

# The Inhibitory Effect of Rivastigmine and Galantamine on Choline Transport in Brain Capillary Endothelial Cells

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**Abstract** – The blood-brain barrier (BBB) transport of acetylcholinesterase (AChE) inhibitors, donepezil and tacrine suggested to be mediated by choline transport system in our previous study. Therefore, in the present study, we investigated the interaction of other AChE inhibitors, rivastigmine and galantamine with choline transporter at the BBB. The effects of rivastigmine and galantamine on the transport of choline by conditionally immortalized rat brain capillary endothelial cell lines (TR-BBB cells) were characterized by cellular uptake study using radiolabeled choline. The uptake of [<sup>3</sup>H]choline was inhibited by rivastigmine and galantamine, with IC<sub>50</sub> values (*i.e.* concentration necessary for 50% inhibition) for 1.13 and 1.15 mM, respectively. Rivastigmine inhibited the uptake of [<sup>3</sup>H]choline competitively with K<sub>i</sub> of 1.01 mM, but galantamine inhibited noncompetitively. In addition, the efflux of [<sup>3</sup>H]choline was significantly inhibited by rivastigmine and galantamine. Our results indicated that the BBB choline transporter may be involved in a part of the influx and efflux transport of rivastigmine across the BBB. These findings should be therapeutically relevant to the treatment of Alzheimer's disease (AD) with AChE inhibitors, and, more generally, to the BBB transport of CNS-acting cationic drugs via choline transporter.

**Keywords:** Rivastigmine, Galantamine, Choline transport, Blood-brain barrier, TR-BBB cells

## INTRODUCTION

Acetylcholinesterase (AChE) inhibitors, such as donepezil, tacrine, rivastigmine and galantamine, are clinically useful in the treatment of Alzheimer's disease (AD). The efficacy of AD treatment drugs depends upon the ability of drugs to cross the blood-brain barrier (BBB) and reach therapeutic concentrations in brain following systemic administration. The BBB is formed by tight junctions of the brain capillary endothelial cells, which are highly restrictive for the entry of both large and small hydrophilic drugs used in the treatment of CNS disorder from the blood to the brain parenchyma cells (Pardridge, 1988). Actually, the clinical failures of most of the potentially effective therapeutics to treat the central nervous system disorders are often due to their poor permeability across the BBB (Ohtsuki and Terasaki, 2007). Therefore, the understanding of transport mechanism of these AChE inhibitors across the BBB would be important for therapeutic optimi-

zation.

It was reported that the brain distribution of these AChE inhibitors such as donepezil and tacrine is relatively high (McNally *et al.*, 1996; Matsui *et al.*, 1999). The concentration of donepezil and tacrine in the brain was several times higher than those in the plasma. These findings suggested that carrier-mediated transport system(s) may be involved in the penetration of these drugs into the brain across the BBB. In our previous study, donepezil and tacrine competitively inhibited choline uptake via organic cation transporter 2 (OCT2) in conditionally immortalized rat brain capillary endothelial cell line (TR-BBB cells) (Kang *et al.*, 2005). In addition, donepezil and tacrine inhibited choline efflux transport at the BBB (Lee and Kang, 2008). These findings indicated that this transporter may be also involved in the transport of basic drugs such as donepezil and tacrine across the BBB. Other reports also suggested that donepezil and tacrine were transported to the brain across the BBB by organic cation transporter (OCT) such as choline transport systems, CHT1 or OCT2 (Sung *et al.*, 2005; Kim *et al.*, 2009).

Rivastigmine and galantamine are also clinically avail-

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able drugs used for the treatment of AD by the inhibition of AChE or butyrylcholinesterase (BChE) like donepezil and tacrine. These drugs are known to cross the blood-brain barrier, but the transport mechanism(s) remains unclear (Jann *et al.*, 2002). Rivastigmine is a carbamate derivative, which has similar structure to acetylcholine and a molecular weight of 400.43. Galantamine is an alkaloid from the bulb and flowers of snowdrop, which is similar to codein. Since these AChE inhibitors are bulky organic cationic drugs in spite of widely differing molecular structures, organic cation transporter (OCT) including the choline transporter may be also involved in the transport of these drugs across the BBB like donepezil and tacrine. The BBB choline transporter has been proposed as a drug delivery system for various amine drugs such as eperisone, scopolamine and thiamine (Kang *et al.*, 1990). Therefore, in the present study, we investigated the BBB transport mechanism of rivastigmine and galantamine by evaluation of the relationship between the choline transporter among the OCTs and these drugs using TR-BBB cells as an *in vitro* model of BBB.

## MATERIALS AND METHODS

### Materials

[Methyl-<sup>3</sup>H]Choline (<sup>3</sup>H]choline, 82.0 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Donepezil hydrochloride was kindly provided by Daewoong Co. (Seoul, Korea). Rivastigmine was provided from Novartis International Pharmaceutical Ltd. (Basel, Switzerland). Galantamine was purchased from Aldrich Chemical (St. Louis, MO, USA). All other chemicals and reagents were commercial products of reagent grade and were purchased from Sigma Chemical (St. Louis, MO, USA).

### Cell culture

The TR-BBB cells were cultured with Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, San Diego, CA, USA) and 15 µg/L endothelial cell growth factor (Roche, Mannheim, Germany) at 33°C in a humidified atmosphere of 5% CO<sub>2</sub>/air. On rat tail collagen type I-coated 24 well culture plates (IWAKI, Tokyo, Japan) initial seeding was done at 1×10<sup>5</sup> cells/well for the uptake study. After incubation for 3 days at 33°C, the cultures became confluent and then used in transport study.

### Transport studies in TR-BBB cells

The [<sup>3</sup>H]choline uptake was performed according to the

previous report (Kang *et al.*, 2005). TR-BBB cells were washed three times with 1 ml extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO<sub>3</sub>, 3 mM KCl, 1.4 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 0.4 mM K<sub>2</sub>HPO<sub>4</sub>, 10 mM D-glucose and 10 mM Hepes (pH 7.4) at 37°C. Uptake was initiated by applying 200 µl containing [<sup>3</sup>H]choline (28 nM) at 37°C in the presence or absence of AChE inhibitors. The cells were then solubilized by incubation overnight in 750 µl of 1 N NaOH at RT. An aliquot (50 µl) was taken for quantification of cellular protein using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The remaining solution (500 µl) was mixed with 5 ml of scintillation cocktail (Hionic-fluor; Packard, Meriden, CT, USA) for measurement of radioactivity in a liquid scintillation counter (LS6500; Beckman, Fullerton, CA, USA).

The efflux of [<sup>3</sup>H]choline by TR-BBB cells was determined by incubating the cells for 60 min at 37°C with [<sup>3</sup>H]choline dissolved in ECF buffer. The medium was then removed, and the cells were washed with ice-cold ECF buffer. The incubation medium alone or containing inhibitors was added for desired times. After appropriate time periods, the incubation medium was removed and the amount of [<sup>3</sup>H]choline remained in the cells was measured.

### Data analysis

To analyze the competitive nature of rivastigmine and galantamine for [<sup>3</sup>H]choline, Lineweaver-Burk plots were generated. The inhibitory constant (*K<sub>i</sub>*) was calculated from equation (1):

$$V = V_{\max} \cdot C/[K_m \cdot (1 + I/K_i) + C] \quad (1)$$

where *I* corresponds to the concentration of rivastigmine and galantamine, respectively. *V*, *C*, *K<sub>m</sub>* and *V<sub>max</sub>* are velocity of saturable uptake, the substrate concentration in the uptake medium, the Michaelis-Menten constant and the maximum velocity of uptake, respectively.

Statistical analyses were carried out by one-way ANOVA with Dunnett's post-hoc test.

## RESULTS

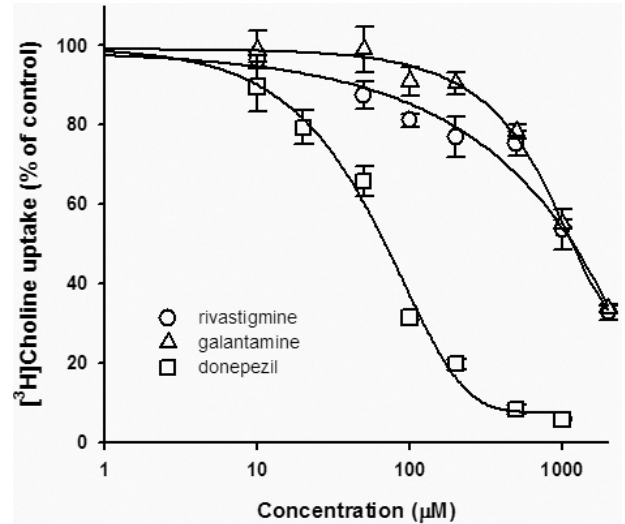
First, the effects of rivastigmine and galantamine on choline influx transport in TR-BBB cells were studied by assessing the ability of these compounds to inhibit [<sup>3</sup>H]choline uptake. [<sup>3</sup>H]Choline uptake in the presence of 2.0 mM rivastigmine and galantamine was decreased to about 30% of the control level (Table I). The inhibition of [<sup>3</sup>H]choline uptake caused by these drugs occurred in a

concentration dependent manner.

The dose-response relationship for the inhibition of [<sup>3</sup>H]choline uptake by donepezil, rivastigmine and galantamine in TR-BBB cells is given in Fig. 1. In our previous report, it was already found that AChE inhibitors such as donepezil and tacrine inhibited choline uptake competitively (Kang *et al.*, 2005). However, the IC<sub>50</sub> value (*i.e.* concentration necessary for 50% inhibition) for donepezil was not calculated, so we also examined the inhibition of [<sup>3</sup>H]choline uptake by donepezil. [<sup>3</sup>H]Choline uptake was decreased by various AChE inhibitors in a dose-dependent manner. Donepezil inhibited [<sup>3</sup>H]choline uptake more strongly than rivastigmine and galantamine. It is indicated that donepezil is a more potent inhibitor of choline uptake than rivastigmine and galantamine. The IC<sub>50</sub> values for donepezil, rivastigmine and galantamine were 0.068, 1.13 and 1.15 mM, respectively (Table II).

Furthermore, we investigated the kinetic nature of the inhibitory effect of rivastigmine and galantamine on choline uptake. Lineweaver-Burk plot of choline uptake in TR-BBB cells showed mutual inhibitory effect by rivastigmine and galantamine (Fig. 2). In the presence of 500 μM riva-

stigmine the K<sub>m</sub> value for choline uptake by TR-BBB cells increased from 39.6 to 51.7 μM, whereas the V<sub>max</sub> did not change significantly. These results indicated that rivastigmine competitively inhibited choline uptake with a K<sub>i</sub> of



**Fig. 1.** Dose-response relationship for the inhibition of [<sup>3</sup>H]choline uptake by rivastigmine, galantamine and donepezil. Uptake of [<sup>3</sup>H]choline was measured in TR-BBB cells with 5 min incubation in the presence of 0-2,000 μM unlabeled rivastigmine (open circle), galantamine (open triangle) and donepezil (open square) at pH 7.4 and 37°C. The data represent the mean ± S.E.M. (n=3-4).

**Table I.** Effect of rivastigmine and galantamine on the [<sup>3</sup>H]choline uptake in TR-BBB cells

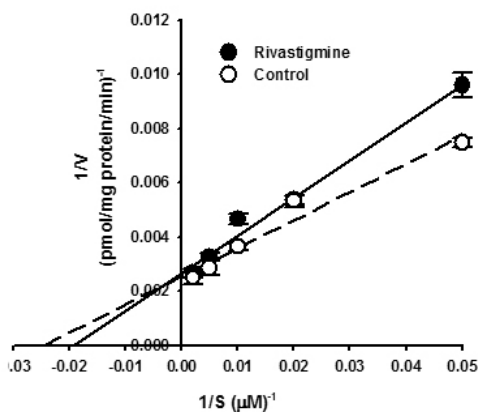
Substrate	Concentration (mM)	Uptake of [ <sup>3</sup> H]choline (% of control)
Control		100 ± 3
Rivastigmine	0.5	75.3 ± 3.1 <sup>a</sup>
	2.0	32.8 ± 1.9 <sup>b</sup>
Galantamine	0.5	78.2 ± 1.9 <sup>a</sup>
	2.0	33.5 ± 0.9 <sup>b</sup>

[<sup>3</sup>H]Choline uptake by TR-BBB cells was measured in the absence (control) or presence of rivastigmine and galantamine for 5 min at 37°C. Each value represents the mean ± S.E.M. (n=3-4). <sup>a</sup>p < 0.05, <sup>b</sup>p < 0.001; significantly different from control.

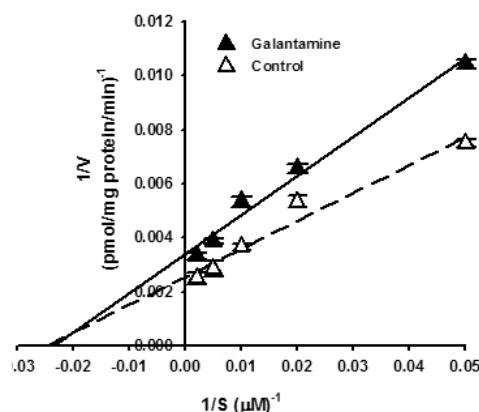
**Table II.** IC<sub>50</sub> and K<sub>i</sub> values of donepezil, rivastigmine and galantamine for choline uptake in TR-BBB cells

AChE inhibitor	IC <sub>50</sub> (mM)	K <sub>i</sub> (mM)
Donepezil	0.068	0.046
Rivastigmine	1.13	1.01
Galantamine	1.15	None

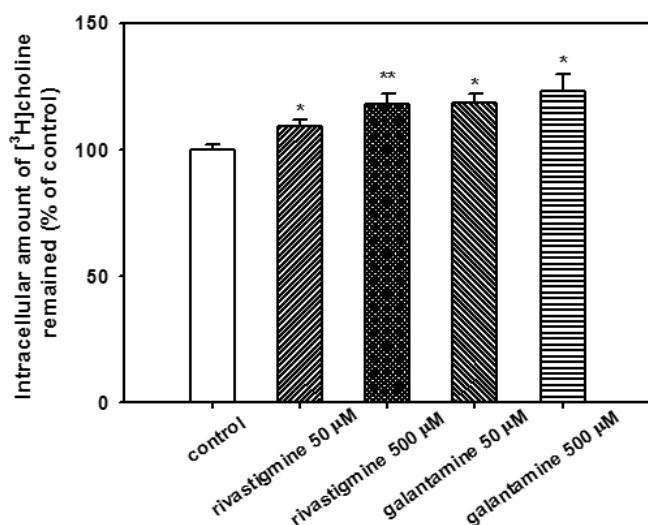
(A)



(B)



**Fig. 2.** Lineweaver-Burk plots of [<sup>3</sup>H]choline uptake by TR-BBB cells showing competitive or non-competitive inhibition by rivastigmine (A) and galantamine (B). [<sup>3</sup>H]choline (28 nM) uptake was performed in the presence of 500 μM rivastigmine (closed circle), 500 μM galantamine (closed triangle) or the absence (open circle, triangle) in TR-BBB cells at pH 7.4 and 37°C for 5 min. The data represents the mean ± S.E.M. (n=3-4).



**Fig. 3.** Effect of rivastigmine and galantamine on efflux of [<sup>3</sup>H]choline in TR-BBB cells. TR-BBB cells were first incubated for 60 min at 37°C with ECF buffer containing [<sup>3</sup>H]choline (28 nM). Then, the incubation medium alone or containing rivastigmine or galantamine was added for 30 min at 37°C. After that, the incubation medium was removed and the amount of [<sup>3</sup>H]choline remained in the cells was measured. Each value represents the mean ± S.E.M (n=3). \**p* < 0.05, \*\**p* < 0.01; significantly different from control.

1.01 mM. In contrast,  $K_m$  value for choline uptake by TR-BBB cells did not change in the presence of 500 μM galantamine. These results indicated that galantamine represent noncompetitive inhibition for choline uptake.

In addition, the effects of rivastigmine and galantamine on choline efflux transport in TR-BBB cells were studied. [<sup>3</sup>H]Choline efflux was inhibited significantly by rivastigmine and galantamines regardless of the drug concentrations in TR-BBB cells (Fig. 3).

## DISCUSSION

In the present study, we investigated the interaction of rivastigmine and galantamine with choline transporter at the BBB using TR-BBB cells as an *in vitro* model of BBB. TR-BBB cells was established from transgenic rats harboring temperature-sensitive SV40 large T antigen gene, exhibited the fastest growth at 33°C. These cells have been shown to express a number of receptors and transporters including glucose transporter-1 (GLUT-1), large neutral amino acid transporter (LAT1) and P-glycoprotein (P-gp) that are considered as specific for endothelial cells (Hosoya *et al.*, 2000). Also, tight junction protein such as claudine-5 and occludin are also expressed in these cells (Hosoya *et al.*, 2002). Further, the BBB permeability clearances of

several compounds estimated in TR-BBB cells appear quite similar to *in vivo* evaluations (Terasaki *et al.*, 2003). TR-BBB cells have already been used to demonstrate that transporters such as taurine transporter (TauT), system A isoform (ATA2) are expressed in rat brain endothelium (Kang *et al.*, 2002; Takanaga *et al.*, 2002). Accordingly, we thought that TR-BBB cells are one of the best *in vitro* models of BBB, allowing for the investigation of the mechanism for transport of diverse compounds including various drugs.

The BBB restricts the transport of cations from the blood to the brain parenchyma by a number of tight junctional proteins and tight junctions of the brain capillary endothelial cells (Pardridge, 1988). Despite these restrictions, it has been shown that the charged quaternary ammonium molecule, choline, is transported across the BBB via a saturable carrier-mediated mechanism (Cornford *et al.*, 1978). The plasma choline concentration in healthy adults is approximately 10 μM (Cohen *et al.*, 1995), which is less than the calculated  $K_m$  for the BBB choline transporter (Sawada *et al.*, 1999; Allen and Smith, 2001; Kang *et al.*, 2005). This fact suggests that this transporter is not saturated under physiological condition and may deliver other compounds to the brain. Actually, the BBB choline transporter has been also reported to play an important role in the brain uptake of other organic cationic compounds such as eperisone, derivatives of lobeline, isoarecolone, and nicotine (Kang *et al.*, 1990; Metting *et al.*, 1998; Allen *et al.*, 2003). Therefore, the BBB choline transporter has been suggested to be suitable as one of drug delivery vectors for cationic drugs.

AChE inhibitors such as donepezil, tacrine, rivastigmine and galantamine are used therapeutic agents for AD by preventing the destruction of synaptic acetylcholine. Among representative AChE inhibitors, donepezil and tacrine have been known to be transported to the brain across the BBB by choline transport system (Kang *et al.*, 2005; Sung *et al.*, 2005; Kim *et al.*, 2009). However, the transport mechanism(s) of rivastigmine and galantamine remains unclear. It has been reported that these drugs cross the BBB and show their pharmacological action (Jann *et al.*, 2002). Rivastigmine and galantamine are also organic cations in spite of having different molecular structures from donepezil and tacrine. Therefore, in this study, we hypothesized that BBB choline transporter may be also involved in transport of rivastigmine and galantamine across the BBB.

In the results of our study, [<sup>3</sup>H]choline uptake in the presence of rivastigmine and galantamine was decreased in a concentration dependent manner (Table I, Fig. 1), and

the  $IC_{50}$  values for donepezil, rivastigmine and galantamine were 0.068, 1.13 and 1.15 mM, respectively (Table II). Also, rivastigmine competitively inhibited choline uptake with  $K_i$  of 1.01 mM, but galantamine represented non-competitive inhibition for choline uptake in the Lineweaver-Burk plot analysis (Fig. 2). These results indicate that rivastigmine as well as donepezil compete with choline for the same binding site on the transporter. It was reported that the  $K_m$  value for [ $^3H$ ]choline uptake by TR-BBB cells were about 26  $\mu$ M (Kang *et al.*, 2005). In the case of donepezil, the  $K_i$  values estimated for choline (45.7  $\mu$ M) was similar to  $K_m$  value for choline, suggesting the occurrence of competition between donepezil and choline for binding to the transporter like the results of previous reports. However, the affinity of rivastigmine for the choline transporter is relatively low compared with that of donepezil. Rivastigmine is a carbamate derivative and has a molecular weight of 400.43 and n-octanol to water partition coefficient of 3.0 (Polinsky, 1998). Based on the chemical properties, it would be expected that BBB transport of rivastigmine is carried out by passive diffusion (Murakami *et al.*, 2000). However, our result proved that BBB choline transport system also involved in the transport of rivastigmine across the BBB. Taken together, we suggest that a part of rivastigmine penetrating into the brain following systemic administration is mediated by the BBB choline transporter and the remaining is the result of passive diffusion.

Choline is an essential nutrient that plays an important role as a precursor for the synthesis of the neurotransmitter acetylcholine (Cornford *et al.*, 1978). Abnormal choline transport and metabolism have been implicated in a number of neurodegenerative disorders such as Alzheimer's and Parkinson's disease. In this respect, the BBB choline transporter is free to transport other cationic molecules without interrupting the supply of choline to the brain. The maximal plasma concentrations of donepezil and rivastigmine at the steady states were 61 ng/ml (Tiseo *et al.*, 1998) and 56 ng/ml (Polinsky, 1998), respectively, and plasma protein binding rates of donepezil, rivastigmine and galantamine were 95%, 40% and 18%, respectively (Jann *et al.*, 2002). These data indicated that plasma concentrations of donepezil and rivastigmine were several times lower than  $K_i$  value of them. Therefore, AChE inhibitors may not significantly inhibit choline transport at the BBB.

In addition, rivastigmine and galantamine also inhibited efflux transport of choline in TR-BBB cells (Fig. 3). In our previous report, it showed that choline underwent efflux from brain to blood across the BBB via a carrier-mediated efflux transport system which has the similar blood-to-

brain influx rate (Lee and Kang, 2006). Therefore, this efflux system is supposed to play an important role in the choline turnover in the brain. Our previous report, donepezil and tacrine inhibited choline efflux via choline transport system *in vivo* and *in vitro* BBB model (Lee and Kang, 2008). Our result suggests that brain-to-blood efflux of rivastigmine and galantamine also occur by BBB choline transporter like donepezil and tacrine. These findings also suggest that these drugs may play a role in maintaining choline concentration in the brain resulting in the increase of acetylcholine synthesis.

In conclusion, it was found that the BBB choline transporter may be involved in a part of the influx and efflux transport of rivastigmine across the BBB. These findings also show that AChE inhibitors, rivastigmine as well as donepezil and tacrine, which are various organic cationic compounds, may exhibit increased brain distribution via the choline transport system. These findings should be therapeutically relevant to the treatment of AD with AChE inhibitors, and, more generally, to the BBB transport of CNS-acting cationic drugs via choline transporter.

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