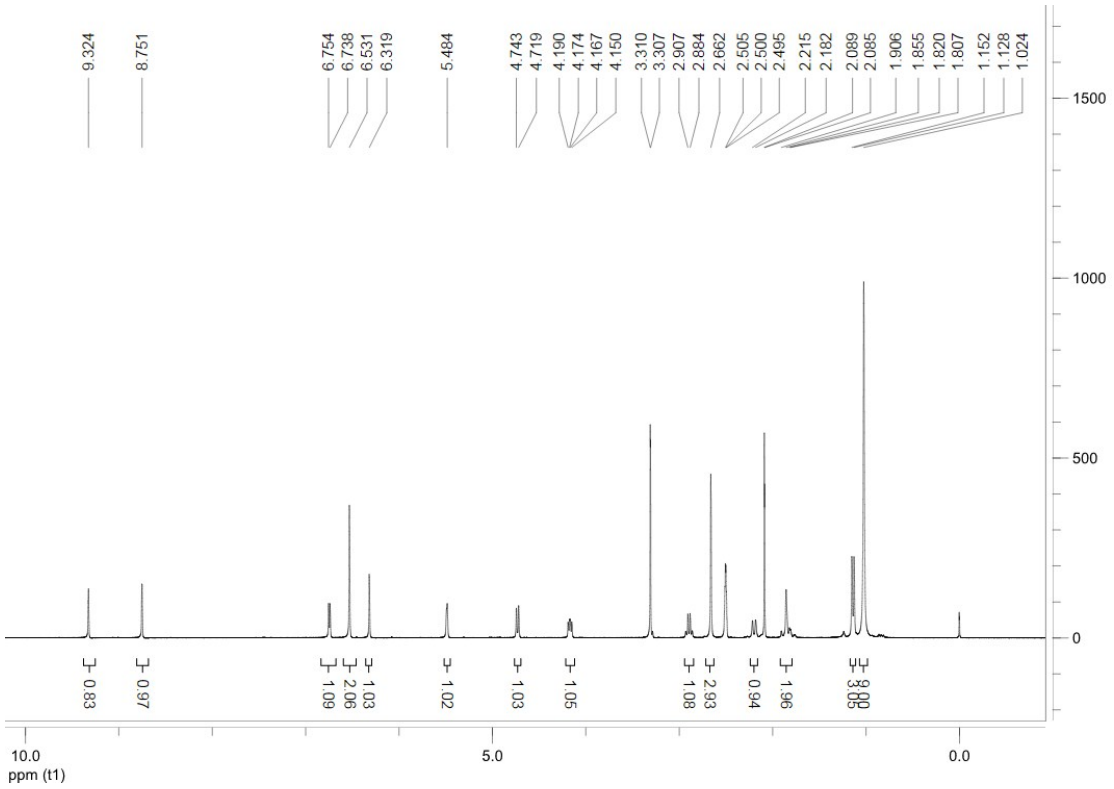


Fig. 2S ^{1}H NMR spectrum of PGB

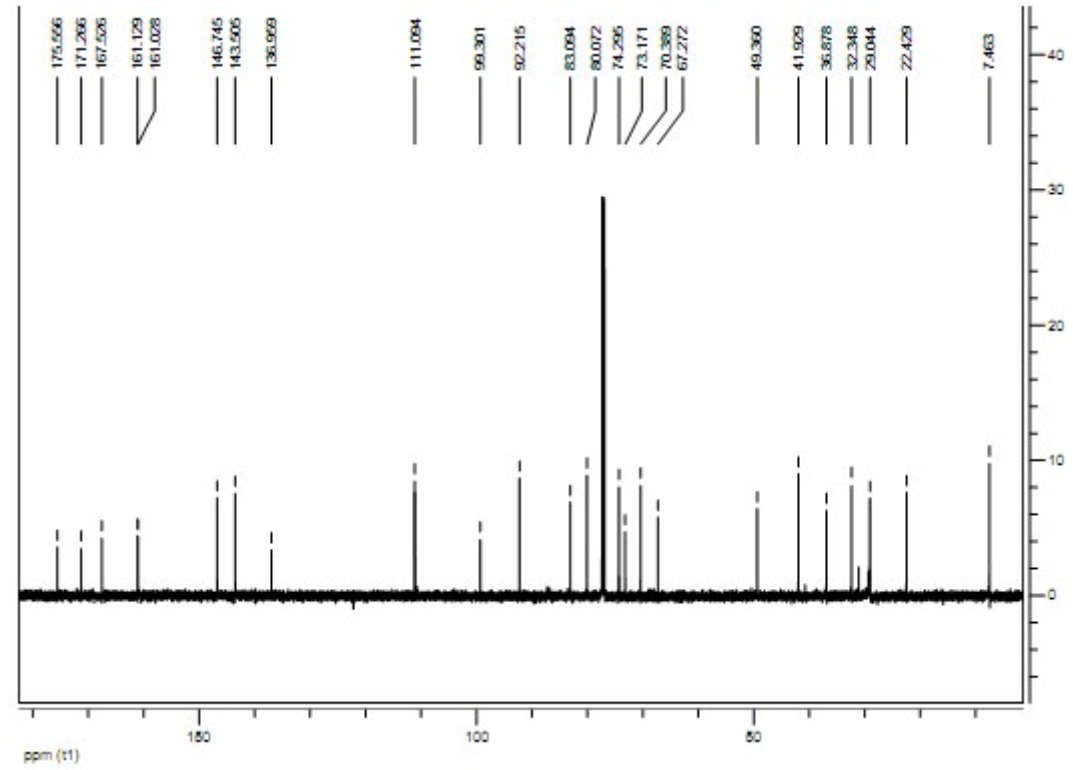


Fig. 3S ^{13}C NMR spectrum of PGB

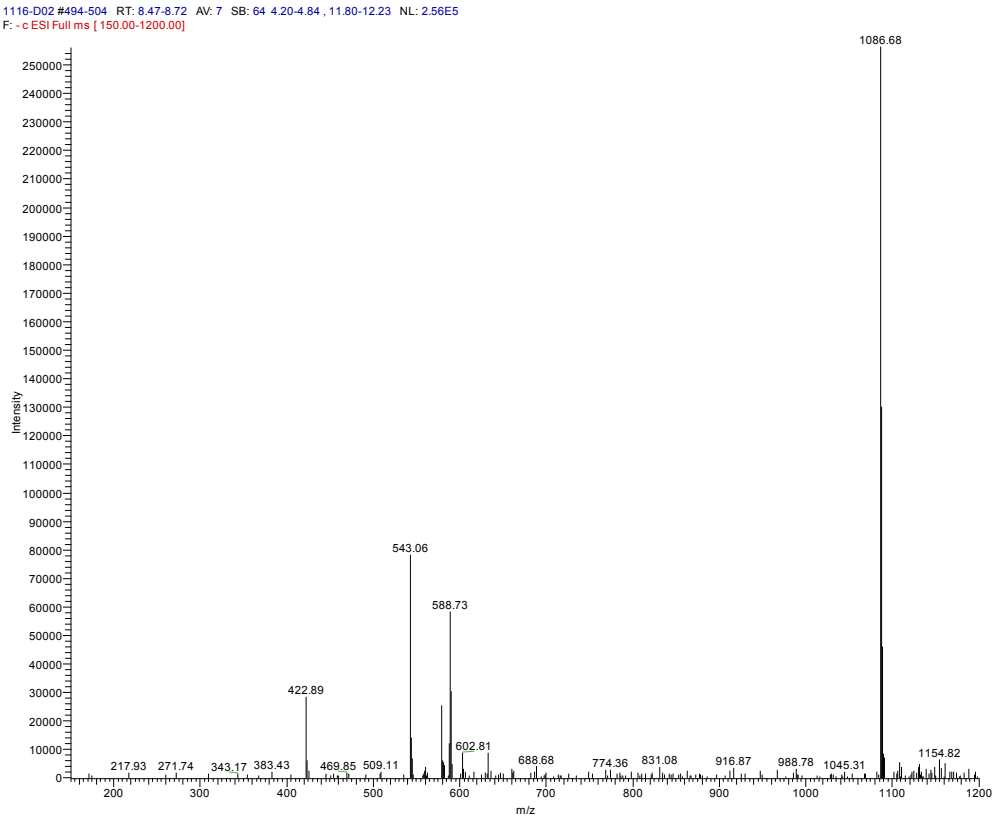
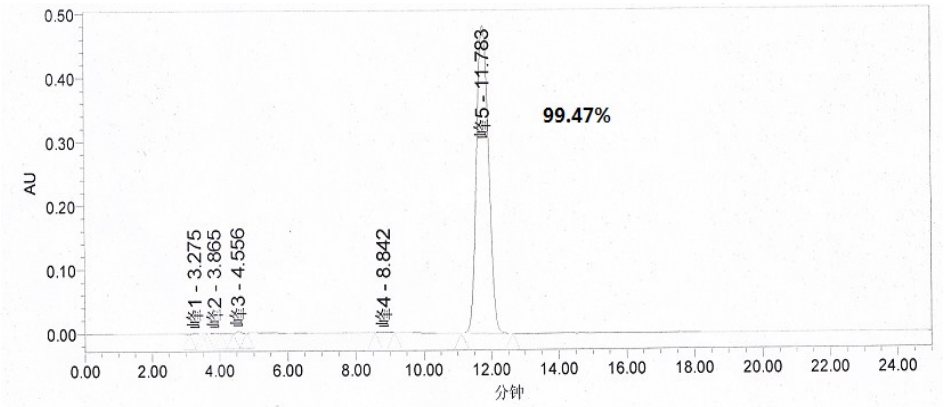


Fig. 4S ESI-MS of PGB

Fig. 5S HRMS screenshot of PGB

The HPLC purity analysis of PGB was performed on a Delta 600 HPLC system with Symmetry Shield RP18 column (150 mm × 3.9 mm i.d. 5µm particle size, Waters, USA) at a temperature of

25°C and a flow rate of 0.8 mL/min. The mobile phase was composed of methanol and water, the ratio was 72:28. Ultraviolet detector was operated at 275 nm for the determination of GB pyrazine derivatives. The HPLC spectra was shown in Fig **6S**.



峰结果

	名称	保留时间 (分钟)	面积 (微伏*秒)	%面积
1	峰1	3.275	11754	0.10
2	峰2	3.865	13727	0.11
3	峰3	4.556	26522	0.22
4	峰4	8.842	11974	0.10
5	峰5	11.783	12033363	99.47

Fig. 6S HPLC-UV purity evaluation of PGB

10-O-cinnamic carboxylic Ginkgolide B (D7 in Fig. 1)

Yield: 58.5%. The characterization data and spectra were reported by Lu et al.[3] ^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (1H, d, *J* = 13.6 Hz, C6H5CH=), 7.65–7.66 (3H, t, C_6H_5), 7.44–7.45 (2H, d, C_6H_5), 6.62 (1H, d, *J* = 16.0 Hz, = CHCO), 6.31 (1H, s, 12-H), 6.27 (1H, s, 10α-H), 5.61 (1H, s, 6-H), 4.63 (1H, d, *J* = 6.4 Hz, 2-H), 4.33 (1H, d, *J* = 6.4 Hz, 1β-H), 3.08 (1H, q, *J* = 7.0 Hz, 14-H), 2.29 (1H, d, *J* = 10.4 Hz, 7β-H), 1.96–2.07 (2H, m, 8-H, 7α-H), 1.26–1.33 (3H, m, 16 -H), 1.07 (9H, s, t-Bu); ^{13}C NMR (100 MHz, CD_3OD) δ 6.97, 27.86, 31.72, 36.70, 41.92, 49.16, 67.46, 69.32, 72.40, 74.04, 79.01, 83.15, 94.74, 99.97, 110.74, 115.60, 128.23, 128.69, 130.68, 134.11, 147.77, 164.85, 169.01, 171.08, 176.98.

10-O-(N-methyl acetate)- 1,4-dihydropyridine carboxylic Ginkgolie B (D2 in Fig. 1)

The characterization data were reported by Zhou et al. [4] ^{1}H NMR (300 MHz, d-DMSO) 7.43(1H, d, *J* = 1.3 Hz, Dihydropyridine), 6.47(1H, m, Dihydropyridine), 6.06 (1H, s, 12-H), 5.30 (1H, d, *J* = 3.7 Hz, 6-H), 5.01(1H, s, 10α-H), 4.85-4.92 (1 H, m, Dihydropyridine), 4.66 (1H, d, *J*=7.2 Hz, 2- H), 4.03-4.06 (1H, m, 1β-H), 3.29 (7H, s, NCH_2, OCH_3, and 2H in Dihydropyridine), 2.84 (1H, q, *J*

= 7.0 Hz ,14-H), 2.10-2.14 (1H, m, 7β-H), 1.92-1.98 (1H, m, 1H, 7α-H), 1.69-1.72 (1H, m, 8-H), 1.10 (3H, d, *J* = 7.0 Hz, 16-H), 1.03 (9H, s, t-Bu).

2. P-gp expression in rBMECs

rBMECs isolation and culture

rBMECs were isolated according to the method of Abbott et al. [5] Briefly, isolated cortex from 1- to 3-day-old SD rats was placed in ice-cold D-Hanks solution. After removal of surface vessels and meninges, cortex gray matter was rinsed with D-Hanks solution. Then, the cortex was minced in cold DMEM and incubated in DMEM solution containing 0.1% collagenase II at 37 °C for 10 min. The sample was centrifugated at 800g for 5 min and the supernatant was removed. The residue was resuspended in phosphate-buffered saline containing 20% bovine serum albumin and centrifuged at 1000g for 20 min. The precipitate was collected and the residue was repeated centrifugation. The above pellet was resuspended and incubated in 0.1% collagenase/dispase at 37 °C for 10 min. DMEM was added and the vessels were finally collected by centrifugation at 800 g for 8 min, then the pellet was cultured in DMEM/F12 (1:1) medium and supplemented with 1% gelatin at 37 °C in a 5% CO_2 humidified atmosphere. After 16 h, the cell impurity without adhesion and old culture medium were sucked out, the fresh culture medium was replaced, then every 3 days for replace.

Cell viability assay and cell treatment

Adherent BMECs was digested by trypsin, then diluted about 1× 10^5/mL cell suspension and inoculated in 96-well flat-bottomed plates. The volume of each hole was 200 μL. The cell adhered to the surface overnight, old culture medium was sucked out and 200 μL fresh medium containing test drug was added. To ensure cells remain viable during treatment, the drug concentrations used were tested applying the MTT assay as described following. In our experiment, test drugs were dissolved in DMSO and further diluted with fresh medium to 5-100 μM. Control cells were exposed to 0.1% DMSO (*V*/*V*) in the absence of drugs.

Cells were incubated for 20 h at 37 °C in a 5% CO_2 humidified atmosphere. Then, 20 μL of 5.0 mg/mL MTT solution in PBS was added and the incubation lasted for another 4 h. The formazan content dissolved in DMSO from each well was determined at 630 nm using ELX80UV microplate reader (Bio-Tek, USA). Cell viability was expressed as the ratio between the

absorbance of treated cells and that of untreated (control) cells, as shown in Fig. 7S.

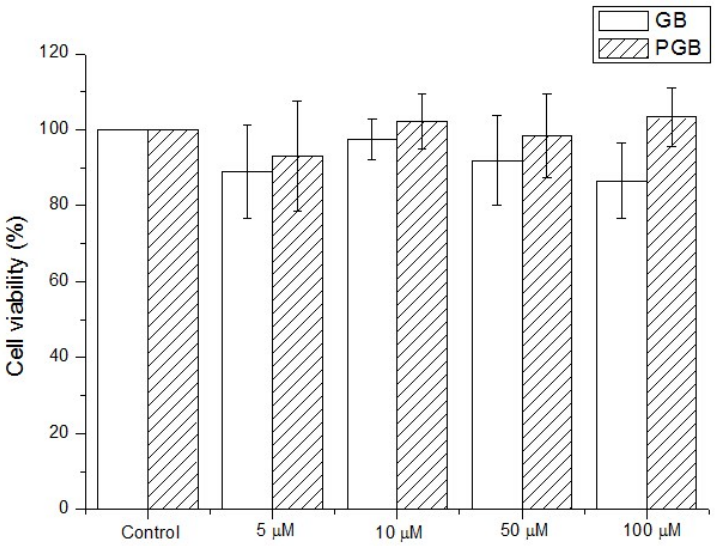


Fig 7S. Cell viability in rBMECs treated with GB and PGB

P-gp western blot analysis in rBMECs

Western blot analysis was used for assessing P-gp expression in rBMECs according to the method of Liu et al.[6] All the cells were exposed to the test drug (5-100 μM) and incubated for 24 h. The medium was removed and the cells were washed three times with ice-cold PBS and collected for the next P-gp expression. All the cells were pelleted by centrifugation and lysed by ultrasonication in ice-cold cell lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM mercaptoethanol, 1% glycerol and protease inhibitor cocktail (1 mM dithiothreitol and 2 mM PMSF). The lysate was centrifugated at 15000 rpm for 30 min, the supernatant was total protein extracted from cells. An aliquot of total protein sample (150 μL) was diluted with 5× sodium dodecyl sulfate (SDS) sample buffer containing 0.1 M Tris-HCl (pH 6.8), 4% SDS, 200 mM DTT, 20% glycerol, and 0.2% bromophenol blue. Then cooked in boiling water for 7-8 min. Proteins were separated by electrophoresis on 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to a PVDF membrane. The membrane was washed three times with TBST for 15 min and blocked in TBST containing 5% dried skim milk for 120 min at room temperature. Then the membrane was incubated with the anti-P-glycoprotein monoclonal antibody C219, diluted 200-fold, overnighted at 4 °C. After washed three times with TBST for 15 min, it was incubated in the HRP-conjugated goat anti- mice secondary antibody at

room temperature for another 2 h and washed three times with TBST. The transferred protein was incubated with ECL substrate solution for 5 min according to the manufacturer's instructions and pressed in the X-ray film, developing and fixing. The relative expression was expressed as the ratio between the gray degree of target protein band and that of β-actin (reference bands).

3. P-gp efflux function in rats brain

Plasma and Brain accumulation of Rh 123

The plasma and brain concentration of Rh 123 were determined by microplate reader at an excitation wave-length of 485 nm and emission wavelength of 546 nm. Briefly, 200 μL of plasma was vortexed with 3 mL of methanol for 5 min. After centrifugation at 12000 rpm for 10 min, 2 mL of the supernatant was collected in quartz cuvette for the detection at Spectra Max M2e microplate reader. The brain was homogenized in 4 volumes of ice normal saline and 400 μL of brain homogenate was vortexed with 1 mL of ethyl acetate for 5 min. After centrifugation, the supernatant was collected and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 3 mL of methanol-water by vortex, and the mixture was contrifuged at 12000 rpm for 10 min. 2 mL of the supernatant was collected and detected directly. Rh 123 concentration was quantified by determining the increase in fluorescence that was subtracted the fluorescence generated in the presence of Rh 123 from the fluorescence generated in the blank plasma or brain homogenate. The recovery of Rh 123 was higher than 80%. The linear ranges of Rho 123 in brain and plasma were 80-2000 ng/g brain (r^2 = 0.995), 400-2000 ng/mL (r^2 = 0.999) respectively.

Reference

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