BIOLOGY

Biochemistry

BIOCHEMICAL STUDIES ON A NOVEL POTENT ACETYLCHOLINESTERASE INHIBITOR WITH DUAL-SITE BINDING FOR TREATMENT OF ALZHEIMER'S DISEASE

Rumiana Simeonova, Vessela Vitcheva, Ivanka Kostadinova, Iva Valkova, Irena Philipova*, Georgi Stavrakov, Nikolai Danchev, Irini Doytchinova

(*Submitted by Academician I. Ivanov on January 12, 2019*)

Abstract

Galantamine (GAL) and other acetylcholinesterase (AChE) inhibitors are the main therapeutic agents used for the treatment of Alzheimer's disease. During the last years many GAL derivatives have been synthesized and tested in vitro for anti-AChE activity. Some of them showed activities significantly higher than the activity of GAL. In the present study, we examine the in vivo anti-AChE activity and the antioxidant properties of a novel potent AChE inhibitor with dual-site binding to the enzyme. The anti-AChE tests showed that although the novel inhibitor is a better binder to AChE in vitro than GAL, in vivo it performs worse. It crosses the blood-brain barrier easily by passive diffusion but might be too bulky to enter any of the active transport systems and this might be a reason for low brain bioavailability. The bulkiness of AChE inhibitors with dual-site binding leading to low brain bioavailabilty should be considered in the design of novel multi-target ligands for treatment of Alzheimer's disease.

Key words: in vivo AChE activity, malondialdehide, glutathione, PAMPA

1. Introduction. The main therapeutic agents used for treatment of Alzheimer's disease (AD) are the acetylcholinesterase (AChE) inhibitors galantamine (GAL), rivastigmine, and donepezil, and the glutamate receptor blocker

memantine [1]. The inhibition of AChE leads to increased levels of acetylcholine (ACh) – the main transmitter involved in the processes of cognition (memory, learning, orientation, judgement and language). During the last years many efforts have been made for the development of strong AChE inhibitors with low toxicity and additional target actions like clearance of amyloid β (Aβ) peptide in the AD brain [2], M- and/or N-acetylcholine receptor modulations [3,4]. In order to act on the brain, the AChE inhibitors should be able to cross the blood-brain barrier (BBB). It was reported that donepezil, tacrine, rivastigmine and GAL are transported across the BBB by choline transport system [5]. LEE and KANG [6] have found that GAL decreases the [3H] choline uptake in a concentration-dependent manner by a non-competitive inhibition.

In a previous study, two newly synthesized GAL derivatives have shown in vitro inhibitory activity 1338 and 1008 times higher than GAL, respectively [7]. The acute toxicity in mice showed that one of the compounds is highly toxic, while the other one is less toxic even than GAL with LD_{50} of 77.46 mg/kg i.p. [8]. In the present study, we examine the ability of the less toxic AChE inhibitor (compound **1**) to cross the BBB and to inhibit the AChE in vivo. The AChE inhibitor is a derivative of GAL (Fig. 1) and it was designed to bind to AChE in two domains: the catalytic anionic site (CAS) and the peripheral anionic site (PAS). PAS is considered a trigger of the AChE-induced amyloid-beta (Aβ) aggregation [9]. Additionally, here we examine the antioxidant properties of the novel inhibitor by in vivo assessment of the levels of malondialdehyde (MDA) and reduced glutathione (GSH).

2. Methods. *2.1. Compound.* The synthesis of compound **1** is described elsewhere [7]. Briefly, 6-bromoheptanoic acid was coupled with phenylalanine methyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole hydrate (HOBT), resulting into bromo-amide. The latter was reacted with norgalantamine in dry acetonitrile, and potassium carbonate as a base to give the target compound.
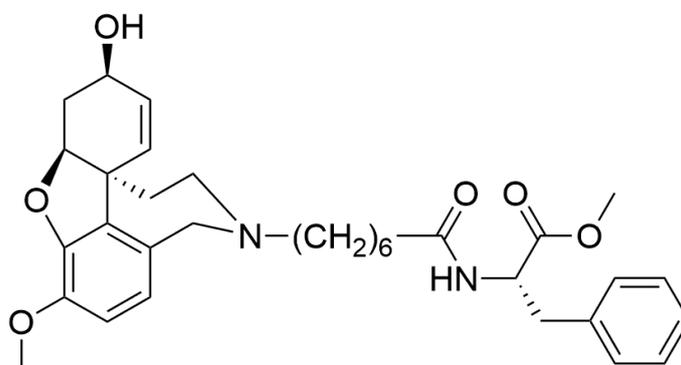

Fig. 1. Structure of the novel AChE inhibitor (compound **1**)

R. Simeonova, V. Vitcheva, I. Kostadinova et al.

2.2. Experimental animals. ICR female and male mice (22–24 g; 6 weeks old) were obtained from the National Breeding Center, Sofia, Bulgaria. The mice were housed in plexiglass cages (six per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature $20 \pm 2\,°C$ and humidity $72 \pm 4\%$) with free access to water and standard pelleted food 53-3, produced according ISO 9001:2008. Seven days acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. The vivarium (certificate of registration of farm No 15320139/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (No A-16-0532/14.10.2016). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) (permission No 187/03.12.2017) and the principles stated in the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123) (Council of Europe, 1991) were strictly followed throughout the experiment. Efforts were done to minimize the suffering of the animals.

2.3. Treatment of animals. Alzheimer's type dementia was induced by i.p. injection of scopolamine (SC) 3 mg/kg to mice for period of 12 days. To assess the effect of compound **1** and GAL on SC-induced changes on brain biochemical parameters, 48 mice were divided into six groups with eight mice in each group ($n = 8$). Group 1 contained control animals, treated with vehicle (0.9% saline i.p.). The animals from group 2 were treated with SC alone (3 mg/kg i.p.) [10]. Groups 3 and 4 comprised animals treated with GAL alone as a positive control (3 mg/kg i.p.) [11] and compound **1** alone (4 mg/kg i.p., which is 1/20 of LD_{50}) [8], respectively. The animals from group 5 were treated with GAL (3 mg/kg i.p.) and SC (3 mg/kg i.p. 30 min after GAL treatment), while the animals from group 6 – with compound **1** (4 mg/kg i.p.) and SC (3 mg/kg i.p. 30 min after the treatment with compound **1**). The treatments were performed once per day for 12 days. The learning abilities of the animals were tested by passive avoidance test and the results were described elsewhere [8]. After the tests, the animals were decapitated under ketamine/xylazine anesthesia (80/10 mg/kg, i.p.). The brains were taken out, measured, and divided into three parts – one for measurement of AChE activity, one for malondialdehyde (MDA) assessment and one for reduced glutathione (GSH) levels assessment. The brain samples were consequently homogenized with the corresponding buffers (see below). The protein content of brain homogenates was measured using the method of LOWRY [12] with bovine serum albumin as a standard.

2.4. Determination of AChE activity in mouse brain. The brains were homogenized with 0.1 M, pH 7.4 phosphate buffer and aliquots of homogenates of the individual mouse brain of the various treatment groups were taken and used to measure AChE activity by ELLMAN's method [13]. AChE activity was calculated and expressed as µmol/min/mg protein, using the molar absorption coefficient of $13.600\ M^{-1}\ cm^{-1}$.

2.5. MDA assessment in mouse brain. The brains were homogenized with 0.1 M phosphate buffer and EDTA, pH 7.4 (1:10). Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as MDA equivalents) as described by DEBY and GOUTIER [14]. MDA concentration was calculated using a molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

2.6. GSH assessment in mouse brain. GSH was assessed by measuring nonprotein sulphhydryls after precipitation of proteins with trichloroacetic acid (TCA) using the method described by BUMP et al. [15]. The absorbance was determined at 412 nm and the results were expressed as nmol/g wet tissue.

2.7. Parallel artificial membrane permeability assay (PAMPA). The intestinal and BBB permeabilities were measured by PAMPA Permeability Analyzer (pION Inc.) at the following settings: wavelength analyzed 250–500 nm, pH 7.4, temperature 25 °C, permeation time 4 h, lipid formulation GIT-0 and stirring 60 rpm and BBB-1 and no stirring, respectively. The intestinal permeability was tested at three pH values: 5.0, 6.2 and 7.4, while the BBB permeability was tested at pH 7.4. The permeability was presented as $- \log$Pe, where Pe is the permeability coefficient (10^{-6} cm/s). Compounds with $- \log$Pe below 5 are considered highly permeable, with $- \log$Pe between 5 and 6 – medium permeable and with above 6 – low permeable [16]. Carbamazepine, ketoprophen and ranitidine were used as controls for intestinal permeability. Theophylline, progesterone and propranolol were used as controls for BBB permeability.

2.8. Statistical analysis. For in vivo experiments statistical program 'MEDCALC' was used. The results are expressed as mean $\pm$ SEM for eight mice in each group. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $p \leq 0.05$ were considered statistically significant.

3. Results. 3.1. Effect of compound 1 on AChE activity in mouse brain. SC administered alone increased the AChE activity by 31%, while the positive control GAL decreased the AChE activity by 31% of the control group (Table 1). Compound **1** decreased the AChE activity by 23%. Both GAL and compound **1** suppressed the AChE activity, induced by SC in mouse brain, by 67% and 53%, respectively, in comparison with the SC-treated group.

3.2. Effect of compound 1 on MDA quantity in mouse brain. SC increased by 23% the quantity of MDA in mouse brain after 12 days of treatment (Table 1). GAL did not affect the quantity of MDA. Compound **1** caused slight increase by 11%. The increase in MDA quantity, induced by SC, was reduced by GAL with 18%, but not by compound **1**.

3.3. Effect of compound 1 on GSH levels in mouse brain. SC decreased the concentrations of GSH in mouse brain by 38%, while GAL and compound **1** increased them by 32% and 28%, respectively, compared with controls (Table 1). Even more, GAL and compound **1** inhibited by 31% and by 23%, respectively, the depletion of GSH induced by SC. These results suggest antioxidant and free radical scavenging activity of the new compound.

R. Simeonova, V. Vitcheva, I. Kostadinova et al.

Effects of multiple-dose administration of SC, GAL and compound **1**
on AChE activity, MDA quantity and GSH level in mouse brain

Group	AChE activity nmol/mg/min	MDA nmol/g	GSH nmol/g
Control	0.226 ± 0.04	5.58 ± 0.41	1.76 ± 0.14
SC	$0.297 \pm 0.03^*$	$6.89 \pm 0.51^*$	$1.09 \pm 0.11^*$
GAL	$0.155 \pm 0.02^{*+}$	$5.24 \pm 0.23^+$	$2.32 \pm 0.2^{*+}$
Compound **1**	$0.173 \pm 0.02^{*+}$	$6.2 \pm 0.32^*$	$2.24 \pm 0.09^{*+}$
GAL + SC	$0.108 \pm 0.01^{*+}$	$5.66 \pm 0.09^+$	$1.43 \pm 0.16^{*+}$
Compound **1** + SC	$0.141 \pm 0.004^{*+}$	6.84 ± 0.21	$1.35 \pm 0.07^{*+}$

$^*p < 0.05$ vs. control group; $^+p < 0.05$ vs. SC-treated group

3.4. GIT and BBB permeability. The permeability of compound **1** tested by PAMPA showed that it is highly permeable through the GIT and BBB. The $-\log$Pe is 4.591 for the BBB permeability and 4.293 for the GIT permeability at pH 6.2. For comparison, GAL has $-\log$Pe 5.060 [7] and 4.268 for BBB and GIT permeability, respectively, which corresponds well to its low ability to penetrate BBB by passive diffusion [17] and to 90% oral bioavailability [18].

4. Discussion. The AChE activity in vivo was decreased by GAL and compound **1** alone or in combination with SC comparing to the control and SC-treated groups. GAL inhibits AChE slightly better than compound **1** (31% vs. 23% for the control group and 67% vs. 53% for the SC-treated group). Considering the better binding of compound **1** than GAL to AChE in vitro (IC_{50} of compound **1** = 1.1 nM vs. IC_{50} of GAL = 1.07 µM) [7] one would expect to observe a similar effect in vivo. As this expectation was not met, insufficient bioavailability in brain could be supposed. The insufficient brain bioavailability of compound **1** might be due to its inability to cross BBB using any of the cation transporters like choline transport system, despite our finding that compound **1** has a better BBB permeability by passive diffusion than GAL. The increase in the molecular size by incorporating of groups binding to PAS leads to inability of the inhibitor to be loaded by the transporter, reduces its bioavailability in brain and decreases the in vivo inhibition of AChE. The low AChE activity in vivo explains the lower effect of compound **1** on learning and memory observed in the passive avoidance test [8].

The antioxidant properties of compound **1** were measured in vivo by assessment of MDA and GSH levels in mouse brain. MDA is one of the most frequently measured biomarkers of oxidative stress related to the lipid peroxidation. The oxidative stress causes degeneration of cholinergic neurons leading to cognition and memory impairments. The suppression of oxidative damage has been offered as a strategy for the treatment of AD [19]. In the present study, we found that

GAL decreases the elevated levels of MDA in SC-treated mice, while compound **1** increases them.

Glutathione exists in two forms: reduced GSH and oxidized GSSG. The thiol group of GSH reacts with oxygen species, neutralizes them, but itself becomes reactive, interacts with another reactive thiol group and a glutathione disulphide is formed. An increased GSSG-to-GSH ratio is indicative of oxidative stress. SC decreases the GSH levels, GAL and compound **1** increase them. The antioxidant activity of GAL depends on the enolic OH group [20]. As this group is conserved in the molecule of compound **1**, it also exhibits a scavenging effect. The better performance of GAL in the antioxidant tests could also be connected with better brain bioavailability in comparison with compound **1**.

In conclusion, the novel AChE inhibitor has in vivo anti-AChE activity and antioxidant properties similar to those of GAL. The enlargement of the molecule decreases its ability to cross the BBB by active transport and leads to low brain bioavailability. This obstacle should be considered in the design of novel bulky AChE inhibitors with dual-site binding.

REFERENCES

[1] SANABRIA-CASTRO A., I. ALVARADO-ECHEVERRIA, C. MONGE-BONILLA (2017) Molecular Pathogenesis of Alzheimer's disease: an update, Ann. Neurosci., **24**(1), 46–54.

[2] TAKATA K., Y. KITAMURA, M. SAEKI, M. TERADA, S. KAGITANI et al. (2010) Galantamine-induced amyloid-β clearance mediated via stimulation of microglial nicotinic acetylcholine receptors, J. Biol. Chem., **285**(51), 40180–40191.

[3] KITA Y., Y. AGO, K. HIGASHINO, K. ASADA, E. TAKINO et al. (2014) Galantamine promotes adult hippocampal neurogenesis via M1 muscarinic and α7 nicotinic receptors in mice, Int. J, Neuropsychopharmacol., **17**(12), 1957–1968.

[4] DAJAS-BAILADOR F. A., K. HEIMALA, S. WONNACOTT (2003) The allosteric potentiation of nicotinic acetylcholine receptors by galantamine is transduced into cellular responses in neurons: Ca^{2+} signals and neurotransmitter release, Mol. Pharmacol., **64**(5), 1217–1226.

[5] KANG Y. S., K. E. LEE, N. Y. LEE, T. TERASAKI (2005) Donepezil, tacrine and alpha-phenyl-n-tert-butyl nitrone (PBN) inhibit choline transport by conditionally immortalized rat brain capillary endothelial cell lines (TR-BBB), Arch. Pharm. Res., **28**(4), 443–450.

[6] LEE N. Y., Y. S. KANG (2010) The inhibitory effect of rivastigmine and galantamine on choline transport in brain capillary endothelian cells, Biomol. Ther. (Seoul), **18**(1), 65–70.

[7] STAVRAKOV G., I. PHILIPOVA, D. ZHELEVA, M. ATANASOVA, S. KONSTANTINOV et al. (2016) Docking-based design of galantamine derivatives with dual-site binding to acetylcholinesterase, Mol. Infor., **35**(6–7), 278–285.

[8] SIMEONOVA R., V. VITCHEVA, I. KOSTADINOVA, I. VALKOVA, I. PHILIPOVA et al. (2019) In vivo studies on novel potent acetylcholinesterase inhibitors with dual-site binding for treatment of Alzheimer's disease (Submitted).

[9] De Ferrari G. V., M. A. Canales, I. Shin, L. M. Weiner, I. Silman et al. (2001) A structural motif of acetylcholinesterase that promotes amyloid beta-peptide fibril formation, Biochem., **40**(35), 10447–10457.

[10] Eduviere A. T., S. Umukoro, A. O. Aderibigbe, A. M. Ajayi, F. A. Adelowe (2015) Methyl jasmonate enhances memory performance through inhibition of oxidative stress and acetylcholinesterase activity in mice, Life Sci., **132**, 20–26.

[11] Sadigh-Eteghad S., J. Mahmoudi, S. Babri, M. Talebi (2015) Effect of alpha-7 nicotinic acetylcholine receptor activation on beta-amyloid induced recognition memory impairment. Possible role of neurovascular function, Acta Cir. Bras., **30**(11), 736–742.

[12] Lowry O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall (1951) Protein measurement with the Folin phenol reagent, J. Biol. Chem., **193**(1), 265–275.

[13] Ellman G. L., K. D. Courtney, J. V. Andre, R. M. Featherstone (1961) A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol., **7**(1), 88–95.

[14] Deby C., R. Goutier (1990) New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases, Biochem. Pharmacol., **39**(3), 399–405.

[15] Bump E. A., Y. C. Taylor, J. M. Brown (1983) Role of glutathione in the hypoxic cell cytotoxicity of misonidazole, Cancer Res., **43**(3), 997–1002.

[16] Di L., E. H. Kerns, K. Fan, O. J. McConnell, G. T. Carter (2003) High throughput artificial membrane permeability assay for blood-brain barrier, Eur. J. Med. Chem., **38**(3), 223–232.

[17] Karasova J. Z., J. Korabecny, F. Zemek, V. Sepsova, K. Kuca (2013) Acetylcholinesterase inhibitors used or tested in Alzheimer's disease therapy; their passive diffusion through blood brain barrier: In vitro study, Afr. J. Pharm. Pharmacol., **7**(22), 1471–1480.

[18] Farlow M. R. (2003) Clinical pharmacokinetics of galantamine, Clin. Pharmacokinet., **42**(15), 1383–1392.

[19] Melo J. B., P. Agostinho, C. R. Oliveira (2003) Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide, Neurosci. Res., **45**(1), 117–127.

[20] Tsvetkova D., D. Obreshkova, D. Zheleva-Dimitrova, L. Saso (2013) Antioxidant activity of galantamine and some of its derivatives, Curr. Med. Chem., **20**(36), 4595–4608.

Faculty of Pharmacy
Medical University of Sofia
2, Dunav St
1000 Sofia, Bulgaria
e-mail: idoytchinova@pharmfac.mu-sofia.bg

*Institute of Organic Chemistry
with Centre of Phytochemistry
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 9
1113 Sofia, Bulgaria

C. R. Acad. Bulg. Sci., **74**, No 2, 2021

225