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Abcb1a but not Abcg2 played a predominant role in limiting the brain distribution of Huperzine A in mice

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ABSTRACT

Huperzine A has been used for improving symptoms of Alzheimer's disease. Its cholinergic side effect is thought to be an exaggerated pharmacological outcome linked to its high brain or CNS concentrations. Although Huperzine A is brain penetrable, its interaction with efflux transporters (ABCB1 and ABCG2) has not been fully investigated. The aim of the present study was to characterize roles of ABCB1 and ABCG2 in the transmembrane transport of Huperzine A and identify a rate limiting step in its brain distribution. Data obtained from stably transfected MDCK II cells showed that Huperzine A is a substrate of ABCB1 but not ABCG2. ABCB1 inhibitors significantly inhibited ABCB1 mediated efflux of Huperzine A. In *Abcb1a*$^{-/-}$ mice, the brain to plasma concentration ratio of Huperzine A was significantly increased as compared to the wild type mice, while there were no obvious differences between the wild type and *Abcg2*$^{-/-}$ mice. Taken together, the present study demonstrated that ABCB1 but not ABCG2 played a predominant role in the efflux of Huperzine A across BBB. The current finding is clinically relevant as changes in ABCB1 activity in the presence of ABCB1 inhibitors or genetic polymorphism may affect efficacy and safety of Huperzine A.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is a major cause for age-related dementia (Prasad, 2017; Wang et al., 2016). AD is generally characterized by cognitive impairment and memory loss, which were associated with elevated levels of pro-inflammatory cytokines and deposition of beta-amyloid plaques and neurofibrillary tangles within the brain and cerebral blood vessels (Masters et al., 2015). Currently, there are more than 40 million people are affected by this disease worldwide and the number will likely be over 140 million by 2050 as the aging population grows. AD has become a heavy healthcare, social and economic burden for AD patients' family and society as a whole (Pahnke et al., 2009; Santos et al., 2016). Therefore, there is a significant unmet medical need to prevent and treat this debilitating disease.

Thus far, there are very few effective therapies for AD-related dementia and no cure for the progression of AD symptoms, despite concerted efforts have been made over the years on basic research on AD pathophysiology. Some of the approved drugs such as donepezil, galantamine and rivastigmine target the cholinergic pathway in central nerve system (CNS), while other such as memantine interacts with the *N*-methyl-D-aspartate receptor and glutaminergic pathway (Graham et al., 2017). Alternatively, dietary supplements rich in natural alkaloid and polyphenolic compounds have been implicated in relieving some AD-related symptoms (Ng et al., 2015). Among those, Huperzine A has been used for treating various diseases including AD in China and is marketed as a dietary supplement in the US (Ha et al., 2011).

Naturally occurring Huperzine A is extracted from *Huperzia serrata*. Because of its low yield, the compound can be produced via chemical and biological processes. It is believed that Huperzine A exerts its pharmacological effects via the inhibition of acetylcholinesterase (Ha et al., 2011). An early pharmacokinetic evaluation of Huperzine A after an oral dose of 0.99 mg in human subjects suggested that the absorption of Huperzine A was rapid with a T_{max} of 80 min and C_{max} of 8.4 ng/mL (Qian et al., 1995). The elimination

Abbreviations: ABCB1, ATP Binding Cassette Subfamily B Member 1; ABCG2, ATP Binding Cassette Subfamily G Member 2; BBB, Blood-Brain Barrier; BCRP, Breast Cancer Resistant Protein (protein encoded by ABCG2 gene); P-gp, P-glycoprotein (protein encoded by ABCB1 gene).

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half life of Huperzine A was estimated to be 288 min after oral administration (Qian et al., 1995). Huperzine A was relatively stable in human liver microsomes and hepatocytes and it appeared not as an inhibitor of cytochrome P450 (CYP) enzymes (Lin et al., 2016). However, Huperzine A might induce the expression of CYP3A4 through the activation of PXR signaling pathway (Zhang et al., 2014). Huperzine A was mostly excreted unchanged via renal elimination after oral administration (Lin et al., 2016).

For CNS therapies, it is generally believed that brain distribution is a key factor governing the efficacy and target-related side effects of drugs. In this aspect, the blood-brain barrier (BBB) plays a conflicting role in protecting the brain from harmful substances in one way while limiting drug efficacy in the other. Huperzine A is well tolerated in low doses in human subjects, however mild to moderate cholinergic side effects have been reported at therapeutic doses (Ha et al., 2011). In dogs, dose-related acute cholinergic toxicity was seen with death observed at 10 mg/kg/day dose level (Little et al., 2008). Since Huperzine A is brain penetrable (Wang et al., 1988), it could be speculated that more severe cholinergic side effects were related to high brain concentrations.

In the present study, we aimed to investigate molecular mechanisms affecting the brain penetration of Huperzine A by employing various in vitro cell lines expressing efflux transporters such as ABCB1 and ABCG2 and in vivo transgenic mouse models such as *Abcb1a*$^{-/-}$, *Abcg2*$^{-/-}$ and *Abcb1a*$^{-/-}$/*Abcg2*$^{-/-}$. Results showed ABCB1 but not ABCG2 played a more predominant role in limiting the brain penetration of Huperzine A. It is anticipated that the current finding would lead to safe and effective use of Huperzine A in treating AD patients.

2. Materials and methods

2.1. Materials

Huperzine A was kindly provided by PharmaResources (Shanghai) Co.,Ltd. (Shanghai, China) with HPLC purity >98%. GF120918, digoxin, propranolol, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Hyclone (Logan, UT). Twelve-well plates and transwell (0.4 μm pore-size, 0.65 mm diameter) polycarbonate inserts were from Corning Co. (Corning, NY). Hanks' balance salt solution (HBSS) containing 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 10 mM HEPES and 5 mM D-glucose was prepared in house. All other reagents and chemicals were of analytical grade or of the highest quality available commercially.

2.2. Cell culture

Caco-2 and MDCK II cells were purchased from ATCC (Manassas, VA) and ECACC (Salisbury, UK). Caco-2 cells were grown in high-glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C with 5% CO_2 and 95% humidity. Human efflux transporters ABCB1 and ABCG2 single-transfected MDCK II cell lines were described previously (Wang et al., 2015). ABCB1/ABCG2 double-transfected MDCK II cell lines were established at PharmaResources (Shanghai) Co.,Ltd. (Shanghai, China). Briefly, the ABCB1 and ABCG2 cDNA, synthesized by Genwiz Co. Ltd. (Suzhou, China), were cloned into pcDNA3.1 (+) and pcDNA3.1 (+)/Hygro (Invitrogen, Carlsbad, CA), respectively. MDCK II cells were transfected with pcDNA3.1 (+) containing ABCB1 cDNA or pcDNA3.1 (+)/Hygro containing ABCG2 cDNA or co-transfected with pcDNA3.1 (+) containing ABCB1 cDNA and pcDNA3.1 (+)/Hygro containing ABCG2 cDNA using Lipofect AMINE 2000 Reagent (Invitrogen, Carlsbad, CA). Single colonies were picked out after the transfected cells were cultured in complete medium containing G418 (0.6 mg/mL) or Hygromycin B (0.3 mg/mL) or G418 and Hygromycin B, respectively. Stable-transfected cells were selected using RT-PCR for the measurement of mRNA expression and transport activity evaluation.

2.3. Bidirectional transport assay using Caco-2 and MDCK II cells

To evaluate the role of efflux transporters involved in the transmembrane transport of Huperzine A. Bidirectional transport assay were conducted in Caco-2 as well as MDCK II cells using similar procedures as previously described (Wang et al., 2015). Briefly, Caco-2 or MDCK II cells were plated on transwell membrane inserts at a density of 2×10^5 or 1×10^5 cells per well and cultured for 21 or 5 days, respectively. On the experimental day, transmembrane resistance was measured and a value of >200 Ω or >500 Ω was considered adequate to make sure the integrity of the monolayer for MDCKII cells or Caco-2 cells, respectively. Huperzine A (10 μM) was loaded to the apical or basal side. Incubations were carried out at 37 °C for 120 min. After the end of the incubation, media from both sides were collected and concentrations of Huperzine A were determined by the LC-MS/MS method described below.

2.4. In vivo studies

Male C57BL/6 mice were obtained from Joinn Laboratories New Drug Research Center Co., Ltd. (Suzhou, China). Male *Abcb1a*$^{-/-}$ knockout mice, *Abcg2*$^{-/-}$ knockout mice, and *Abcb1a*$^{-/-}$/*Abcg2*$^{-/-}$ double knockout C57 BL/6 mice were generously provided by Nanjing Galaxy Biopharmaceutical Co.,Ltd. (Nanjing, China) and single-knockout mice strains were generated according to previously reports (Jonker et al., 2005; Schinkel et al., 1995) and double knockout mice were obtained through cross-breeding with *Abcb1a*$^{-/-}$ knockout mice and *Abcg2*$^{-/-}$ knockout mice and BBB transport activity was confirmed using sorafenib according to the published report (Agarwal and Elmquist, 2012). All mice were 8–10 weeks old and maintained under controlled temperature with a 12-h light/dark cycle. Food and water were available ad libitum. All animal studies were reviewed and conducted according to the guidelines of Animal Care and Use Committee at Soochow University.

Huperzine A was dissolved in DMSO and further diluted with saline to obtain a final concentration of 0.05 mg/mL. Animals were divided into four groups (WT, *Abcb1a*$^{-/-}$, *Abcg2*$^{-/-}$, and *Abcb1a*$^{-/-}$/*Abcg2*$^{-/-}$) and each received an oral dose of 0.5 mg/kg of Huperzine A. Plasma and brain samples were collected at 0.25, 1, 2, 4 and 8 h after Huperzine A administration. Blood samples were immediately centrifuged and plasma was collected and stored at −80 °C until analysis. The brain samples were rinsed with cold saline to get rid of blood contamination, petted dry with paper towel, weighed and homogenized with three times of ice-cold saline with a tissue homogenizer (Fluko, Germany). The levels of Huperzine A in the plasma samples and brain homogenates were quantified by LC-MS/MS.

2.5. Quantitation of Huperzine A by LC-MS/MS

Concentrations of Huperzine A were determined by reverse-phase liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatographic separation was achieved using a reverse phase C_{18} column (50 × 2.1 mm, 5 μm particle size; Agela, Tianjin, China) and linear gradient of mobile phase A containing 10 mM