P-Glycoprotein (ABCB1) Limits the Brain Distribution of YQA-14, a Novel Dopamine D₃ Receptor Antagonist

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YQA-14 is a promising agent for treating addiction to cocaine and opioids. However, previous studies have showed there is marked contrast between the relatively small differences in pharmacological action in vivo and the large differences in their respective receptor binding properties in vitro. We hypothesized that the conflict between the in vivo and in vitro outcomes was attributable to poor brain exposure to YQA-14 caused by drug efflux transporters. To address this issue, we investigated the directional flux of YQA-14 across Caco-2 cells at 37°C or 4°C and the bidirectional transport in the presence and absence of transporter chemical inhibitors. These phenomena were further investigated by an in vivo determination of the brain and blood pharmacokinetics (PK) profile of YQA-14 following intraperitoneal administration with and without inhibitor. The efflux ratio of YQA-14 on Caco-2 cell monolayers was 2.39 and the efflux was temperature-dependent. When co-incubated with GF120918 or LY335979, the efflux of YQA-14 was markedly decreased. However, there was no significant difference in the permeability of YQA-14 when the cells were treated with Ko143. In vivo experiments showed that the brain-to-plasma ratio increased by more than 75-fold and 20-fold with co-administration of GF120918 and LY335979, respectively. Use of Ko143 did not change the brain-to-blood ratio of YQA-14. The results indicate that the brain distribution of YQA-14 was restricted because of active efflux transport at the blood brain barrier. In addition, P-glycoprotein (P-gp) played a dominant role in limiting the distribution of YQA-14 to the brain.

Key words YQA-14; P-glycoprotein (P-gp); blood–brain barrier (BBB); breast cancer resistance protein (BCRP)

Drug abuse is a serious health problem worldwide, yet there are no widely effective medications available to treat this disease.1–3 YQA-14 (Fig. 1) is a novel compound with potential for the treatment of cocaine and opioid abuse; it displays high potency and selectivity for dopamine (DA) D₃ relative to other DA receptors.2,3 The pharmacological actions of YQA-14 on the central nervous system (CNS) have been reported in several animal models, including in self-administration, drug seeking behavior and behavior sensitization.2,5 To date, SB-277011 A has been the most extensively-studied D₃ receptor antagonist in multiple animal models of addiction. But further development of SB-277011 A has been terminated due to problematic pharmacokinetics.6–8 Previous in vitro studies showed that YQA-14 has two binding sites with high affinities (Kᵢ-high=0.68×10⁻⁴ nM; Kᵢ-low=2.11 nM) for human D₃ receptors. The affinities of YQA-14 for both binding sites were over 10⁵ (Kᵢ-high) and 5-fold (Kᵢ-low) higher, respectively, than that of SB-277011 A (Kᵢ=11.2 nM) for human D₃ receptors.2 However, there are sharp contrasts between the relatively small differences in its respective receptor binding properties in vivo and big differences in their pharmacological action of YQA-14 in vivo, and the large differences in their respective receptor binding properties in vitro. The reasons underlying these observed conflicting findings in vivo and in vitro are still unclear. Song et al. proposed that this may be caused by significant species differences in D₃ receptors expressed between humans and rodents, or by receptor heteromerization, which may block YQA-14 binding to one of the binding sites.2

YQA-14 is a typical DA D₃ receptor-selective fluorenyl amide, such as BP-897, SB-277011 A, NGB 2904, JJC 4-077 and PG 01037.9–12 The c Log D values and brain-to-plasma concentration ratios of those compounds have proven satisfactory. For example, the overall brain-to-plasma exposure ratio (AUCbrain/AUCplasma) was 11.81 and 8.73 for NGB 2904 and JJC 4-077 after intravenous administration, respectively.13 Previous studies showed that the Log D₃ of YQA-14 was 2.15, and the apparent permeability coefficient (P_app) of YQA-14 in vivo have showed there is marked contrast between the relatively small differences in pharmacological action in vivo and the large differences in their respective receptor binding properties in vitro. We hypothesized that the conflict between the in vivo and in vitro outcomes was attributable to poor brain exposure to YQA-14 caused by drug efflux transporters. To address this issue, we investigated the directional flux of YQA-14 across Caco-2 cells at 37°C or 4°C and the bidirectional transport in the presence and absence of transporter chemical inhibitors. These phenomena were further investigated by an in vivo determination of the brain and blood pharmacokinetics (PK) profile of YQA-14 following intraperitoneal administration with and without inhibitor. The efflux ratio of YQA-14 on Caco-2 cell monolayers was 2.39 and the efflux was temperature-dependent. When co-incubated with GF120918 or LY335979, the efflux of YQA-14 was markedly decreased. However, there was no significant difference in the permeability of YQA-14 when the cells were treated with Ko143. In vivo experiments showed that the brain-to-plasma ratio increased by more than 75-fold and 20-fold with co-administration of GF120918 and LY335979, respectively. Use of Ko143 did not change the brain-to-blood ratio of YQA-14. The results indicate that the brain distribution of YQA-14 was restricted because of active efflux transport at the blood brain barrier. In addition, P-glycoprotein (P-gp) played a dominant role in limiting the distribution of YQA-14 to the brain.

Key words YQA-14; P-glycoprotein (P-gp); blood–brain barrier (BBB); breast cancer resistance protein (BCRP)

Fig. 1. Chemical Structure of YQA-14

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on Caco-2 cells was larger than $10^{-2}$; both of these results indicate a high permeability of YQA-14 through the intestinal membrane and blood–brain barrier (BBB). The limited entry of drugs into the brain has been attributed to the tight junctions and active efflux transport mechanisms at the BBB. Based on this, we hypothesized that the conflicting in vivo and in vitro dose efficiency of YQA-14 could be attributed to drug efflux transporters and subsequent poor brain exposure of YQA-14.

In order to validate this inference, we investigated the directional flux of YQA-14 across Caco-2 cells at 37°C or 4°C, and its bidirectional transport in the presence and absence of transporter inhibitors (GF120918, LY335979 and Ko143). The brain and blood pharmacokinetic profiles of YQA-14 in vivo were then determined after intraperitoneal administration with and without these inhibitors.

**Experimental**

**Materials**  YQA-14 (>99%) was synthesized at the Beijing Institute of Pharmacology and Toxicology. SB-277011A (>99%) was synthesized at MegaPharma (Budapest, Hungary). Dual P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) inhibitor GF120918, P-gp inhibitor LY335979, P-gp substrate Rhodamine 123 (Rho123), BCRP inhibitor Ko143 and BCRP substrate topotecan were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Methanol and acetonitriles used for analysis were of analytical or HPLC grade (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

**Animals**  BALB/c mice (20–25 g, male) were supplied by the Academy of Military Medical Sciences Animals Center (Beijing, China) for pharmacokinetics (PK) studies. The animals were acclimatized at a temperature of 25±2°C with relative humidity of 70±5% under natural light/dark conditions for 1 week, and were given food and water ad libitum. Prior to the experiment, the animals were kept under a fasting condition overnight. All experimental procedures conformed to the ethics and regulation of animal experiments of the Academy of Military Medical Sciences, China.

**Cell Culture**  Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured as described previously. Briefly, the cells were maintained at 37°C in 5% CO2 at 90% humidity in Dulbecco's modified Eagle's medium (DMEM) high glucose medium containing 15% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin and 1% streptomycin; the medium was exchanged every 3 or 4 d. For the flux studies, the cells were seeded onto polyethylene terephthalate Millipore® cell culture inserts (0.4 µm pore size, 6.5 mm diameter; Millipore Corporation, Billerica, MA, U.S.A.) at a density of $3\times10^5$ cells per well. The culture medium was refreshed on the day after seeding, after which it was refreshed every other day and on the day before the transport experiment. The cells were cultured for 20–22 d after seeding and then evaluated by measuring the transepithelial electrical resistance (TEER) (Millipore ERS®, Millipore Corporation) before our experiments. Only cultures with TEER values exceeding 400Ω·cm² were used for the permeability studies.

**In Vitro Studies**

**Directional Flux across Caco-2 Cells at 37°C or 4°C**

Before the initiation of transport studies, the cell monolayers were rinsed twice with warm (37°C) Hanks’ balance salt solution (HBSS) and pre-incubated at 37°C or 4°C for 20 min. After pre-incubation, the cell monolayers were incubated with YQA-14 (100 µM) in fresh incubation medium from either the apical (AP) or basolateral (BL) side at 37°C or 4°C for another 90 min. The volume of incubation medium on the AP and BL sides was 0.4 mL and 1.2 mL, respectively, and a 50 µL aliquot of the incubation solution was withdrawn from the receiver compartment.

**P-gp and BCRP Inhibition Assays**  The inhibiting effects of several compounds on YQA-14’s flux on Caco-2 cells were investigated by adding the corresponding concentration of each inhibitor to both sides of the cell monolayers. When an inhibitor was used in the flux study, the cell monolayers were preincubated with the inhibitor for 30 min, after which was added the YQA-14 solution (100 µM) to either the AP or BL side, followed by co-incubation for another 90 min. A 50 µL aliquot of this incubation solution was withdrawn from the receiver compartment. The concentrations used were 1 µM for LY335979, 200 nm for Ko143, and 2 µM for GF120918. All compound solutions were made by dilution using dimethyl sulfoxide (DMSO) (final DMSO concentration <0.1%). A positive control, Rho123 for P-gp or topotecan for BCRP, was used in all inhibitory experiments.

**In Vivo Studies**  Brain Distribution of YQA-14 in Mice

The dose formulation of YQA-14 was prepared on the day of the experiment at the concentration of 2.5 mg/mL. YQA-14 was dissolved in normal saline containing 25% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) then injected intraperitoneally into mice at the dose of 25 mg/kg. Blood
and brain samples were collected at 0.08, 0.25, 0.5, 1, 2, and 4 h after intraperitoneally (i.p.) injection of YQA-14 (n=5 at each time point).

### Influence of Efflux Transporter on Brain Distribution of YQA-14

To evaluate the effect of P-gp and/or BCRP inhibitor on the brain distribution of YQA-14, the mice were divided into five groups of 24 animals each. YQA-14 (2.5 mg/mL) was dissolved in normal saline containing 25% HP-β-CD and intraperitoneally injected into the mice at the dose of 25 mg/kg. This dose came from previous pharmacology studies. Inhibitor solutions were prepared by dissolving each inhibitor in a vehicle containing DMSO, propylene glycol, and saline, 2:2:1 (v/v/v). The control group received i.p. blank vehicle (0.2 mL) 30 min prior to YQA-14. GF120918 (10 mg/kg), LY335979 (25 mg/kg) and Ko143 (10 mg/kg) were pre-treated to each group in the same manner as the control group, respectively. Blood and brain samples were collected at 0.08, 0.25, 0.5, 1, 2, 4 h after the i.p. injection of YQA-14 (n=5 at each time point).

### Collection and Quantitation of Samples

Mice were anesthetized with sevoflurane, then blood was collected by cardiac puncture using heparinized Microtainers (BD Biosciences, San Jose, CA, U.S.A.). After exsanguination, whole brains were harvested and homogenized with two volumes of bovine serum albumin using a tissue homogenizer (IKA, T10 basic S25, Germany). Protein of samples in vivo and in vitro (50 µL) were precipitated with 200 µL of a precipitating agent (acetonitrile–methanol 1:1, v/v, containing 1 µM internal standard (IS). The combined samples were vortex-mixed for 1 min then centrifuged at 10,000 rpm for 10 min. The upper organic phase (100 µL) was transferred to HPLC auto-sampler vials, and 5 µL was injected into the LC-MS-MS system. All samples were prepared for analysis by LC-MS-MS as described previously.14
monolayers are shown in Fig. 2. The AP-to-BL and BL-to-AP permeability of YQA-14 was investigated at concentrations of 5 μM. The $P_{app}$ (AP-to-BL) of YQA-14 at 37°C was $3.09 \times 10^{-5}$ cm/s, which was in agreement with our previous study, but contradicted the poor brain distribution of YQA-14.$^{14}$ The BL-to-AP transport of YQA-14 was more than two-fold (2.39) that in the opposite direction at 37°C. The efflux transport of YQA-14 at 4°C was significantly reduced in comparison with the efflux transport at 37°C ($p<0.01$).

**Effect of Several Inhibitors on YQA-14 Transport** In order to characterize the mechanisms involved in the BL-to-AP transport of YQA-14, the effects of several compounds on YQA-14 transport were examined (Fig. 3). As mentioned above, the BL-to-AP permeability of YQA-14 in the Caco-2 cells was significantly higher than the permeability in the AP-to-BL direction ($p<0.01$), yielding an efflux ratio of 2.39. However, after the cells were treated with GF120918 and LY335979, this difference in directional transport was abolished and the efflux ratio reduced to 0.49 and 0.63, respectively. In addition, when the cells were treated with Ko143, the permeability of YQA-14 in both directions was similar to the control group (ER = 2.28).

**Brain Distribution of YQA-14 in Mice** Brain distribution after a single i.p. dose of 25 mg/kg of YQA-14 was determined in BALB/c mice. The concentration of YQA-14 in the brain was significantly lower than in blood ($p<0.01$), and the brain-to-blood concentration ratios were less than 0.05 at all the measured time points (Fig. 4A). The $AUC_{brain}$/AUCblood was 3.97% (Fig. 4B).

**Dual P-gp and BCRP Inhibitor Elacridar (GF120918) Enhances Brain Distribution of YQA-14** We investigated whether the inhibition of P-gp and BCRP at the BBB could enhance the brain exposure of YQA-14 in BALB/c mice. The dual P-gp and BCRP inhibitor GF120918 was used for this purpose. Brain YQA-14 concentrations were greater in the GF120918 treated group compared with the control ($p<0.01$). The brain-to-plasma ratio and $AUC_{brain}$/AUCblood increased by more than 75-fold and 25-fold, respectively, when GF120918 was administered along with YQA-14 (Figs. 5, 6). This indicates that the brain distribution of YQA-14 can be significantly improved by concurrent administration of a dual inhibitor such as GF120918.

**Enhancement of Transport Across the BBB** The transport characteristics of YQA-14 across Caco-2 cell monolayers are shown in Fig. 2. The AP-to-BL and BL-to-AP permeability of YQA-14 was investigated at concentrations of 5 μM. The $P_{app}$ (AP-to-BL) of YQA-14 at 37°C was $3.09 \times 10^{-5}$ cm/s, which was in agreement with our previous study, but contradicted the poor brain distribution of YQA-14.$^{14}$ The BL-to-AP transport of YQA-14 was more than two-fold (2.39) that in the opposite direction at 37°C. The efflux transport of YQA-14 at 4°C was significantly reduced in comparison with the efflux transport at 37°C ($p<0.01$).

**Discussion**

Table 1 shows the receptor binding affinity, $c$ Log $D$ values, brain-to-plasma (blood) exposure ratios and in vivo effective dose of SB-277011A, NGB 2904, PG 01037 and YQA-14. There were sharp contrasts between the relatively small differences in pharmacological action in vivo and big differences in their respective receptor binding properties in vitro. This apparent discrepancy was probably caused by the different
brain-to-plasma exposure ratios of these D₃ receptor inhibitors. As can be seen from the data, the $AUC_{\text{brain}}/AUC_{\text{plasma}}$ of NGB 2904 was 7.55. However, as a P-gp substrate, the brain exposure ratio of PG 01037 was 2.93. The brain concentrations of YQA-14 were generally 20-fold lower than the blood concentrations.

Penetration of drugs into brain tissue from the circulatory system is limited by the BBB, which is formed by the tight connection between endothelial cells and adjacent cells. Caco-2 (human colonic adenocarcinoma) cells share similar characteristics with human small intestinal epithelial cells, and have been widely used to evaluate drugs’ intestinal absorption and brain distribution in humans. A previous study showed that the $P_{\text{app}}$ (AP-to-BL) of YQA-14 across Caco-2 cells was greater than $10^{-5}$ cm/s, and the gastrointestinal absorption of YQA-14 was significant in preclinical animals, which contradicted the poor brain distribution of YQA-14 in the present study. Therefore, we used the Caco-2 cell model in this study to test the roles of transporters in YQA-14 brain distribution. The transport of YQA-14 from the BL-to-AP side of Caco-2 cell monolayers was greater than (more than 2-fold) that in the opposite direction (Fig. 2). The efflux of YQA-14 was temperature-dependent. This suggests that the efflux of YQA-14 is likely mediated by active ATP-binding cassette (ABC) efflux transporters. Members of this superfamily use ATP as an energy source, and pump substrates against the concentration gradient. The most commonly investigated ABC family transporters are P-gp, multidrug resistance-associated protein 2 (MRP2) and BCRP. Substrates of MRP2 are glutathione, glucuronide sulfate, heavy metal conjugates, and unconjugated organic anions, therefore P-gp and BCRP are more likely to be involved in the efflux of YQA-14. These potential transporters, as involved in the efflux of YQA-14, were further characterized in our study.

In the present study, after co-incubation with GF120918 or LY335979, the efflux of YQA-14 decreased and the influx increased correspondingly (Fig. 3). However, when the cells were treated with Kol43, there was no significant difference in the permeability of YQA-14 in both directions (Fig. 3). These results imply that the transcellular transport of YQA-14 from the basolateral membrane was mediated by P-gp, but not by BCRP. The efflux ratio of YQA-14 was decreased obviously (<1) when co-incubated with the P-gp inhibitor, suggesting the involvement of the uptake transporter in the transport of YQA-14, which was reported to be expressed in the AP membrane of Caco-2 cells. However, further studies are needed to confirm the involvement of the influx transporter.

An in vivo experiment showed that YQA-14 transfer into the CNS across the BBB was significantly limited. This result agreed with our assumption that the sharp contrast between the effective in vivo and in vitro dose was due to poor target tissue exposure. Co-treatment with GF120918 enhanced the concentration of YQA-14 in the brain. This finding demonstrates that the increase resulted from the pharmacological inhibition of active efflux at the BBB. Next, we investigated the relative contribution of P-gp and BCRP to the active efflux of YQA-14. Pharmacological inhibition of BCRP by Kol43 did not result in any significant increase in brain levels of YQA-14. However, brain concentration and brain-to-blood ratios increased more than 20-fold when P-gp was inhibited by LY335979, where the brain-to-blood ratio approached 1. This indicates that YQA-14 efflux from the brain occurs mainly via the P-gp efflux transporter, whereas the involvement of other efflux transporters was negligible. This inhibitory effect in vivo was more apparent than in vitro, which may be caused by the difference between the in vitro model (Caco-2 cell) and the real BBB. Brain endothelial cells are connected more tightly than intestinal cells, and the endogenous expression levels of the transporters were obviously higher in BBB. In addition, the extremely high plasma protein binding of YQA-14 (99%) also contributes to its poor brain distribution.

Animal models are used to evaluate anti-drug abuse effects in ethology, such as self-administration, drug seeking behavior and behavior sensitization, yet these models are generally
complicated and not easy to measure. Therefore, we did not investigate the influence of co-administration with inhibitors on the effective dose in vivo of YQA-14. Nevertheless, in the behavior study of administration with or without an inhibitor, mice were in deep slumber without other adverse effects after the intraperitoneal administration of YQA-14 (25 mg/kg) and LY335979 (25 mg/kg) (data not shown). Deep slumber should be caused by a high concentration of YQA-14 in the CNS (>5 µg/g), which would inhibit DA receptors and stimulate the effects of norepinephrine, yielding an increase in the production and release of melatonin.29)

In conclusion, our results show that the brain distribution of YQA-14 is restricted because of active efflux transport at the BBB. P-gp plays a dominant role in limiting the distribu-
tion of YQA-14 to the brain. This could explain the conflicting findings observed in vivo and in vitro in previous pharmacological studies of YQA-14.

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Conflict of Interest The authors declare no conflict of interest.

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