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Full Length Research Paper

Acetylcholinesterase inhibitors used or tested in Alzheimer's disease therapy; their passive diffusion through blood brain barrier: *In vitro* study

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Although the information about Alzheimer's disease (AD) etiology is still unclear; acetylcholinesterase inhibitors still play a major role in symptomatic treatment of AD. Unfortunately, a relevant argumentation is complicated since information about real drug concentration in the brain or time-dependent blood-brain barrier (BBB) distribution studies are still quite rare. In this *in vitro* study, high-performance liquid chromatography (HPLC) method with special (IAM – immobilized artificial membrane) column was used to determine the ability of cholinesterase inhibitors to penetrate through BBB. Set of 8 structurally different cholinesterase inhibitors applicable to AD treatment was evaluated throughout this study. According to our method, all molecules are able to penetrate BBB by passive transport. However, some molecules such as huperzine A and galanthamine have lower ability to penetrate the BBB directly. These molecules may be delivered into the brain via active transport. Other molecules probably use passive transport to permeate into the central nervous system; tacrine and 7-methoxytacrine exert the highest passive permeation from this set of compounds.

Key words: Blood-brain barrier, central nervous system, acetylcholinesterase, Alzheimer disease.

INTRODUCTION

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease with many cognitive and neuropsychiatric manifestations that result in progressive disability (Grossberg, 2003; Schwarz et al., 2012). Loss of basalocortical cholinergic neurons in the hippocampus and the presence of β-amyloid protein in extraneuronal plaques and tau protein in neurofibrillary tangles are the characteristic histopathological features of AD (Bi, 2010; Braak and Del Tredici, 2013). Cholinergic neurons are slowly depleted, and consistent deficit of acetylcholine (ACh) is responsible for insufficient cholinergic neurotransmission (Castellani et al., 2010; Van der Zee et al., 2011).

Two enzymes are closely involved in ACh fate: human choline acetyltransferase (hChAT; EC 2.3.1.6) partaking in ACh synthesis and human acetylcholinesterase (hAChE; EC 3.1.1.7)) in the degradation of ACh in neurons (Racchi et al., 2004). Interestingly, hAChE activity decreases progressively in certain brain regions from mild to severe stages of AD to reach 10 to 15% of normal values. While butyrylcholinesterase (BChE; EC 3.1.1.8) activity is unchanged or even increased by 20%, therefore a large pool of BChE is available in glia, neurons and neuritic plaques (Becker and Giacobini, 1997).One way to improve cholinergic transmission in AD patients is inhibition of hAChE. Lower degradation leads to increased

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availability of ACh to stimulate nicotinic and muscarinic recaptors within the brain. Although the cholinesterase inhibitors (ChEI) application may be considered as simple symptomatic treatment, they still represent the cornerstone of AD therapy (Giacobini, 2004).

The effectiveness of ChEI in AD treatment is limited by their ability to penetrate through the blood-brain barrier (BBB). BBB is dynamics and complex interface between blood substances and central compartment that play a key role in brain protection and homeostasis (Tsukita et al., 2001; Turksen and Troy, 2004). BBB is not only physical but also a metabolic barrier where imbedded enzymes are involved in the catabolism of xenobiotics (Lorke et al., 2008). Smaller lipophilic substances have some access to the central nervous system by diffusion, whereas other substances can cross the BBB by carrier-mediated influx transport, receptor mediated transcytosis and absorptive-mediated transcytosis (Terasami and Ohtsuki, 2005; Edvinsson and Tfelt-Hansen, 2008).

Tacrine, donepezil, rivastigmine and galanthamine were found among the commonly used ChEI for the symptomatic treatment of patients suffering from mild to moderate AD (Moussa et al., 2005). The other potential compounds are huperzine A and 7-methoxytacrine (7-MEOTA) (Zhao et al., 2002). The penetration into central nervous system (CNS) is commonly confirmed by their therapeutic effect (improved cognitive and memory functions, improved behavioral deficits) or by their potency to inhibit cholinesterase in brain (de los Rios, 2012).

The aim of this study is to experimentally characterise and predict the ability of some ChEI to penetrate the BBB. For this purpose, we have chosen *in vitro* high-performance liquid chromatography (HPLC) method, which employs immobilized artificial membrane (IAM) (Karasova et al., 2010a). The IAM chromatography was used earlier for the prediction of the passive drug transport across biological barrier and for that reason it could be also used as a screening method for the prediction of BBB permeation (Yoon et al., 2006; Karasova et al., 2010b).

This method was validated on a set of twenty-one therapeutic compounds (Table 1) (Yoon et al., 2006) and consequently used for a set of eight structurally varying ChEI. Set of these ChEI consists of commonly used anti-AD drugs (donepezil, rivastigmin, galanthamine) and also some other aqiured drugs with promising central inhibition potency (tacrine, huperzine A, 7-MEOTA). The last tested compounds was quaternary pyridostigmine. All structures are depicted in Table 2.

MATERIALS AND METHODS

The experimental part such as used chemicals, apparatus and chromatographic conditions were published and reviewed previously (Karasova et al., 2010a, b). The results of validation process were slightly changed as described below.

Chemicals

Atenolol, β-estradiol, caffeine, cefuroxime, chlorpromazine, cimetidine, corticosterone, desipramine, enalapril, hydrocortisone, ibuprofen, imipramine, lomefloxacin, loperamide, nadolol, piroxicam, progesterone, promazine, propranolol, and testosterone were purchased from Sigma Aldrich (Prague branch, Czech Republic). Acetonitrile gradient grade LiChrosolv was purchased from Merck (Darmstadt, Germany). KH_2PO_4, Na_2HPO_4, KCl, and NaCl were purchased from Lachema (Neratovice, Czech Republic). AChE reactivators were synthetized previously in our laboratory. Their structures are shown in Table 1. Water was reverse osmosis pure.

Apparatus and chromatographic condition

The HPLC system consisted of a P200 gradient pump (Spectra-Physics Analytical, Fremont, USA), a 7125 injection valve – 10 ul loop (Rheodyne, Cotati, USA), an UV1000 detector (Spectra-Physics Analytical, Fremont, USA) and a CSW Chromatography Station 1.5 software (DataApex, Praha, Czech Republic). For analyses an IAM.PC.DD 2 (150 × 4.6 mm; 12 µm) column (Regis Technologies, Morton Grove, IL) was used. The mobile phase was 80% phosphate buffered saline (PBS) and 20% acetonitrile (v/v) with pH 7.4 using Na_2HPO_4. The PBS was prepared with 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8.1 mM Na_2HPO_4. The flow rate was 1.2 ml/min. The absorbance was measured at 210 nm. All chromatograms were obtained at 37°C.

Procedure

In this study, we determined k_{IAM} (capacity factor of this special column) for AChE reactivators. The capacity factor was calculated according to below mentioned formula.

$$k_{IAM} = \frac{t_r - t_0}{t_0}$$

t_r is the retention time of the drug and t_0 is the hold-up time of the column.

The k_{IAM} was determined for twenty-one reference drugs mentioned before. The knowledge about penetration of these drugs through the BBB was compared with measured k_{IAM}. The last step was correction of result by power function of molecular weight according to below mentioned formula.

$$X = \frac{k_{IAM}}{MW^4}$$

The obtained results of standards were correlated with known physico-chemical constants – logarithm of partition coefficient (LogP), molecular polar surface area (PSA) and molecular weight (MW) of standards (Table 1). These constants were also calculated for tested AChE inhibitors (Table 2) (Yoon et al., 2006). The calculated physico-chemical parameters were used for correlation standard compounds results (X) (Table 1) and showed the validity of this method (Figures 1 and 2).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version

Table 1. Structures and descriptors of standards.

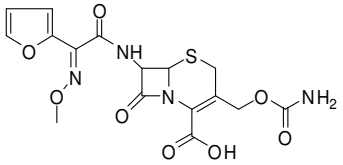
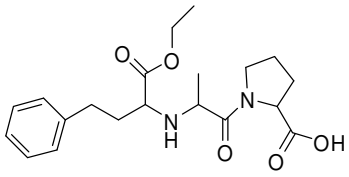
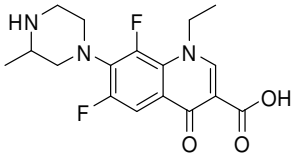
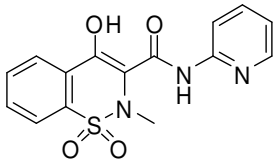
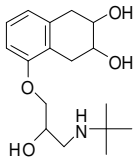
S/No	Compound name	MW	Structure	pKa	log P	PSA (Å^2)	t_r (min)	Result (X)
1	Cefuroxime CNS-	424		3.15	-1.44	201.89	1.64	0.38
2	Enalapril CNS-	376		3.18/5.19	2.10	98.77	1.77	0.71
3	Lomefloxacine CNS-	352		5.65/8.70	2.06	80.29	3.09	2.11
4	Piroxicame CNS-	331		4.27	1.02	110.81	1.87	1.31
5	Nadolol CNS-	309		9.76	0.37	86.53	8.56	11.78

Table 1. Contd.

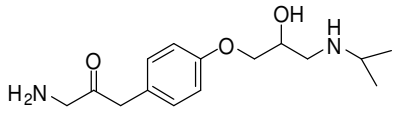
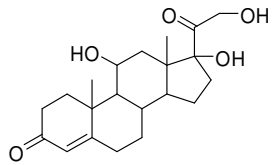
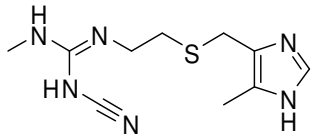
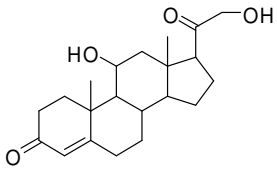
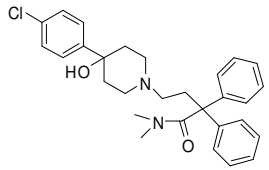
6	Atenolol CNS-	266		9.67	0.26	84.58	4.81	11.19
7	Hydrocortisone CNS-	362		12.74	1.47	94.83	5.90	4.13
8	Cimetidine CNS-	252		6.92	0.24	114.19	2.66	6.57
9	Corticosterone CNS-	346		-	2.48	74.60	9.33	8.23
10	Loperamide CNS-/CNS+	477		9.41	4.71	44.98	359.06	94.95

Table 1. Contd.

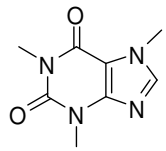
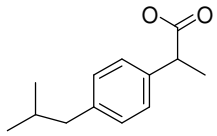
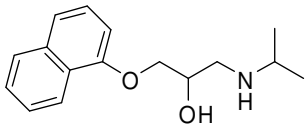
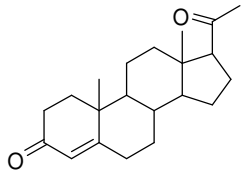
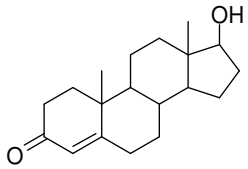
11	Caffeine CNS+	194		1.50	-0.79	58.44	2.06	12.85
12	Ibuprofene CNS+	206		4.86	3.83	40.13	2.81	15.83
13	Propranolol CNS+	259		9.67	2.50	46.07	99.61	301.41
14	Progesterone CNS+	314		-	4.63	34.14	61.74	86.10
15	Testosterone CNS+	288		-	3.54	37.30	26.24	50.87

Table 1. Contd.

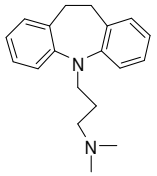
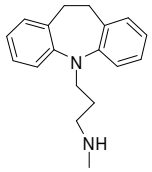
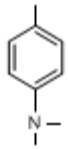
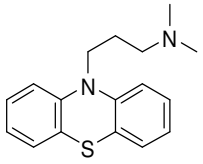
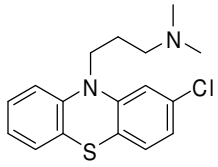
16	Imipramine CNS+	280		9.19	4.01	7.68	229.98	511.62
17	Desipramine CNS+	266		10.02	3.64	19.85	240.37	656.61
18	p-Toluidine CNS+	107		5.46	2.78	3.24	4.10	352.56
19	Promazine CNS+	284		9.20	4.04	32.98	313.41	659.96
20	Chlorpromazine CNS+	319		9.19	4.56	32.98	644.08	863.00

Table 1. Contd.

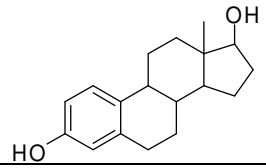
21	β-estradiol CNS+	272		10.33	3.71	40.46	94.39	218.23

Table 2. Structures of AChE inhibitors.

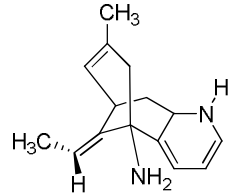
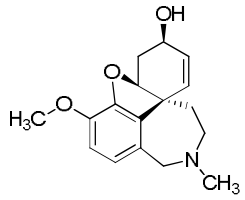
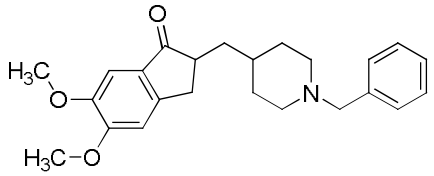
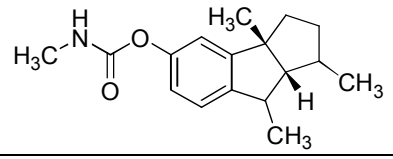
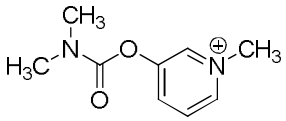
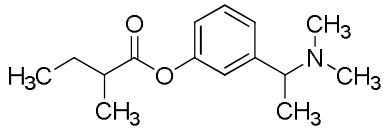
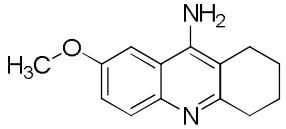
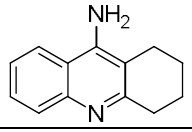
No.	Compound name	MW	Structure	pKa	log P	PSA (Å^2)	t_r (min)	Result (X)
1	Huperzine A	242		9.10 11.40	0.62	56.74	4.02	13.15
2	Galanthamine	287		8.90	1.16	43.13	7.26	13.20
3	Donepezil	379		8.60	4.21	39.97	29.48	19.11
4	Physostigmine	275		6.60	2.23	44.81	9.28	20.50

Table 2. Contd.

5	Pyridostigmine	181		-	-3.47	33.42	3.866	40.09
6	Rivastigmine	250		8.90	2.41	33.98	14.15	47.14
7	7-MEOTA (7-mehtoxytacrine)	228		8.90	1.88	49.39	59.11	296.37
8	Tacrine	198		8.95	2.63	40.16	34.436	300.84

5.0 (GraphPad Software, San Diego, California). Marvin was used for drawing, displaying and characterizing chemical structures, substructures and also for calculating of physico-chemical properties (pKa, log P, PSA), Marvin 5.1.0, 2008, ChemAxon (http://www.chemaxon.com).

RESULTS

Over the last decade polar surface area (PSA) has become a ubiquitous term in medicinal and computational chemistry. As shown previously, it correlated with human intestinal and other biological barrier permeation, especially BBB (Palm et al., 1998; Zhu et al., 2002; Clark, 2011). However, good correlation was observed between PSA and k_{IAM}/MW^4 for drugs used in calibration in this study. Correlation being 0.741 at the mobile phase of pH 7.4 (Figure 2) was found. The correlation between log P, the standard pharmacokinetics descriptor, and k_{IAM}/MW^4 was 0.706. Both results show stronger dependency between respected physicochemical descriptors and drug ability to penetrate through the BBB. Based on this result this method may be accepted for *in vitro* prediction of BBB permeation. On the other hand, the results of standards were also used for determination of border between CNS- and CNS+ compounds. For this, the known data about BBB penetration of standards from human studies were used.

According to the reported method, the CNS^- drugs (drugs with low passive penetration through the BBB) showed evident inability to bound to the phosphatidylcholine column and have permeability values less than 9.48, whereas the CNS^+ drugs (drugs with higher passive penetration through the BBB) proved to bound much better and their permeability values were higher than 17.6. If compounds reach values over 17.6, they can penetrate the BBB. However, values below 9.48 predict that compounds stay in the periphery. These conclusions were achieved during the correlation of physical parameters with the results of assay of twenty-one structurally different therapeutics set. After the method validation, a set of eight structurally different ChEIs was measured three times using similar conditions. K_{IAM} values were calculated from the

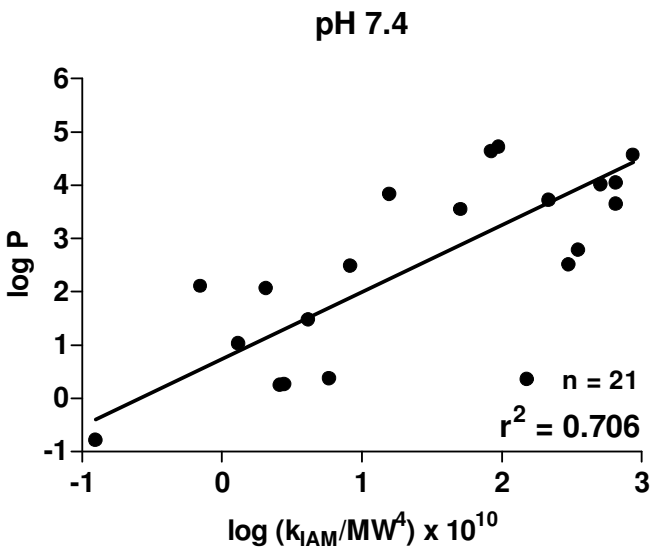


Figure 1. Correlation between log P and k_{IAM}/MW^4 for tested 21 drugs. Good correlation was found with the correlations coefficient (r^2) being 0.706 at pH 7.4.

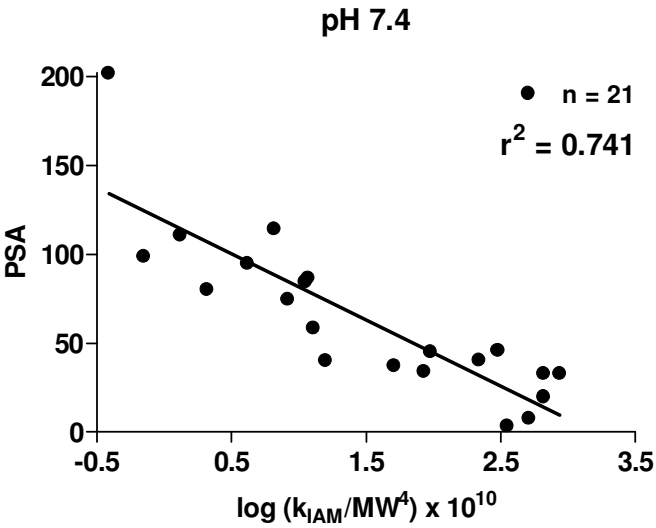


Figure 2. Correlation between polar surface area (PSA) and k_{IAM}/MW^4 determined at the mobile phase pH of 7.4 for the tested drugs.

retention times of ChEIs and after that, results were correlated by molecular weights. Using this approach, the permeability values for tested drugs were found.

In accordance with the acquired values characterizing tested compounds BBB permeability, we can discuss features influencing this process. All results obtained in this study are shown in Table 2. The structure of the tested ChEIs and also molecular weight are the most important factors, which may influence passive transport of these molecules into the brain. According to our methodology, the molecules such as huperzine A and galanthamine have lower ability to go through the BBB directly, which points to the possibility that these molecules could be delivered into the brain via active transport. Donepezil and physostigmine are able to use passive transport to permeate into CNS. Interestingly, rivastigmine is able to penetrate throught the BBB 2.5 fold more than donepezil. The key role in this discrepancy probably plays molecular weight.

The passive penetration into the CNS is strongly influenced by the presence of charge in molecule. It can be seen as a surprise that molecule as pyridostigmin is able to pass the BBB by passive penetration. The last two ChEIs molecules, tacrine and 7-MEOTA seem to mainly exploit the passive penetration to overcome the BBB.

DISCUSSION

All drugs that are successfully used in the therapy of AD should be considered as CNS+ targeted. There are many markers that may help us to estimate their ability to penetrate through the BBB. Physico-chemical descriptors are the most recent and most convenient prediction indicators used at the moment. Among them, lipophilicity (expressed by logP) was the first suitable parameter. The optimal BBB penetration value of logP is in 1.5 to 2.7 range (Hansch and Leo, 1979). From the group of tested ChEIs, physostigmine, rivastigmine, tacrine and 7-MEOTA fulfil this condition.

MW also plays important role in passive penetration through the BBB. Smaller molecules (MW < 400 Da) have significantly better passive lipid-mediated transport into CNS. The commonly used centrally active drugs have the mean value of MW 310 Da (Leeson and Davis, 2004). Better descriptor than MW is PSA; PSA is the sum of surfaces of polar atoms such as oxygens, nitrogens and attached hydrogens, in a molecule (Chen et al., 2009; Lanevskij et al., 2009); and is successfully used as a predictor for BBB passive penetration which is successfully used as a predictor for BBB passive penetration by many investigators (Yoon et al., 2006; Feng, 2002). Drugs aimed at the central compartment tend to have lower PSA than peripherally acting therapeutics. The higher PSA value convenient for CNS penetration was estimated at 60 to 70 $Å^2$. The upper limit of PSA for molecule to penetrate the brain is around 90 $Å^2$ (Kelder et al., 1999). All tested ChEIs ranged from 34.0 (rivastigmine) to 56.7 $Å^2$ (huperzine A).

Although these descriptors are able to confirm ability of tested drugs to penetrate through the BBB, it is still necessary to compare them with *in vivo* studies (Amourette et al., 2009; Geerts et al., 2005; Karasova et al., 2011; Polinsky, 1998; Wilson et al., 2008; Yue et al., 2007). Previously published *in vivo* data show many contradictory results. Many of them confirmed the BBB

penetration by certain indirect methods such as decrease of cholinesterase level (Ellman's method) in chosen brain parts. These studies may be loaded by numerous mistakes. A better way on how to follow this pharmacokinetic parameter is to measure the real brain concentrations directly (mainly by HPLC) (Geerts et al., 2005; Wilson et al., 2008; Yue et al., 2007).

It is peculiar that results of this kind of studies are so rare. One of the useful studies was published by Geerts et al. (2005) where author suggested that donepezil had a better brain penetration than galanthamine. The donepezil distribution curves in the brain have tendency to decrease more slowly that the galanthamine levels, suggesting a higher retention rate.

In another study, peak brain concentration was reached 15 (donepezil) and 30 min (galanthamine) after s.c. administration (Geerts et al., 2005).

According to our results, donepezil and galanthamine have lower ability to penetrate BBB under passive diffusion like molecules of Huperzine A and physostigmine. The real concentration of Huperzine A in brain was also measured (Yue et al., 2007). According to these results, it was demonstrated that Huperzine A is capable to cross the BBB readily under passive diffusion mechanism. The maximal concentration was reached after 5 min (intravenous application) and 30 min (intranasal application). This rapid permeation may also confirm integration of some BBB active transport system into this process.

Molecules such as rivastigmine and pyridostigmine were evaluated to have better potency to pass through the BBB via passive transport. According to *in vivo* results (Polinsky, 1998), rivastigmine is noted in cerebrospinal fluid (CSF) 30 min after application and its concentration quickly grew up till the maximal concentration was achieved (120 min after administration). Rivastigmine elimination from CSF was slow. The brain action of pyridostigmine is still unclear. Some results exist but they are based only on changes in ChE activities. Although pyridostigmine is a quaternary molecule, some evidence about BBB penetration for this type of molecules exist. The application of this ChE inhibitor dose induced a 7% depression in brain ChE activity. This was subsequently confirmed by radioactivity measurement in selected brain areas (Amourette et al., 2009). It is not the first evidence of quaternary molecules penetrating into the central nervous system (Karasova et al., 2011). Among the tested molecules, tacrine and 7-MEOTA were evaluated as structures with highest potency to penetrate through the BBB by passive diffusion. According to our results, we found out their ability to pass into central nervous system in comparable concentrations. Some *in vivo* results with tacrine were published by Wilson et al. (2008). The tacrine BBB penetration was confirmed, the real concentration was in 10^{-8} order (g/ml of brain homogenate). The brain concentration of 7-MEOTA and other pharmacokinetics data are still missing.

Conclusions

All tested ChEIs are able to penetrate the BBB. This ability is the cornerstone in AD therapy. Some of them should mainly use passive transport system; others may partially pass under active transport. Unfortunately, a relevant argumentation is complicated since information about real drug concentration in the brain or time-dependent BBB distribution studies are still quite rare; especially *in vivo* studies.

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